Investigation into the potential for key biomarkers to predict the outcome of periodontal treatment

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ABSTRACT

Various biomarkers in gingival crevicular fluid (GCF) have been examined in an attempt to obtain a specific and sensitive marker for periodontal disease progression and response to treatment. To date none have been found to be particularly reliable on their own. Therefore, the aim of this longitudinal clinical study was to determine whether key host enzymes (Matrix metalloproteinase-8, Cathepsin G, Elastase) and bacterial enzymes (Trypsin-like activity, Sialidase) detectable in GCF plus levels of key bacteria (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Fusobacterium nucleatum)* detectable in subgingival plaque can be used in combination to provide an improved prognostic "finger print" for the outcome of treatment in patients with chronic periodontitis.

Methods: 89 subjects were recruited to a longitudinal study. At baseline, 3-month and 6-month samples of GCF and subgingival plaque were collected from 3 representative sites: healthy (≤ 3mm), deep non-bleeding (DNB) (≥ 6mm) and deep bleeding (DB) (≥ 6mm) using a Periopaper® strips and a periodontal curette. In addition full mouth clinical data (pocket probing depth, clinical attachment loss, plaque index and bleeding on probing) were also recorded and patients received standard non-surgical periodontal treatment. GCF samples were assayed for each of the above enzyme activities using colourimetric/fluorometric substrates and subgingival plaque samples were assayed for the levels of each of the above bacterial species by qPCR. Data were analysed on a site-by- site basis using logistic regression for enzyme and bacterial profiles predictive of ≥ 2mm improvement in pocket probing depth (PPD) 6 months after treatment.

Results: 77 individuals completed the 6-month interval phase. Full mouth clinical data showed statistically significant reduction in response to treatment, however, one third of DNB and DB sites showed less than 2mm improvement in pocket depth. The average levels of all biomarkers (enzymes and bacteria) were significantly higher in diseased sites than healthy sites and overall they decreased through the course of the study except *Fusobacterium nucleatum*. Matrix metalloproteinase-8 (MMP8), elastase, sialidase, *Porphyromonas gingivalis* and *Tannerella forsythia* showed the greatest reductions. The levels of MMP8, elastase and sialidase at baseline significantly correlated with the initial

PPD as follows: MMP8 (r= 0.58), elastase (r=0.51) and sialidase (r=0.5). Using threshold enzyme levels that were the values with the highest sensitivity and specificity MMP8 (94ng/µl), elastase (33ng/µl) sialidase (2.3ng/µl), *Porphyromonas gingivalis* (0.23%) and *Tannerella forsythia* (0.35%), ROC curve analysis demonstrated that the baseline levels of these five biomarkers at sites are reliable diagnostic biomarkers as they differentiated healthy sites from diseased sites with degree of sensitivity and specificity above 77%. Furthermore, logistic regression showed that the combination of MMP8, elastase and sialidase provided accurate predictions of treatment outcome (81.3% for DNB, 80.3% for DB), which was significantly better than each enzyme alone (62.5%). When combined with the levels of *Porphyromonas gingivalis* and *Tannerella forsythia* the prediction value increased to 92% for DNB sites and 93.3% for DB sites. The biomarker values of MMP8, elastase and sialidase were also shown to be reliable after validating with an independent cohort. Preliminary tests showed that these three enzymes can be translated in to a simple chair-side test but this requires further development.

Conclusion: Combined profiles of the above biomarkers offer a significantly improved indication of a site's likely response to non-surgical periodontal treatment.

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List of abbreviations

A. actinomycetemcomitans	*Aggregatibacter actinomycetemcomitans*
AUC	Area under the curve
BANA	N-benzoyl-DL-arginine-2-naphthyle amide
BAPNA	N-α-benzoyl-DL-arginine-p-nitroanilide
BOP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment loss
CI	Confidence interval
DB	Deep bleeding
DNB	Deep non bleeding
F. nucleatum	*Fusobacterium nucleatum*
GCF	Gingival crevicular fluid
Ig	Immunoglobulin
IL	Interleukins
MMP	Matrix metalloproteinase
OR	Odds ratio
PBS	Phosphate buffered saline
PPD	Probing pocket depth
P. gingivalis	*Porphyromonas gingivalis*
P. intermedia	*Prevotella intermedia*
PI	Plaque index
PMNs	Polymorphonuclear leukocytes
ROC	Receiver operating characteristic
T. forsythia	*Tannerella forsythia*
T. denticola	*Treponema denticola*
TIMP	Tissue inhibitors of metalloproteinases
TNF	Tumor necrosis factors

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Chapter 1- Literature review

Chapter 1- Literature review

1.1 General introduction

Periodontal diseases are a group of related inflammatory diseases that affect the supporting tissues of teeth, and are characterized by inflammation of gingival tissues, the formation of periodontal pockets, gingival recession, increased tooth mobility and alveolar bone resorption. The World Health Organization has reported that severe forms of periodontitis cause tooth loss in about 5-15% of the population worldwide, and as a consequence it is considered to be amongst the most common global health problems (Newman et al., 2012).

Periodontitis is multifactorial in its aetiology and whilst the destruction of tissue is largely irreversible, treatment can halt and stabilize the condition. Consequently, early and accurate diagnosis is imperative to improve the prognosis and focus expensive and time-consuming treatment on patients and diseased sites that would benefit most (Greenstein, 1997).

Oral bacteria are considered to be primary etiological factors in periodontal disease, although most of the tissue destruction is thought to be due to the host's response (Schenkein 2006). However, other factors are also known to increase the risk of periodontal disease such as smoking (Baharin et al., 2006) and diabetes mellitus (Mealey and Oates, 2006). The severity of the disease varies, not only between teeth within the same person but also between sites around the same tooth (Baelum et al. 1996). These characteristics pose considerable diagnostic and prognostic difficulties for clinicians, which may result in inappropriate treatment.

In contemporary clinical practice, diagnosis is almost entirely dependent upon the assessment of clinical measurements including clinical attachment loss (CAL), probing pocket depth (PPD), tooth mobility index, plaque index (PI), bleeding upon probing (BOP) and radiographical findings. These all provide information about past periodontal tissue destruction, rather than demonstrating the current state of disease activity or predicting future disease progression and likely outcome following treatment (Chapple 2009). Therefore, a major challenge in the field of periodontology is to discover methods that have improved diagnostic and prognostic capability.

There have been considerable efforts made over the years to search for biomarkers of chronic periodontitis. Such biomarkers have been identified in saliva, plaque and

gingival crevicular fluid (GCF). While some progress has been made, there has, however, been relatively little progress in identifying markers that have value in predicting the response to treatment. Owing to the complex nature of periodontitis, it is unlikely that a single clinical or laboratory test could address all issues related to the diagnosis and prognosis mentioned above (Kinney et al., 2014, Ramseier et al., 2009).

Amongst the most investigated biomarkers are levels of key enzymes in GCF and load of specific bacteria in plaque, particularly those that are thought to lead to or be involved in tissue destruction. For example, elastase activity is considered to be a useful quantitative measure of gingival inflammation (Herrmann et al. 2001), cathepsin G and Matrix metalloproteinase-8 (MMP-8) have been correlated with disease severity (Kinney et al. 2014, Mailhot et al. 1998), and trypsin-like activity (mainly of bacterial origin) has been significantly correlated with the gingival Index, PI and PPD (Beighton and Life 1989). Certain other bacterial enzymes are just beginning to be studied in this context. For example, sialidases are produced by a number of periodontal pathogens and that of *Tannerella forsythia* has been found to promote biofilm formation (Roy et al. 2011). Also, sialidase activity has been found to be higher in people with periodontitis than gingivitis and this activity has been correlated with the clinical parameters (PI, BOP, PPD and CAL) (Beighton et al. 1992).

In terms of bacteria, *Porphyromonas gingivalis* levels remain high in sites that respond poorly to conventional periodontal treatment (Choil et al., 1990, Winkelhof et al., 1988). Moreover, active diseased sites showed higher levels and increased frequency of *T. forsythia* than quiescent sites (Tanner, 2014, Dzink et al., 1988) and the number of *T. forsythia* cells present correlated with the degree of periodontal tissue breakdown (Lai et al., 1987). The prevalence of *Fusobacterium nucleatum* increased with increasing PPD and severity of the disease (Yang et al., 2014, Riep et al., 2009, Moore and Moore, 1994) and the number of *F. nucleatum* cells has been found to be higher in biofilm samples from periodontitis patients than healthy subjects. These three bacterial species, therefore, are potentially useful biomarkers for periodontal disease.

The finding of biomarkers with diagnostic and prognostic value in GCF and plaque would be a valuable clinical tool to help dentists determine whether active periodontal tissue destruction is present, what the likely response to treatment is and thereby assist in patient management. However, so far the search for a single marker has been

unsuccessful. This longitudinal clinical study, therefore, was directed at investigating whether a 'biomarker profile' has greater prognostic value than each single biomarker alone in patients with chronic periodontitis.

1.2 Periodontal diseases

As described earlier (1.1 General introduction), periodontal diseases constitute a group of related inflammatory diseases caused primarily by formation and maturation of bacterial plaque on the surface of the teeth. These diseases are characterized by gingival tissue inflammation, periodontal pocket formation, recession of gingival tissue, increased tooth mobility and resorption of alveolar bone. A key characteristic of the disease is loss of collagen from the supporting tissues. (Newman et al., 2012).

1.3 Classification of periodontal disease

Periodontal diseases are actually a group of related but distinct conditions and they seem to have subtly different aetiologies, thus it is important that they are correctly defined and classified so that different studies can be compared. The currently used classification of periodontal diseases was developed at the 1999 International Workshop for the Classification of Periodontal Diseases and Conditions. It is mainly based on the onset and pattern of infection and the host response (Armitage, 2002). It consists of eight major categories and numerous subcategories. The major categories of classification are as follows: I. Gingival disease, II. Chronic periodontitis, III. Aggressive periodontitis, IV. Periodontitis as a manifestation of systemic diseases, V. Necrotizing periodontal disease, VI. Abscesses of the periodontium, VII. Periodontitis associated with endodontic lesions, and VIII. Development of acquired deformities and conditions.

This classification scheme is not based on the etiology and pathology of periodontal diseases as this needs more information on the interactions between the host and microbes, and environmental factors behind them. Instead it reflects the current understanding of periodontal disease and should be modified when new knowledge becomes available (Armitage, 2002).

Categories I and II cover the most common forms of periodontal disease, with the greatest prevalence. Category I includes gingival diseases, and category II consists of the chronic forms of periodontitis.

1.3.1 Gingivitis

Almost all individuals suffer periodically from gingivitis, which is a reversible inflammation of the gingiva. The main aetiology behind the inflammation is accumulation of bacterial biofilm, termed dental plaque, on the tooth surfaces when oral hygiene measures are insufficient. The signs of inflammation appear with increasing load of bacteria, such as redness, loss of surface texture (loss of stippling) with oedema, bleeding upon probing the sulcus or tooth brushing. There is no alveolar bone resorption or clinical attachment loss in gingivitis. During the accumulation of dental plaque, neutrophils begin to aggregate and within a few days the clinical signs of inflammation can be seen with an increase in flow of a serum transudate called gingival crevicular fluid (Schroeder and Listgarten, 1997). When the plaque is removed and the reasons for accumulation eliminated (i.e. insufficient oral hygiene or iatrogenic factors), the gingival tissues return to a healthy condition (Loe et al., 1965). What is not known, however, is why gingivitis progresses to destructive periodontitis in some individuals but not in others. Thus, defining the risk of tissue deterioration is still impossible, although there are some known risk factors that should be taken into account when evaluating the risk.

An accumulation of neutrophil leukocytes exists in the connective tissue beneath the epithelial lining of the gingival crevice, even when the tissues are healthy. Since microbes regularly challenge the gingival tissues, these inflammatory cells in the lamina propria directly underneath the junctional epithelium are considered to be an essential defence system for the tissues and are a part of normal homeostasis (Schroeder and Listgarten, 1997). During the early stage of progression from health to gingivitis, there is an increased GCF level of interleukin 8 (IL-8), which further attracts neutrophils to the inflamed sites (Tonetti et al., 1998). To make space for the neutrophils leaving blood vessels and migrating into the connective tissue, many gingival collagen fibers are broken down. Later, toward established gingivitis, plasma cell infiltration increases (10-30% of infiltrated inflammatory cells), and when this becomes established then a destructive periodontitis lesion (clinical attachment loss and alveolar bone loss) develops and plasma cells form more than 50% of inflammatory cells (Lindhe et al., 2008).

1.3.2 Chronic periodontitis

Chronic gingivitis may remain for a long period without progressing to periodontitis. Albandar (2002) as well as Page and Schroeder (1976) showed that only a proportion of individuals and sites with gingivitis develop irreversible periodontal tissue loss (periodontitis). There are a number of possible factors that trigger this transition, such as disruption of host defences, introducing new pathogens to the biofilm, or activation of a destructive immune-inflammatory process in susceptible patients (Samaranayke, 2002), possibly by not dampening down the inflammatory response appropriately (Campbell et al., 2011). Additionally, there are some risk factors that increase the risk for periodontitis, including smoking (Baharin et al., 2006), diabetes mellitus (Mealey and Oates, 2006), genetic predisposition (Michalowicz et al., 1991), poor oral hygiene (Baltacioglu et al., 2006) and presence of specific bacterial species (Kinane et al., 1999).

Clinically, chronic periodontitis is characterized by clinical attachment loss, alveolar bone loss, true pocket formation and inflammation of gingiva (Flemmig, 1999). Furthermore, signs of bleeding upon probing, gingival recession, gingival enlargement and increased mobility of the tooth may also be apparent (Page and Schroeder, 1976). Chronic periodontitis mainly presents in adults and generally it is slow in progression, but periods of rapid destruction are also known to occur, although little is known about what precipitates these. It is thought that in general terms the subgingival bacteria have relatively weak virulence and it is the host's response that is responsible for much of the damage. Indeed, in response to the inflammation, the normal periodontal tissue converts to granulation tissue with infiltration of plasma cells and it is thought that the shift in balance of local cytokines drives the loss of collagen and so loss of the attachment to the tooth (Darveau et al., 1997).

1.4 Dental plaque

There is consensus that periodontal disease is linked with the microbial biofilms that form on the tooth surfaces, however, various host and environmental factors have been found to be associated with the onset of the disease (Socransky and Haffajee, 1993). The dental biofilm has been defined as a matrix-enclosing a diverse population of bacteria adherent to each other and/or surfaces (Marsh, 2003a). The oral cavity contains a large number of distinct habitats, each of which has different growth patterns, and gradually a distinctive complex biofilm develops which corresponds to their different ecological

niches (Marsh, 1989). Formation of biofilm on any surface requires attachment of bacterial cells to surfaces. Salivary glycoproteins form a conditioning film or pellicle that alters the physical and chemical properties of the oral surfaces which provides a degree of selective recruitment for oral bacteria and this is considered as the first and second steps in dental biofilm formation. This is followed by co-aggregation and growth and development of the adherent bacterial community and production of the biofilm matrix (third step). Dispersion is the final step, in which bacterial cells disperse from the surface of the mature biofilm and spread to colonise other sites (Huang et al., 2011) (Figure 1.1).

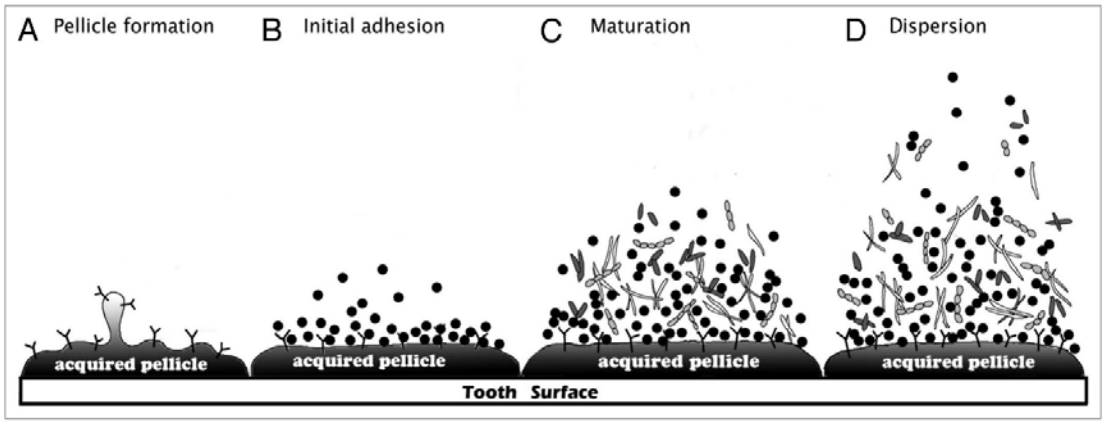


Figure 1.1. Diagram showing the steps of oral biofilm formation (Huang et al., 2011) (Permission obtained to reproduce here).

The pioneer colonisers of the tooth surface include streptococci, actinomycetes and *Neisseria* species. *Streptococcus oralis*, *Streptococcus sanguinis* and *Streptococcus mitis* are the most abundant bacteria in the early stage of plaque formation (Li et al., 2004). There are numerous forms of interaction between streptococci and pellicle, for example, *Streptococcus sanguinis* adheres to host molecules on the cell membrane and salivary glycoprotein via terminal sialic acid residues (McBride and Gisslow, 1977). During this stage, bacteria are more likely to colonise on surface defects (Marsh, 2003b). The primary colonisers alter the environmental conditions in different ways, such as by oxygen consumption, reducing compounds and further nutrient production. These changes cause the site to become more favorable for the growth of fastidious species (secondary colonisers) (Lamont and Rosan, 1990). The primary coloniser is followed by secondary colonisers that were not previously attached to the tooth surface. *Fusobacterium* species are considered to be the most important species amongst the secondary colonisers, as they are able to coaggregate with many oral species, such as

streptococci and *P. gingivalis*, and is consequently known as a key component of dental biofilm that bridges the early and later colonisers (Kolenbrander et al., 2002). *Provetella intermedia*, *Eikenella corrodens*, *Leptotrichia buccalis* and *Actinomyces naeslundii* also belong to the secondary coloniser group, and these bacteria make extracellular matrix, such as the fructans and glucans, that are necessary to maintain the integrity of the plaque (Dibdin and Shellis, 1988) (Figure 1.2). The mature plaque creates a favorable environment for its inhabitants, such as through greater resistance to environmental stress, hosting a broader range of habitats, further increasing virulence potential, and enhancing metabolic efficiency (Lemos et al., 2005). Plaque composition varies from one tooth surface to another, depending on the anatomical site, with proximal, gingival and fissure sites representing distinctive ecosystems. Moreover, shear forces and nutrient supply vary from one tooth surface to another. The lower shear force areas offer higher bacterial density, such as in the gingival margins, which again protects the biofilm from removal forces (Marsh et al., 2009).

Bacterial species in the dental biofilm interact with each other by physiological and metabolic means and through cooperative or competitive routes. The exchange of information within dental biofilms takes place by genetic exchange, metabolic communication and, most importantly, quorum-sensing (Chalmers et al., 2008). The genes involved in quorum-sensing control expression of key genes responsible for modifying physiological features suitable for growth and survival in the biofilm environment.

Oral bacteria obtain their nutrients from sources such as saliva, GCF, host diet and metabolic products from other bacteria (Hojo et al., 2009). Metabolic communication happens when the byproducts of one bacterium are used as a source of nutrients by another or through destruction of a substrate by the extracellular enzymes of one species that produces other substrates for different species (Kolenbrander et al., 2002). Quorum-sensing is known to be the most important form of chemical communication amongst bacteria, and occurs in response to cell density. It has impact on different bacterial functions such as acid tolerance, virulence and formation of biofilm (Hojo et al., 2009).

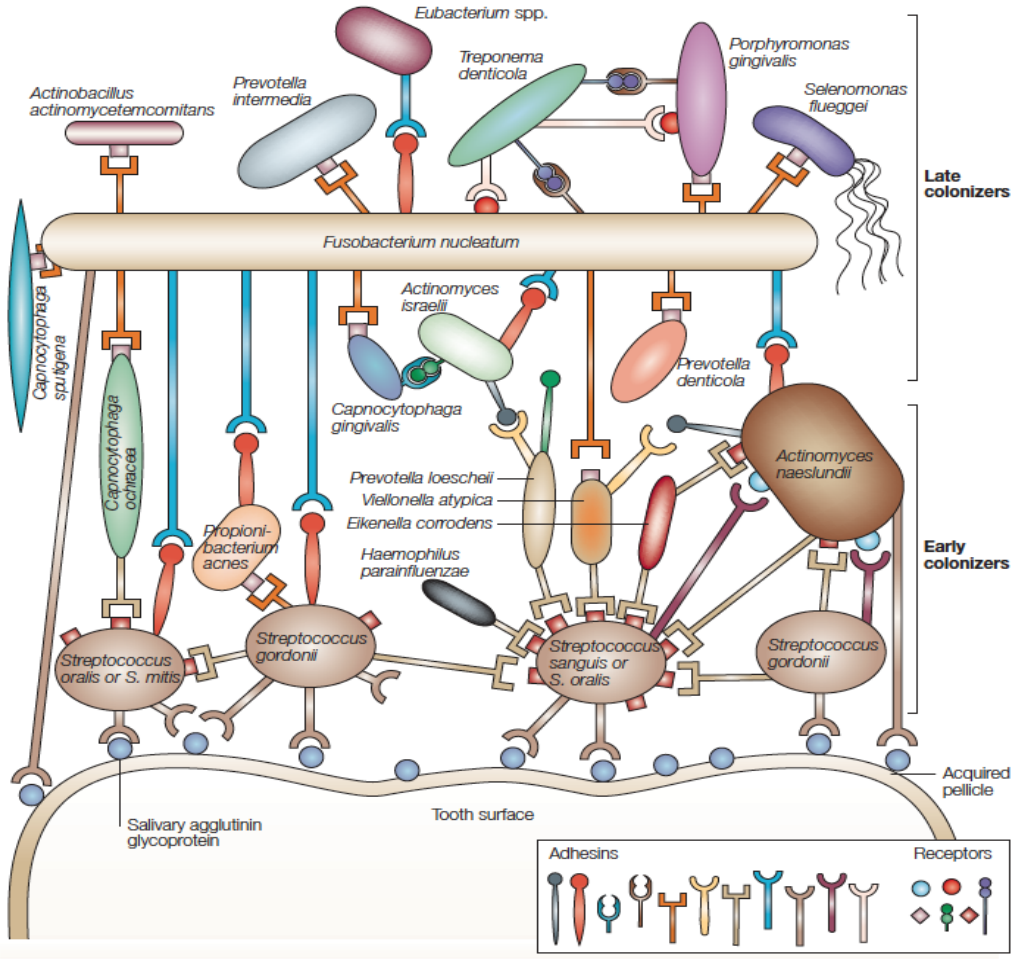


Figure 1.2. Schematic showing co-aggregation pairings in dental biofilm to demonstrate some of the mechanisms involved in bacterial colonisation of the tooth surface (Bakaletz, 2004) (Permission obtained to reproduce here).

1.4.1 Supra gingival plaque

Supra gingival plaque refers to the biofilms that reside above the gingival margins and these include all plaque that forms on exposed tooth surfaces in the oral cavity. It develops mainly in the absence of oral hygiene measures. The accumulation of supragingival biofilm is restricted by its constant subjection to disruption by salivary flow and masticatory forces (Sissons et al., 1995). Generally, supragingival biofilm contains greater percentages of facultative anaerobes than subgingival biofilm (Signoretto et al., 2006), and Gram-positive bacteria are more prevalent than Gram-negative bacteria (Rozkiewicz et al., 2005). Furthermore, various parts of the host defence system can also be found in supragingival biofilm including enzymes, immunoglobulin A and lactoferrin

and these are thought to contribute to suppressing the growth of supragingival biofilm organisms to a degree.

1.4.2 Subgingival plaque

Subgingival plaque forms in unique locations that are protected from the shear forces that are usually associated with most supragingival sites. It is confined by the tooth surface on one side and the gingival tissue on the other (Darveau et al., 1997). The subgingival site is bathed in GCF, which is advantageous to the subgingival biofilm and although it contains components of host defence, it also provides unique nutrients for subgingival microbiota (Cimasoni, 1983). These support the growth of a most diverse bacterial microbial community comprising in general, larger amounts of Gram- negative anaerobic species (Socransky et al., 1998).

1.5 Microbiology of periodontal disease

Strong evidence has emerged to support the view that the dental biofilm is the cause of most periodontal disease (Socransky and Haffajee, 1992, Socransky and Haffajee, 1991, Socransky and Haffajee, 1990), and this evidence derives from such sources as: (1) studies that show correlation between presence of dental biofilm and periodontal disease; (2) a body of evidence showing that removal of the dental biofilm being associated with clinical improvement; (3) in vivo and in vitro studies demonstrating that various micro-organisms within the dental biofilm exhibit virulence properties (Zambon, 1996).

Different hypotheses have been developed to support the notion that bacteria are the primary aetiological factor of periodontal disease. These studies have employed improved laboratory techniques that have allowed better understanding of the composition and organization of the dental biofilm. Before the emergence of the concept of individual bacterial species within dental biofilm as a causative factor of periodontal disease, gross accumulation of dental biofilm was considered to be an aetiological factor of periodontal disease. This theory, described as the non-specific plaque hypothesis, assumed that periodontal disease resulted in continuous accumulation of plaque to the point that it overwhelmed the host defences (Theilade, 1986). This theory is no longer accepted as more than 500 species have now been found in dental biofilm, each with different characteristics, and it could therefore be presumed that they would each perform different roles, some of which may be essential for disease progression (Dahlén, 1993).

Consequently, the specific plaque hypothesis was formulated, suggesting that only a few bacterial species are responsible for periodontal disease; these species can be also found in healthy sites but at lower levels compared to periodontal disease sites (Loesche, 1975). This hypothesis has been supported by clinical cross-sectional and longitudinal studies, demonstrating that although periodontal disease is a mixed infection, only a few bacterial species are disease-related (Haffajee and Socransky, 1994, Theilade, 1986). The third hypothesis, known as the ecological plaque hypothesis, is a modified version of the specific plaque hypothesis, which proposes that besides presence of periodontopathic bacteria, changes in the local environment, such as in pH or availability of essential nutrients, are also necessary for development of periodontal disease in susceptible individuals (Marsh, 1994). Thus, interference by both microbial and environmental factors, for instance, through increase of periodontal pocket redox potential by oxygenating and redox agents, can halt disease progression. The microbial aetiology of periodontal disease is not currently certain but a few species have been considered as more important periodontopathogenes than others (Socransky et al., 1998). This argument is supported by the latest hypothesis, termed the "keystone plaque hypothesis", which suggests that certain species, including *P. gingivalis,* are able to shift microbial diversity even though they themselves are present in low numbers (Hajishengallis et al., 2012). *P. gingivalis* as a keystone pathogen has virulence factors capable of incapacitating parts of the local defensive response, including possession of potent proteolytic enzymes, fimbriae for adhesion and metabolic products that directly or indirectly lead to periodontal destruction.

1.5.1 Comparison of the microbiota associated with healthy and diseased periodontium

The healthy periodontium has limited subgingival space for bacterial growth. Therefore, healthy sites exhibit relatively low bacterial loads (10^2 to 10^3 microorganisms) comprising a relatively high proportion of Gram-positive bacteria such as *Streptococci* and *Actinomyces*. About 15% of isolated bacteria from healthy sites appear to be Gram-negative species (Darveau et al., 1997, Tanner et al., 1996). Meanwhile, gingivitis is characterised by an increase in the local bacterial load (10^4 to 10^6 microorganisms) and a shift in isolated species towards a greater proportion of Gram-negative bacteria (approximately 15-50%) (Lai et al., 1987, Moore et al., 1987) (Table 1.1). Furthermore,

a greater total bacterial load is detected in periodontitis sites compared to gingivitis and healthy sites, (between 10^5 - 10^8 microorganisms; (Darveau et al., 1997) and the higher prevalence of microorganisms has been directly correlated with probing pocket depth (Wolff et al., 1997). Amongst these species, several have been implicated as putative pathogens of periodontitis (Table 1.1) as they have strong association with the severity of periodontitis (Haffajee and Socransky, 1994) and they generally decrease after successful periodontal treatment (Tanner and Izard, 2006, Simonson et al., 1992).

Studies on the subgingival biofilm have shown that a number of species usually occur in complexes (Socransky and Haffajee, 2005) (Figure 1.3). For instance, red complex bacteria are strongly correlated with the clinical signs of periodontitis. *F. nucleatum* belongs to the so called orange complex which collectively has been strongly associated with the breakdown of periodontal tissues (Socransky and Haffajee, 2002).

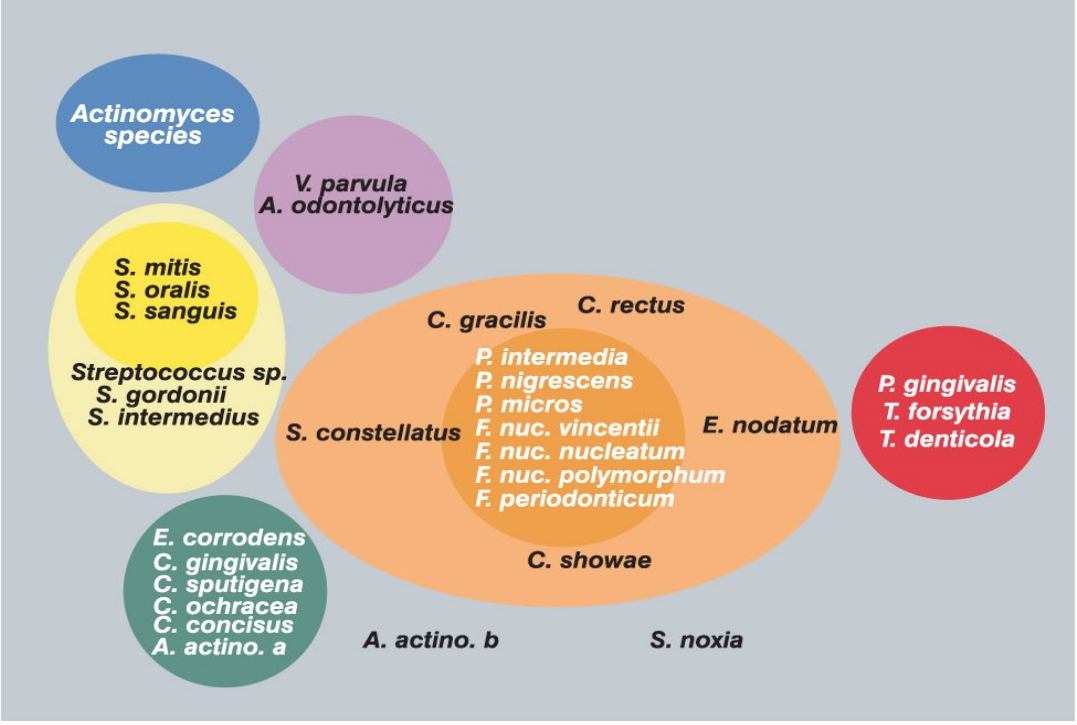


Figure 1.3. Complexes described by Socransky and Haffajee (Socransky and Haffajee, 2005) (Permission obtained to reproduce here).

Table 1.1. Predominant bacterial species identified in healthy periodontium, gingivitis and periodontitis sites. Species arranged from the most likely to the least likely to be detected by culture. (Adapted from Darveau et al. (1997).

Periodontal health	**Gingivitis**	**Periodontitis**
Streptococcus oralis	*Streptococcus oralis*	*Porphyromonas gingivalis*
Streptococcus sanguinis	*Streptococcus sanguinis*	*Aggregatibacter actinomycetemcomitans*
Streptococcus mitis	*Streptococcus mitis*	*Tannerella forsythia*
Streptococcus gordonii	*Streptococcus intermedius*	*Spirochaetes*
Streptococcus mutans	*Capnocytophaga ochracea*	*Treponema denticola*
Streptococcus anginosus	*Capnocytophaga gingivalis*	*Prevotella intermedia*
Streptococcus intermedius	*Campylobacter gracilis*	*Prevotella nigrescens*
Gemella morbillorum	*Prevotella loescheii*	*Campylobacter rectus*
Actinomyces naeslundii	*Eubacterium nodatum*	*Fusobacterium nucleatum* subspecies *vincenti*
Actinomyces gerencseriae	*Actinomyces naeslundii*	*Fusobacterium nucleatum* subspecies *nucleatum*
Actinomyces odontolyticus	*Actinomyces israelii*	*Selenomonas noxia*
Parvimonas micra	*Campylobacter concisus*	*Selenomonas flueggeii*
Eubacterium nodatum	*Actinomyces odontolyticus*	Enteric species
Capnocytophaga ochracea	*Fusobacterium nucleatum* subspecies *nucleatum*	*Filifactor alocis*
Capnocytophaga gingivalis	*Eubacterium brachy Eikenella corrodens*	*Lactobacillus uli*
Campylobacter gracilis	*Aggregatibacter actinomycetemcomitans*	*Veillonella parvula*
Fusobacterium nucleatum subspecies *polymorphum*		

For the purpose of this study, three species have been investigated as biomarkers of periodontal disease. Two, *P. gingivalis* and *T. forsythia,* belong to the red complex of pathogens and there is now considerable evidence to implicate these two species as

putative periodontal pathogens (Hajishengallis and Lambris, 2011, Tanner and Izard, 2006). *F. nucleatum* belongs to the orange complex and it acts as an important bridge organism between early and late coloniser species. Further information about these species is given in Chapter 6.

1.6 Pathogenesis of periodontitis and periodontal infection

In periodontitis, a shift in the bacterial population in the gingival crevice towards more complexity, including significant increases in gram-negative anaerobic species, stimulates a chronic inflammatory response. The inflammatory cascade begins with the consequent increase in local concentration of lipopolysaccharides. Monocyte/macrophage cells release inflammatory modulators in response to the lipopolysaccharides, such as prostaglandin E2, IL-1, IL-6, IL-8, tumour necrosis factor alpha (TNFα) and collagenases (MMPs) (Offenbacher et al., 1993). IL-1β and TNFα can activate fibroblasts to increase export and activation of matrix metalloproteinases and secretion of prostaglandin E2. Other cells, such as neutrophils, endothelial and epithelial cells also secrete MMPs and these can mediate destruction of tissue matrix molecules like collagen (Reynolds and Meikle, 1997), whereas prostaglandin E2 can stimulate bone resorption (Roberts et al., 2004). On the other hand, released IL-8 acts as a chemotactic factor for neutrophils to the site, which produce various proteolytic enzymes. For example, MMP-8, cathepsin G and elastase are secreted mainly by neutrophils and play an important role in destruction of collagen (Jin et al., 2002, Bickel, 1993, Baggiolini et al., 1989) (Figure 1.4).

Besides its role in providing attachment between the tooth and underlying connective tissues, junctional epithelium takes part in host defence mechanisms (Bosshardt and Lang, 2005). Detachment of junctional epithelium from the tooth surface and adjacent junctional epithelial cells has been attributed to the formation of periodontal pockets (Pollanen et al., 2003). This structural disintegration of junctional epithelium can be further exacerbated by bacterial invasion, release of bacterial products, increased number of neutrophils, monocyte/macrophage, T-lymphocyte and B-lymphocytes (Bosshardt and Lang, 2005, Schroeder and Listgarten, 1997). Furthermore, junctional epithelium expresses several cell adhesion molecules, for example, intercellular cell adhesion molecule-1, which is thought to contribute to a chemotactic gradient for neutrophils (Tonetti et al., 1998, Tonetti, 1997). Other relevant molecules can be also expressed by

junctional epithelium in response to bacterial challenge, including IL-8, IL-1α, IL-1β, and TNFα, particularly in the coronal half (Miyauchi et al., 2001). Dale (2002) also reported that this epithelium responds to bacteria by signaling further host responses, enhancing proliferation, modifying differentiation, and cell death, and integration of innate and acquired immunity.

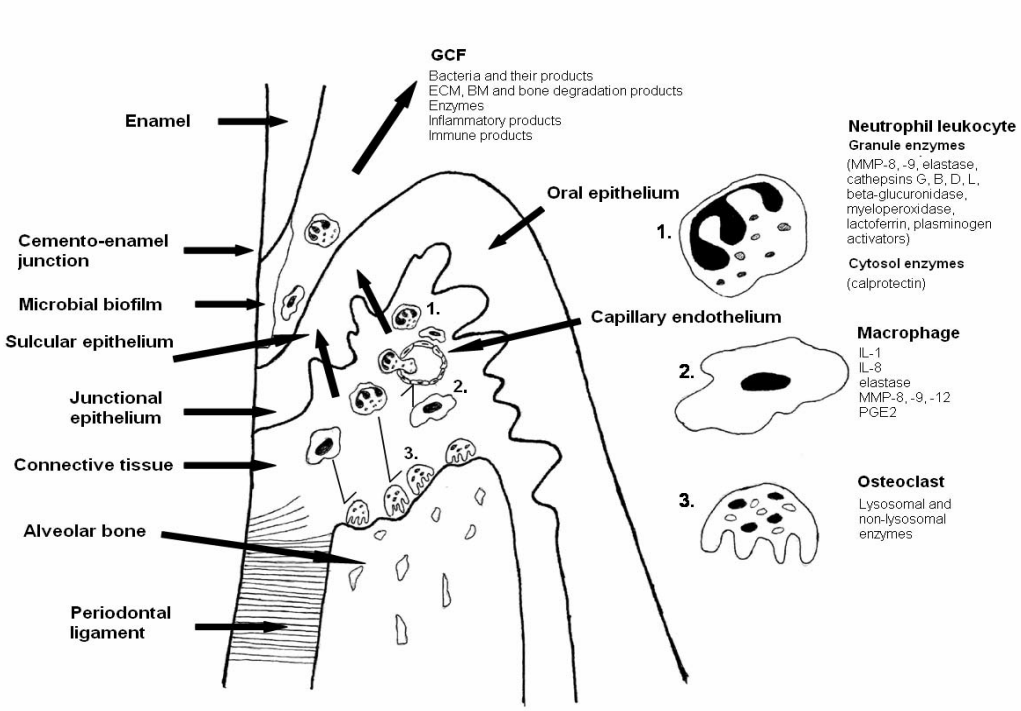


Figure 1.4. Schematic view of pathogenesis of periodontal disease (Ozmeric, 2004) (Permission obtained to reproduce here).

As already mentioned, several studies have implicated the host response as playing a key role in the pathogenesis of periodontitis, since the expression of microbial virulence factors alone may be insufficient to cause periodontitis (Darveau et al., 1997, Birkedalhansen, 1993a). It is true that tissue breakdown can be initiated directly by bacterial products but damage can also be indirect by triggering host-mediated responses (Jain et al., 2008). For example, in active sites of chronic periodontitis, a number of key periodontal pathogens are commonly found, including *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *T. forsythia*, *Prevotella intermedia* and *F. nucleatum* species (Moreno et al., 1999).

These produce virulence factors that are capable of causing direct damage to the extracellular matrix, such as proteinases, epitheliotoxin, cytolethal distending toxin, haemolysin and cytotoxic factors such as ammonia, and hydrogen sulfide (Haffajee and Socransky, 1994). Sorsa et al. (1987) demonstrated that *P. gingivalis* can produce trypsin-like proteases that can be considered as virulence factors in periodontitis and *T. forsythia* can release sialidase that modifies extracellular matrix glycoproteins as well as host cell membranes to provide nutrition for their growth (Severi et al., 2007). On the other hand, Kornman (2008) reported that lack of regulation between host derived factors such as MMPs and their tissue inhibitors, and between pro-inflammatory cytokines such as IL-α, IL-1β and TNFα, prostaglandins and anti-inflammatory cytokines leads to greater degradation of connective tissue.

Having said that, there is ample evidence to show that an integrated host response is generally protective of the periodontal tissues and it is felt that it is the imbalance in the process that leads to disease.

1.7 Current clinical methods for diagnosis

Contemporary diagnosis of periodontal diseases is based on the assessment of clinical inflammatory signs as well as clinical and radiographical evidence of periodontal tissue destruction; of course the latter is a historical record of previous disease progression (Armitage, 2004b). The clinical parameters assessed include: PI, gingival index, BOP, PPD, CAL and radiographic evidence of alveolar bone levels. Signs of inflammation such as swelling, redness and loss of contour are often associated with marginal gingivitis, while bleeding upon probing provides information on pocket epithelium and indicates inflammation and ulceration. Advanced forms of periodontitis are more likely to be associated with pain and suppuration. Loss of periodontal soft tissue is commonly measured by PPD and CAL (Greenstein, 1997, Goodson, 1992), whereas dental radiographs can be used to determine the degree of alveolar bone loss due to periodontitis. Accurate diagnosis of disease progression can really only be achieved by serial longitudinal evaluation of dental radiographs and probing depths rather than single cross sectional measurement. Sites undergoing active destruction can be detected in this way based on increasing clinical attachment loss over a period of time and this information impacts significantly on treatment and prognosis. Even now, PPD, CAL and alveolar bone loss are considered as gold standards against which new diagnostic tests

are evaluated. However, the historical context of what is being evaluated leaves clinicians guessing as to whether a periodontal site will deteriorate or not.

1.7.1 Limitations of current diagnostic methods and need for biomarkers

Clinical and radiographical examination plus detailed periodontal history provide enough information to allow diagnosis and classification of different types of periodontal diseases as described above (1.7 Current clinical methods for diagnosis). Although the current clinical diagnostic methods are easy to use, cheap and relatively non-invasive procedures, they exhibit a number of limitations. For example, as mentioned earlier (section 1.7), these clinical methods mostly provide information about past periodontal history rather than the current status of disease, they lack the ability to estimate the response to treatment before therapy and they are poor predictors for future tissue breakdown in highly susceptible patients (Korte and Kinney, 2016, Greenstein, 1997, Fine, 1992, Goodson, 1992). Furthermore, these clinical methods are subjective. For example, probing depth measurement is susceptible to error because it is affected by the type of probe used, probe angulation, probing force and inter- or intra-examiner variability (Listgarten, 1980). Additionally, radiographs can only detect bone loss after bone demineralization of approximately 30% has occurred and this has very little predictive value. Finally, bleeding upon probing, which is taken as an indicator of local inflammation, is not a reliable marker for identifying the activity of disease, predicting future tissue destruction and the tissue response to periodontal treatment. For example, it has been estimated that only approximately 30% of sites that bleed on probing on each of 4 successive occasions are likely to undergo further disease progression (Goodson, 1992, Haffajee et al., 1991, Haffajee et al., 1983). It is true that some refinements to these methods have been introduced, such as automated periodontal probes and subtraction radiography, and these have improved accuracy and allowed minute changes in the height of alveolar bone to be determined, but these techniques are mostly used for research purposes rather than routine clinical practice.

Considering the limitations of current clinical methods, there is a need for alternative diagnostic methods that provide accurate information beyond the traditional examination (periodontal history and clinical examination), such as the use of biomarkers (Korte and Kinney, 2016). Due to the presence of sites that do not respond to treatment and the

episodic nature of periodontitis, McCulloch (1994) emphasized the necessity of identifying the period of active disease before the destruction of periodontal tissue becomes detectable clinically. Indeed, finding suitable diagnostic biomarkers would be very advantageous (summarized in Table 1.2). However, according to a systematic review by Buduneli and Kinane (2011), no biomarker to date has been found that can predict further disease progression or the response to treatment.

Table 1.2. Advantages of diagnostic biomarkers.

1- Identify the presence of disease and correlate with degree of severity.
2- Predict subsequent clinical course and prognosis after treatment.
3- Estimate the tissue response before therapy.
4- Evaluate actual response to treatment after completion.

As destructive periodontitis is irreversible, early diagnosis and treatment is very important. The ideal purpose of periodontal diagnosis is, therefore, to determine the severity, type and location of periodontal destruction. These data provide the basis for efficient treatment planning and enough information to monitor the maintenance phases of treatment (see Figure 1.5) (Zia et al., 2011). While it is acknowledged that the activity of periodontal disease can usually only be determined if these measurements are assessed at two time-points, having information on the disease activity at the first appointment helps the clinician to make critical decisions. However, despite dozens of studies there are as yet no decisive answers (Buduneli and Kinane, 2011).

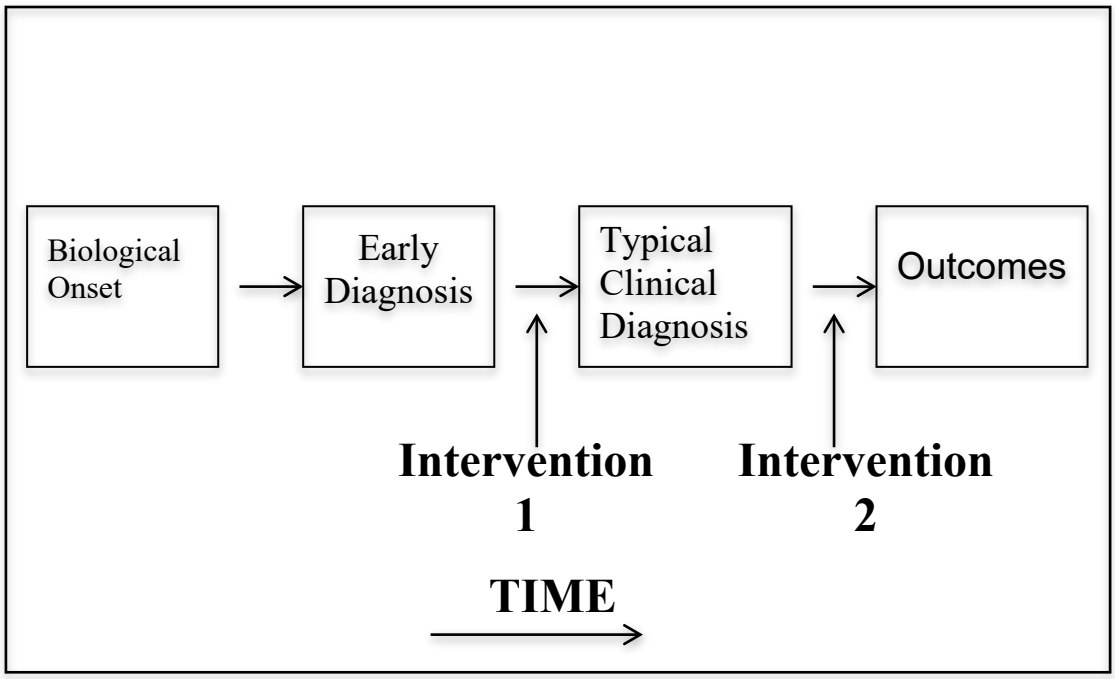


Figure 1.5. Diagrammatic illustration of natural history of periodontal disease (adapted from McCulloch (1994). The aim for use of biomarkers is to provide as early a diagnosis as possible (i.e. shifting toward the left side of the figure). The sooner the diagnosis, the less invasive and the less costly the treatment procedure is likely to be with better prognosis (Permission obtained to reproduce here).

1.7.2 Development of a pathway for diagnostic tests based on host and bacterial biomarkers

The development of diagnostic tests for periodontal disease started with the finding of potentially accurate biomarkers. Generally, these biomarkers can be from different sources such as bacteria and immune cells (Figure 1.6). GCF is considered to be an inflammatory exudate and the levels of these biomarkers increase in concentration in association with the disease status. As the volume of GCF that can be collected is generally less than 2µl, the next step must focus on achieving high sensitivity assays of these molecules, e.g. in the pico- or nano-gram range. ELISA and enzyme-substrate-based assays usually meet this requirement. The third step is to perform a cross-sectional or short-term longitudinal study for evaluation of the biomarkers in different types of periodontal disease. These kinds of studies are used to assess the short-term effect of the

periodontal treatment and represent the proof of principal stage, which is necessary to proceed to the fourth step. This is a longitudinal study, initially in a small number of patients, to determine whether there is a relationship between the biomarkers and clinical parameters (commonly CAL, PPD and radiographic bone loss) or not. If these trials are successful in providing favorable results, then the next step is to perform a large longitudinal study. The penultimate step is translating the assays to a simple chair side test, whilst the final step is post-marketing evaluation of the tests, which serves to identify any unexpected clinical outcomes and reassess the utilization of the test appropriately (Lamster, 1997).

1.8 Biomarkers of periodontal disease

Generally, biomarkers are substances that can be determined and quantified with objectivity, and evaluated as indicators of different biological statuses such as normal, diseased and as a response to therapeutic intervention. The World Health Organization defined biomarkers as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" (Louis, 2006). The majority of biological functions, such as production of energy, battling disease, destruction of waste products, are carried out by proteins, which is why they are targeted as a potential biomarkers in exploration studies (Feng et al., 2006).

An ideal diagnostic biomarker should have the following properties: firstly, the capacity to identify susceptible subjects before extensive clinical damage has happened; secondly, the capacity to discriminate between active and non-active sites; thirdly, it should have prognostic value; and lastly, it should be able to monitor the response to treatment (Buduneli and Kinane, 2011) (as shown in Figure 1.7).

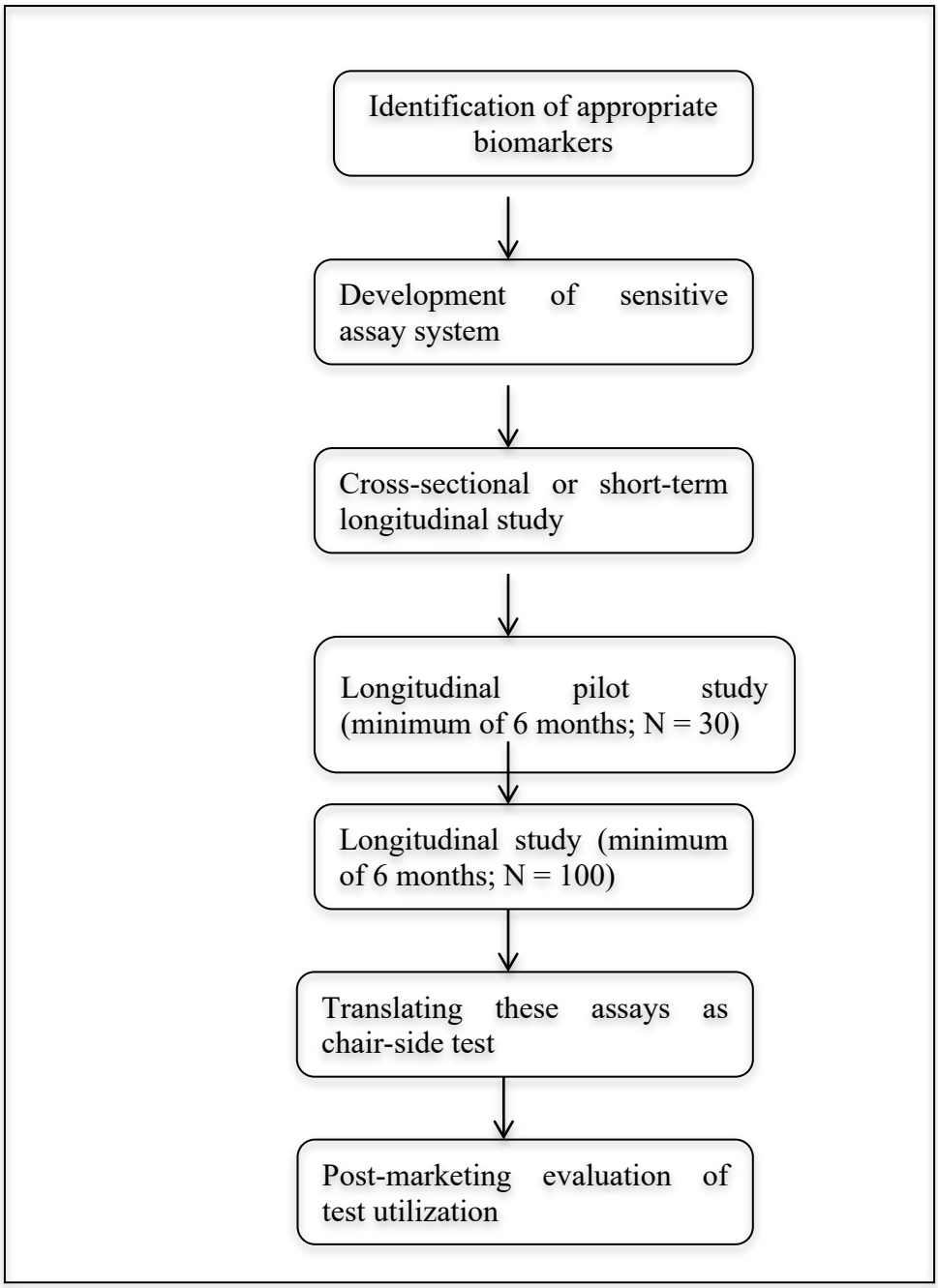


Figure 1.6. Pathway for developing of diagnostic test for periodontal disease using host and bacterial biomarkers (Lamster, 1997).

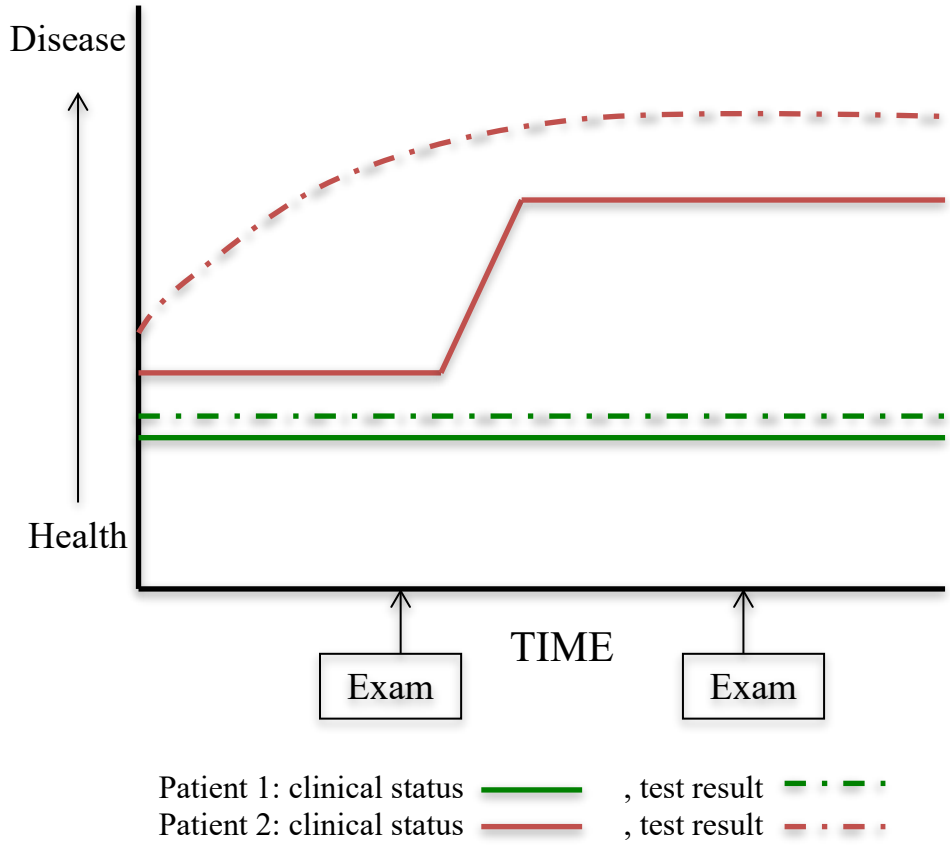


Figure 1.7. The ideal biomarker would predict development and further progression of disease before emergence of clinical signs and symptoms (Zia et al., 2011). In this hypothetical scenario where two patients present at the first examination and both patient 1 (green) and 2 (red) would have similar levels of disease clinically, the results of a biomarker test would be different with patient two being higher than patient one. At the second examination, the level of disease in patient 1 neither improved nor deteriorate, while in patient two the disease has worsened clinically. The result of a good biomarker test would be that its level remains elevated for patient 2.

Several available criteria require consideration before embracing a test for clinical use (as shown in Table 1.3).

Table 1.3. Criteria for assessment of suitability of a diagnostic test, adapted from Chapple (2009) and McCulloch (1994).

1- Comparable with the gold standard of diagnosis
2- Able to evaluate wide spectrum of disease.
3- Highly specific and sensitive.
4- Rapid and simple to perform as chairside test.
5- Quantitative and reproducible.
6- Noninvasive and cost-effective.
7- Responds appropriately to changes by therapy.
8- Has prognostic value.
9- Discriminates active and non-active sites.

1.8.1 Sources of biomarkers of periodontal disease in the oral cavity

Periodontitis is a site specific disease and its severity varies, not only between the teeth of the same patient but also between sites surrounding the same tooth (Baelum et al., 1996). These characteristics pose considerable diagnostic and prognostic dilemmas for clinicians, which may result in inappropriate treatment. Taking this into consideration, GCF contains the most relevant potential biomarkers for reflecting changes in the metabolic status of periodontal tissue (Taylor and Preshaw, 2016). However, therapeutic intervention is mostly patient based, which means saliva is another source of biomarkers as it represents the full mouth status of the disease. The current study places emphasis more on biomarkers of periodontal disease in GCF.

1.8.1.1 Saliva as a source of biomarkers

Saliva is excreted mainly by three major salivary glands plus minor salivary glands that are distributed in the oral cavity. It contains products of salivary glands, blood, serum, GCF and plaque (Edgar, 1992). In fact more than a thousand proteins have been detected in saliva that are not found in the saliva itself when secreted. These proteins mostly

derive from other sources, such as bacteria, host immune cells and diet. This resultant complex nature of saliva reflects the disease status at patient level. Furthermore, there are valid reasons to use saliva as a source of biomarkers as it contains both local and systemically derived biomarkers of periodontal disease (Jaedicke et al., 2016). Saliva also meets the criteria of being easy to collect, cheap, non-invasive to collect and more comfortable for sampling patients (Wong and Segal, 2008).

The disadvantages, as mentioned earlier, are that saliva represents the severity of the disease at patient level and cannot identify the sites that are at high risk of disease initiation and further progression; neither can it identify sites that will not respond to treatment. Furthermore, when levels of salivary biomarkers are correlated to full mouth clinical measures, there is a risk of masking important information by averaging as the severity, progression and outcome of treatment varies from one site to another. With these views in mind, it was decided to look to other sources of biomarkers in oral fluids.

1.8.1.2 Plaque as a source of biomarkers

As bacteria are considered as a primary etiological factor in periodontal disease (Lang, 2014), plaque is considered as another source of biomarkers. Subgingival plaque is more closely linked to periodontitis and among several hundred known species, only a few are regarded as key periodontal pathogens associated with the initiation and progression of periodontal disease (Socransky and Haffajee, 1992, Socransky and Haffajee, 1991). The species that are mostly examined as biomarkers of periodontitis are red complex bacteria (*P. gingivalis*, *T. forsythia* and *T. denticola*), along with *A. actinomycetemcomitans*, *F. nucleatum* and *P. intermedia*. However, the mere presence of these species is not enough to cause or induce further progress of the disease. Microbial biomarkers are of value to identify sites with compromised treatment outcomes from conventional treatment. The roles of microbial biomarkers in plaque are explained in more detail in chapter 6.

1.8.1.3 Gingival crevicular fluid as a source of biomarkers

Gingival crevicular fluid can be defined as a serum transudate or inflammatory exudate that seeps into a gingival sulcus or pocket. It holds a vast array of potentially diagnostic or prognostic biomarkers of the biological state of the periodontal tissues in health and disease (Barros et al., 2016, Embery et al., 2000). The volume of GCF at a site is very small in the healthy periodontium and this can be considered as a transudate that has

similar protein concentration to interstitial fluid (Curtis et al., 1988), whereas under inflammatory conditions it becomes a true exudate with protein concentration similar to serum, especially during inflammation (Griffiths, 2003). The production of GCF has been investigated by a number of researchers; Brill (1960) and Egelberg (1966) suggested that increase in capillary permeability results in GCF production, while Pashley (1976) postulated that GCF represented interstitial fluid traversing into the gingival crevice as a result of differences in osmolarity.

Whatever the driving force, the main route for GCF diffusion is through the basement membrane and then through the relatively wider intercellular spaces of the junctional epithelium. As this fluid travels through the capillary circulation, then through the tissues and lastly into the gingival crevice or pocket, it can contain molecules that are involved in destructive processes and local metabolic tissue byproducts. Examples include such as inflammatory mediators, tissue breakdown products, antibodies, bacteria, bacterial enzymes and byproducts (Armitage, 2004a, Griffiths, 2003). These cellular and biochemical mediators reflect the metabolic status of periodontal tissues at that time, especially as GCF is closely approximated to the specific site of the periodontium. The progression of periodontitis occurs at the individual site level (Champagne et al., 2003), thus GCF is considered to be a good material to monitor the pathological processes of periodontitis in a site-specific manner and to provide more information than can be obtained through traditional clinical examination (Wassall and Preshaw, 2016, Lamster et al., 1985). Use of GCF as a diagnostic material has the advantages that its collection is a minimally invasive, simple process and samples can be taken repeatedly from individual periodontal sites or from all sites (McCulloch, 1994). In this thesis, GCF is used as a source for biomarker discovery and hence its collection is now explained in more detail.

1.8.1.3.1 Methods of collection

The collection of GCF can be accomplished using a variety of methods, each with distinct advantages and disadvantages. Usually the method is selected based on the objectives of the study. There are three commonly used techniques for collecting GCF: gingival washing, capillary tubes and absorbent filter papers.

Chapter 1- Literature review

1- Gingival washing method

In this technique a fixed volume of an isotonic solution such as Hanks' Balanced Salt Solution is used to perfuse the gingival crevice. Usually a customized acrylic stent is used for isolation of the gingival tissues from the rest of the oral cavity (Oppenheim, 1970). The fluid collected obviously is a diluted form of GCF and contains cells and soluble constitutes. This technique is mostly employed for harvesting cells, however, it has a number of disadvantages, including the complexity of using an acrylic stent, the fact that GCF from individual sites cannot be analyzed and, most importantly, the total fluid may not be recovered during injection and aspiration procedures. As a result, GCF volume and its composition cannot be measured precisely as there is difficulty in determining the dilution factor exactly (Griffiths, 2003).

2- Capillary tubing or Micropipettes

An alternative method for GCF collection is insertion of capillary tubes of known internal diameter, length and volume into the entrance of a gingival crevice, after isolation and drying of the site. Capillary action enhances the fluid's migration into the tube and the tube calibration makes fluid quantification very simple. The advantage of this technique is that it provides undiluted GCF for analysis. The disadvantages of this technique are that it is time consuming, especially at healthy sites (though these would not be tested in normal clinical practice), and due to the long time needed for collection, the gingival tissue can become traumatized. Finally, the most serious problem with this technique is the difficulty in recovering all the fluid from the tube can ultimately affect the volume of GCF obtained (Griffiths, 2003).

3- Absorbent filter paper strips

This is the most commonly employed technique (Wassall and Preshaw, 2016). A periopaper strip® is inserted into or placed at the entrance of the gingival crevice, following isolation and gentle drying of the desired site. Generally, collection methods are divided into extracrevicular and intracrevicular techniques. In the case of the first technique, the strip is overlaid on the gingival crevice region to reduce trauma, while, the second technique can be further subdivided according to whether the strip is inserted deeply until minimal resistance is felt or placed at the entrance of the crevice or pocket (Griffiths, 2003, Loe and Holm-Pedersen, 1965). This technique is relatively simple,

quick, minimally invasive and can be performed for individual sites. This method has been reported to be useful for collecting metabolically active components such as proteolytic and glucuronidase enzymes (Waddington et al., 1996, Waddington et al., 1994). Its drawbacks relate to the maximum volume of fluid that the paper can hold, measuring accurately the volume absorbed and the quantitative elution of material absorbed into the paper.

1.8.1.3.2 GCF volume estimation

In early studies the volume of GCF was determined by linear migration of the fluid on the periopaper. A greater level of accuracy was obtained by evaluating the area of the strip wetted with GCF or by staining the strip to assess the area wetted by the GCF sample. The disadvantages of these methods include difficulty in application at the chairside. Delay in measuring the volume increases the chance that some of the fluid will evaporate leading to volume error. Moreover, the staining of the strip is a barrier to further laboratory investigations of GCF components. An alternative method to assess volume is to weigh the strips before and after sample collection. This method provides a reasonable degree of accuracy, but requires a very sensitive balance to estimate very small volumes of fluid and evaporation is also a problem of this technique (Griffiths, 2003).

Nowadays, an electronic measuring device (Periotron) can offer an accurate way to measure the volume of GCF samples. This device measures the electrical conductance of the wetted strips. It is rapid, simple and has no effect on the composition of the GCF sample (Wassall and Preshaw, 2016). Additionally, the effect of evaporation can be minimized if it is conducted at the chairside. The limitation of this device is the limited range of fluid volume that can be measured.

1.9 Potential biomarkers of periodontal disease

A lot of research has been conducted into discovering biomarkers that can determine the current status of periodontal disease and as possible predictors for future periodontal disease progression and likely outcomes of treatment. However, few such studies have shown promising results (Kinney et al., 2014, Buduneli and Kinane, 2011, Loos and Tjoa, 2005). Generally, these biomarkers can be classified into four main groups (see

Table 1.4) (Chapple, 1997). In this section, these biomarkers are further described and their potential value is discussed in more detail.

Table 1.4. Main categories of periodontal disease biomarkers.

Biomarker category	Examples
Bacterial biomarkers	*T. forsythia*, *P. gingivalis* and *T. denticola*
Biomarkers of tissue damage	Osteocalcin, collagen telopeptide and fibronectin
Biomarkers of inflammation	IL-1β, TNFα, immunoglobulins
Biomarkers involved in disease process	MMP8, elastase, cathepsin g and trypsin-like enzymes

1.9.1 Bacteria as biomarkers

More than 600 bacterial species are known to be present in the oral cavity but only a few of them are thought to play a causal role in pathogenesis of periodontal diseases in susceptible host i.e. the so called "red complex" bacteria *T. forsythia (formerly forsythensis)*, *P. gingivalis*, and *T. denticola* (Socransky and Haffajee, 2002). The rationale for use of microbial analysis as a diagnostic/prognostic test has been suggested to help determine (1) the specific periodontal disease (2) the sensitivity of the bacteria to antibiotics and (3) to be associated with disease (Taba Jr et al., 2005).

Several methods are available for detecting bacteria in dental plaque. These include simple culturing, the use of molecular biological techniques to identify bacterial DNA or RNA, and immunologic assay.

The use of bacterial assessment as a detection tool presents some difficulties. Periodontal diseases are caused by mixed infection, which makes identification of the species present challenging (Haffajee and Socransky, 1994). Furthermore, some species are difficult to cultivate and some species do not even have any pathological role. Immunologic methods can be used to detect specific bacteria, however, antibodies are available only for a few of those bacteria that have been identified (Sanz et al., 2004). Furthermore, Haffajee and Socransky (1994) reported that presence or absence of periodontal pathogens in dental biofilm could not differentiate healthy sites from diseased sites. In

addition, Listgarten and Loomer (2003) stated that there is no strong evidence supporting the use of bacterial tests in diagnosis of periodontal disease. Finally, these tests are costly, require sophisticated laboratory techniques and are time consuming, so although they are not currently practical for routine clinical use, they do provide potentially useful information about a site.

1.9.2 Tissue breakdown products as biomarkers

Several components of collagen breakdown products and other gingival connective tissue structures have been investigated to measure the periodontal connective tissues and basement membrane catabolism as a possible biomarker of bone resorption and periodontal disease activity.

One of the major features of periodontitis is the destruction of the extracellular matrix (i.e. collagen) of the periodontium. Type I and III collagens are the predominant collagen types present in the periodontium.

The following molecules are the most studied as biomarkers:

- **Osteocalcin** is a non-collagenous protein mainly found in extracellular matrix bone produced by osteoblasts and has an important role in bone formation (Ducy et al., 1996). It has been examined as a biomarker of periodontal destruction, however no significant difference was found in the level of osteocalcin between healthy and diseased sites (Lee et al., 1999).

- **Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen** is a sensitive biomarker of bone resorption which is released by digestion of collagen by trypsin or bacterial collagenases (Risteli et al., 1993). It is found at higher levels in deep diseased sites than in shallow sites and the level decreases in response to treatment (Al-Shammari et al., 2001).

- **Hydroxyproline**-containing peptides are released during the degradation of fibrillar collagen.

- **Fibronectin** is involved in cell adhesion of connective tissue and can be found in GCF but it is also present in serum.

These molecules have been reported as ubiquitous in GCF samples, so do not necessarily make ideal biomarkers (Embery et al., 2000). Moreover, it has also been reported that diagnoses based on collagen breakdown products have not been satisfactory as these products may reflect rapid collagen synthesis/turnover instead of identifying the actual destruction associated with the disease, although these biomarkers have been of value to monitor healing following treatment (Talonpoika and Hämäläinen, 1994).

1.9.3 Inflammatory mediators as biomarkers

Periodontal disease is characterized by increase in blood flow, enhanced vascular permeability and influx of inflammatory cells (Madianos et al., 1997). Consequently, B cells and T lymphocytes increase at the site of infection. These cells produce various cytokines (IL-1β, IL-6, TNFα) and antigen-specific immunoglobulins (Offenbacher, 1996). GCF has been extensively studied for release of inflammatory biomarkers and the following are the inflammatory molecules most widely studied as biomarkers:

- **IL-1:** Activated macrophage, B cells, neutrophils, monocytes, fibroblast and epithelial cells produce IL-1. It is considered as a potent bone resorption stimulator and is involved in the pro-inflammatory process and matrix destruction (McCauley and Nohutcu, 2002).

- **IL-8:** is a strong activating and chemotactic factor for polymorphonuclear leukocytes, and it is believed that it plays a role in providing protection against periodontal infection by stimulating host defence mechanisms (Jin et al., 2002).

- **IL-6:** is a cytokine originating from T-lymphocytes- and macrophages. Accumulation of IL-6 in the neighbouring connective tissue of periodontal pockets is thought to enhance pocket healing (Guillot et al., 1995).

- **TNF-α:** is a pro-inflammatory cytokine that mainly derives from monocytes and macrophages, and enhances the synthesis of proteolytic enzymes and osteoclast activity (McCauley and Nohutcu, 2002).

- **Immunoglobulins (Ig):** are potential immune mediators in periodontal pathology. The role of Ig in GCF may be protective against further progression of periodontal tissue destruction (Grbic et al., 1999).

- **Prostaglandin E2:** is a metabolic product of arachidonic acid and a potent biochemical mediator of inflammation with many effects. Extracellular matrix components of connective tissue can be indirectly destroyed by the actions of prostaglandin E2 through initiating vasodilatation, increasing permeability of capillaries and stimulating infiltration of inflammatory cells. It has also been found to enhance the release of collagenase from inflammatory cells (Lamster, 1997).

Generally, inflammatory biomarkers are not good biomarkers of periodontal disease. Prostaglandin E2 cannot fully distinguish between gingivitis and periodontitis, or between active and inactive sites (Offenbacher et al., 1993, Offenbacher et al., 1986). Immunoglobulins cannot distinguish stable sites from progressive sites as they do not correlate with severity of periodontal disease, i.e. severity may be lower in progressive sites than in stable sites (Figueredo et al., 1999). They also cannot identify patients who are at high risk of active disease or predict active sites (Eley and Cox, 1998). Champagne et al. (2003) reported that prostaglandin E2 and IL-1β increase in both stable and progressing sites. This may be due to the fact that the host response is more systemic. Although some of these biomarkers show some promising results, none of them have proven to be specific to periodontal disease. Another problem with inflammatory biomarkers is that they often require the use of time consuming and complex technologies that cannot easily be performed in the clinic, and as yet no rapid chair side test based on inflammatory biomarkers has been developed.

1.9.4 Host derived enzymes as biomarkers

As described earlier (1.4 Dental plaque), periodontal diseases are initiated by the response of the host against offending microorganisms that are present within pockets. Identification of those molecules that are involved in the progression of the disease (especially enzymes) has been an area of study, particularly over the last decade. Most of these biomarkers originate from neutrophils, monocytes/macrophages, mast cells and T lymphocytes; the first two types of cells form the most essential part of the host's inflammatory response in GCF. Leukocyte-derived proteases have the ability to degrade almost all components of the extracellular matrix and basement membrane as well as extracellular proteins such as immunoglobulins, complement components, clotting factors and pro- and, anti-inflammatory mediators (Gueders et al., 2005, Owen and Campbell, 1999b).

On the other hand, a number of periodontopathogenic bacteria produce proteolytic enzymes as important virulence factors. Thus, part of the proteolytic activity in GCF can be considered as bacterial proteases, for example trypsin-like activity from *P. gingivalis* and *T. denticola* has been suggested to have predictor value for periodontal attachment loss (Eley and Cox, 1996a). However neutrophils that are found in large numbers at a site are the main source of proteolytic activity (Sorsa et al., 2004).

Besides MMP8, cathepsin G, elastase, trypsin-like activity and sialidase, which will be described later in further detail, the following enzymes have also been investigated widely:

- **Alkaline phosphatase** is mainly secreted by neutrophils and plays a role in normal bone turnover. The diagnostic value of this enzyme is limited and it has been found that the sensitivity of alkaline phosphatase is about 30% (Loos and Tjoa, 2005).

- **Aspartate aminotransferase and lactate dehydrogenase** are cytoplasmic enzymes that are released extracellularly upon cell death. However it is impossible to know from which type of cells they derive (McCulloch, 1994). Persson et al. (1990) demonstrated that the level of aspartate aminotransferase is associated with clinical attachment loss. However, as the test exhibited low predictive value, the relationship is not conclusive. It is also reported that these enzymes have no potential diagnostic or prognostic value (Loos and Tjoa, 2005). Measurements of these enzymes indicate cell death and may not be in keeping with cellular and biochemical processes that result in tissue destruction.

- **β-glucoronidase** is a lysosomal enzyme released by activated neutrophils. Positive correlation between β-glucoronidase, on the one hand, and both pocket depth and bone loss on the other have been reported earlier (Lamster et al., 1991a, Lamster, 1990). It is important to mention that measurement of β-glucoronidase may provide information on neutrophil degranulation but not necessarily the activation system required for periodontal disease progression.

- **Lysozyme** is derived from macrophages and neutrophils. The literature regarding the relation between lysozyme and severity of periodontal disease is conflicting. Some authors (Nord et al., 1971, Brandtzaeg, 1965) found a positive correlation between the amount of this enzyme and gingival inflammation, while others

(Modeer and Twetman, 1979, Van Palenstein Helderman, 1976) reported the opposite. As there are no recent data in the literature supporting the relation between lysozyme and periodontal disease, one can conclude that it is likely to be a poor predictor of periodontal disease status.

- **Myeloperoxidase** is released from primary granules of neutrophils and can create hypochlorous acid from H_2O_2 and Cl^- that is toxic to bacteria and may be important in activation of latent MMP (Epstein and Weiss, 1989). While the level of myeloperoxidase is positively correlated with inflammation and pocket depth and decreases following periodontal therapy (Cao and Smith, 1989), there are no longitudinal data available confirming the relation of this enzyme with disease progression.

1.10 Potential biomarkers examined in this study

1.10.1 Matrix metalloproteinase-8

Matrix metalloproteinases are among the most important host proteinases and are classified on the basis of their affinity for specific substrates and molecular structures. They are categorized into the following groups: collagenases, gelatinases, stromelysins and membrane type MMPs (Mantyla et al., 2004, Sorsa et al., 2004, Ryan and Golub, 2000). Matrix metalloproteinase-8 (MMP-8) or collagenase-2 or neutrophil collagenase, as it is also known, is a host enzyme that belongs to the collagenase family of calcium and zinc-dependent neutral proteinases. MMP-8 is characterized by a potent catalytic ability in extracellular matrix and basement membrane degradation. It is also seen in normal tissue remodeling, such as embryonic development and wound healing (Sorsa et al., 2011, Llano et al., 1997). MMP-8 is also called neutrophil collagenase because polymorphonuclear leucocytes (PMNs) mainly secrete it, and in those cells it is stored inside specific (secondary) granules. However, other cells, such as oral epithelial cells, plasma cells and fibroblasts, also secrete MMP-8 (Van Lint and Libert, 2006).

The molecular weight of MMP-8 varies depending on degree of glycosylation and whether the enzyme is of active or latent form (Hanemaaijer et al., 1997). PMN-type MMP-8 is secreted in a latent 75-80 kD form and converted to a 65 kD active form upon PMN degranulation (Ding et al., 1997, Ding et al., 1996). The non-PMN-type 55 kD latent MMP-8 isoform is converted to a 45 kD active species upon activation (Moilanen

et al., 2003, Moilanen et al., 2002). MMP-8 can also be found in high molecular weight forms (>100 kD) when complexed with other molecules. For example, the high molecular weight immunoreactivity is probably the result of connection of MMP-8 to its endogenous inhibitors, i.e. α2-macroglobulin and tissue inhibitors of matrix metalloproteinase (TIMP) (Ingman et al., 1996). The low molecular weight form (<30 kD species) most likely represents degraded fragments of MMP-8 (Apajalahti et al., 2003).

Levels of active MMPs are very low in normal tissue because their production is strongly controlled by growth factors and cytokines, such as TNFα, transforming growth factor beta and interleukins (IL-1, IL-4, and IL-6) at the level of transcription and secretion (Tervahartiala et al., 2000, Birkedalhansen et al., 1993). MMP-8 is secreted mainly by neutrophils in response to various stimulating factors, such as IL-1, TNFα, various bacteria and their virulence factors (Ryan et al., 1996, Sorsa et al., 1992, Weiss and Reddy, 1989). Imbalance between MMP-8 and their inhibitors leads to structural protein destruction.

MMP-8 is usually produced in a latent (non-active) form called proMMP-8. Activation of the enzyme is necessary for enzyme function (Sorsa et al., 2004) and this is achieved by a number of proteolytic enzymes such as plasmin, cathepsin G, MMP-2, MMP-3, MMP-10, MMP-13, MMP-14 and bacterial proteases (Beklen et al., 2006, Uitto et al., 2003, Okamoto et al., 1997, Knaeuper et al., 1993). Activity of MMP-8 is controlled by non-specific endogenous inhibitors such as α2-macroglobulin and specific TIMP, especially TIMP-1 and TIMP-2 (Brew et al., 2000). The known substrates of MMP-8 are types I, II, III, VII and X collagen (Uitto et al., 2003).

1.10.1.1 MMP-8 as a biomarker of periodontal disease

GCF and saliva are well known to contain large amounts of inflammatory mediators, serum proteins, host tissue degradation products, microbial metabolites and enzymes (Kinney et al., 2007a, Sorsa et al., 2006). Amongst these MMP-8 is the most abundant of the MMPs found in GCF and has been suggested to be useful as a biomarker of disease to identify susceptible sites and subjects at risk of developing periodontal attachment loss (Sorsa et al., 2011). Although MMP-8 is one of the most efficient enzymes at degrading type I collagen in periodontal tissues (Sorsa et al., 2004), its protective role against periodontal tissue destruction has also been analyzed (Kuula et al., 2009). It is

worth mentioning that collagenases from other sources (monocyte, macrophage, epithelia cells and fibroblast) play less important roles in this context (Golub et al., 1995).

Many studies have demonstrated the relationship between MMP-8 and periodontal disease (Mantyla et al., 2003, Kinane et al., 2003, Kiili et al., 2002, Golub et al., 1997). An imbalance between MMP-8 and its inhibitors is known to cause irreversible connective tissue breakdown (Soell et al., 2002, Ingman et al., 1996) and the level of MMP-8 in GCF decreases after scaling and root planing. Indeed, periodontal pockets showing regularly elevated levels of MMP-8 are thought to have a greater risk of irreversible tissue destruction (Sorsa et al., 2010). In contrast the MMP-8 level in GCF of healthy sites is barely detectable (Romanelli et al., 1999).

In gingivitis, the level elevates slightly, but mostly in latent or inactive forms of the enzyme. In contrast, in active chronic periodontitis, the MMP-8 level in GCF is considerably increased and has been reported to be almost completely in active form (Mantyla et al., 2003, Kivela-Rajamaki et al., 2003). Pozo et al. (2005) also found that levels of collagenase activity in GCF were higher in patients with progressive loss of periodontal connective tissue in comparison to those whose condition was stable or only present as gingivitis. Additionally, association between the level of MMP-8 and subsequent loss of attachment has been demonstrated (Lee et al., 1995), while no association was found between MMP-8 levels and alveolar bone loss (Reinhardt et al., 2010). Based on these discoveries, MMP-8 appears to be a useful biomarker for the destructive phase of periodontal disease. Also, recently, MMP8 has been reported to be a useful biomarker for predicting periodontal disease progression in combination with other biomarkers (Kinney et al., 2014) as well as a predictor of treatment outcome (Sorsa et al., 2016, Leppilahti et al., 2015).

Despite the above evidence, other studies have failed to find any relationship between the level of MMP-8 and periodontal disease. For example, Yakob et al. (2012) found no significant difference in the level of MMP-8 in subjects with or without periodontitis and this may be due to the fact that the method of collecting GCF was by an intra-crevicular washing technique instead of the periopaper method used by numerous other studies. It has been continually demonstrated that outcomes of assays depend on the antibodies used. The antibodies used in the DentoAnalyzer, immunofluorometric assay and dip-

stick have high affinity to identify both neutrophil and fibroblast MMP-8 and particularly their active form, whereas the Amersham ELISA method detects all forms of MMP-8 (Leppilahti et al., 2011, Gursoy et al., 2010, Sorsa et al., 2010). It seems that there are conflicting data in the literature on the usefulness of MMP-8 as a biomarker of periodontal disease progression, and this requires further clarification.

1.10.2 Elastase

Neutrophil elastase, also called elastase 2, is a neutral serine protease stored inside azurophilic granules of PMNs that acts at neutral or slightly alkaline pHs (Weiss, 1989, Janoff, 1985a). Elastase is a member of the chymotrypsin superfamily of serine proteases that intracellularly participates in degradation of foreign organic molecules phagocytosed by neutrophils, but when released excessively it can destroy elastin, collagen, proteoglycan and fibronectin and plays an important role in connective tissue destruction during inflammatory processes (Cimasoni, 1983). In this regard an important function is to facilitate migration of neutrophils through subendothelial and subepithelial basement membranes (Dallegri and Ottonello, 1997, Watanabe et al., 1990).

In addition to connective tissue proteins, many plasma proteins, such as immunoglobulins, clotting factors and complement proteins, can be hydrolyzed by elastase (Janoff, 1985b). For example, the Fc and Fab fragments of IgG produced by the action of elastase regulate the functions of stimulated PMNs, including chemotaxis, oxidative burst and enzyme release. The Fc fragments have an inhibitory effect on inflammation by decreasing chemotaxis and oxidative burst, while perversely the Fab fragments further enhance elastase-mediated degradation of IgG (Eckle et al., 1992). In contrast to the classical notion that neutrophil elastase is a proinflammatory factor, other studies suggest that it is capable of degrading various proinflammatory cytokines, including IL-1β, TNFα (Owen et al., 1997), IL-2 and IL-6 (Bank et al., 1999). Kawabata et al. (2002) suggested that neutrophil elastase-induced release of transforming growth factor β, an anti-inflammatory cytokine, may be important in the remodeling of inflamed tissue.

Elastase can be released during phagocytosis and cell lysis (Weiss, 1989), and various factors can stimulate elastase release, such as TNF-α, IL-8 and lipopolysaccharide, by increasing membrane-bound neutrophil elastase by about 20 fold. Although these

enzymes bind to the cell membrane through ionic interactions, they retain their proteolytic activity (Owen and Campbell, 1999a). The proteolytic effect of neutrophil elastase is enhanced by the presence of other neutrophil-derived proteins, such as lysozyme and cathepsin G (Boudier et al., 1981). Since the degradation products of certain substrates can amplify destruction of host tissues (Senior and Campbell, 1983) (e.g. elastin fragments) through increased recruitment of leukocytes by chemotaxis, the effects of this enzyme clearly have to be kept under tight control by the host.

The natural defence mechanisms for preventing injury by elastase involve circulating inhibitors. The most prominent and specific inhibitors of this family of proteases are the serpins (Travis and Salvesen, 1983). Alpha-l proteinase inhibitor, initially named α-1-antitrypsin because of its ability to inactivate pancreatic trypsin (Travis and Salvesen, 1983), α-2-macroglobulin and secretory leukocyte protease inhibitor, are present in large quantities in serum to neutralize proteases. Alpha-1 antitrypsin is the main inhibitor of neutrophil elastase, which is also regulated by α-2-macroglobulin and secretory leukocyte protease inhibitor (Weiss, 1989). The main source of α-1-antitrypsin is the liver but also it is synthesized and secreted locally in the tissues by macrophages (Koj et al., 1978). These inhibitors form a complex with the enzyme, which is then rapidly eliminated by the reticuloendothelial system, from the circulation by the liver and from the local inflammation by accumulated phagocytes (Travis and Salvesen, 1983). Alpha-1 antitrypsin is irreversibly bound to elastase while α-2-macroglobulin is reversibly bound to elastase due to instability of the elastase-α-2 macroglobulin complex (Kennett et al., 1997, Giannopoulou et al., 1992).

Elastase activity can be measured using small molecular weight substrates. These can be degraded not only by free elastase but also by elastase-α-2 macroglobulin complex (Giannopoulou et al., 1992, Wewers et al., 1988). Under normal conditions, there is a balance between neutrophil elastase and elastase inhibitors, but in the presence of marked inflammation and tissue infiltration by neutrophils, the relatively large quantity of elastase released disrupts this fine balance, leading to significant tissue damage (Uitto et al., 2003). Additionally, substantial breakdown mediated by elastase can occur even when there is a low intensity of inflammation, especially in pericellular microenvironments, and this may in part be due to the fact that membrane bound elastase is remarkably resistant to naturally occurring proteinase inhibitors and oxidative inactivation of proteinase in such environments (Owen et al., 1995).

1.10.2.1 Elastase as a biomarker of periodontal disease

PMNs provide a continuous source of elastase for GCF (Smith et al., 1995). Other sources include mast cells (Seppa and Jarvinen, 1979), fibroblasts and epithelial cells (Bourdillon et al., 1980). In the periodontium, it is expected that elevated levels of the enzyme are likely to be present when PMN-mediated tissue destruction is occurring (Birkedalhansen, 1993b, Uitto, 1987). Indeed, it has been shown that crevicular fluid PMNs release up to five times more elastase than peripheral blood PMNs in patients with periodontitis, as a result of an exaggerated or hyperactive PMN response because of exposure to bacterial lipopolysaccharide (Lamster et al., 1991b). Consequently, presence of elastase in GCF is thought to reflect the increased physiological and pathological destruction of periodontal extracellular matrix that characterizes disease progression and so is a good candidate for a biomarker of periodontitis (Ingman et al., 1994).

Elastase is found in GCF in different forms; these include free enzymes, enzymes complexed with α-1 antitrypsin, or α-2 macroglobulin and enzymes inside PMNs (Smith et al., 1995). Immunohistochemical analysis using monoclonal anti-human elastase antibody has been used to show that the enzyme is widely distributed in gingival tissue, but this method detects both active and inactive enzyme (Kennett et al., 1995). Therefore enzyme activity assays would be more informative as a local measure of potential enzyme insult. Elastase activity can be measured by hydrolysis of low molecular weight peptide substrates, but only free elastase and elastase-α-2 macroglobulin complex can degrade such substrates, while elastase bound to the α-1 antitrypsin cannot (Giannopoulou et al., 1992).

Previous researchers have shown that elastase activity in GCF is readily detectable by enzyme assay (Kennett et al., 1995, Cox and Eley, 1989), while others have failed to identify such activity (Giannopoulou et al., 1992). It is possible of course that at least some of the elastase activity in GCF could be of bacterial origin, but this seems unlikely since that which has been detected has a clear preference for valine-containing substrates (typical of PMNL–derived elastase), while oral bacterial elastase-like enzymes prefer alanine-containing substrates (Bieth et al., 1974).

The relationship between neutrophil elastase and periodontal disease has been studied in the last three decades (Lamster, 1997). Nieminen et al. (1993) found that the activity of elastase in saliva correlated significantly with the number of deep gingival pockets

(PPD> 6mm) and with gingival index and BOP, and the presence of some putative periodontopathogenic (*P. gingivalis*, *P. intermedia*) bacteria has been found to correlate with GCF elastase levels (Zafiropoulos et al., 1991). Also, higher levels of elastase activity were found at sites of intermediate probing depths than at healthy sites (Darany et al., 1992), although other studies have shown decreasing concentration with increasing pocket depth and bone loss (Skaleric et al., 1986). Thus, while there is not total agreement it does seem that the consensus is that elastase is acting as a biomarker of periodontal disease activity through being a marker of PMN activity at the periodontal site. Elastase activity has been reported to reflect both severity of periodontal disease and progression (Lamster, 1997). Eley and Cox (1996b) demonstrated that increased elastase in GCF is a predictive biomarker of periodontal attachment loss. Furthermore, Palcanis et al. (1992) suggested that higher levels of elastase may serve as a predictor of bone and attachment loss and other studies have indicated that sites with higher levels of elastase are at a considerably greater risk of bone loss than sites with low levels (Jin et al., 1995, Armitage et al., 1994).

Ideally any biomarker of periodontal disease should have predictive value for the future degeneration of the site in question and the diagnostic predictive ability of elastase is felt to be significantly higher than that of some other markers of tissue damage, such as lactate dehydrogenase (Lamster et al., 1988) and aspartate aminotransferase (Chambers et al., 1991). While these latter markers are potentially useful measures, Eley and Cox (1996b) reported higher sensitivity and specificity for elastase than the other two enzymes. Moreover, while elastase activity was elevated in both patients with gingivitis and periodontitis, the activity was higher in patients with periodontitis (Gustafsson et al., 1994), which makes elastase a potentially useful diagnostic biomarker of periodontal disease (McCulloch, 1994).

1.10.3 Cathepsin G

Cathepsin G is a serine proteinase belonging to the family of chymotrypsins, stored mainly inside azurophilic granules of PMNs, but it can be expressed by monocytes and mast cells as well (Polanowska et al., 1998, Campbell et al., 1989). It comprises up to 18% of the azurophilic granule protein content and 1-2% of total PMN proteins (Bank and Ansorge, 2001, Watorek et al., 1988). Similar to MMP-8, its optimal activity is neutral to slightly alkaline pH (Uitto et al., 2003). Cathepsin G consists of 235 amino

acid residues with a molecular weight of 26-30kD (Starkey and Barrett, 1976). A C-terminal pro-peptide is responsible for the latency of the enzyme upon release, which is removed by a single peptidase (Korkmaz et al., 2010, Bode et al., 1986).

The main physiological role of cathepsin G is associated with the early stages of the host's immune response. Despite its ability to proteolytically degrade engulfed foreign organisms and dead tissues during inflammatory reactions, cathepsin G also displays various pathophysiological capabilities (Bank and Ansorge, 2001, Tkalcevic et al., 2000). For example, numerous studies have shown that different components of extracellular matrix are degraded by cathepsin G, such as collagen (type III and IV), elastin, fibronectin and fibrinogen (Uitto et al., 2003). Additionally, cathepsin G is attributed to several processes, such as platelet activation (Gramse et al., 1980), regulation of monocyte and neutrophil chemotaxis (Lomas et al., 1995), converting angiotensin I to angiotensin II (Reilly et al., 1982), increase in vascular permeability, degradation of IgG (Baici et al., 1982a) and IgM (Baici et al., 1982b). It also displays potent antimicrobial activity against certain Gram-positive and Gram-negative pathogens independent of its proteolytic activity (Bangalore et al., 1990). Zasloff (2002) stated that due to its positive charge, cathepsin G binds tightly to bacterial membranes and this may lead to inhibition of protein synthesis. Lastly, it plays a crucial role in tissue remodelling at sites of injury (MacIvor et al., 1999) and so would be expected to be active at periodontally diseased sites.

Cathepsin G is mostly released into extracellular environments during phagocytosis and cell lysis (Pham, 2006). However, other factors, for instance, platelet-activating factor, TNF-α and IL-8, are also known to enhance cathepsin G secretion (Owen and Campbell, 1999a, Si-Tahar et al., 1994). When released, some of the cathepsin G remains attached in an active form to the neutrophil plasma membrane and in that form resists inhibition by proteinase inhibitors (Owen et al., 1995). One or more of the serine proteinase inhibitors known as serpins control the activity of cathepsin G (Martins et al., 2009) and endogenous inhibitors of cathepsin G are α2-macrglobulin, α1-protease inhibitors, secretory leukocyte protease inhibitor and α1-antichymotrypsin (Doumas et al., 2005, Janciauskiene, 2001, Travis, 1988, Calvin and Price, 1986). However, during inflammation, an imbalance between cathepsin G and its inhibitors tends to occur, resulting in tissue damage (Martins et al., 2009).

1.10.3.1 Cathepsin G as a biomarker of periodontal disease

Proteolytic enzymes are considered as potent contributing factors to the progression of periodontal destruction. Although some of these are bacterial in origin, it is thought that the major activity is of host origin and among these cathepsin G is thought to play a major role, especially in the absence of its inhibitors (Starkey et al., 1977)

Two forms of cathepsin G are found in GCF of patients with periodontitis. The high molecular weight of cathepsin G (86kD) appears when it is complexed with α1-antichymotrypsin or α1-protease inhibitors, while the low molecular weight form (26kD) represents the free cathepsin G enzyme (Kunimatsu et al., 1995). As mentioned above (section 1.10.3), cathepsin G plays a role in tissue destruction but it also could have a protective role. It is certainly the case that the presence of PMNs at sites of inflammation is believed to play a central role in host defence mechanisms. Consequently, the existence of high levels of cathepsin G in the early stages of inflammation is thought to be associated with net defence. However, the progressive increase of cathepsin G levels in the late stages of gingivitis is thought to be related to net periodontal tissue destruction (Kunimatsu et al., 1995). Furthermore, according to Kunimatsu et al. (1995), in healthy gingival tissue only a few cells that secrete cathepsin G are found and this suggests that cathepsin G is generally attributed to periodontal inflammation rather than normal tissue remodelling processes.

The other possible role of cathepsin G in periodontal disease is its action on other proteolytic enzymes that are capable of mediating connective tissue degradation. For example, cathepsin G is found to promote tissue destruction by activating pro-MMPs such as proMMP-3, proMMP-8 and proMMP-9 (Kahari and Saarialho-Kere, 1999, Okada and Nakanishi, 1989). In addition, this activation and the destruction of TIMPs by cathepsin G further enhances MMP activity (Rice and Banda, 1995). Moreover, cathepsin G has been reported to act synergistically with neutrophil elastase to increase elastin degradation. Boudier et al. (1981) reported this to be enhanced 5 fold, while Reilly et al. (1984) reported that the cathepsin G and elastase combination resulted in only a two fold increase in elastin degradation. However, Suomalainen (1992) observed poor correlation between cathepsin G and elastase or cathepsin G and collagenase after scaling and root planing in initially deep periodontal pockets, although it was noticed

that there was a statistically significant correlation between cathepsin G and collagenase before treatment.

Several studies have reported a relationship between cathepsin G and periodontal disease. For example, Pederson et al. (1995) demonstrated elevated concentrations of cathepsin G in saliva and this was closely associated with increasing severity of periodontal disease. Additionally, immunohistochemical analysis in chronic periodontitis patients' gingival tissue specimens showed the presence of cathepsin G in association with infiltrating neutrophils (Tervahartiala et al., 1996). Kunimatsu et al. (1997) observed that the number of cells immunoreactive for cathepsin G (mainly neutrophils but some macrophages) was higher in periodontitis groups of subjects than control groups. Furthermore, the GCF content of cathepsin G determined by immunoassay displayed a positive correlation with GCF volume, which is often taken to reflect the degree of inflammation at a site, since GCF is partly a serum transudate. Similar correlations were also found between cathepsin G concentration and pocket depth, and gingival index, but not with tooth mobility (Kunimatsu et al., 1995). Periodontal treatment, including oral hygiene instruction, scaling and root planing play a crucial role in decreasing the concentration of cathepsin G within GCF; again this result suggests the association of cathepsin G with exacerbation of periodontitis (Kunimatsu et al., 1993). Lastly, there is growing evidence that cathepsin G exhibits antibacterial activity against certain periodontopathogenic bacteria, such as *P.gingivalis* (Bangalore et al., 1990) and *A. actinomycetemcomitans* (Johansson et al., 2000).

Thus, the evidence suggests that cathepsin G is a useful biomarker of inflammation at a periodontal site, particularly when present at high levels, since this would indicate that net tissue destruction is likely to be occurring. However, difficulties in identifying and quantifying cathepsin G by activity measurements have meant that some studies have failed to clarify the relationship between the level of cathepsin G and status of periodontal disease. The difficulty may be due to the fact that cathepsin G mainly exists in high molecular weight form (86kD) in GCF, in which it is conjugated with α1-antichymotrypsin or α1-protease inhibitors. Therefore, it is more likely that a considerable part of the cathepsin G activity locally in the periodontium is blocked by these inhibitors. Despite this, however, it is possible to find the enzyme and its inhibitors separately in different microenvironments of the gingival sulcus (Kunimatsu et al., 1993).

1.10.4 Trypsin

Trypsin is a serine protease that cleaves proteins and peptides at the carboxyl side of arginine or lysine amino acids. The pH optimum of the enzyme is slightly alkaline and in the range 7.5-8.5 (Rawlings and Barrett, 1994). Mammalian trypsin is mainly produced by the pancreas in the form of trypsinogen (pro-enzyme) and secreted to the small intestine where it is activated. Within the intestine it plays a crucial role in digestion by proteolysis.

Human trypsin is a strong matrix degrading proteinase that directly degrades various components of the extracellular matrix and basement membrane, such as fibronectin and collagen types 1, II and IV (Stenman et al., 2005, Koshikawa et al., 1992). Furthermore, some latent forms of MMPs are efficiently activated by trypsin *in vitro*, such as MMP-1, -3, -8, -9 and -13 (Moilanen et al., 2003, Sorsa et al., 1997). On the other hand, there are several inhibitors that control the activity of trypsin to minimize inappropriate effects and these include α-1 antitrypsin and α-2 macroglobulin (Ohlsson, 1988). However, trypsin is present within human serum of normal healthy individuals at approximately 80 units/l (Lemoine et al., 1994), which implies that it may also be present in GCF. Furthermore, as *P. gingivalis* can destroy inhibitors of trypsin this may lead to release of the enzymes bound to the inhibitors, although as yet no study has proved that. That is why attention has been mostly drawn toward trypsin-like activity from bacterial sources.

1.10.4.1 Bacterial Trypsin-like enzymes

Several periodontopathic bacteria elaborate enzymes with trypsin-like activity (Loesche et al., 1990, Laughon et al., 1982a). The main producers of trypsin-like enzymes are *P. gingivalis* and *T. denticola* but other species, such as *Capnocytophaga, P. intermedia*, *T. forsythia* and *A. actinomycetemcomitans,* also release trypsin-like proteases (Eley and Cox, 2003, Wang et al., 1999, Suido et al., 1986, Laughon et al., 1982b, Slots, 1981).

The trypsin-like protease of *T. denticola* has a molecular mass of 69kD and it is known to break down synthetic substrates containing arginine or lysine bonded to p-nitroanilide, but natural proteins such as casein and gelatin cannot be cleaved by this enzyme (Ohta et al., 1986). Similarly, trypsin-like protease of *T. forsythia* cannot hydrolyze native proteins. This suggests that these enzymes mostly take part in small peptide degradation to completion, although when acting in concert with enzymes from other bacteria large

proteins will be hydrolyzed. However, the view is that the role of trypsin-like proteases of *P. gingivalis* and *T. denticola* in protein breakdown appears to be greater than that of *T. forsythia* (Grenier, 1995). Among other periodontally significant bacteria, three species of *Capnocytophaga* possess trypsin-like activity (*C. gingivalis, C. ochracea* and *C. sputigena*), with *C. gingivalis* showing the greatest activity (Gazi et al., 1997, Laughon et al., 1982a). *P. intermedia* has a very weak trypsin-like protease but it has been shown to be able to reduce the effectiveness of the host response and inflammatory defence through degradation of IgG and fibronectin (Eley and Cox, 2003, Gazi et al., 1997).

Trypsin-like proteases contribute actively to the destruction of periodontal tissue either directly or indirectly (Uitto et al., 1989) as activators of host proteinases, such as pro-MMP-8, pro-MMP-2 and pro-MMP-1 (Grayson et al., 2003, DeCarlo et al., 1997). Indeed, such actions offer one method by which the organism can enhance virulence (Bretz et al., 1990) and provide additional nutrition (Holt and Bramanti, 1991). Moreover, trypsin-like proteases of these bacteria participate in evasion of host defence mechanisms through destruction of complement factors (Sundqvist et al., 1988) and immunoglobulins (Kilian, 1981). Finally, Carlsson et al. (1984) found that trypsin-like protease can further enhance tissue destruction and disease progression by modulating the normal tight control of host proteases. Destruction of intrinsic protease inhibitors, such as α1-antitrypsin and α2-macroglobulin, has been observed, however, the situation is complex since different proteases have different efficiencies and are produced in different quantities by the various bacteria present (Eley and Cox, 2003) (see Table 1.5). For example, some workers have reported that the trypsin-like proteases are sensitive to serum protease inhibitors (Sorsa et al., 1987, Sundqvist et al., 1987, Tsutsui et al., 1987), whereas others have found the opposite (Fletcher et al., 1998, Okamoto et al., 1998, Lantz et al., 1991) and in fact reported that these were degraded by trypsin-like enzymes (Carlsson et al., 1984). There are two methods that are commonly used for quantifying trypsin-like activity. These employ the synthetic substrates N- benzoyl-DL-arginine-2-naphthylamide (BANA) and N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Laughon et al., 1982a, Haverback et al., 1960).

Table 1.5. Comparison of bacterial trypsin activities of putative periodontal pathogens (Eley and Cox, 2003).

Bacteria	Trypsin-like activities
P. gingivalis	High
T. denticola	Medium
Capnocytophaga gingivalis	Medium
Capnocytophaga sputigena	Low
Capnocytophaga ochracea	Low
T. forsythia	Low
P. intermedia	Very low
A. actinomycetemcomitans	Very low

It is thought that *P. gingivalis* is one of the major causative bacteria associated with active periodontal disease sites, since it is either absent from or present in very low number in inactive or healthy periodontal sites (Haffajee and Socransky, 1994). This bacterium produces large amounts of proteolytic enzymes as virulence factors, which are thought to play a significant role in periodontal disease progression. The trypsin-like proteases of *P. gingivalis* are collectively known as gingipains (Kesavalu et al., 1996, Grenier and Mayrand, 1987). Gingipains are a group of cysteine endopeptidases that are believed to constitute at least 85% of general proteolytic activity and 100% of trypsin-like activity of *P. gingivalis* (Potempa et al., 1997, Potempa et al., 1995a). *P. gingivalis* produces two main types of trypsin-like enzymes, called arg-gingipain and lys-gingipain, that are cysteine proteases responsible for cleavage of substrates containing arginine and lysine peptide bonds, respectively (Potempa et al., 1995b). Arg-gingipain is also referred to as gingipain-R and is encoded by the *rgpA:B* genes, whereas lys-gingipain is referred to as gingipain-K and is encoded by the *kgp* gene.

Gingipains have been found to exhibit the following pathogenic activities, which may play a role in the progression of periodontal diseases. These are summarized in Figure 1.8.

1- Kallikrein/Kinin system activation

Hinode et al. (1992) and Kaminishi et al. (1993) were the first to describe the activation of the kallikrein/kinin system by *P. gingivalis*. Arg-gingipain was found to enhance vascular permeability and potentially increase GCF production and oedema formation at sites of *P. gingivalis* infection and, in so doing, providing nutrients necessary for bacterial growth. On the other hand, arg-gingipain acts synergistically with lys-gingipain and together they lead directly to the release of bradykinin from high molecular weight kininogen (Imamura et al., 1995). Bradykinin may be involved in resorption of alveolar bone through stimulating prostaglandin formation by osteoblasts (Rahman et al., 1992) and periodontal ligament cells (Ransjo et al., 1998).

2- Blood clotting system activation

Arg-gingipain plays a significant role in shortening plasma clotting time through activation of factors IX (Imamura et al., 2001a), X (Imamura et al., 1997) and prothrombin (Imamura et al., 2001b). Thrombin is the final product of the coagulation system that converts fibrinogen to fibrin. It is also known that thrombin induces vascular permeability (Demichele et al., 1990) and leukocyte chemotaxis (Bizios et al., 1986). It is believed that at sites with periodontal inflammation, these activities are involved in increasing GCF production and accumulation of leukocytes. Furthermore, IL-1 production by macrophages and prostaglandin secretion by osteoblast cells have been found to be stimulated by thrombin (Jones and Geczy, 1990, Partridge et al., 1985). The increase of these two factors in GCF of patients with chronic periodontitis is related to the destructive process of periodontal disease (Masada et al., 1990, Offenbacher et al., 1986). In addition, thrombin has been reported to enhance bone resorption by osteoclasts through the prostaglandin-dependent pathway (Hoffmann et al., 1986). Thus, it can be said that uncontrolled release of thrombin is likely to be associated with periodontal disease progression and alveolar bone resorption.

3- Fibrinogen and fibrin degradation

Bleeding upon probing is a well-known clinical sign of periodontitis and it provides a useful criterion for diagnosis of gingival inflammation. Lys-gingipain exerts potent fibrinogenolytic activity and this activity may contribute to the bleeding tendency at sites with periodontitis (Albandar et al., 1990).

4- Disturbance of host defence system

Pathogenic microorganisms employ different strategies to protect themselves from the host immune system. Regarding *P. gingivalis*, the gingipains play a crucial role in evasion of the host defence system. Gingipains protect *P. gingivalis* from lysis by the complement system through activation of the system via production of C3 convertase, which results in consumption of its components (Schenkein, 1991). Also, gingipains can easily degrade C3, which is the central factor in the complement system, resulting in inadequate production of C5, which acts as a major phagocyte chemo-attractant (Wingrove et al., 1992). Moreover, gingipains can destroy immunoglobulins (Gregory et al., 1992) and monocyte receptors for bacterial lipopolysaccharide (Sugawara et al., 2000); the latter results in reduced release of cytokines such as IL-8 (Potempa et al., 1997, Potempa et al., 1995a). IL-6 is normally elevated in inflamed gingival tissue over 6 mm away from the infected pocket (McGee et al., 1998), whereas the gingival tissue adjacent to the dental plaque contains very low concentrations of IL-6, and this may be due to the fact that gingipains can also degrade this cytokine effectively (Imamura, 2003). In addition, gingipains can degrade IL-1β (Fletcher et al., 1998), TNF-α (Calkins et al., 1998) and interferon gamma (Yun et al., 1999).

5- Imbalance of host serine proteinase inhibitors

Under normal conditions the activities of proteases released by PMNs are controlled by serine protease inhibitors, such as α1-antitrypsin and α2-macroglobulin. The destruction of these inhibitors by gingipains can, therefore, further increase tissue destruction and progression of the disease (Carlsson et al., 1984).

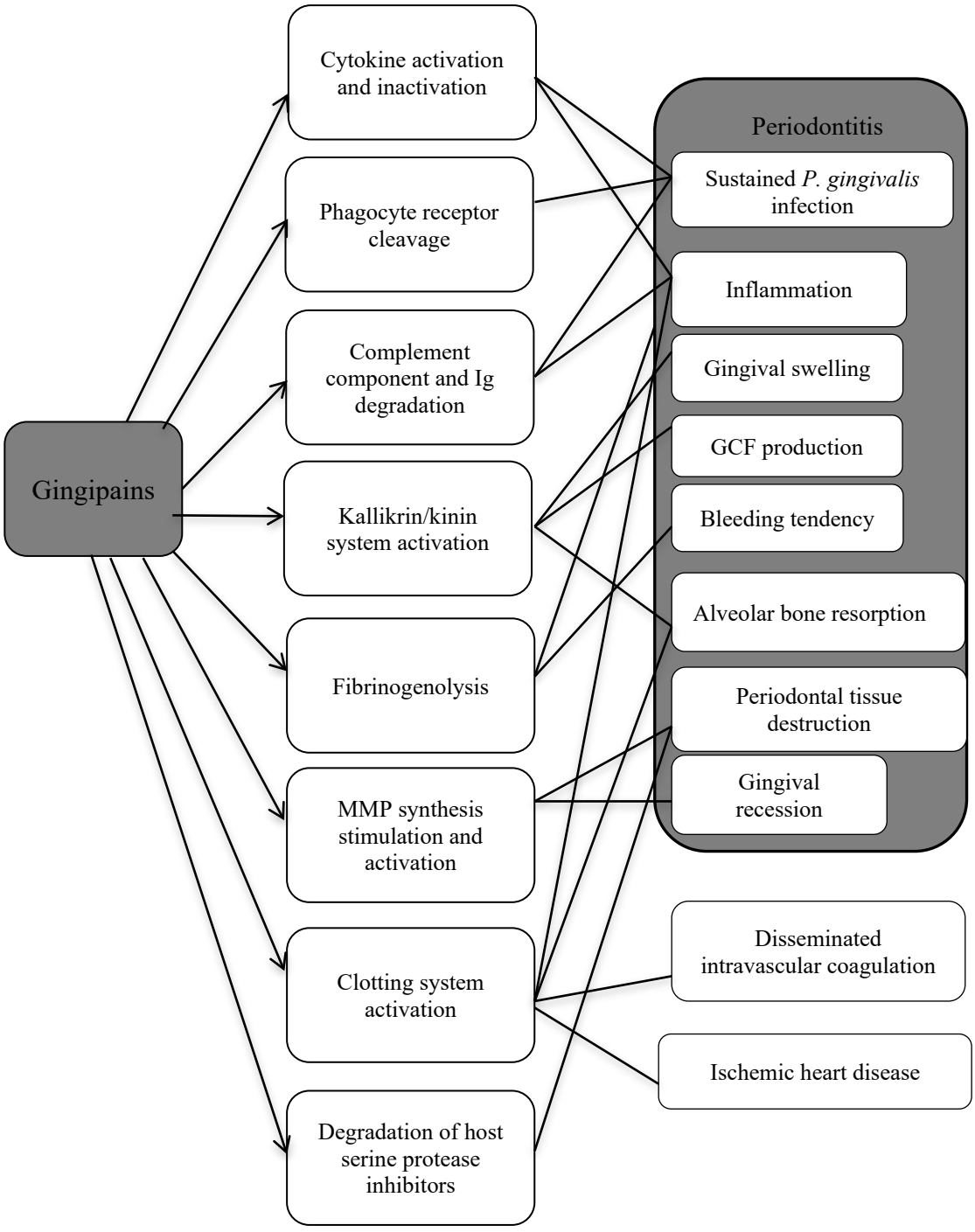


Figure 1.8. Pathogenic activities of gingipains and their association with periodontitis [modified from Potempa et al. (2000)] (Permission obtained to reproduce here).

1.10.4.2 Trypsin-like protease as a biomarker of periodontal disease

As described above (1.10.4.1 Bacterial Trypsin-like enzymes), trypsin-like proteases from various microorganisms are involved in inflammatory processes through destruction of periodontal tissues (Travis et al., 1997, Nakamura et al., 1991). The ability of some periodontopathic bacteria to hydrolyze synthetic trypsin substrates (BANA and BAPNA) has been used to develop simple and rapid methods for diagnosis of periodontitis, for monitoring the effects of periodontal therapy and even to predict future attachment loss. Indeed, the number of bacteria with positive trypsin-like activity has been reported to be 100-fold higher in diseased sites than in healthy sites (Takada and Hirasawa, 2000, Gusberti et al., 1986). Furthermore, the trypsin-like activities detected in plaque samples from patients with chronic periodontitis have been significantly correlated with the levels and proportion of periodontopathic bacteria present, especially *P. gingivalis*, *T. denticola* and *T. forsythia* (Socransky et al., 1998). Moreover, higher levels of trypsin-like activity within GCF samples from chronic periodontitis patients than from healthy subjects have been reported (Eley and Cox, 1992a, Radford et al., 1992).

The clinical parameters of periodontal disease are positively correlated with levels of trypsin-like protease enzymes. A two-year longitudinal study by Eley and Cox (1996c) showed that trypsin-like protease activity of *P. gingivalis* is positively correlated with periodontal attachment loss and it can be used as a predictor of attachment loss. Similarly, pocket depth has been shown to correlate strongly with trypsin-like activity (Yucekal-Tuncer et al., 2003, Smith et al., 1998) and this is significantly reduced following periodontal treatment. Various workers have found that surgical periodontal treatment of deep pockets significantly reduced the concentration of the enzyme activity compared with non-surgical periodontal treatment (Eley and Cox, 1992a, Eley and Cox, 1992b, Radford et al., 1992, Zambon et al., 1985). These findings suggest that non-surgical periodontal treatment cannot remove all of the bacteria that release trypsin-like activity from the periodontal site and so they can re-grow at the site, regenerating the enzyme levels over time. Strength of correlation of trypsin-like protease with the different clinical parameters generally follows the order: pocket depth> clinical attachment loss> gingival index> bleeding index (Cox and Eley, 1992a). Amongst the clinical parameters, pocket depth has the strongest correlation with levels of trypsin like enzyme. However, neither plaque index nor bleeding index exhibited correlation with

concentration of trypsin-like protease (Cox and Eley, 1992a). Similarly, gingival index showed no correlation with trypsin-like protease (Yucekal-Tuncer et al., 2003, Smith et al., 1998).

With regard to the above reports, there is some disagreement regarding sources of this trypsin-like activity, accuracy of BANA and BAPNA test, suitable samples for detecting this enzyme and using enzyme activity or enzyme concentration to relate to the clinical parameters. The trypsin-like proteases of *P. gingivalis* and *T. denticola* are cell bound and released in soluble form or contained in vesicles. Considering this, GCF would contain less cell-associated activity than plaque would itself, Loesche et al. (1987) thought it may contain more soluble enzyme. It is possible that bacterial enzymes produced in subgingival plaque might be detectable in saliva, but salivary levels of these enzymes further decline after periodontal treatment and this makes the detection of the enzymes more complicated as it requires the saliva to be centrifuged and the analysis conducted on salivary sediment (Zambon et al., 1985). Furthermore, Loesche et al. (1987) reported that using the chromogenic BANA substrate could not demonstrate trypsin-like activities in GCF; their sampling method was to use intra-crevicular washings. In contrast, Beighton and Life (1989) used filter paper for sampling of GCF and found trypsin-like activity in the majority of samples collected from the sites with only mild to moderate gingivitis. Furthermore, Syed et al. (1984) stated that BANA is the more appropriate substrate for reflecting severity of periodontal disease and it could be used in the diagnosis and monitoring of periodontal disease.

Considering these discrepant reports, further work is necessary to verify the potential diagnostic and prognostic value of trypsin-like enzymes, especially in terms of quantifying the enzyme and relating it to the clinical parameters in a longitudinal study.

1.10.5 Sialidases

Sialidases (neuraminidases, N-acetylneuraminosyl-glycohydrolase, EC 3.2.1.18) are the key enzymes responsible for sialic acid catabolism. They are exo-glycosidases that cleave the α-glycosidic (α-ketosidic) linkages between terminal sialic acid and the penultimate sugar of the glycan chain of sialyated glycolipids, glycoproteins and oligosaccharides in higher animals and some microorganisms (Corfield and Schauer, 1982). Sialidases are widely distributed in nature in organisms of various species,

including viruses, bacteria, protozoa, and fungi, through eukaryotes (Bairoch and Apweiler, 2000). The viral and bacterial sialidases have been studied extensively since they are virulence factors. In spite of the remarkable diversity in their distribution, amino acid sequences and biological properties, sialidases still demonstrate considerable molecular and structural similarities that characterize the sialidase superfamily (Roggentin et al., 1989).

The term sialic acids includes the group of nine carbon neuraminic acids that are found widely on the cell surface. Sialic acids have been implicated in various biological processes (summarized in Table 1.6) (Schauer, 1985) and sialidases, therefore, have significant effects on many cellular processes. In general microorganisms lack sialic acid and so obtain this by the action of sialidases (Schauer, 1982).

1.10.5.1 Bacterial sialidases

The molecular weight of bacterial sialidases varies from 40-120kDa. Bacterial sialidases can either be in the free form or cell bound. The main physiologically functional form is the excreted form (Corfield, 1992), while the cell bound form could either be a stored form before release and possibly located in the periplasm, or it could be surface located and so play a role in interaction of the bacteria with the host (Guzman et al., 1990). A potentially important feature is that the activity of sialidases can be enhaced by the local availablility of sialic acid, sialoglycoconjugates and polysaccharides (Corfield, 1992).

A wide range of bacteria can produce sialidases and they are considered as one of the many virulence factors secreted by bacteria that participitate in important diseases (Tang et al., 1996, Dwarakanath et al., 1995). For bacterial species that live in close contact with higher animals, sialidases play a central role in scavenging host sialic acid as a carbon and energy source and this is important for both pathogenic and non-pathogenic bacteria (Severi et al., 2007, Corfield, 1992). Consequently, many bacteria possess systems for sialic acid transportation and pathways for sialic acid catabolism inside the cell (see Figure 1.9) (Galen et al., 1992). Also, demasking underlying host cell epitopes provides receptors for microbial adhesion (Tong et al., 2000) and this can mediate initial attachment for subsequent biofilm formation and provide nutrition through utilization of host glycoproteins in the biofilm state (Honma et al., 2011, Roy et al., 2011).

Table 1.6. Functions of sialic acid, based on (Corfield, 1992, Schauer, 1985).

Functional characteristic	Examples of effects
Negative charge	Mutual repulsion of circulating blood cells
	Viscoelasticity of mucins
	Direct intramolecular interactions influencing molecular shape and form
	Binding/transport of positively charged compounds
	Aggregation and disaggregation of cells
Dictates biological function	Involved in blood clotting
	Involved in complement activation
	Regulation of molecular and cellular recognition
Anti-recognition	Affects half-life of RBCs, thrombocytes lymphocytes, and sialoglycoconjugates in circulation
	Exposure of T-antigen
Receptor functions	Macrophage- lymphocyte interactions
	Neutrophil migration by selectin binding
	Attachment of microorganisms, often via fimbriae to host cells
	Essential components of receptors
Conformation of cell surface glycoproteins	Maintains activity of glycoprotein enzymes
	Resistance to proteases

For some microbial species, sialidases assist their invasion and spread within the host (Corfield, 1992). This can be because removal of sialic acid allows the underlying carbohydrate chains as well as the protein backbone to become easily accessible for either attachment or further degradation (Godoy et al., 1993). Furthermore, amongst the host molecules that are targetted by bacterial sialidases are a number of immune-related functions, which modulates the host's ability to respond to infection (Schauer, 1982, Drzeniek, 1972). For example, removal of sialic acid from immunoglobulin G causes

reduced ability to bind to complement factor C1. Also, interferon becomes susceptible to proteolytic degradation on desialation and sialidase is known to reduce collagen synthesis (Aalto et al., 1974).

Despite the evidence that sialidases play a role in pathogenicity, non-pathogenic species produce these enzymes as well. It is likely then that sialidases are not particularly effective virulence factors on their own, although they may facilitate colonisation and survival, but their release along with other toxic factors leads to tissue damage (Muller, 1976, Muller, 1974). Interestingly, in general terms pathogenic species produce considerably higher levels of sialidases in comparison to non-pathogenic species (Grossi et al., 1994), which is consistent with the notion that massive release of sialidases is important in pathogenicity.

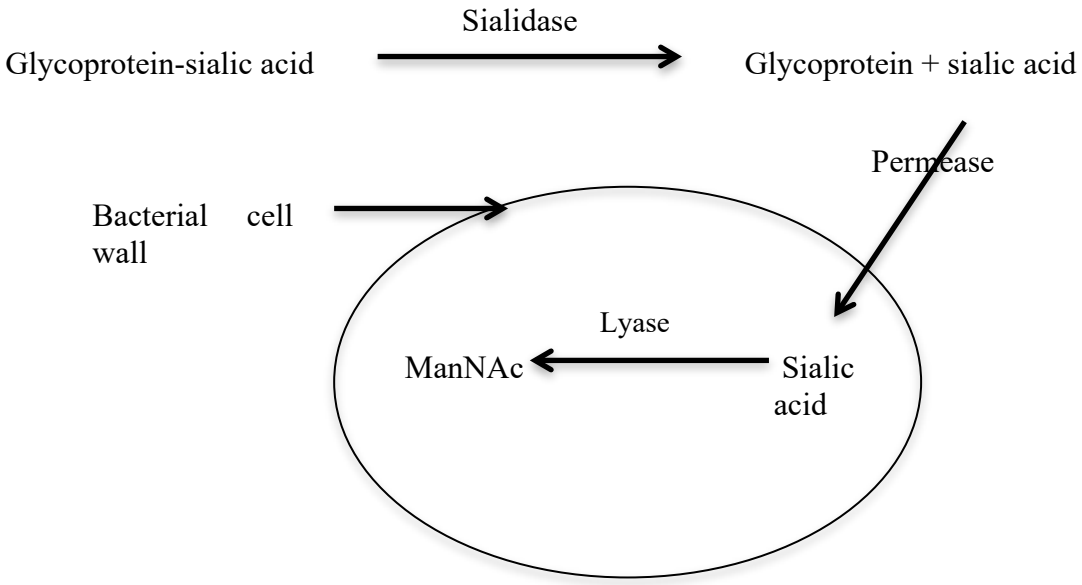


Figure 1.9. Bacterial metabolism of sialic acid. The sialidase removes sialic acid from sialo-glycoprotein and transports it via sialic acid permease into the cell, where it is degraded by acetylneuraminate pyruvate lyase to N-acetylmannosamine (ManNAc) (Corfield, 1992) (Permission obtained to reproduce here).

Another interesting group of sialidases are mammalian sialidases. Four kinds of human sialidases have been identified and distinguished on the basis on intracellular location. They include lysosomal sialidase (Pshezhetsky et al., 1997), cytosolic sialidase (Monti et

al., 1999), plasma membrane-associated sialidase (Monti et al., 2000) and mitochondrial or lysosomal membrane- associated sialidase (Monti et al., 2004).

1.10.5.2 Sialidases as biomarkers of periodontal disease

Since bacterial sialidases are common, it is to be expected that oral bacteria and those associated with periodontal diseases may possess this important trait and this opens the possibility that sialidase activity could be a useful biomarker of local disease challenge. Indeed, sialidase activity has been observed in several oral bacteria (Tuyau and Sims, 1975) and Table 1.7 shows the percentage of isolates in a range of species that are positive for sialidase activity. The red complex bacteria (*P. gingivalis, T. forsythia* and *T. denticola*) each express sialidase activity. *T. forsythia* produces two types of sialidase that are encoded by SiaHI (Ishikura et al., 2003) and NanH genes (Thompson et al., 2009), with the latter being the most abundant type released. Li et al. (2012) showed that sialidase activity enhances the pathogenicity of *P. gingivalis* through influencing capsule biosynthesis when the bacterium acquires sialic acid from GCF or saliva. On the other hand, a study by Aruni et al. (2011) suggested that *P. gingivalis* sialidase activity might be attributed to regulation of other enzymes that are secreted by this bacterium, such as the gingipains. In contrast, little is known about the *in vivo* function of the sialidase produced by *T. denticola*, Hirai et al. (1997), though it is reasonable to speculate that its role is similar to that in *T. forsythia* and *P. gingivalis.*

Other oral bacteria that possess strong sialidase activity include *Streptococcus oralis, Streptococcus mitis* and *Actinomyces viscosus,* but their relative roles in disease are not currently known (Corfield, 1992, Fukui et al., 1971). However, the formation of dental plaque and the course of periodontal disease has been suggested to be affected by sialidase activity (Leach, 1963, Lura, 1961). Furthermore, pre-treatment of rabbit mucosa with sialidase increased the penetration of a dye into the tissue (Adams, 1975) and this may be an important factor in periodontal disease as the sialidase may influence penetration of gingival epithelium by bacterial antigens.

Clearly then, there are numerous bacterial species that produce sialidases which are known to grow subgingivally and several studies have identified sialidase activity not only in dental plaque and GCF but also in salivary secretions. Rogers et al. (1979) found sialidase activity in saliva to be approximately double the activity found in dental plaque

and so if a sialidase is to be of value as a biomarker of periodontal destruction, saliva must be excluded from any samples (e.g. GCF) taken from diseased sites.

Table 1.7. Percentage incidence of sialidase activity in positive isolates of periodontopathogenic bacteria (Holt and Bramanti, 1991, Beighton and Whiley, 1990).

Species	Percent positive isolates (%)
Porphyromonas spp.	
Porphyromonas gingivalis	100
Other Bacteroides spp.	
Prevotella melaninogenica	100
Bacteroides loescheii	100
Prevotella denticola	100
Prevotella oralis	55
Prevotella bivia	98
Prevotella buccae	29
Prevotella buccalis	100
Prevotella disiens	79
Bacteroides capillosus	100
Tannerella forsythia	100
Bacteroides levii	100
Capnophilic spp.	
Capnophilic ochracea/ Capnophilic sputigena	100
Streptococcus spp.	
Streptococcus intermedius	100
Streptococcus mitis	66
Streptococcus oralis	100

It is generally accepted that salivary constituents affect the oral microflora and of course saliva contains an abundance of glycoproteins such as mucin and fetuin that contain sialic acid. Consequently workers have tried to correlate salivary sialic acid and sialidase activity with disease status. A study by Ito et al. (1975) demonstrated that salivary sialic acid in patients with periodontal disease was significantly higher than among

periodontally healthy subjects and that the sialic acid significantly correlated with severity of gingivitis. A similar correlation was reported by Shinohara et al. (1994), which begs the question of where this sialic acid is coming from. Does it derive from periodontally damaged sites due to the action of bacterial sialidases or does it merely reflect a different supragingival or mucosal flora with elevated sialidase activity?. A study by Perlitsh and Glickman (1967) failed to find any relationship between sialidase and periodontal disease. However, Kitawaki (1983) reported that sialidase is considerably higher in periodontally diseased patients than in subjects with clinically healthy periodontium and that the activity was significantly correlated with the clinical parameters (PI, Gingival index, PPD) of periodontal disease. Another study by Kitawaki et al. (1983) showed 12-13 fold higher sialidase activity in GCF from patients with periodontal disease than in fluid from a control group. This has been supported by further work by Beighton et al. (1992) on subjects with periodontitis or gingivitis, and it seems biologically likely that the sialidase activity detected is of bacterial origin rather than host origin, given the pH in a periodontal pocket and the pH optimum of bacterial sialidases (Kitawaki et al., 1983). Nonetheless, ideally, correlation between sialidase levels in GCF and significant presence of sialidase-producing bacteria at that site should be sought.

Thus, in summary, there have been insufficient studies on the relationship between sialidase and the progression of periodontal diseases and further studies are necessary. Although oral microorganisms have been considered to be the primary source of salivary sialidases, cellular components, for example, epithelial cells and leukocytes, have been suggested to be another source of this enzyme and this makes saliva an unsuitable sample. The studies on GCF sialidases that have been performed were cross sectional in nature and no attempt has been made to identify the effect of periodontal therapy on local sialidase levels or the presence of sialidase-producing bacteria at the site. It is appropriate then to investigate the potential of sialidases as biomarkers for predicting the outcome of periodontal therapy.

1.11 Detection methods for biomarkers in periodontitis

Biomarkers should serve as the basis for early detection of disease, better treatment planning and providing effective periodontal therapy with better prognosis. A particularly convenient material to examine for potential biomarkers is GCF, which contains large numbers of serum proteins, inflammatory mediators, degradation products of host cells

and products of microbial metabolites. Researchers have investigated these molecules intensively as they may afford useful information about the critical processes that mediate the destruction of periodontal tissues (Barros et al., 2016, Lamster, 1997, Golub and Kleinberg, 1976). Many highly sensitive and specific enzyme assays have been developed for certain enzymes that require only microliter quantities for accurate analysis and this is beneficial to overcome the substantial limitation that only small volumes of GCF can be obtained from periodontal sites. The small quantities of enzymes likely to be present could be measured as protein molecules using specific immunological assays, however, this tells us nothing about the activity of that enzyme at a site and so whether it is likely to be responsible for any destructive effect. Alternatively the enzyme could be quantified by its activity towards a suitable substrate. It would not be possible to describe the detail of all enzyme assays and procedures that have been used for detection of enzymes in GCF but the following outlines and compares the principle methods employed, to help identify which methods are likely to be most useful for this study.

1.11.1 Immunological assays

The quantities of GCF enzymes are usually very low, especially in healthy sites and despite their level increasing during the course of periodontal disease, they remain fairly low. Consequently, to obtain meaningful analysis, highly sensitive and specific reagents and tests are required. In cases of low abundance of molecules in various biological fluids, antibody-based detection systems are often used for specific and accurate measurement (Uitto et al., 2003). Different types of assay systems based on immunological reagents have been developed and these include enzyme-linked immunosorbent assay (ELISA), immunoblot, immunodot, time-resolved immunofluorescence and other related assays that utilize specific antibodies against the target enzyme. The fundamental commonality between these assays is the requirement of high-affinity, specific antibodies (Sorsa et al., 1999, Yoshida, 1993) (Table 1.8).

The presence of proteolytic enzymes in GCF has been measured by immunological assays such as ELISA (Ingman et al., 1996) and immunoblotting (Sorsa et al., 2004, Kiili et al., 2002, Sorsa et al., 1994), all using high-affinity antibodies to recognize the given enzyme. Immunoblotting used with chemiluminescence is a sensitive method for recognition of target molecules particularly when differentiation between closely related forms is required (e.g. active and latent forms) but quantification of the target molecule is

often difficult (Romanelli et al., 1999). However, there are now some commercial kits that quantify against a set of standard concentrations of antigen. Furthermore, immunoblotting is relatively expensive because of the cost of antibodies and its time consuming nature. Also, it is not very adaptable to the chairside because it requires specialized equipment and is a complicated procedure.

ELISA can be used to test multiple markers and their levels in single samples. Various approaches can be taken but commonly two different antibodies are used: one to capture the enzyme antigen from a mixture of molecules and the other to detect the captured enzyme (Uitto et al., 2003). Compared to other enzyme assays, ELISA is more sensitive, is quantitative and is sufficiently flexible to permit testing of more than one enzyme in a single sample (Lein et al., 1997, Ingman et al., 1996). While on the face of it ELISA is more adaptable to the clinic than some other assays, it still requires specialized equipment and is an involved procedure. Also, ELISA cannot discriminate between active and inactive forms of enzymes, yet in periodontal diseases, the progression of a lesion is strongly associated with the active form of enzymes present rather than the total amount of enzyme level (Lee et al., 1995). Clearly, a range of antibodies that specifically recognize the activated forms of enzymes that are relevant to periodontal tissue destruction would be required (Sorsa et al., 1999).

1.11.2 Substrate degradation assays

These assays primarily measure the ability of the enzyme to degrade a substrate, and in most instances only measure active forms of the enzyme. Several different types of reporter substrate can be employed, such as release of radioactivity from radiolabelled substrate, change in absorbance in colorimetric assay and fluorescence in fluorimetric assays based on synthetic fluorogenic substrate. As shown in Table 1.8, the different substrate assays exhibit considerable variation in sensitivity, speed and utility, depending on the type of the assay. In general, substrate degradation assays are more time consuming than immunological assays (Yu and Stamenkovic, 2000) because of the low level of the enzymes in GCF samples and so time is required to degrade sufficient substrate to allow detection by simple methods. However, this type of assay can detect low levels because it is an amplifying system (i.e. one molecule of enzyme will convert several molecules of substrate) and, it is likely to be more adaptable for use in the clinic,

although some specialized equipment may be required to read the enzyme-reaction product.

Table 1.8. Comparison of commonly used methods for detection of enzymes in GCF.

Methods	Sensitivity	Substrate specificity	Speed	Specialized equipment requirement	Utility	Clinic adaptability
Immunoblot	**3**	**NA**	**1**	**3**	**1**	**1**
ELISA	**3**	**NA**	**2**	**3**	**2**	**2**
Radiolabelled substrate	**1**	**3**	**1**	**3**	**1**	**1**
Fluorescent substrate	**3**	**1**	**1**	**3**	**1**	**1**
Colorimetric substrate	**2**	**1**	**1**	**3**	**1**	**1**

NA: not applicable. Estimates are given in a range from 3 (high) to 1 (low). Sensitivity: the ability of the assay to measure low amount of enzyme in small volumes, 3 is high, 1 is low. Specificity is the ability of assay to differentiate enzyme based on substrate used. Speed: 1 means needing overnight duration and 2 means less than 4 hr. Utility refers to the ability of non-trained staff to run the assay efficiently. Clinic adaptability refers to the suitability of the assay to be conducted in the clinic.

One example of a relatively complex assay is the degradation of collagen in solution, which can be analyzed using a radiolabelled substrate in an SDS-PAGE fluorography (Lee et al., 1991, Overall and Sodek, 1987). This method takes seven days to complete and it uses radioactive isotopes and thus is unsuitable for clinical use.

Photometric assay is the most widely used enzyme assay and, based on the type of substrate used, it can be classified into colorimetric (spectrophotometric) assay and fluorometric assay. In spectrophotometric assay the enzyme activity can be calculated by measuring the change in absorbance of the reaction product. Colour change in the assay can be seen if the product is within the visible range (400-700 nm), and this is called a colorimetric assay. The enzyme will react with its substrate and the increase of product or

decrease in concentration of substrate can be followed spectrophotometrically. Whereas, in a fluorometric assay, the molecule emits light of a different wavelength from that used to excite the fluorophore. Generally, this type of assay is more sensitive than spectrophotometric assays and this is particularly beneficial when only a small volume of sample is available.

To sum up, the advantages of substrate assays are that they can be used for quantitative analyses and can easily discriminate active and non-active forms of the enzymes. As the whole procedure depends on degradation of the substrates, users should have an idea of the range of enzyme concentration in order to adjust the conditions of the assay to achieve maximum sensitivity.

1.12 Periodontal chairside test kits

Many test kits have been developed since the 1990s as prototype tests or for commercial use based on microbiological, chemical and immunological techniques for detection of biomarkers. The rationale behind developing these tests is that they enable earlier detection of active disease and are less invasive, less time consuming, and less costly in terms of the treatment required. Hopefully they can also provide information on the disease prognosis for patients (Kinane, 2000). In this section the applicability and usefulness of these test kits are reviewed through examining studies that have evaluated them.

Chairside test kits can be generally classified into three groups.

i. Microbiological test kits

Microbiological tests have potential to aid the diagnosis of different types of periodontal disease such as gingivitis, chronic periodontitis and aggressive periodontitis. Potentially they can also be used to detect disease initiation, progression and identify sites that are at high risk for further disease progression. These tests are mostly directed toward identifying the main known periodontopathic bacteria, for example, *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia* and *T. denticola*. Microbiological tests also assist in monitoring the periodontal treatment used to reduce or completely eradicate the periodontal pathogens (Malagi, 2012, Bhatia et al., 2011). Examples of these tests are shown in table 1.9.

ii. Biochemical test kits

Biochemical tests are mainly used to analyze potential biomarkers in GCF. Molecules such as host/bacterial enzymes, inflammatory mediators and components of extracellular matrix are excreted from GCF and these molecules reflect alteration of the tissues. These constituents have been evaluated for their biomarker value and some have been translated as chairside tests (Table 1.9)

iii. Genetic test kits

The periodontal susceptibility test (PST®) is the only test used to determine genetic variation of two interleukin genes (IL-1α and IL-1β). These genes do not cause or initiate the disease but they might increase the severity and earlier development of the disease (Kornman et al., 1997).

All of the biomarkers analyzed by these commercial kits and those that are under development have shown the ability to determine disease activity. However, some of these kits are no longer available and few clinicians are willing to use such kits in their practice as they can add little further information than conventional clinical diagnostic measures. This has been the case with all chairside tests developed so far. As is shown in Table 1.9, none of these test kits are able to predict the onset and progression of periodontitis, or the likely outcome of treatment, which is the information that clinicians and patients really want to have. Ideally, the use of biomarker kits at the chairside should enable identification of the onset and progression of the disease as well as the outcome of treatment, simply through the presence of the biomarker or its presence above a certain threshold. Clinicians would then be able to incorporate such tests into their practice as diagnostic tools and they would become standard care in determining appropriate delivery of periodontal treatment. Furthermore, Chapple (2009) reported that these tests have been expensive, not user friendly, complex, time consuming to perform, sometimes difficult to interpret and, most importantly, they did not have any prognostic value.

Table 1.9. Summary of diagnostic biomarker kits (Grover et al., 2014, Reddy et al., 2011).

Assay	Kit	Sample	Availability	Detection
Microbial test kits	**Evalusite test** (Kodak,Eastman company, Switzerland)	GCF	No	Immunological detection of antigens of *Aa*, *Pi* and *Pg*.
Microbial test kits	**PerioScan** (Oral B Laboratories)	Subgingival plaque	No	It utilizes the BANA test for bacterial trypsin-like proteases.
Biochemical test	**Prognostik** (Dentsply)	GCF	No	Serine proteinases and elastase
Biochemical test	**Pocketwatch** (SteriOss, San Diego, CA, USA)	GCF	No	Detects aspartate aminotransferase through colorimetric detection
Biochemical test	**Periogard** (Colgate)	GCF	No	Detects the presence of aspartate aminotransferase
Biochemical test	**Periocheck** (ASTech) (CollaGenex Pharmaceuticals, Newtown, PA)	GCF	No	Detects presence of neutral proteinases (collagenase)
Biochemical test	**Biolise** (SLT,Labinstruments, Crailsheim, Germany)	GCF	No	Aids in detection of elastase
Biochemical test	**TOPAS** (Affinity Labelling Technologies, USA)	GCF	Yes	Detects toxins derived from anaerobic metabolism and measures GCF protein level
Biochemical test	**PerioMarker®** (GlaxoSmithKline)	GCF	Yes	Activated MMP8
Genetic test kits	**PST®** Genetic susceptibility test	Oral epithelial scraping	Yes	Analyzes two interleukin (IL- α and IL-1β) genes for variations

Aa: *A. actinomycetemcomitans*, *Pi*: *P. intermedia, Pg*: *P. gingivalis*

1.13 Summary and Challenges

Various molecular biomarkers in saliva and GCF have been examined in an attempt to obtain a specific and sensitive marker for periodontal disease destruction and progression. GCF enzymes are amongst those molecules that have been investigated by immunological or biochemical means to reveal the events occurring in the periodontal microenvironment. Many of these tests have lacked sensitivity and, owing to the complex nature of periodontitis, it is unlikely that a single clinical or laboratory examination can

address all issues concerning diagnosis and prognosis (Sorsa et al., 2016, Xiang et al., 2010, Van Der Velden, 2005). Buduneli and Kinane (2011) stated that after nearly 30 years of research using sophisticated methods, no single or combination of markers exists that can exactly determine periodontal tissue destruction, which is why the clinical parameters are still relied upon. Furthermore, the diversity in sample collection, storage and analysis methods makes it difficult to evaluate biomarkers for periodontal disease diagnosis and longitudinal studies to evaluate disease prognosis on a site-basis are difficult to perform because of the requirement to treat patients. Lastly, it is noticeable that individual responses to specific causative factors vary from one individual to another (Buduneli and Kinane, 2011).

In general, previous studies exhibit four main limitations. Firstly, most of these studies looked at a single biomarker of periodontal disease (Leppilahti et al., 2015, Leppilahti et al., 2014b), yet periodontal disease is a complex pathological process and an individual biomarker cannot address all the related issues. However, a combination of several biomarkers may be more effective than a single biomarker in providing valuable information to predict the progression of periodontal disease (Sorsa et al., 2016, Kinney et al., 2007b). Only recently has it been reported that a combination of biomarkers in plaque, saliva and GCF can predict future disease progression (Kinney et al., 2014).

Secondly, many such studies have examined the diagnostic value of these biomarkers and described the correlation of biomarkers with the periodontal condition (Leppilahti et al., 2014a, Buduneli and Kinane, 2011). However, this adds little additional information to that derived from conventional clinical and radiographic diagnostic measures used to examine patients with periodontitis. What such diagnostic tools have failed to do is to identify the prognostic value of these biomarkers in relation to disease progression and/or the outcome of treatment.

Thirdly, most of these biomarkers were analyzed at the patient level by correlating biomarkers from saliva and pooled GCF to full mouth clinical measures (Kinney et al., 2014, Buduneli and Kinane, 2011); however, as periodontitis is site specific in nature, the severity, progression and response to treatment can vary not only between different teeth but also between sites surrounding the same tooth. In fact, one of the major concerns of the clinician is how to differentiate sites that will respond to the treatment from those that will not and/or those at high risk of further disease progression. Lastly, the success of

treatment is evaluated on the basis of improvements in pocket depth, which is a subjective measure and therefore liable to error. Hence it is important to detemine a threshold point that will enable differentiation between successful and compromised treatment outcomes.

Consequently, this study has taken these limitations into consideration and tried to address them as follows: firstly, a combination of enzymes (from host and bacteria) were examined as biomarkers instead of a single biomarker; secondly, both the diagnostic and the prognostic value (outcome of treatment) of these biomarkers was studied. Thirdly, these biomarkers were analyzed and correlated to corresponding site-specific clinical measures at each time point. Lastly, a threshold point of 2mm improvement in PPD, which is beyond measurement error, was considered, as a significant change that would meet with the approval of most clinicians (Goodson et al., 1982).

1.14 Aims and objectives of the Study

1.14.1 Aims

The aim of this longitudinal clinical study was to determine whether key host enzymes (MMP-8, elastase and cathepsin G) and bacterial enzymes (trypsin-like activity and sialidase) detectable in GCF can be used in combination as:

1. Diagnostic biomarkers for the clinical condition.
a. To differentiate between health and disease based on conventional criteria such as PPD.
2. Prognostic biomarkers of the outcome of treatment in patients with chronic periodontitis.

1.14.2 Objectives

1. Obtain ethical approval for the study.

2. Determine the best recovery elutant for GCF enzymes.

3. Develop suitable colourimetric or fluorimetric assays.

4. Collect samples in each time point and analyse them.

5. Identify potential biomarkers in GCF.

6. Test the diagnostic utility of these identified biomarkers.

7. Test the prognostic utility of these identified biomarkers.

8. Check the ability of these experiments to be translated as a chairside test.

Chapter 2- Materials and Methods

Chapter 2- Materials and Methods

2.1. Materials

All chemicals in this study were from Sigma-Aldrich Company Ltd, Dorset, UK unless otherwise stated.

2.2 Method

This clinical study investigated the presence and quantity of 5 enzymes (MMP8, cathepsin G, elastase, trypsin like enzyme and sialidase) in GCF collected from patients with chronic periodontitis.

2.3 Design

A quasi-experimental design was employed in this study.

2.4 Patient recruitment

Recruitment was conducted among patients attending the Periodontology Clinic in the Charles Clifford Dental Hospital. Potential study participants were screened by the clinical investigators (Professor A. Rawlinson and Professor G. Griffiths) and then assessed for periodontal disease, general inclusion and exclusion criteria before being invited to join the study. A detailed patient information sheet (Appendix 1) was given to those interested and they had the opportunity to discuss the study further before agreeing to participate. Volunteers wishing to take part in the study were asked to sign a consent form (Appendix 2). A copy of the patient information sheet and consent form was retained by the patient and by the study lead.

Inclusion criteria

- Aged 18 years and over
- Systemically healthy
- Diagnosis of chronic periodontitis with at least one healthy site (≤ 3mm), one deep bleeding and one deep non-bleeding periodontal pocket (pocket ≥ 6mm).
- Radiographic evidence of bone loss with clinical attachment loss.
- Available for the duration of the study.

In addition:

Smoking status was noted in pack years (Grossi et al., 1995) (current number smoked daily and years smoked).

Exclusion criteria

- Patients who do not have the capacity to consent for themselves.
- Pregnant and lactating females.
- Patients who have received periodontal treatment and antibiotics during the previous month.
- Patients with a history of systemic disease or medication that may affect the periodontal condition.
- Non-English speaking participants.

2.5 Ethical approval

The study protocol was approved by the National Research Ethic Service Committee Yorkshire and Humberside (study number 13/YH/0114) (Appendix 3), registered with Sheffield Teaching Hospital NHS for Research Governance (STH 17158) and insured by Sheffield University (Appendix 4).

2.6 Clinical measurements

All patients were examined for suitability and selection of representative sites. Full mouth clinical measurements of periodontal pocket probing depth, plaque scores, bleeding on probing and clinical attachment loss were taken in accordance with routine clinical practice for the assessment of periodontal health and these measurements were recorded by the clinical staff for new and review patients on a clinical data capture form (Appendix 5). Two staff dental hygienists (Nivan Al- Hammouri and Claire Vallance-Owen) collected clinical data and samples for this study, with S. Gul in attendance. Presence or absence of plaque was identified by using a Langer curette and this information was recorded. The University of North Carolina 15 probe was used to measure PPD and CAL. Presence or absence of bleeding was recorded within 30s after probing with the University of North Carolina 15 probe. The number of diseased sites with PPD $\leq$ 3mm, PPD = 4-5mm and PPD with $\geq$ 6mm was determined and total number of teeth was used to find the percentage of sites with above PPD for each patient.

Three representative sites were selected in each patient:

A healthy non-bleeding site ≤ 3mm (control).

A deep non-bleeding site (DNB) ≥ 6mm (non-inflamed).

A deep bleeding site (DB) ≥ 6mm with bleeding on gentle probing (actively inflamed).

For diseased sites, the deepest and most diseased sites that were accessible for sampling were selected, and an accessible healthy site was selected. Clinical parameters from the patients' full mouth and 3 representative sites plus GCF samples were collected at baseline and the 3 month and 6 month appointments (Figure 2.1). Sites showing reduction in pocket depth of ≥ 2mm or more from the baseline measure were considered as having responded to the periodontal treatment.

2.7 Periodontal treatment

Standardized treatment was provided in accordance with the departmental protocol for the treatment of periodontal diseases. This included advice on plaque control, scaling and root surface debridement under local anaesthesia for sites ≥ 4mm in depth. The patients were reviewed after one month of the treatment to reinforce the oral hygiene measures. The effects of treatment were reviewed no less than 3 months after the treatment had been completed and the need for further treatment assessed at that stage. After treatment, irrigation of the pockets with chlorhexidine digluconate (0.2%) was carried out. Following root surface debridement, patients with a poor response to the treatment having residual deep pocket bleeding and/or suppuration sites received a 3 day course of oral Azithromycin (500mg, once a day) as an adjunct to further root surface debridement. All treatment per patient was provided by a single staff hygienist, which ensured standardised care.

2.8 Quality control (inter and intra examiner calibration)

Intra- and inter-examiner reproducibility of PPD was assessed on 20 patients (720 sites) not included in the study, using University of North Carolina 15 probe between the three examiners. For intra examiner calibration the data was recorded and re-measured at an interval of not less than 30 minutes. The data was entered on a Microsoft Excel worksheet and compared for ±1mm agreement. The data revealed that inter- and intra-

examiner reliability with ±1mm was high (≥92%). A total of 90% of readings of PPD within ±1mm difference is accepted to be good intra- and inter-examiner agreement (Badersten et al., 1984).

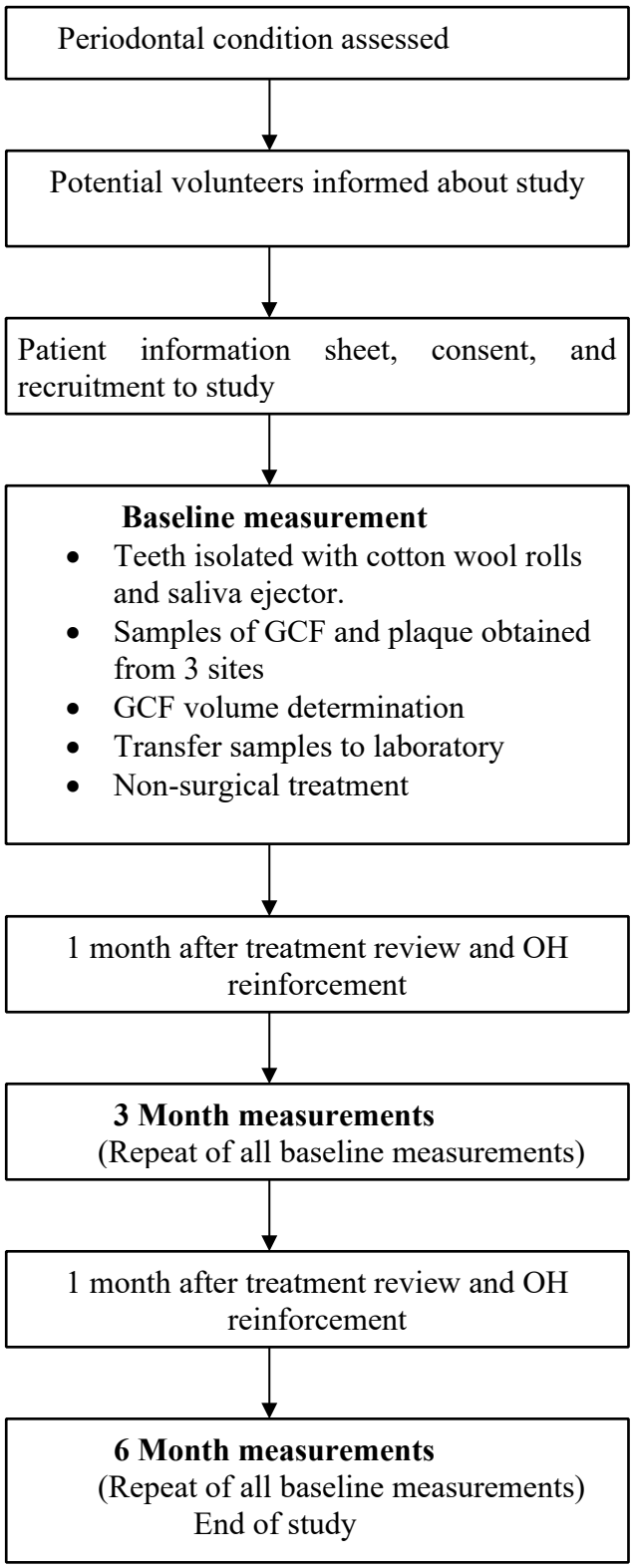


Figure 2.1. Flow chart indicating participants' involvement throughout the course of the study.

2.9 GCF collection

GCF collection was done before clinical examination to avoid altering the natural crevicular fluid flow and avoid contamination with blood. The sites selected (three representative sites) for sampling were isolated and dried with cotton wool, and protected from salivary and blood contamination. Supragingival plaque was removed, the tooth air-dried and collection of GCF was carried out using Periopaper® strips (Oraflow Inc., Plainview, NY, USA), placed in the entrance of the periodontal crevice or pockets for 30 sec (Wassall and Preshaw, 2016) (Figure 2.2). The GCF volumes were immediately determined using the Periotron 8000 instrument or by weighing. The GCF samples were analysed immediately as described below (2.12 Laboratory measurements).

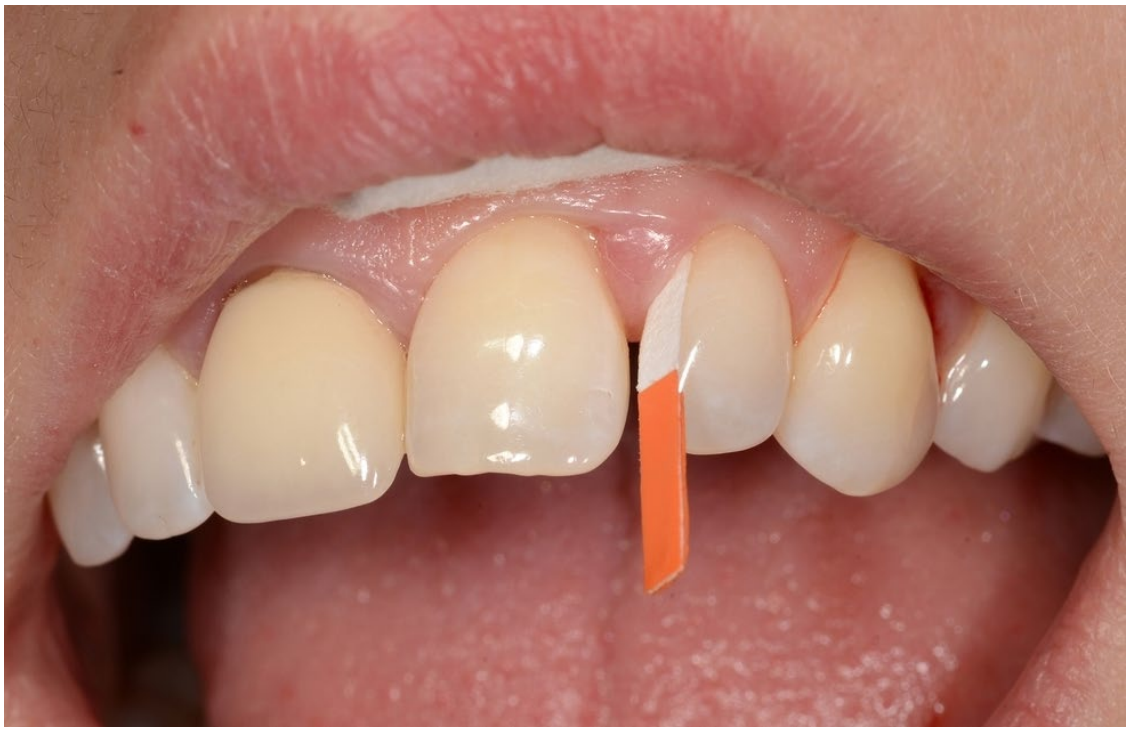


Figure 2.2. GCF collection by Periopaper strips®. The strip was placed in the entrance of the crevice/pocket and the GCF absorbed by capillary action.

2.10 GCF volume determination

The volumes of GCF collected were immediately determined by two methods depending on the volume of GCF.

2.10.1 Periotron instrument

The Periotron 8000 (Oraflow Inc., Plainview, NY) was used to determine the volume of GCF on Periopaper® strips (Figure 2.3) and then the instrument readings were converted to an actual volume (µl) by reference to a standard curve of Periotron reading versus volume. This standard curve was constructed using human serum pipetted onto Periopaper® strips and measured immediately with the Periotron 8000 as described by Wassall and Preshaw (2016) and Chapple et al., (1999).

2.10.2 Weighing method

The Periotron 8000 was found to lack accuracy above 1.7µl and so weighing was investigated as an alternative method of volume determination. Again a standard curve was prepared using serum as a surrogate for GCF over the volume range 1.7-2.5µl. For GCF samples, the weight of each Periopaper® strip was measured before sampling and retained dry until needed. Also, for each sample an eppendorf tube containing appropriate elution buffer was weighed. Once the GCF sample had been taken and placed in the eppendorf tube, it was re-weighed to determine the volume of GCF adsorbed. The density of GCF was determined to be 1.0mg/µl.

2.11 Enzyme recovery

To recover the enzymes from the samples, the Periopaper® strips were eluted in 105µl of sterile phosphate buffered saline (PBS, pH 7.3) containing 1% w/v bovine serum albumin (BSA) for 1 hour. A Centrifugal Filter Unit tube (Millipore Ltd. UK) containing another tube was used (the filter part was removed) (Figure 2.4) to centrifuge the samples at 10,000g for 15 minutes.

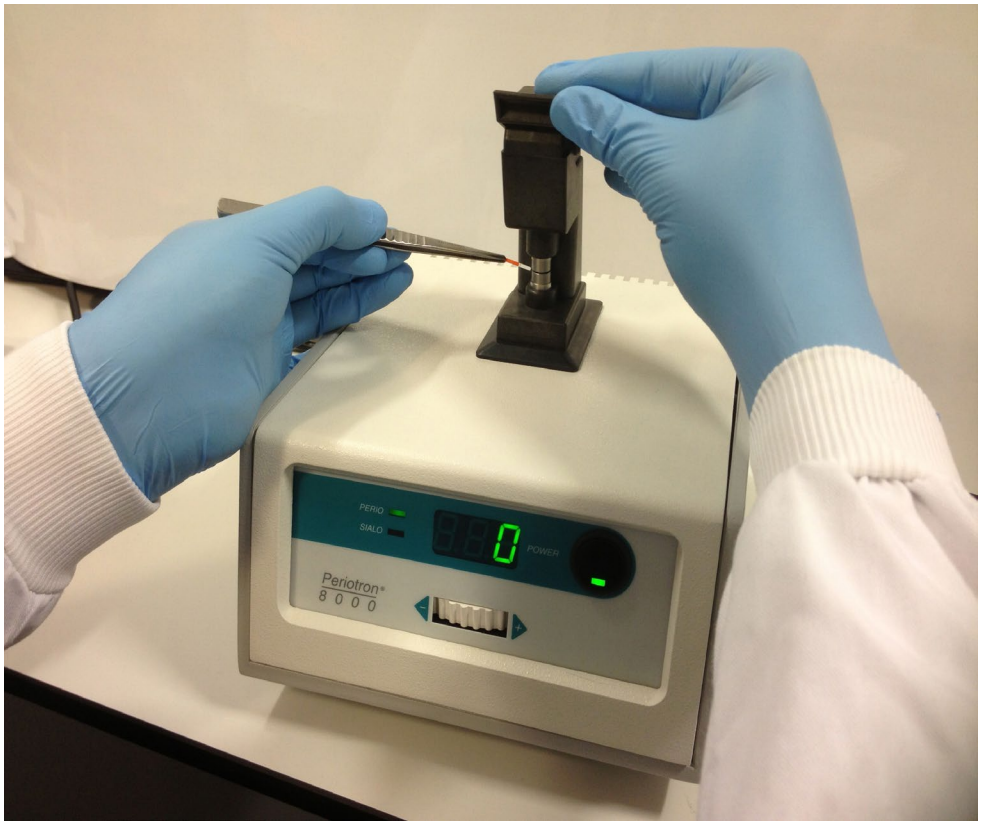


Figure 2.3. Using Periotron to determine GCF volume.

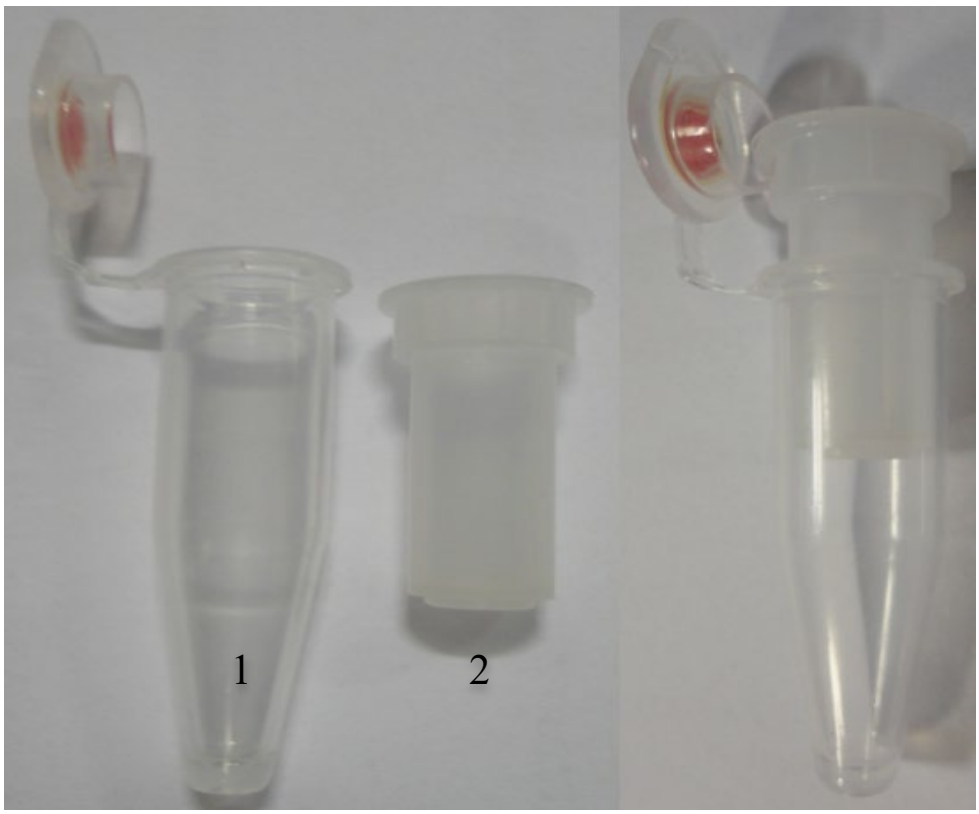


Figure 2.4. Centrifugal Filter Unit tube (1= Eppendorf tube, 2= Filter part).

Chapter 2- Materials and Methods

2.12 Laboratory measurements

Methods of analysis for laboratory measures

The clinical samples were subjected to conventional assay for each of the 5 enzyme activities using fluorescent or chromogenic substrates and each reaction was measured in a microplate reader (FLUOstar Galaxy; BMG Labtech GmbH, Offenburg, Germany). To determine the concentration and total amount of each enzyme in GCF samples the dilution factor was computed according to the equation given below.

$$\text{Dilution factor} = \frac{\mathbf{105}}{\mathbf{X}} \times \mathrm{Y}$$

Where X is the GCF volume, 105µl is the volume of the eluent buffer and Y is the dilution in to the reaction volume; for MMP8= 1, for elastase and cathepsin G= 9, for Trypsin = 5 and for sialidase = 10. In each case duplicate 10µl portions were used for each enzyme assay. The enzyme concentration was then determined from standard curves and multiplied by the dilution factor.

2.12.1 MMP8 assay

2.12.1.1 Standard curve

In the first well of sterile flat bottom 96 well plates, 5µl of stock solution (100ng/µl) Pro-MMP8 (from human neutrophils) (Enzo Life Sciences Inc. Lausen, Switzerland) were added to 14µl PBS, and activated by the addition of 1µl of 20 mM 4-amino-phenyl-mercuric acetate. This starting sample was then serially diluted into 10 µl PBS down 6 wells. Directly after serial dilution, 45µl MMP8 substrate (Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2; Enzo Life Sciences Inc. Lausen, Switzerland) (stock solution 125µM; final concentration 102µM) was added to all wells and incubated at 37°C for 4 hours. The fluorescence generated was monitored at 340nm excitation and 380nm emission wavelengths. The negative control well consisted of 9 µl PBS, 1µl of 20 mM 4-amino-phenyl-mercuric acetate and 45µl MMP8 substrate (Figure 2.5).

2.12.1.2 Clinical samples

10µl of eluted clinical samples (from healthy site, deep non-bleeding site and deep bleeding site) were added to duplicate wells and then 45µl of MMP8 substrate (125µM

stock solution) was added, the plates were incubated for 4 hours and then processed as above. The mean of the duplicate wells was calculated and compared to the standard curve.

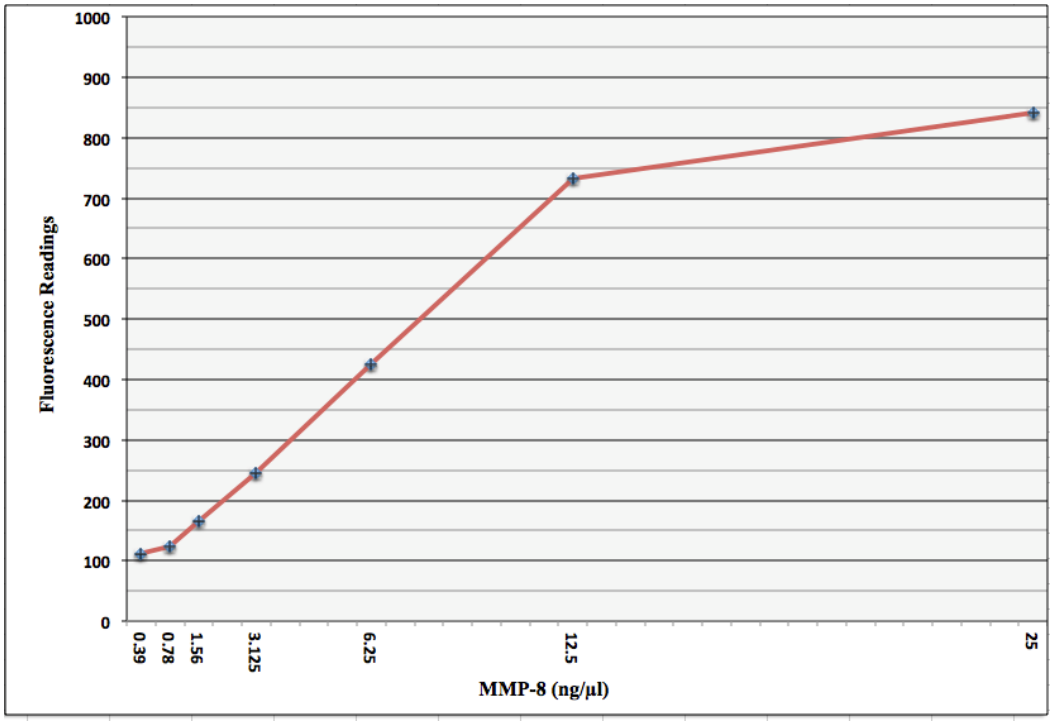


Figure 2.5. MMP8 standard curve.

2.12.2 Elastase and Cathepsin G assays

2.12.2.1 Standard curves

The elastase and cathepsin G (from human leukocytes) standard curves were prepared by adding 25µl of human neutrophil elastase (144 ng/µl = 1 unit; Stock solution) and human neutrophil cathepsin G (115.2 ng/µl = 1 unit; stock solution) to 155µl of PBS buffer in the first well of a sterile flat bottom 96 well plate. The final concentrations of elastase and cathepsin G in the first wells were 20 ng/µl and 16 ng/µl respectively. These starting concentrations were serially diluted down 6 wells containing 90µl of PBS buffer. Then 10µl of relevant substrate (20mM) was added to all wells and the plates were incubated for 18 hours at 37°C before being read at 405nm excitation. The elastase substrate was N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, and the cathepsin G substrate was N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide. The absorbance readings were plotted against the concentrations of the enzymes and the results expressed as ng/µl. The negative

control samples were prepared through adding 90µl of PBS and 10µl of relevant substrate (Figure 2.6 and 2.7 respectively).

2.12.2.2 Clinical samples

After elution of clinical samples from each site, 10µl of sample were added in duplicate to wells containing 80µl PBS buffer and 10µl of relevant substrate. The plates were incubated at 37°C for 18 hours, then the average readings of clinical samples were examined against the corresponding standard curve to identify the concentration of elastase and cathepsin G.

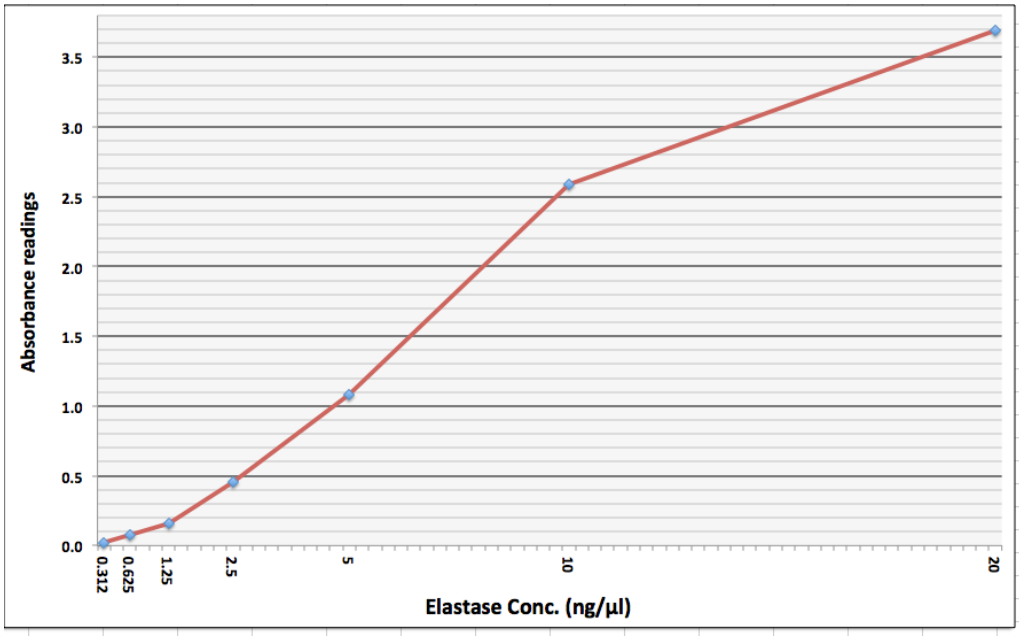


Figure 2.6. Elastase standard curve.

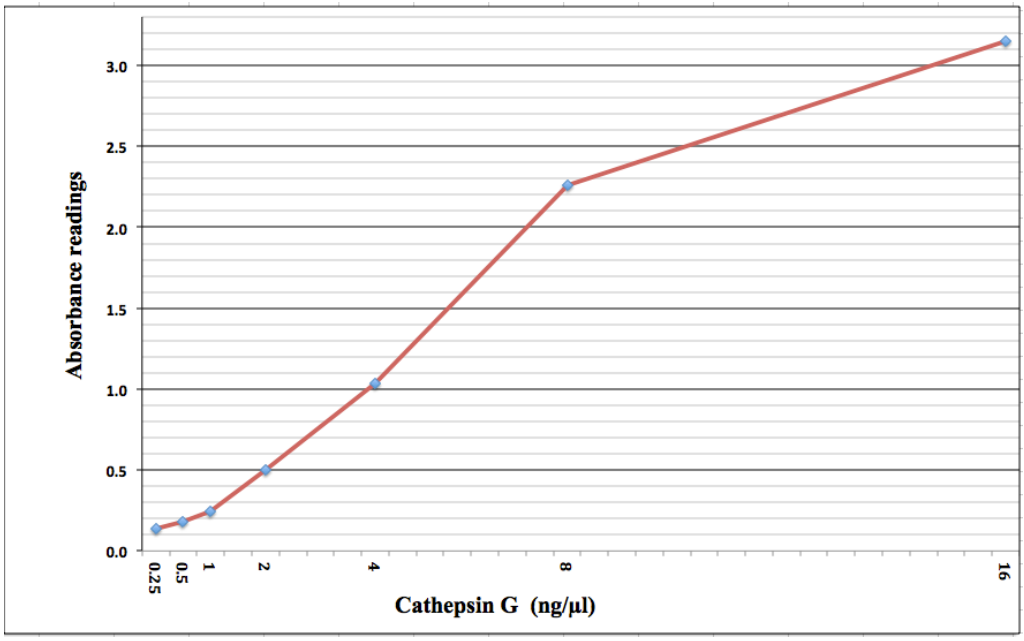


Figure 2.7. Cathepsin G standard curve.

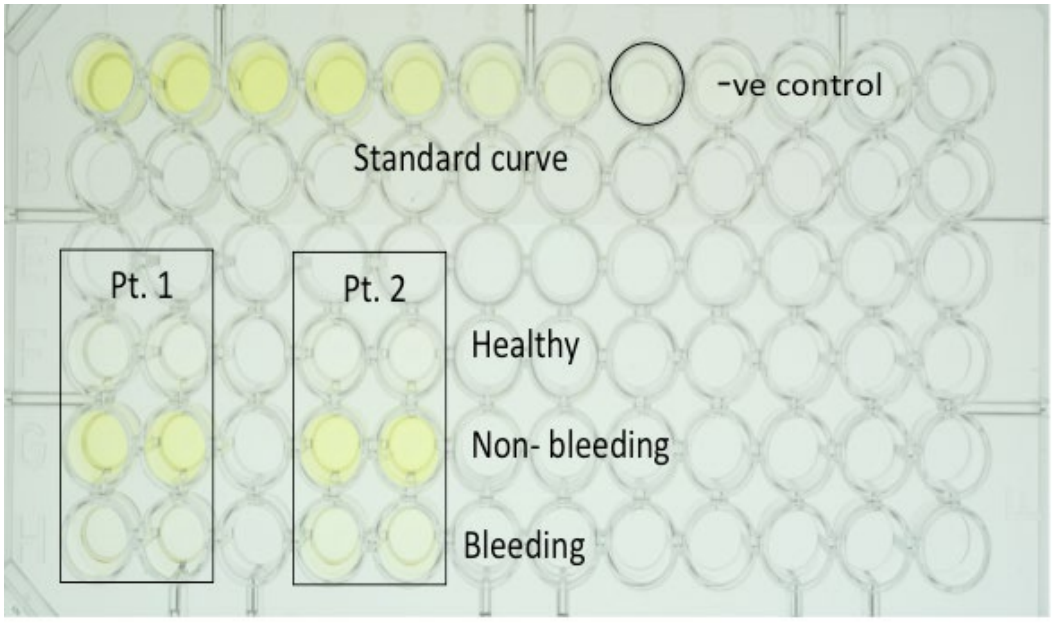


Figure 2.8. Appearance of typical standard curve assay and elastase determination of example clinical samples.

2.12.3 Trypsin assay

2.12.3.1 Standard curve

A stock solution of bovine pancreatic trypsin (100ng/µl in PBS) was prepared and 5mM

di-thiothreitol added. Enzyme was then added (100µl) to the first well of flat bottom 96 well plate and two fold serially diluted in PBS down 7 wells. Then 50µl trypsin substrate solution (5.2mg/ml N-α-benzoyl-DL-arginine-p-nitroanilide hydrochloride (BAPNA) in Dimethyl Sulfoxide plus Tris buffer (0.05M + 0.2M NaCl pH7.5) was added to all wells and incubated at 37°C for 18 hours. The resultant colour was then read at 405nm excitation. The negative control samples consisted of 50µl PBS and 50µl BAPNA (Figure 2.9).

2.12.3.2 Clinical samples

Aliquots (10µl) of eluted clinical samples were added to 40µl of PBS (with 5mM di-thiothreitol) in duplicate and 50µl of trypsin substrate (BAPNA) were added to all wells. The samples were then incubated at 37°C for 18 hours and then the colour generated read at 405nm emission.

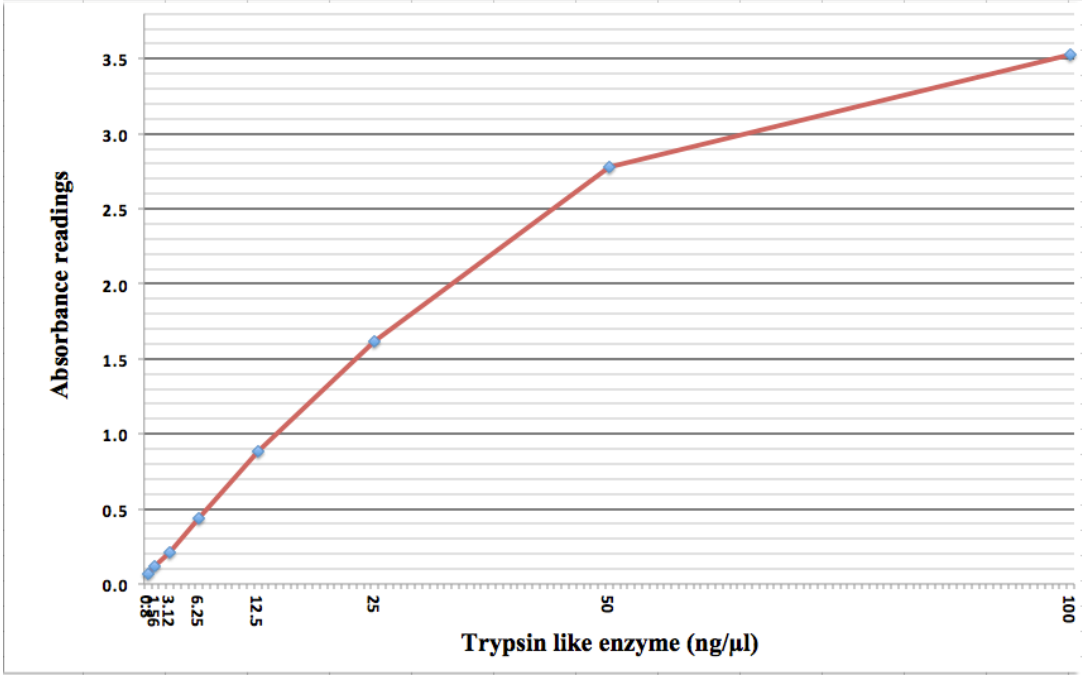


Figure 2.9. Trypsin like enzyme standard curve.

2.12.4 Sialidase assay

2.12.4.1 Standard curve

The standard curve for sialidase activity was prepared by adding 20µl of *Clostridium perfringens* sialidase (3ng/µl in PBS) to 180µl of PBS in the first well of 96 well flat

bottom plates. Two fold serial dilution was made along 7 wells, 10µl of substrate solution (2mM 4-methylumbelliferyl-n-acetyl-α-d-neuraminic acid salt sodium in H_2O; (Neu5Ac); Carbosynth Ltd. Compton, Berkshire, UK) was added to all wells and they were then incubated for 18 hours at 37°C. The fluorescence generated was measured at 355nm excitation and 430nm emission (Figure 2.10).

2.12.4.2 Clinical samples

The concentrations of sialidase activity in clinical samples were measured by adding 10µl of eluted clinical sample to 90µl of PBS in duplicate then adding 10µl sialidase substrate as described above (2.12.4.1 Standard curve). The plates were incubated for 18 hours at 37°C and activity was calculated from the standard curve. The negative control samples consisted of 100µl PBS and 10µl sialidase substrate.

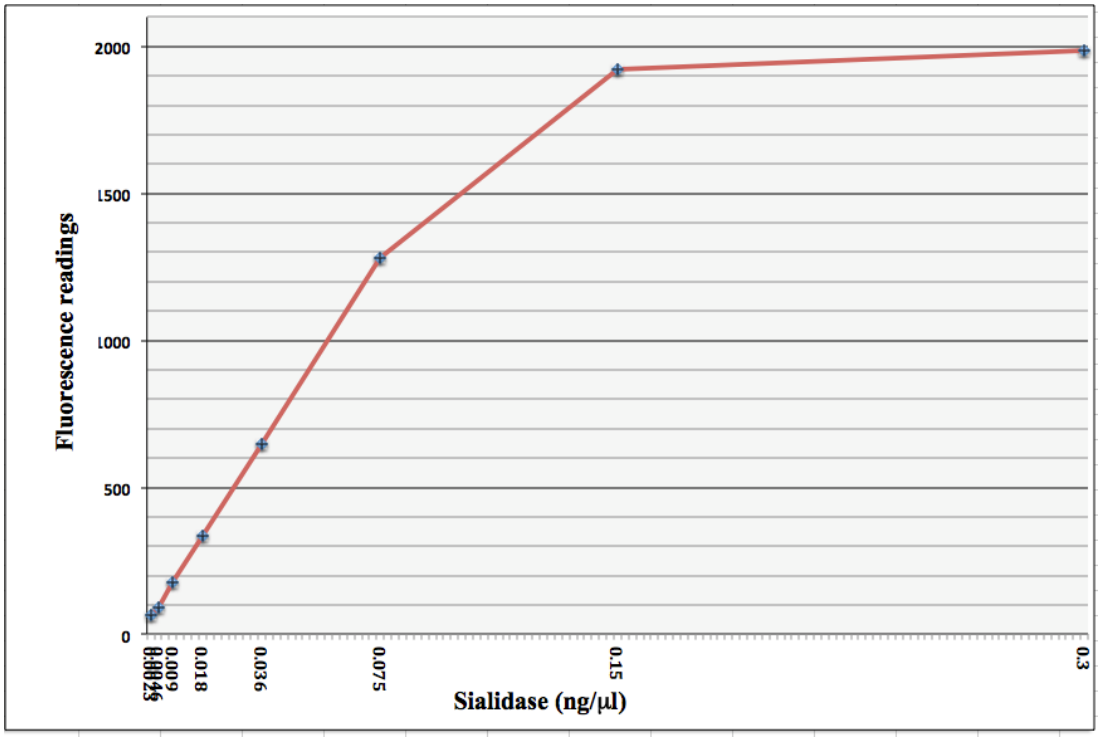


Figure 2.10. Sialidase standard curve.

2.13 Translating to chairside test

The feasibility of translating these tests into a suitable chairside test was examined using enzyme reactions directly in the sample. This approach was based on adding substrate to Periopaper® strips and adding the enzymes to the substrate at the predetermined threshold point. The substrates used for MMP8 and sialidase were the same substrate described earlier, whereas, the elastase substrate used for the chairside test here was the fluorescent substrate N-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin as it was easier to visualize on the paper background. The Periopaper® strip was then incubated at 37°C in a Petri dish with a humid atmosphere for 15, 30, 45 and 60 minutes before being exposed to UV light (wavelength = 300-350 nm) and photographed.

2.14 Power calculation and statistical analysis

2.14.1 Power calculation

Advice was sought from the Statistical Services Unit, The University of Sheffield. Regression analysis was undertaken to investigate the correlation of the clinical data and the enzymes identified. Using this method it was recommended that 10 patients should be recruited for each factor. There are 9 factors in the proposed analysis: 2 variables for the sites being investigated (deep non-bleeding and deep bleeding site), 5 for the enzymes, 1 for the subject factor and 1 factor for the change from baseline, which for this analysis was the change at 6 months. Therefore, a total of 90 patients was needed to be recruited to this study to investigate all 5 enzymes and ensure the strength of the study.

2.14.2 Methods of analysis for demographic and clinical measures

The distribution of volunteers according to age, gender and smoking status was presented in tabular form. The clinical parameters of PPD and CAL, plaque index and bleeding upon probing at baseline for each type of site were compared with these measures at each interval in the study. This enabled the clinical outcomes of treatment to be assessed and compared with the outcomes for the enzymes measured.

The “continuous” data were tested for normal distribution and thereafter subjected to appropriate parametric/non-parametric testing (Shapiro-Wilk test). The following relationships were investigated and a p-value of <0.05 was taken to be statistically significant. Kruskal Wallis test was used to find the statistically significant differences of biomarker values in three selected sites and biomarker values in each time point. Correlations between clinical measures and biomarkers values were evaluated with Spearman’s correlation.

2.14.3 Methods to detect predictive value of laboratory data

Two-millimeter improvement in probing pocket depth was considered to be clinically relevant and was used to dichotomise the outcome variable (PPD at 3 months and 6 months). The areas under the curves (AUCs) of the receiver operating characteristic curve (ROC) were estimated non-parametrically. Threshold points for enzyme levels were selected from the ROC curves as the values with the highest sensitivity and specificity. Baseline continuous values of enzyme biomarkers (as predictors) were analysed by logistic regression against binary outcome measures (PPD as being improved by 2mm or not) to find predictive values of the biomarkers (as dependent variables) at 6 months after treatment. Regression analysis with backward stepwise technique was used to exclude redundant biomarkers. All variables included in the final multivariate model were determined to be independent through the assessment of their co-linearity. Odds ratio (OR) estimates and their confidence intervals (CI) were calculated and statistical significance was defined as $P \leq 0.05$. All calculations were performed using the SPSS software package (version 20; SPSS Inc., Chicago IL, USA).

Chapter 3- Results

3. Results

In order to characterise GCF samples collected from patients, a series of experiments were conducted to be able to set the parameters for accurate measurement of enzyme activities. GCF samples were to be collected from sites using Periopaper® strips and so determination of the volume of the GCF absorbed and the efficiency of its recovery was required.

3.1 GCF volume determination

The volume of GCF was to be determined and two methods are generally used: use of the Periotron instrument and measurement by weight of sample.

3.1.1 Periotron machine

Fluid volume was determined by adding different volumes of serum as a surrogate for GCF (0.1, 0.2,0.3,0.4, 0.5, 0.7, 1.0, 1.3, 1.5, 1.7, 2.0 2.3 and 2.5µl) to Periopaper® strips and placing them between the electrodes of the Periotron instrument. The mean of 9 separate readings for each volume was plotted against the relevant serum volume to prepare a standard curve (Figure 3.1).

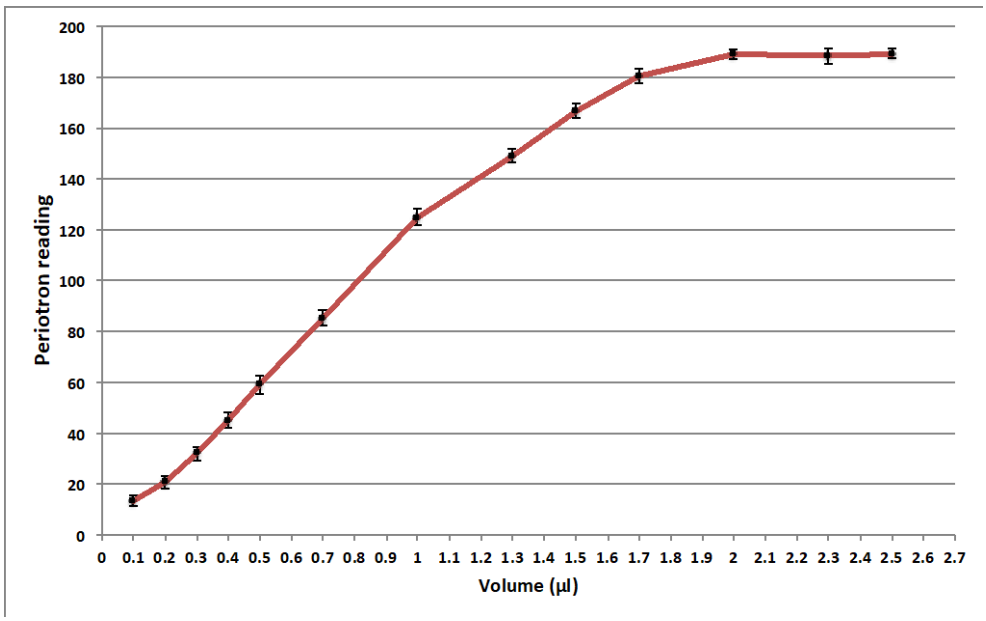


Figure 3.1. Standard curve for volume measure using the Periotron instrument. Values are means of Periotron reading versus volume applied (n=9); error bars show standard deviation.

As seen in Figure 3.1, the sensitivity of the Periotron instrument is limited and it does lose accuracy when volumes are above 1.7µl. However, it is not known whether Periopapers® would be capable of absorbing volumes above that value. Consequently, the maximum volume that could be absorbed by the paper was determined.

3.1.1.1 Maximum volume absorbance by Periopaper®

The maximum volume of serum absorbable by Periopaper® strips was determined by weight. The Periopaper® strips were weighed using a microbalance and then the strips were dipped into serum until the paper appeared saturated, then reweighed. The net difference in weight was 2.45±0.4 mg (mean of five determinations). Varying volumes of serum were weighed and it was concluded that the weight of serum was 1.0 mg/µl, therefore the maximum absorbed volume of GCF was 2.45±0.4µl.

3.1.2 Weighing method

Given the accuracy limitation of the Periotron instrument for measuring volumes above 1.7µl, weighing was tested to see if that provided a better method for sample volume determination. A standard curve of weight versus volume is shown in Figure 3.2. Periopaper® strips were weighed before and after application of varying volumes of serum and the means of 9 determinations plus standard deviation plotted (Figure 3.2).

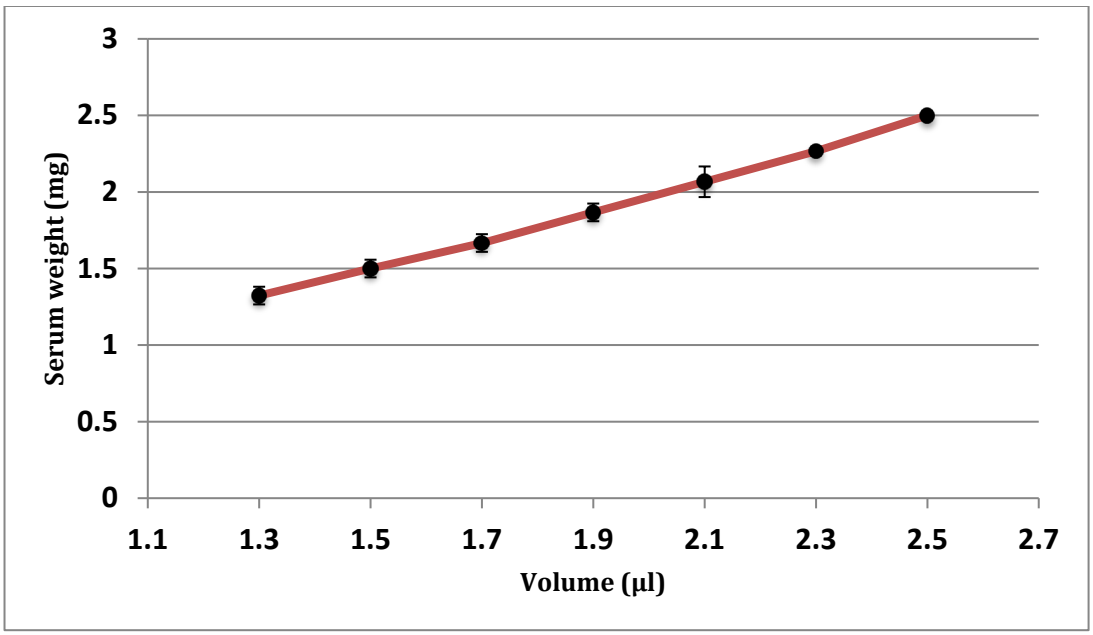


Figure 3.2. Standard curve for determination of volume by weight.

From these studies it was decided that Periotron papers that gave a reading equivalent to 1.7µl or greater would be weighed to establish the volume.

3.2 Enzyme assays

3.2.1 Sensitivity of enzyme assay

The standard curves for each enzyme investigated were prepared according to the method described in Chapter 2 of the Methods. These curves are shown in Figures 2.5, 2.6, 2.7, 2.9 and 2.10. The minimum concentrations detectable for each enzyme were as follows:- MMP8 0.39ng/µl, Elastase 0.312ng/µl, cathepsin G 0.25ng/µl, Trypsin 0.8ng/µl and sialidase 0.004ng/µl.

3.2.2 Optimisation of the enzyme assay reaction time

The optimum incubation time for each enzyme assay was determined. The product of each enzyme reaction increased with enzyme concentration up to and including 4h incubation time but there was no significant increase in product between 4hrs and 18hrs. The results for each of the five enzymes are shown in Figure 3.3 A - E.

The standard curve for each tested enzymes with four-hour incubation is shown in (Figures 2.5, 2.6, 2.7, 2.9 and 2.10).

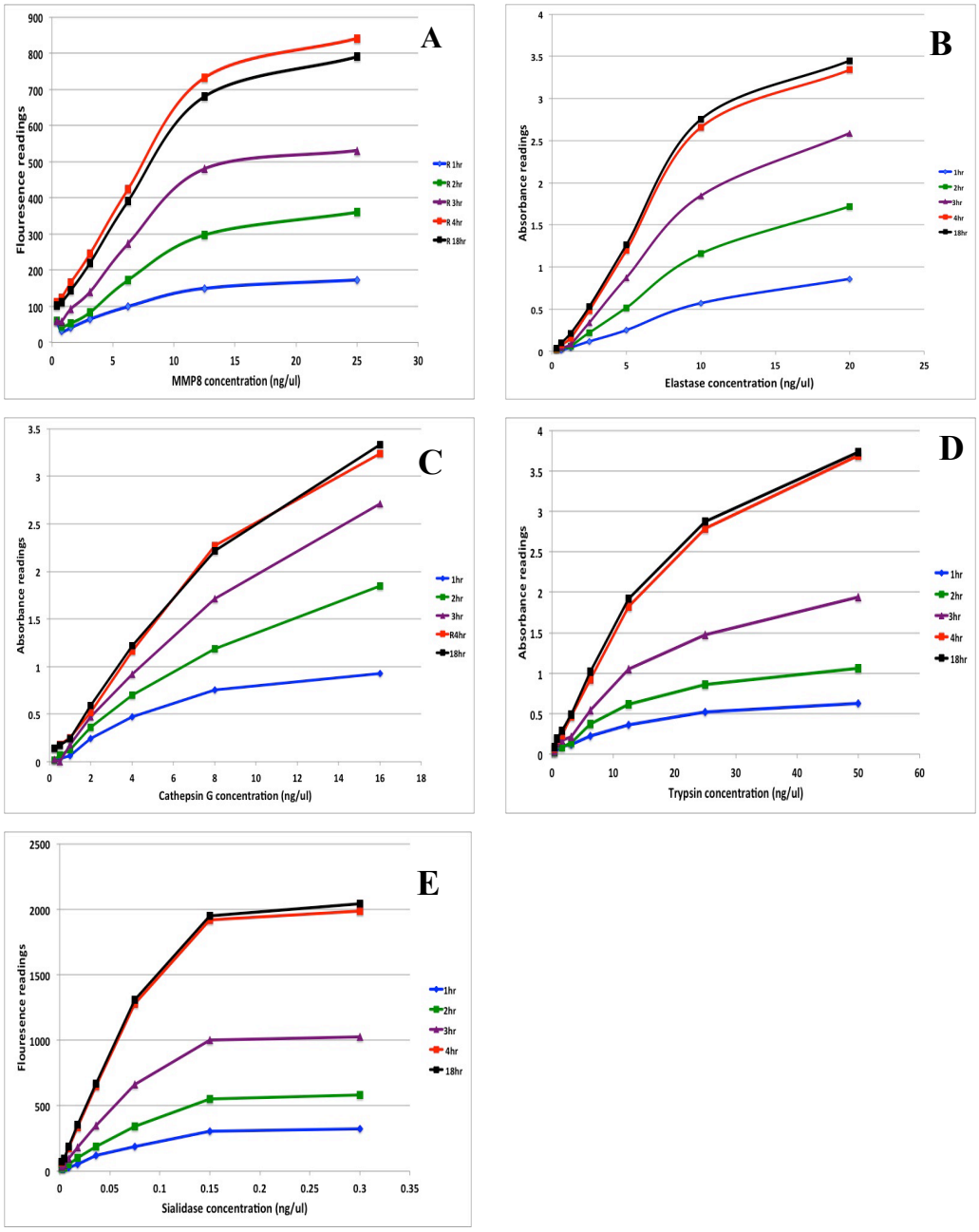


Figure 3.3. Example comparison between different time points of MMP8 (A), elastase (B), cathepsin G (C), trypsin like enzyme (D) and sialidase (E) standard curves readings.

3.2.3 Reproducibility of standard curve

Using a four-hour incubation time the reproducibility of all standard curves was checked by comparing the curves obtained for each of the five enzymes on 3 different occasions. No statistically significant differences between them were identified using one way ANOVA (P> 0.05) (Figure 3.4).

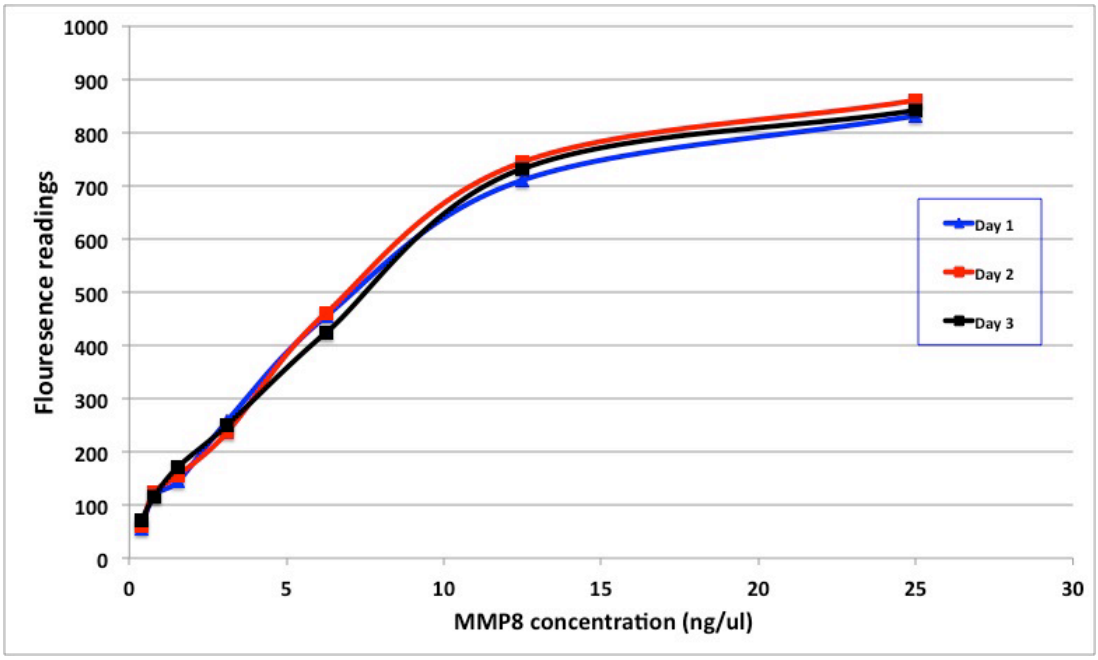


Figure 3.4. Example repeats standard curves for MMP8. Similar results were obtained for each of the other four enzymes.

3.3 Enzyme recovery from Periopapers

Although the GCF sample size can be determined by the above methods, the efficiency of sample recovery from the Periopapers® was not known. Consequently, the recovery of the five targeted enzymes (MMP8, elastase, cathepsin G, trypsin like enzyme and sialidase) from Periopapers® was assessed using 6 different elutants. These were the non-ionic detergents 0.1% (V/V) Triton X-100, 0.1% (V/V) Tween 20, 0.1% (W/V) Brij 35 and 0.1% (W/V) cetylpyridinium chloride and the protein solution 1% (W/V) bovine serum albumin (BSA) in phosphate buffered saline (PBS) and PBS alone. Varying amounts of each pure enzyme were applied to Periopapers®. Enzyme recovery amounts were assessed by elution both in the presence of serum (conditions mimicking GCF) and in buffer alone.

The percent recovery of the enzymes was investigated by comparing the activity of a known concentration of enzyme added to Periopaper® strips in either PBS or human serum and then eluting by various agents with the same amount of enzyme placed directly into the elutant solution. Serum was used since GCF has a similarly high protein content which may influence non-specific binding of enzymes to the Periopapers® and so

the final percent recovery. Values reported are the means of triplicate experiments, which were then expressed as a percentage of the same amount of enzyme directly added to the elutant (Table 3.1). Finally as the MMP8 demonstrated zero recovery with cetylpyridinium chloride from Periopapers® strips, MMP8 enzyme plus substrate was directly added to cetylpyridinium chloride which totally inhibited the MMP8 enzyme activity. This indicates that the effect of cetylpyridinium chloride buffer was not to reduce recovery of MMP8 from Periopapers® strips but rather to inactivate the enzyme.

3.4 Effect of blood on GCF enzyme level

Sometimes blood contaminated the GCF samples (especially in DB sites) and so it was necessary to determine whether the colour of blood affected the absorbance and fluorescence readings of the product of the enzyme assays. Horse blood (2.5µl) was added to 105µl PBS plus 1% BSA and 10µl portions added to serially diluted enzymes as setup for a standard curves, including a negative control. The horse blood volume selected (2.5µl) was because this was the maximum amount of fluid that could be absorbed by Periopaper®. The absorbance and fluorescence readings in the presence of blood were almost identical to the standard curve without blood showing that at the wavelength of the assay and with dilution of around 50 fold (2.5µl in 105µl), there was no contribution or interference to the absorbance/fluorescence reading by the blood.

3.5 Effect of elutant buffer on neutrophils

The analyses of the GCF samples aimed to measure the five key enzyme activities in their free form as they had been in the patient. Since three of these are neutrophil enzymes, it was important to determine whether cellular enzyme would be released during the sample preparation and so add to any free enzyme giving an artificially high level. Blood neutrophils were therefore incubated with the buffer used as elutant for the Periopaper samples. The results showed that cathepsin G, elastase and MMP8 were not present in the supernatant when incubated in the elutant buffer. It was concluded, therefore, the elutant buffer did not lyse neutrophils and so would not contribute to the free enzyme levels detected.

Table 3.1 Shows that recovery of enzymes was generally better in the presence of a high protein environment. Cetylpyridinium chloride provided a high percent of recovery for all of the enzymes except MMP8. BSA also gave a consistently high recovery for all enzymes except cathepsin G and overall was slightly better than the other detergents. Consequently 1% BSA in PBS was used as the elutant for clinical GCF samples from the Periopapers (eluted value have the serum value subtracted).

Serum %	Elutant	PBS		Triton X-100		Tween 20		Brij 35		BSA		CPC	
		From PBS	From Serum	From PBS	From Serum	From PBS	From Serum	From PBS	From Serum	From PBS	From Serum	From PBS	From Serum
2	Elastase	0	84±14	85±13	91±9	87±3	91±9	16±8	86±6	90±10	93±7	100±0	100±0
1	Cathepsin G	0	76±11	5±6	85±7	24±8	83±4	5±6	85±9	22±9	88±5	88±10	92±7
2.8	Trypsin	93±3	77±5	95±5	97±2	95±4	79±3	96±3	98±1	100±0	90±6	88±6	94±5
0.2	Sialidase	30±8	94±5	86±5	96±3	89±3	96±3	90±3	94±3	94±6	99±1	91±5	94±5
4	MMP8	0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	100±0	0	0

CPC – cetylpyridinium chloride

3.6 Demographic and clinical summary of sample population

Consecutive patients attending the periodontology clinic at Charles Clifford Dental Hospital between 2013 and 2015 were screened for their suitability; from these, 101 patients were invited to join the study. Of these, 12 failed to attend further appointments, declined to participate, or were excluded according to the inclusion/ exclusion criteria. This left 89 patients (44 male and 45 female) recruited, with a mean age 49.7±8.9 years (ranging from 30 to 70 years) (30% 30-49, 30% 40-49, 20% 50-59 and 20% 60 or above), of whom 83 completed the 3-month cycle and 77 completed the full study (summarised in Figure 3.5). Altogether 12 patients failed to attend further appointments with the exception of one participant who died during the period of the study. The demographic characteristics of the subjects are shown in Table 3.2. The mean PPD for DNB and DB sites in subjects who dropped out were 6.66±1.15 mm and 6.41±0.66 mm, respectively, this is not statistically significantly different from those completed the study (as shown in Figure 3.6). The median levels of the enzymes in DNB and DB sites in patients who dropped out from the study were as follows: MMP DNB (218ng/µl), MMP DB (201ng/µl), elastase DNB (161ng/µl), elastase DB (149ng/µl), cathepsin G DNB (15.2ng/µl), cathepsin G DB (14.4ng/µl), trypsin like enzyme DNB (39ng/µl), trypsin like enzyme DB (42ng/µl), sialidase DNB (20ng/µl) and sialidase DB (30ng/µl). There were not statistically significant differences in the baseline enzyme levels between those who dropped out from the study and those who completed the study (as shown in Table 3.6).

Table 3.2. Characteristics of patients according to age, gender, smoking habit and antibiotic prescription.

Characteristics	Patients completed the study (77 patients)	Patients dropped out (12 patients)
Age	49.7±8.9	49.6±9
Gender (male/female)	38/39	6/6
Smoking (smoker/non-smoker)	8/69	1/11
Antibiotic (received/ not received)	17/60	7/5

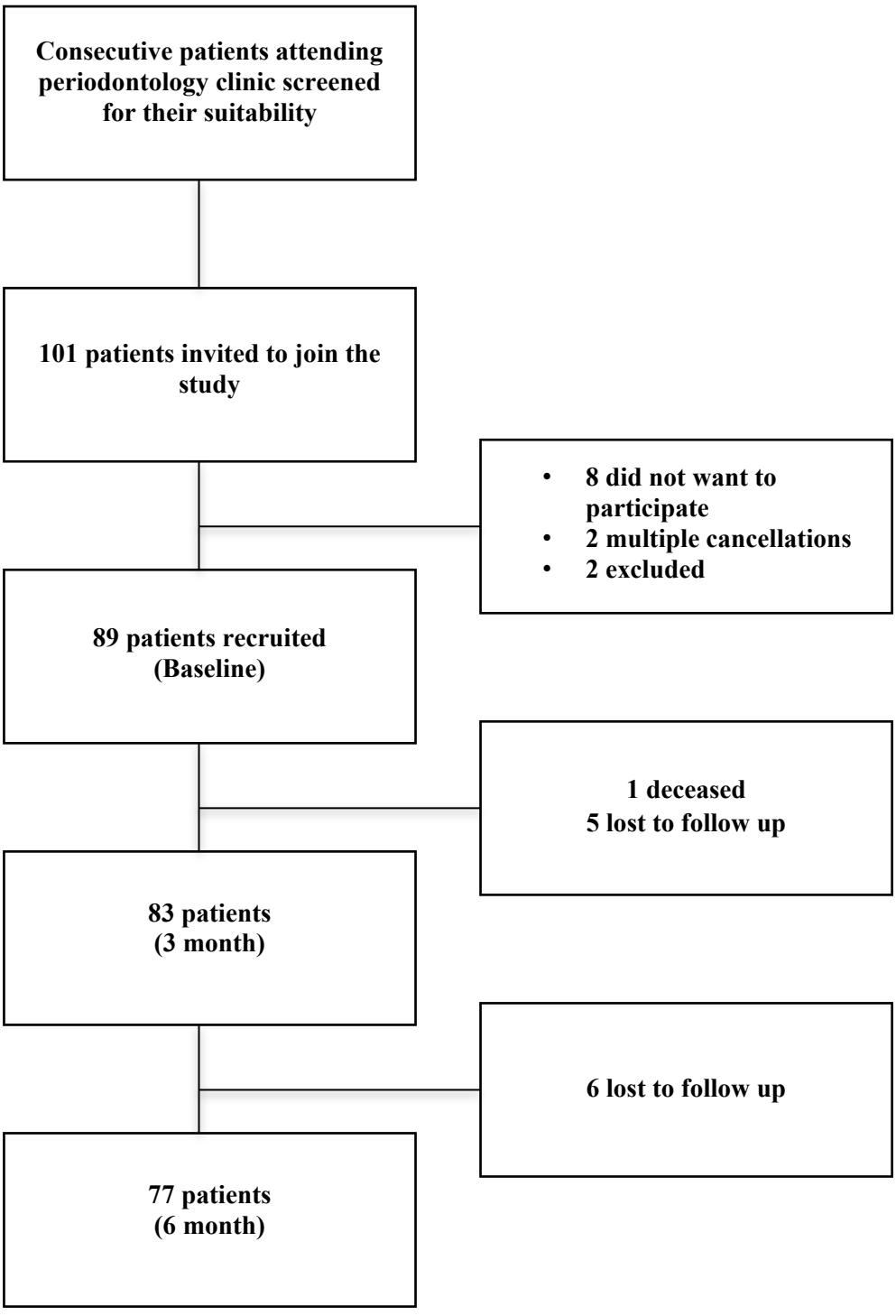


Figure 3.5. Study timeline and recruitment/enrollment activities of the study.

Chapter 3- Results

3.7 Clinical measurements

3.7.1 Changes in full mouth clinical data at study time points

The severity of periodontal disease amongst recruited patients ranged between moderate and severe chronic periodontitis and the distribution ranged between localised and generalised forms of the disease according to the 1999 periodontal disease classification system (Armitage, 1999). Comparisons of the changes in full mouth clincal measures (PPD in particular) were performed in order to assess the effect of non-surgical periodontal treatment and check whether the patients gained benefit from the treatment. Following the baseline treatment, statistically significant reductions in the mean percentage of full mouth PI and BOP were observed from 60.6±27.1% to 39.8±26.7% for PI and 22.6±16.2% to 15.7±18.6% for BOP. Retreatment at the 3 month time point resulted in further reductions in mean percentage of full mouth PI to 34.1±24.8% and BOP to 7.7±10.6% (Table 3.3).

At baseline, the mean percentage of sites with PPD≤3 mm was 67.26±14.2%, the mean percent of sites with PPD of 4-5mm was 17.6 ±9.72% and the remaining 15.1±9.3% of sites had PPD of ≥6 mm (Table 3.3). After one cycle of treatment and at the 3 month time point, the mean percentage of sites with PPD between 4-5 mm had increased by 1.9%. For the same period of time the mean percentage of sites with PPD ≥6 mm showed a marked reduction to 7.9±7.2% (as shown in Table 3.3). At the 6 month re-examination and compared to the baseline measures, additional significant improvements were noticed in which the mean percentage of healthy sites had increased to 81.6±11.1% and the mean percentage of sites with PPD ≥6 mm had decreased to 3.5±4.1%. Meanwhile, the mean percentage of sites with PPD of 4-5mm had reduced to 14.9±9.1% (Table 3.3).

3.7.2 Changes in clinical measures at sampled sites at study time points

At baseline, supragingival plaque was present adjacent to 80% of both DNB and DB sites and the sites with supragingival plaque had significantly reduced to 37% for DNB and 53% DB at 3 months and further reductions were not noticed at the end of the study (39% and 47% at DNB and DB sites) (Table 3.4). On the other hand, plaque was also present at 48% of healthy sites. Regarding BOP, the prevalence of bleeding in DB sites showed

continuous reduction throughout the course of the study. However, in DNB sites the trend was nearly the opposite (Table 3.4).

Table 3.3. Full mouth clinical data at study time points.

Variable	Baseline (% sites ± sd)	3 months (% sites ± sd)	P value* (baseline vs 3 month)	6 months (% sites ± sd)	P value* baseline vs 6 month)
PI	60.6± 27.1	39.8 ± 26.7	**0.0001**	34.1± 24.8	**0.0001**
BOP	22.6 ±16.2	15.7 ± 18.6	**0.004**	7.7± 10.6	**0.0001**
Mean PPD ≤3 mm	67.2±14.2	72.5±16.1	**0.02**	81.6±11.1	**0.0001**
Mean PPD 4-5 mm	17.6 ±9.7	19.6± 11.2	0.61	14.9 ± 9.1	**0.043**
Mean PPD ≥ 6 mm	15.1 ± 9.3	7.9 ± 7.2	**0.0001**	3.5±4.1	**0.0001**

* t-test

Table 3.4. Site specific clinical data at study time points.

Variable	Baseline (% sites)	3 months (% sites)	P value* (baseline vs 3 month)	6 months (% sites)	P value* (baseline vs 6 month)
PI (healthy site)	48	24	**0.009**	24	**0.009**
PI (DNB site)	80	37	0.5	39	0.6
PI (DB site)	80	53	0.7	47	0.8
BOP (healthy site)	0	10	NA	4.5	NA
BOP (DNB site)	0	40	NA	25	NA
BOP (DB site)	100	60	NA	39	NA

* Chi-squared test, NA= not applicable

The outcome of non-surgical periodontal treatment of diseased sites that were sampled was assessed by measurement of PPD (≥2mm improvement in PPD). At baseline, the mean PPDs for DNB and DB sites were 6.73±1.08 mm and 6.79±1.19 mm, respectively, whereas at 3 months after the initial treatment phase the mean PPDs for both types of sites showed statistically significant decreases of 1.48mm to 5.25±1.68 mm (p= 0.0001) and 1.47mm to 5.32±1.48 mm (p= 0.0001), respectively. The second treatment phase at

the 3 month time point, resulted in further decreases by the 6 month time point of 0.81mm to 4.44±1.65 mm (p= 0.0001), and 0.85 mm to 4.47±1.73 mm (p= 0.0001), respectively (Figure 3.6).

On the other hand, at site level, the proportion of sites responding with "≥2mm improvement of PPD" after initial treatment was 44% for DNB sites (Figure 3.7) and 38.5% for DB sites (Figure 3.8). At the 6 month follow up examination 51 sites in both DNB and DB sites (66.2%) presented a ≥2mm reduction in PPD. The sites showing improvement in PPD of ≤2mm included 8 DNB sites (10.4%) and 14 DB sites (18.2%). Additionally, 13 DNB sites (16.9%) and 8 DB sites (10.4%) did not show any improvement and interestingly 5 DNB sites (6.5%) and 4 DB sites (5.2%) demonstrated further disease progression (Figures 3.9 and 3.10). Among the subjects who smoked, 10 diseased sites (DNB and DB sites) (62.5%) improved by ≥2mm in PPD over the period which was very similar to that found amongst the non-smoker group. Comparing the patient-based percentages, 72.1% of sites with PPD of ≥ 6mm had improved by 2mm, 16.5% had reduced by less than 2mm, 8.7% did not show any change and the remaining 2.7% demonstrated further deterioration.

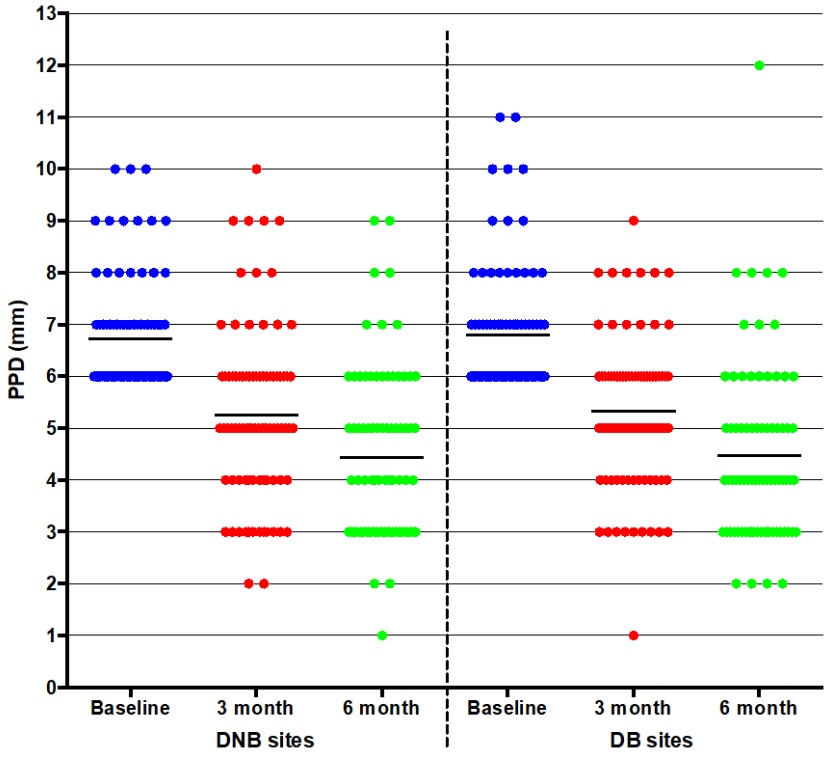


Figure 3.6. Changes in PPD throughout the course of the study in DNB and DB sites.

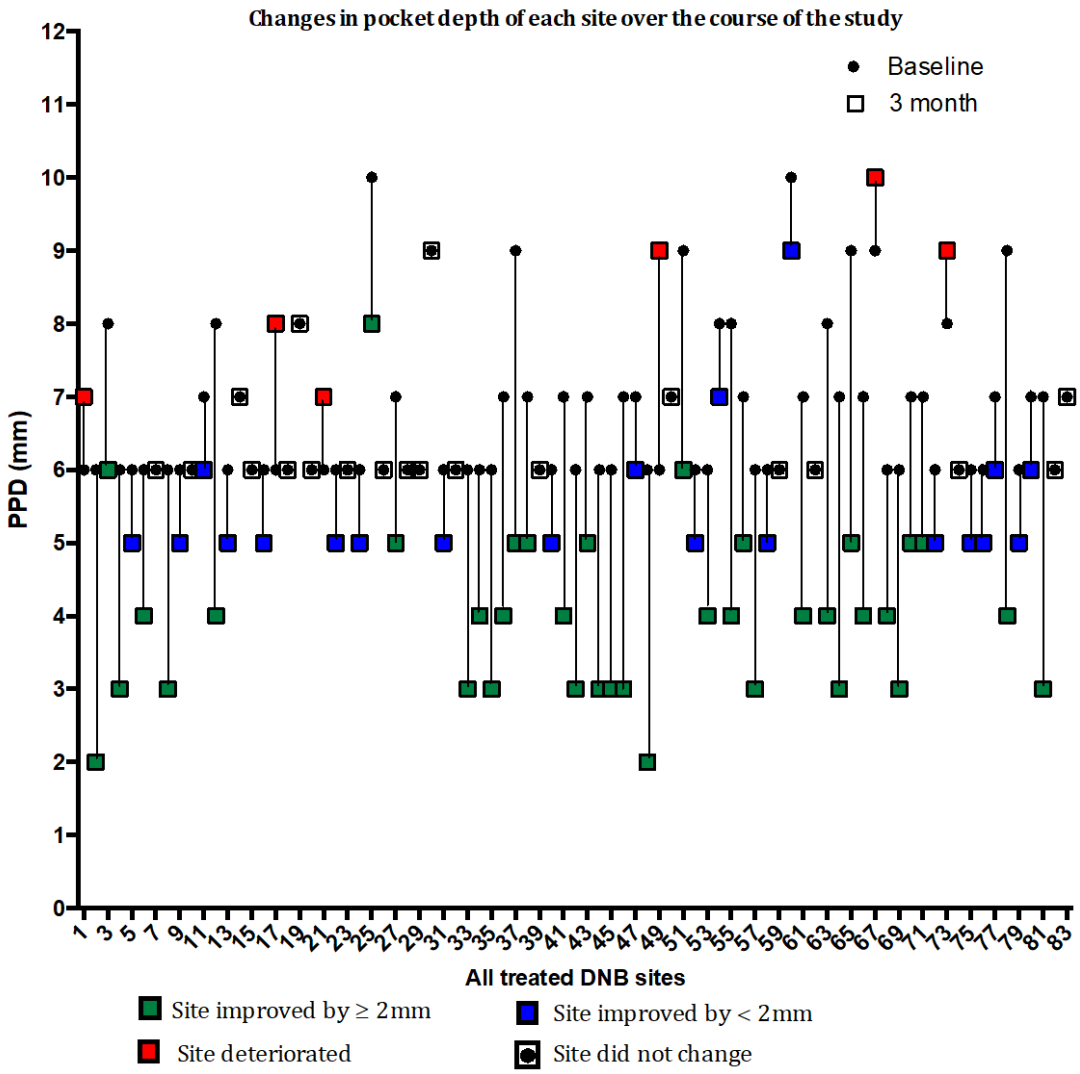


Figure 3.7. Changes in PPD of each DNB site from baseline to the 3 month time point.

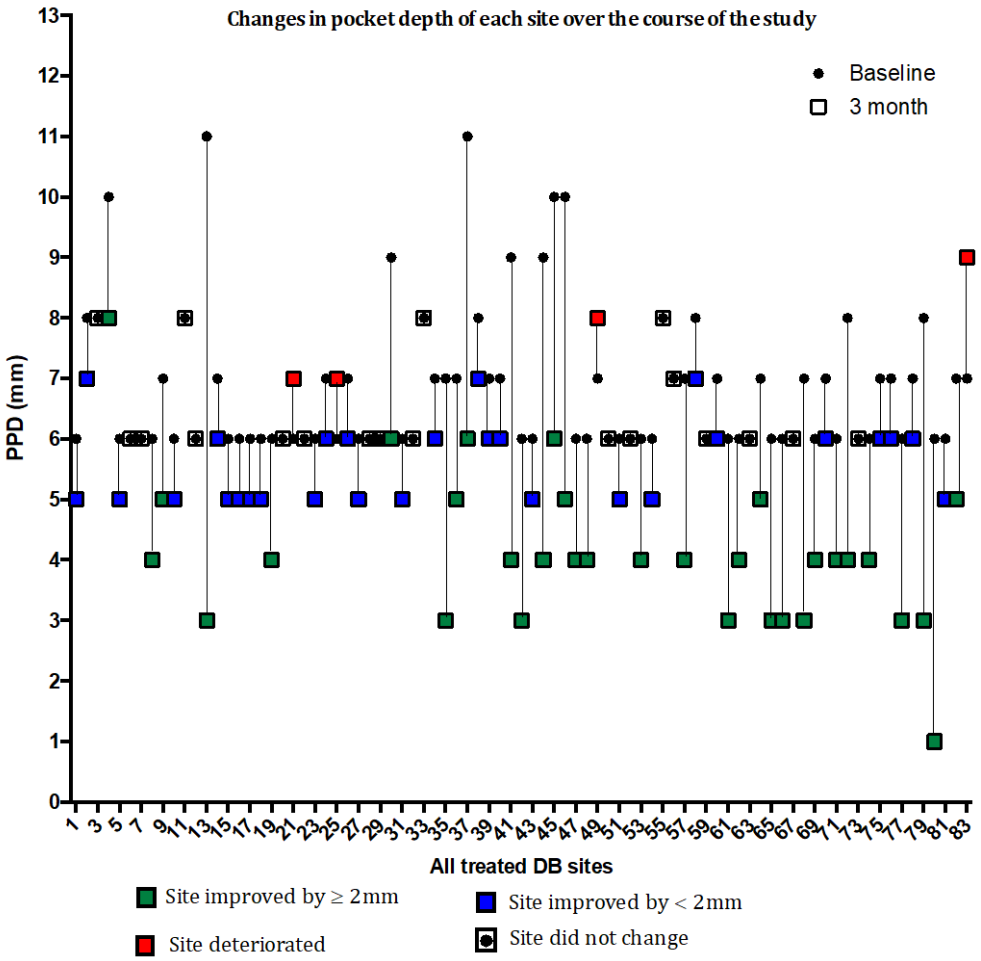


Figure 3.8. Changes in PPD of each DB site from baseline to the 3 month time point.

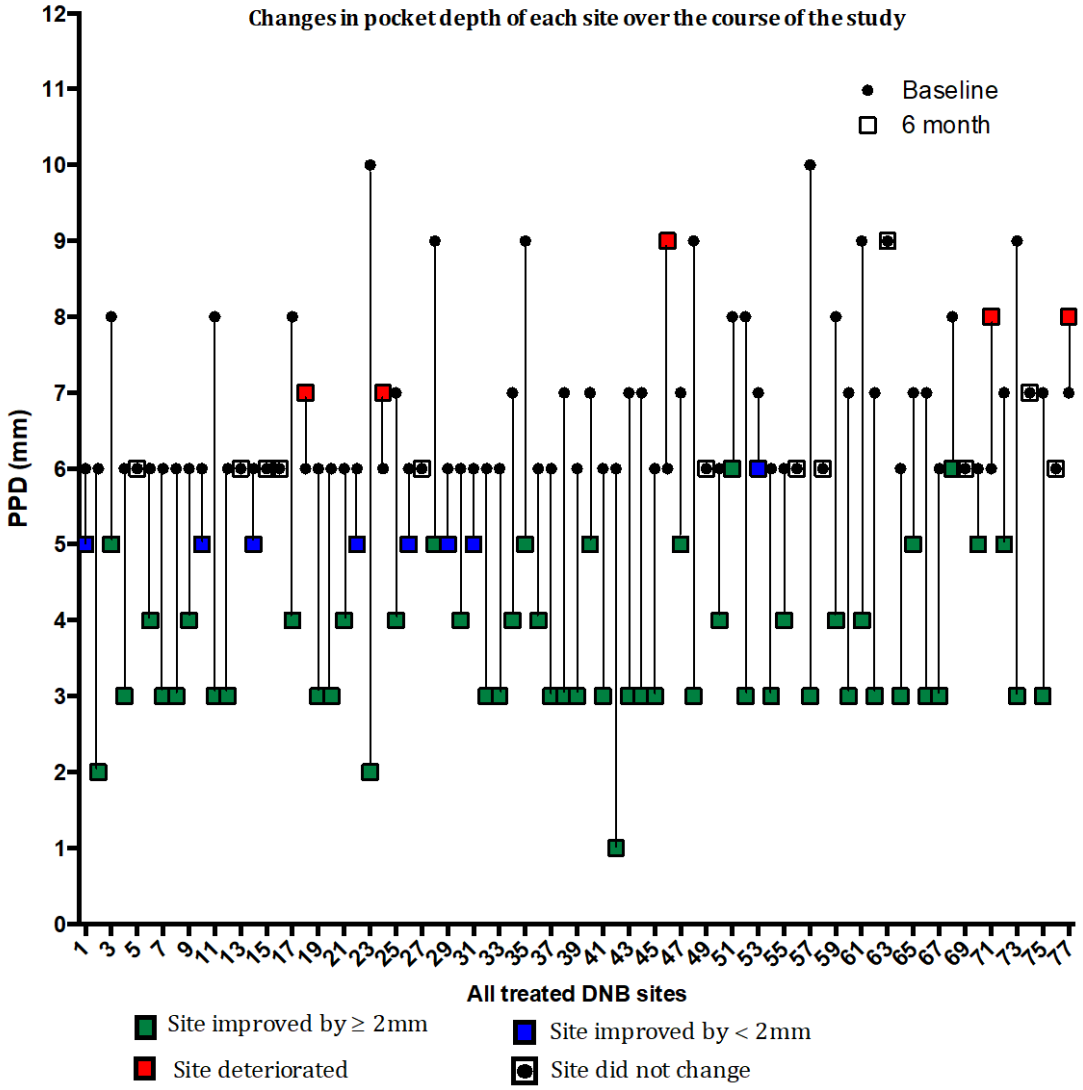


Figure 3.9. Changes in PPD of each DNB site from baseline to the 6 month time point.

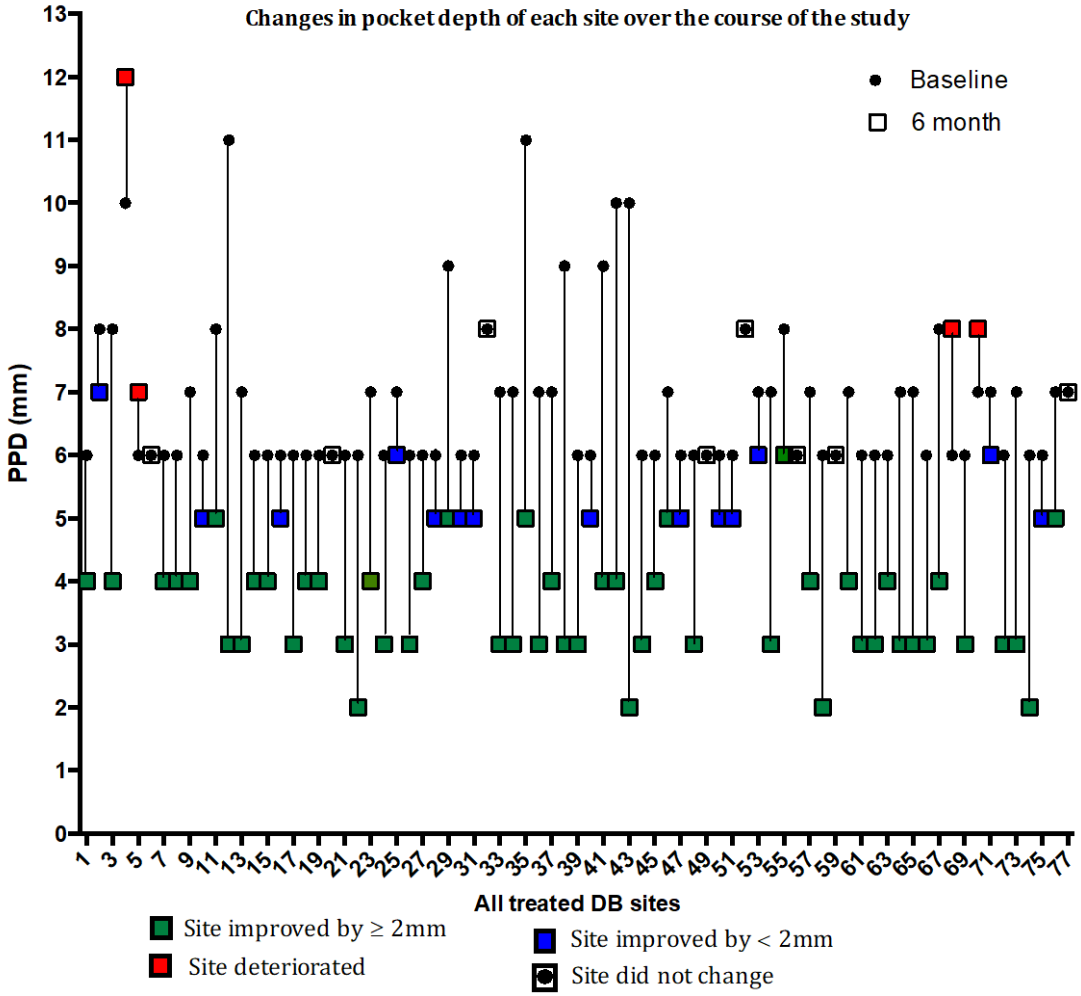


Figure 3.10. Changes in PPD of each DB site from baseline to the 6 month time point.

Comparing the changes in CAL measurements over the study period, from baseline to 3 months, the mean change in CAL at DNB sites was 0.4mm (from 6.66± 2.88 mm to 6.19±2.79 mm) but this change was not statistically significant (p =0.35). Similarly, at DB sites and for the same time period, the average change in CAL was 0.15mm (from 6.44±2.84 mm to 6.35± 2.63 mm) and again it was not statistically significant (p= 0.7). At 6 months post treatment, further gains in attachment were achieved at both DNB (5.75±2.8 mm) and DB (5.75± 2.88 mm) sites. However, these differences were again not statistically significant (p= 0.059, 0.083, respectively) (Figure 3.11).

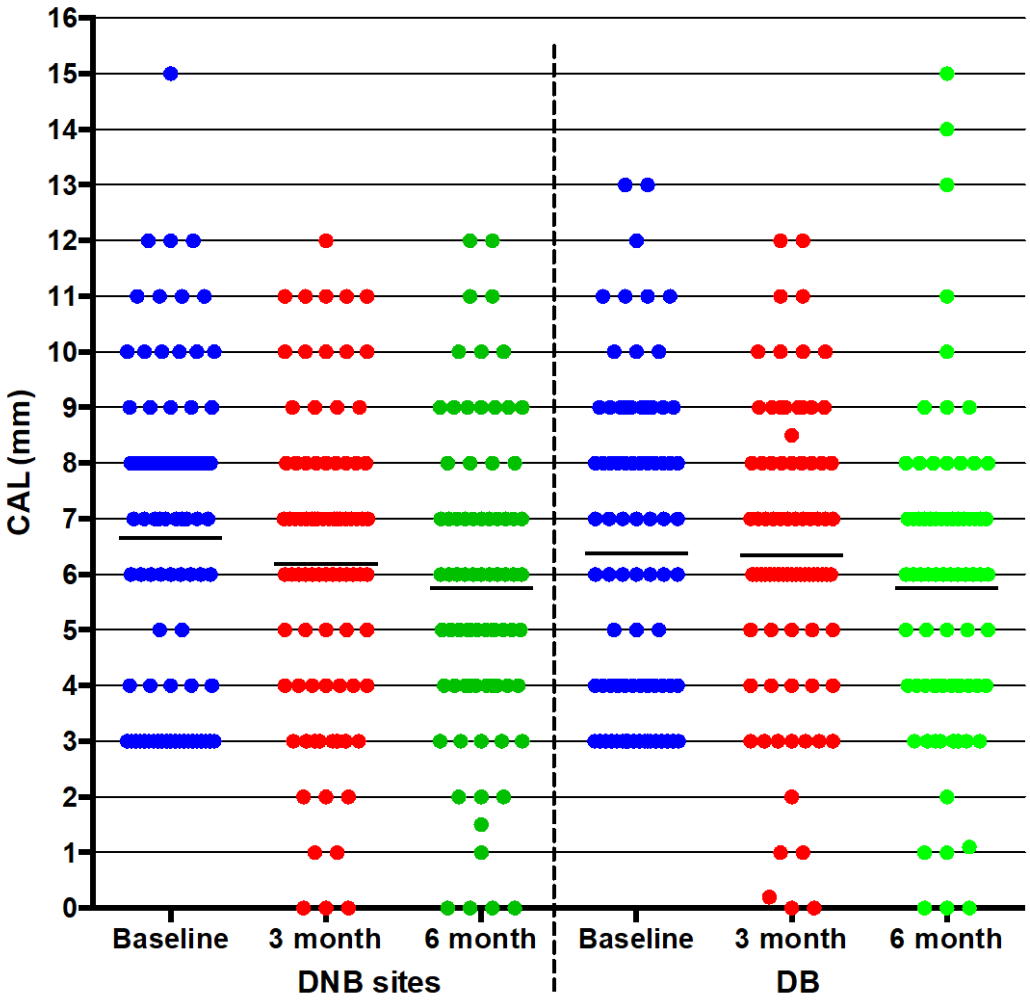


Figure 3.11. Changes in CAL throughout the course of the study in DNB and DB sites.

As described earlier (3.1 GCF volume determination) the Periotron reading and weighing methods were used to determine the volume of GCF in clinical samples. The majority of the GCF volumes were within the range of the Periotron machine (i.e. <1.7µl), and the GCF volumes of only 38 samples out of 747 samples had to be determined by the weighing method. Generally, GCF volume was also detected at significantly higher levels in both DNB (1.25±0.44µl) and DB (1.32±0.44µl) sites than in healthy sites (0.34µl) at baseline (P= 0.0001). The first cycle of treatment resulted in significant reduction of mean GCF volume to 0.92µl and 1.01µl for DNB and DB sites, respectively. Further reductions were observed after the second cycle of treatment in that the mean GCF volume decreased to 0.77µl at DNB sites and 0.85µl in DB sites (p= 0.0001). However,

on a site specific basis, there were still high levels of GCF in both types of diseased sites (Figure 3.12).

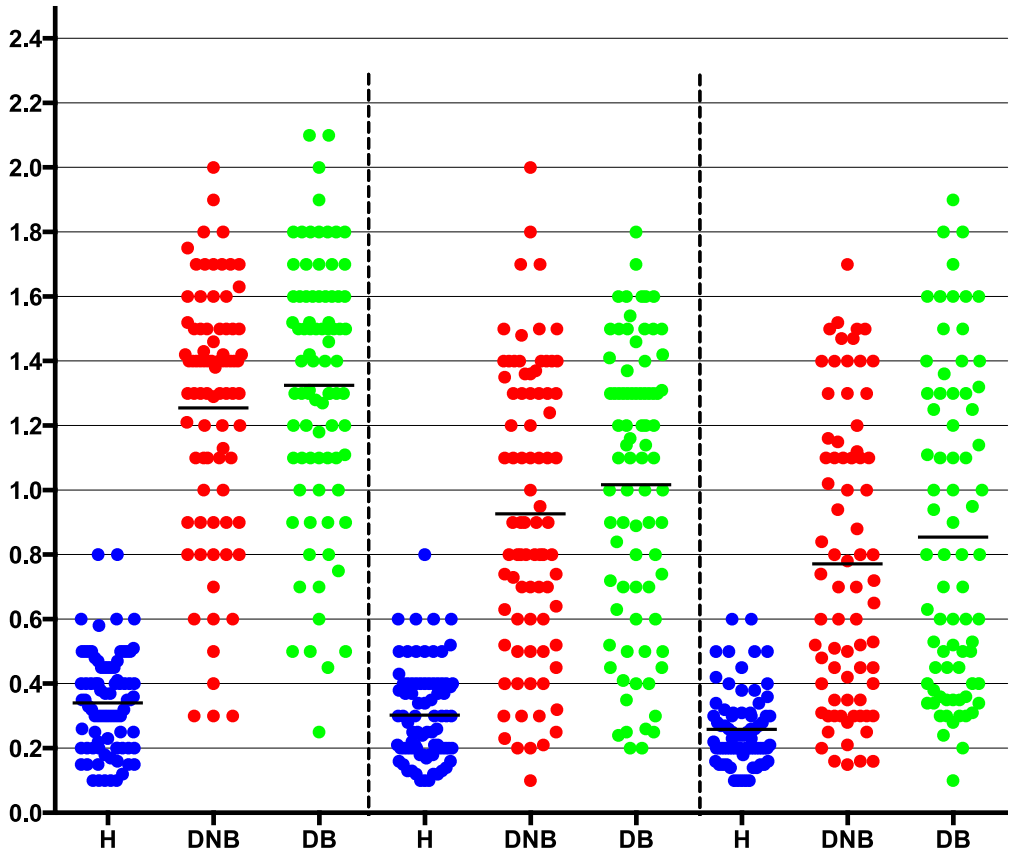


Figure 3.12. Changes in GCF volume throughout the course of the study in sampled sites.

3.8 Enzyme levels at study time points

After collecting and performing the enzyme assays for all clinical samples (747 GCF samples) from the 77 patients who completed the study, most of the biomarkers were detected in the majority of samples from diseased sites. However, in healthy sites these enzymes were detected less commonly. The amount of each enzyme was calculated and the data are expressed as ng/µl, which is calculated from the volume measure as described in section 2.12.

Chapter 3- Results

Generally, the levels of investigated enzymes were greater at diseased sites (DNB and DB sites) than at healthy sites. The Kruskal-Wallis test for the five diagnostic variables revealed significant differences in the average levels of DNB and DB sites compared to healthy sites. Tables 3.5 and 3.6 as well as figures 3.13 - 3.17 which illustrate the enzyme levels in all sampled sites at the different study time points. Comparing diseased sites at baseline, there were no statistically significant differences in any of the enzyme levels in the DB and DNB sites (Table 3.5). However, on an individual basis, there were patients with statistically higher levels of these enzymes in DNB sites than DB sites and vice versa.

Table 3.5. Comparison of median levels of five enzymes (ng/µl) in 3 selected sites at baseline.

Variable	Baseline	P value* (Health vs DNB)	P value* (Health vs DB)	P value* (DNB vs DB)
MMP8 (Health)	22.2	**0.0001**	**0.0001**	0.095
MMP8 (DNB)	191.5			
MMP8 (DB)	225.8			
Elastase (Health)	10.5	**0.0001**	**0.0001**	0.14
Elastase (DNB)	169.6			
Elastase (DB)	199			
Cathepsin G (Health)	7.2	**0.0001**	**0.0001**	0.17
Cathepsin G (DNB)	15.3			
Cathepsin G (DB)	14			
Trypsin like (Health)	0.18	**0.0001**	**0.0001**	0.72
Trypsin like (DNB)	30.5			
Trypsin like (DB)	29.2			
Sialidase (Health)	0.1	**0.0001**	**0.0001**	0.27
Sialidase (DNB)	21.8			
Sialidase (DB)	30.1			

*Kruskal Wallis-test

After one cycle of treatment and at the 3 month time point, the Kruskal-Wallis test revealed that only MMP8, elastase and sialidase biomarkers had significant reductions in their levels compared to baseline (except elastase in DNB sites). By 6 months, continued

significant reductions of all enzymes (except trypsin-like activity in DB sites) were noticed. Furthermore, all enzyme levels (except trypsin like and sialidase in DB sites) were also significantly reduced at the 6 month compared to the 3 month time point. Overall, all biomarkers decreased through the course of the study but MMP8, elastase and sialidase showed the greatest reductions (Table 3.6).

Spearman's correlation analyses between all biomarkers versus PPD at baseline and PPD at 6 months were performed and significant correlations ($p< 0.05$) were shown with MMP8, elastase and sialidase. The levels of MMP8, elastase and sialidase at baseline significantly correlated with the initial PPD as follows: MMP8 ($r= 0.58$), elastase ($r=0.51$) and sialidase ($r=0.5$). Similarly, the levels of these three enzymes at baseline significantly correlated with treatment outcome at the 6 month review and the biomarkers were ordered from greatest to lowest correlation value as follows: MMP8 ($r= -0.61$), elastase ($r= -0.59$) and sialidase ($r= -0.55$), giving a ranking of significance for each biomarker. Further to this, there were also statistically significant correlations between MMP8, elastase and sialidase (Table 3.7).

Table 3.6. Comparison of median levels of five enzymes (ng/µl) in diseased sites at all study time points.

Variable	Baseline	3 month	P value* (baseline vs 3 month)	6 month	P value* (3 month vs 6 month)	P value* (baseline vs 6 month)
MMP8 (DNB)	191.5	96.4	**0.02**	21.2	**0.001**	**0.0001**
MMP8 (DB)	225.8	77.8	**0.001**	51.7	**0.03**	**0.0001**
Elastase (DNB)	169.6	93.2	0.16	48.3	**0.02**	**0.001**
Elastase (DB)	199	110.6	**0.01**	63.1	**0.01**	**0.001**
Cathepsin G (DNB)	15.3	19.2	0.5	6.6	**0.01**	**0.02**
Cathepsin G (DB)	14	18.2	0.2	6.6	**0.01**	**0.03**
Trypsin like (DNB)	30.5	33.1	0.9	20.6	**0.03**	**0.04**
Trypsin like (DB)	29.2	31.5	0.9	20.6	0.06	0.1
Sialidase (DNB)	21.8	7.1	**0.001**	1.4	**0.02**	**0.001**
Sialidase (DB)	30.1	6.5	**0.001**	3.3	0.4	**0.001**

* Kruskal-Wallis test

Table 3.7. Spearman's correlation analyses between all biomarkers and PPD.

Variable	MMP8	Elastase	Cathepsin G	Trypsin-like	Sialidase
MMP8		**0.48**	0.18	0.3	**0.5**
Elastase			0.24	0.21	**0.53**
Cathepsin G				0.22	0.17
Trypsin-like					0.28
PPD (Baseline)	**0.58**	**0.51**	0.2	0.32	**0.5**
Treatment outcome (6 month)	**-0.61**	**-0.59**	-0.16	-0.25	**-0.55**

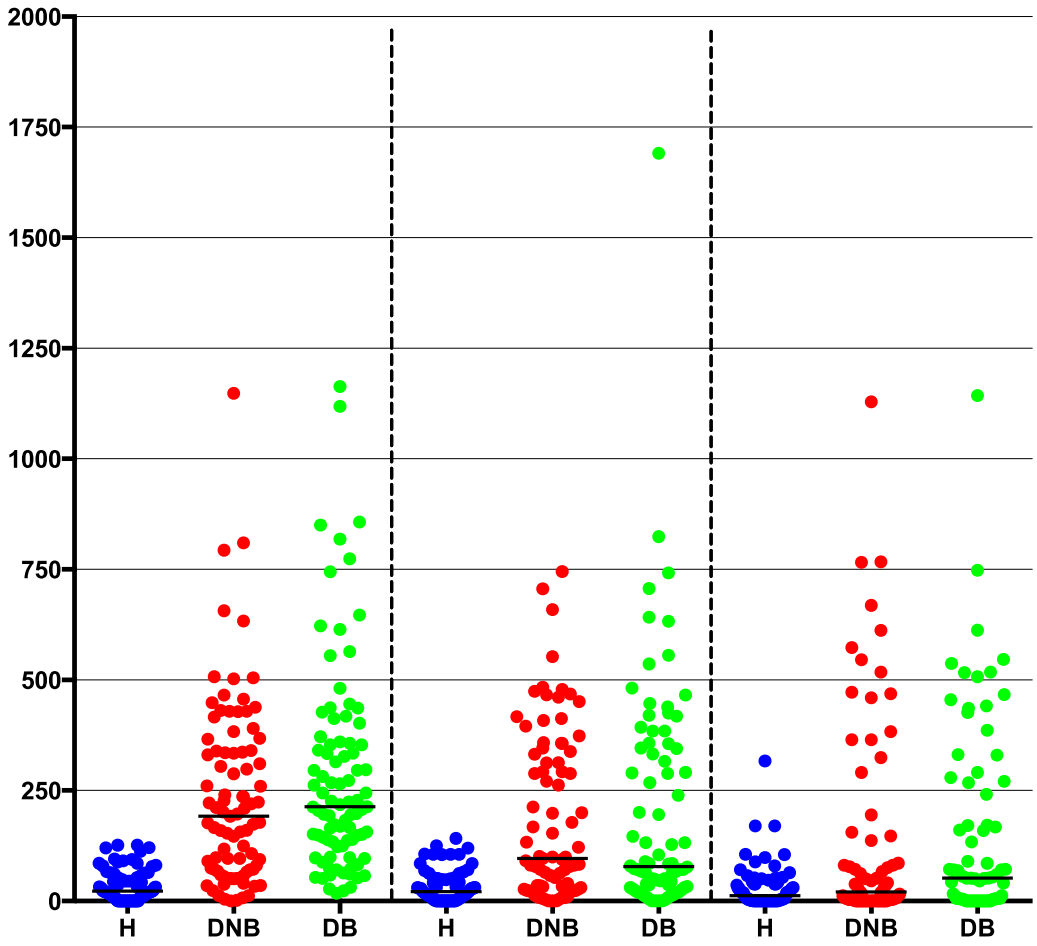


Figure 3.13. Scatter plot of MMP8 in representative sites at different study time points. (Horizontal bar= median, H= healthy site, DNB= deep non bleeding and DB= deep bleeding).

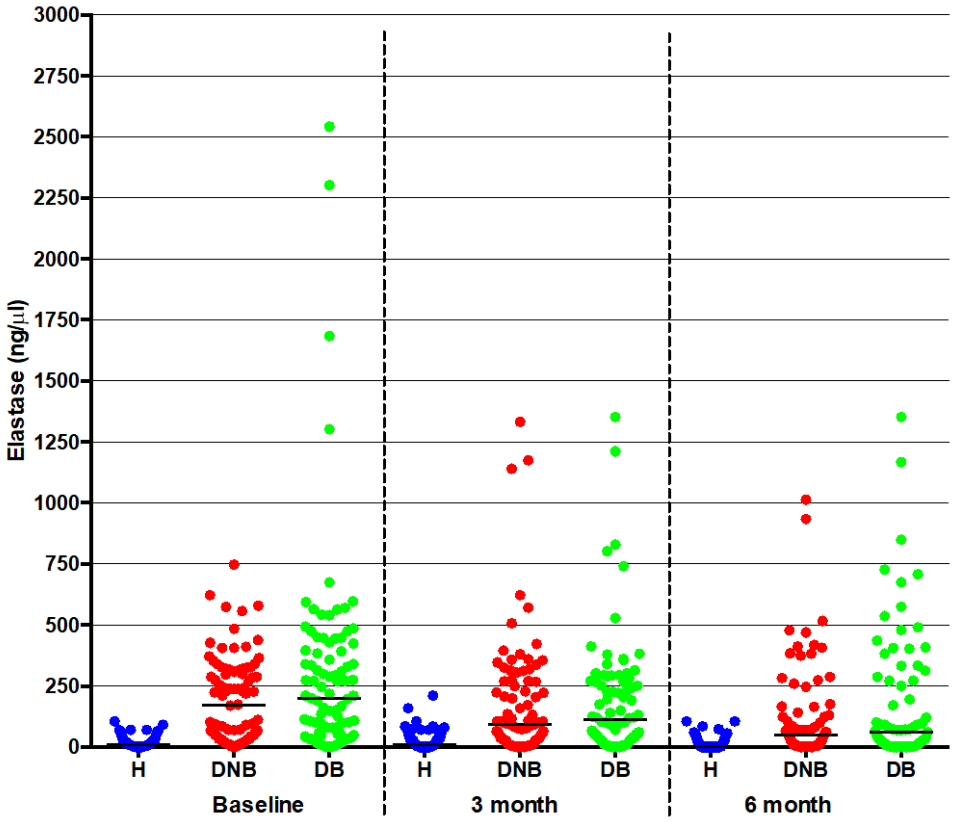


Figure 3.14. Scatter plot of elastase in representative sites at different study time points.

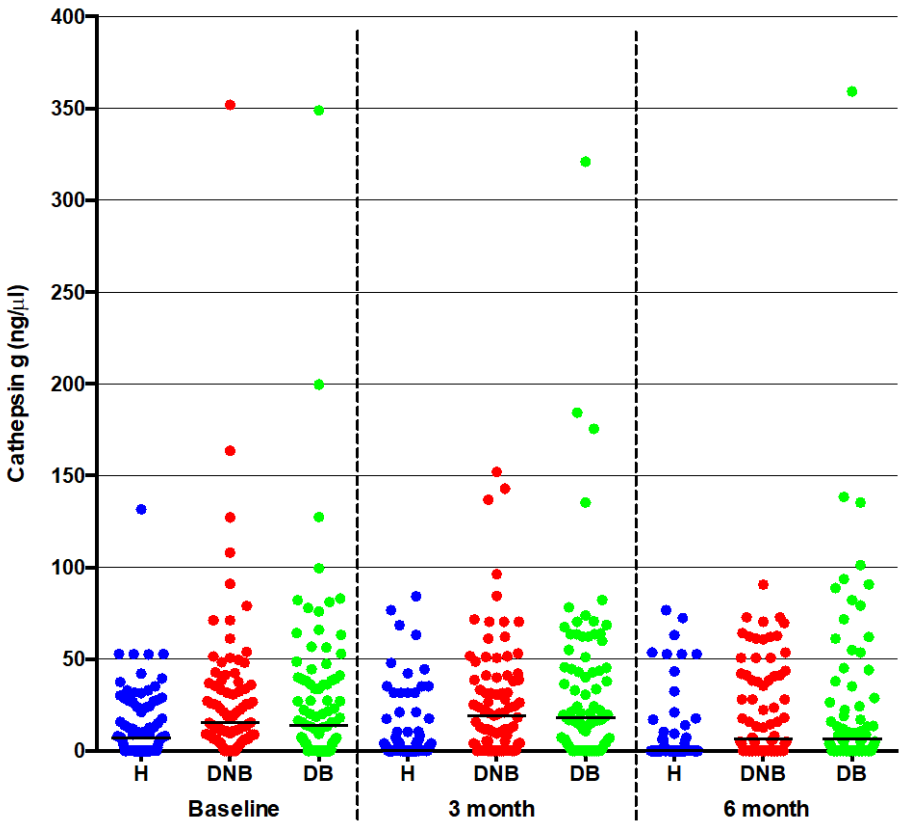


Figure 3.15. Scatter plot of cathepsin G in representative sites at different study time points.

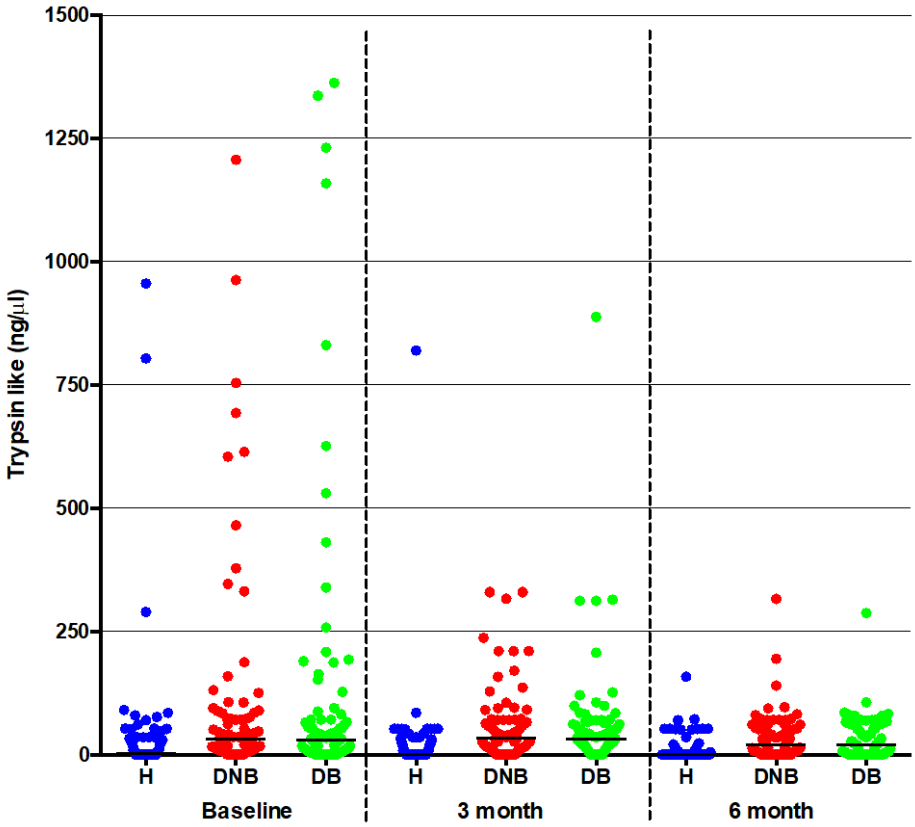


Figure 3.16. Scatter plot of trypsin-like activity in representative sites at different study time points.

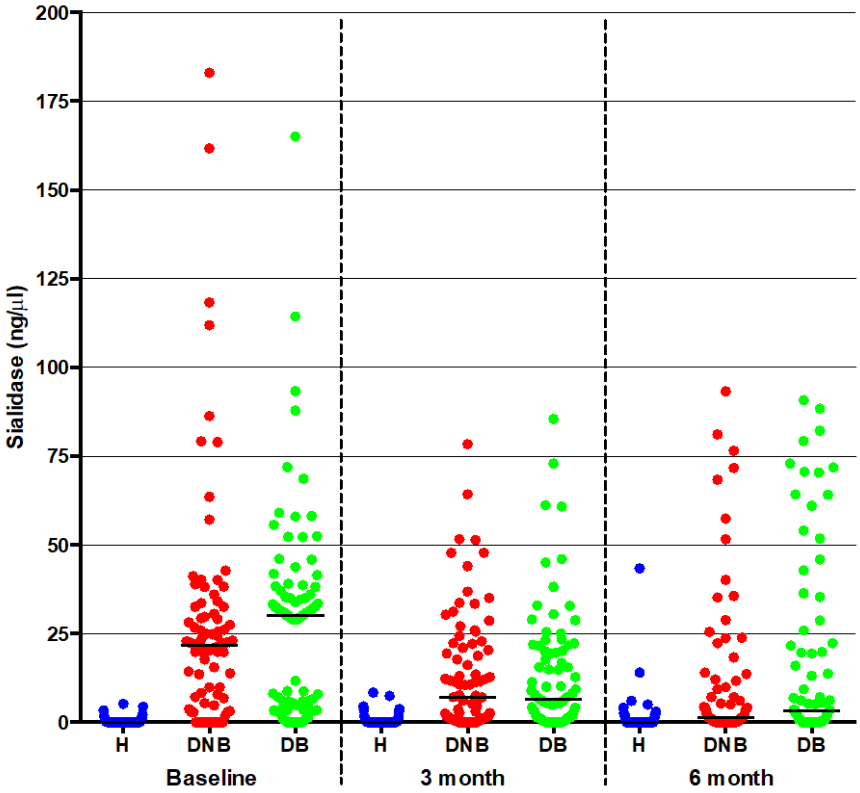


Figure 3.17. Scatter plot of sialidase in representative sites at different study time points.

3.9 Effect of antibiotics on clinical and laboratory measures

Among the patients who completed the study, antibiotics were prescribed for 17 (22%) of them that had a poor response to treatment at the 3 month time point. At baseline, there was no statistically significant difference between clinical measures of the full mouth or sites from which GCF was sampled in the patients who received antibiotics and those that did not (except BOP and PPD≤3mm). However, in those not receiving antibiotic initial treatment resulted in significant improvement in all full mouth clinical measures except for the plaque index when compared to antibiotic group (Table 3.8). Furthermore, PPD in DNB sites demonstrated significant reduction (P= 0.01), but not for DB sites. The second cycle of the treatment plus antibiotic therapy (at the 3 month time point) in those patients having a poor response to root surface debridement resulted in further improvement in full mouth clinical measures by the 6 month review. For example, the mean percentage of sites with PPD ≤3mm was increased by 22% (from 55±15 at 3 month to 77±13 at 6 month time point). Interestingly, pocket depths of ≥ 6mm appeared to gain more benefit

from the antibiotic as the mean percentage of sites with PPD depth ≥ 6 mm was reduced from 14.9±8.6 to 1.8±2.9%, which was statistically significantly lower than in patients who did not receive antibiotic (4.02±4.3%, p= 0.02) (Table 3.8).

The average PPD of both DNB and DB sites demonstrated no statistically significant difference at six month between patients who received antibiotics and patients who did not receive antibiotics. At a site-specific level, 82% of patients who received an antibiotic (28 out of 34 sites) showed ≥2mm improvement of PPD in both DNB and DB sites, which was a statistically significantly higher result than for patients not receiving antibiotic (74 out of 120) (61% presented ≥2mm improvement in both DNB and DB sites).

Similarly, at baseline there was no statistically significant difference in median levels of any of the enzymes tested except cathepsin G and elastase in DNB sites. Whereas at the 6 month time point, using the Mann Whitney test, a statistically significant difference was observed in median levels of MMP8, elastase and sialidase at both representative sites between patients who received antibiotics and those who did not receive antibiotics (Table 3.9).

Considering the enzyme responses during the 6 month period of the study, comparison of the baseline enzyme values with those seen at 3 months in patients that did not receive antibiotic (non-antibiotic group), it was noted that there was a significant reduction in MMP8 and sialidase (both DB and DNB sites) but not in elastase. In contrast, there was no significant change in any enzyme level by 3 months in those that went on to receive antibiotic (antibiotic group) (Table 3.10). It should be noted though that this group had not received their antibiotic therapy at that stage; they did so immediately after the 3 month review. Comparing baseline enzyme levels with those found at 6 months showed that both antibiotic group and non antibiotic groups had a significant reduction in MMP8, elastase and sialidase at both DB and DNB sites (except elastase DNB in non antibiotic group). Furthermore, MMP8, elastase and sialidase levels continued to fall between 3 and 6 months in DB and DNB sites in the antibiotic group but there was not further change in enzyme levels over that period in the non-antibiotic group (Table 3.10).

Table 3.8. Comparison of full mouth and site specific clinical data between antibiotic and non- antibiotic group.

	Variable	Baseline		p value* (Antibiotic vs non-antibiotic at baseline)	3 months		p value* (Antibiotic vs non-antibiotic at 3 month)	6 months		p value* (Antibiotic vs non-antibiotic at 6 month)
		Non antibiotic n=60	Antibiotic n=17		Non antibiotic n=60	Antibiotic n=17		Non antibiotic n=60	Antibiotic n=17	
Full mouth data (% sites ± sd)	PI	58±27	67± 23	0.14	37± 26	47±28	0.21	31± 23	42±28	0.16
	BOP	20± 17	27±11	**0.03**	9.8 ± 9.6	34±26	**0.001**	7.9± 11.5	7.03±7.4	0.69
	PPD ≤ 3mm	69± 12	61±15	**0.02**	77±12	55±15	**0.0001**	82±10	77±13	0.18
	PPD 4-5 mm	17±16	21 ±10	0.28	16 ± 8.7	29±12	**0.0001**	13.3 ± 7.8	20.1±11.2	**0.02**
	PPD ≥ 6 mm	14± 8	17± 9	0.17	5.7±5.1	14.9±8.6	**0.0001**	4.02±4.3	1.8±2.9	**0.02**
Site specific data (mean ± sd)	PPD mm (DNB)	6.7±1.1	6.6±0.9	0.4	4.9±1.5	6.1±1.7	**0.01**	4.3±1.6	4.5±1.8	0.7
	PPD mm (DB)	6.8± 1.2	6.7± 1.02	0.6	5.2±1.5	5.6±1.2	0.19	4.6±1.8	3.9±1.06	0.06

* t-test

Table 3.9. Comparison of site specific median enzyme levels (ng/µl) between patients receiving antibiotic treatment (n= 17) and those that did not (non- antibiotic group, n=60).

Variable (ng/µl)	Baseline		P value* (Antibiotic vs non-antibiotic at baseline)	3 month		P value* (Antibiotic vs non-antibiotic at 3 month)	6 month		P value* (Antibiotic vs non-antibiotic at 6 month)
	Non antibiotic	Antibiotic		Non antibiotic	Antibiotic		Non antibiotic	Antibiotic	
MMP8 (DNB)	211	156	0.5	46	196	**0.043**	43	3	**0.003**
MMP8 (DB)	204	232	0.8	32	233	**0.01**	70	4	**0.002**
Elastase (DNB)	84	254	**0.03**	83	221	**0.004**	62	3	**0.003**
Elastase (DB)	160	217	0.3	93	199	0.13	72	1	**0.0001**
Cathepsin G (DNB)	13	26	**0.03**	19	15	0.98	7	6	0.6
Cathepsin G (DB)	14	15	0.7	20	15	0.19	7	7	0.8
Trypsin like (DNB)	30	18	0.9	29	40	0.44	14	30	0.8
Trypsin like (DB)	24	42	0.5	28	35	0.72	21	12	0.9
Sialidase (DNB)	20	25	0.08	4.5	20	**0.01**	3	0.15	**0.004**
Sialidase (DB)	29	30	0.5	4	15	0.35	5	0.1	**0.002**

* Mann Whitney test.

Table 3.10. Further comparison of site specific median enzyme levels (ng/µl) between patients receiving antibiotic treatment (n= 17) and patients that did not (non- antibiotic group, n= 60).

Variable	Baseline		3 month		P value* Non antibiotic (baseline vs 3 month)	P value* Antibiotic (baseline vs 3 month)	6 month		P value* Non antibiotic (baseline vs 6 month)	P value* Antibiotic (baseline vs 6 month)	P value* Non antibiotic (3 vs 6 month)	P value* Antibiotic (3 vs 6 month)
	Non antibiotic	Antibiotic	Non antibiotic	Antibiotic			Non antibiotic	Antibiotic				
MMP8 (DNB)	211	156	46	196	**0.0001**	0.9	43	3	**0.0001**	**0.0001**	0.8	**0.0001**
MMP8 (DB)	204	232	32	233	**0.0001**	0.45	70	4	**0.0001**	**0.0001**	0.4	**0.0001**
Elastase (DNB)	84	254	83	221	0.13	0.69	62	3	0.19	**0.0001**	0.7	**0.0001**
Elastase (DB)	160	217	93	199	0.13	0.61	72	1	**0.02**	**0.0001**	0.9	**0.0001**
Cathepsin G (DNB)	13	26	19	15	0.65	0.29	7	6	0.25	0.13	0.1	0.33
Cathepsin G (DB)	14	15	20	15	0.13	0.46	7	7	0.44	0.37	0.016	0.13
Trypsin like (DNB)	30	18	29	40	0.84	0.34	14	30	0.1	0.32	0.07	0.13
Trypsin like (DB)	24	42	28	35	0.85	0.69	21	12	0.17	0.57	0.08	0.42
Sialidase (DNB)	20	25	4.5	20	**0.002**	0.79	3	0.15	**0.002**	**0.0001**	0.46	**0.0001**
Sialidase (DB)	29	30	4	15	**0.009**	0.61	5	0.1	**0.031**	**0.004**	0.84	**0.009**

* Kruskal-Wallis

3.10 Diagnostic Value of the enzymes

The aim of using diagnostic biomarkers is to determine the cut-off point that differentiates healthy sites from diseased sites. Using the area under the curve (AUC) from the receiver-operating characteristic (ROC) curves, as described in the statistical methods (section 2.14.3), enabled us to evaluate the ability of biomarkers in GCF to identify whether the site is diseased or healthy (Figure 3.18). Each threshold point characteristically has its own degree of sensitivity and specificity. In this study, the threshold points with the highest sensitivity (positive prediction value) and specificity (negative prediction value) were selected for each of the enzymes at baseline (Table 3.11). In general, MMP8 elastase and sialidase were demonstrated to be more reliable diagnostic biomarkers as they differentiated healthy sites from diseased sites with sensitivity and specificity values above 78% at threshold points of 94ng/µl for MMP8 (AUC= 0.92, 95% CI= 0.89 to 0.95, P< 0.001), 33ng/µl for elastase (AUC= 0.87, 95% CI= 0.83 to 0.91, P< 0.001) and 2.3ng/µl for sialidase (AUC= 0.79, 95% CI= 0.72 to 0.83, P< 0.001). Meanwhile, the sensitivity and specificity percent of trypsin-like enzyme and cathepsin G ranged from 60%-69%.

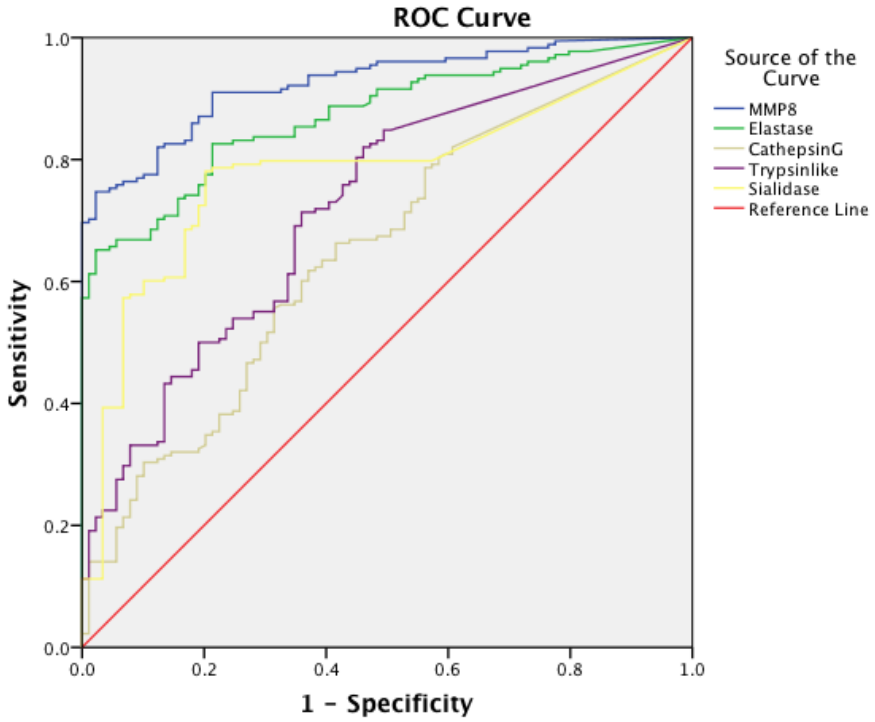


Figure 3.18. ROC for all tested biomarkers at baseline differentiating between healthy and periodontitis sites.

Table 3.11. Diagnostic properties of specific thresholds of the five GCF enzymes.

Variable	Threshold (ng/µl)	Sensitivity%/ Specificity%	Area under the curve	95% CI for OR		P value
				LCL	UCL	
MMP8	94	86/83	0.92	0.89	0.95	**0.0001**
Elastase	33	78/80	0.87	0.83	0.91	**0.0001**
Cathepsin G	10.7	64/60	0.65	0.58	0.71	**0.0001**
Trypsin like	11.2	65/69	0.72	0.65	0.78	**0.0001**
Sialidase	2.3	79/79	0.79	0.72	0.83	**0.0001**

3.11 Usefulness of the enzyme profile as a prognostic tool

As was explained in the previous section, most of the improvement (≥2mm) in PPD was identified at the 6 month time point of the study and that is why the regression analysis was conducted against the outcome of treatment at 6 months. In order to evaluate the usefulness of the enzyme profile as a prognostic tool that can predict treatment outcome, logistic regression was performed with baseline enzyme levels (as independent variables) versus ≥2mm improvement of PPD at the 6 month time point (as dependent variable). The analyses revealed that when all enzymes were combined in the analysis, they were able to predict treatment outcome with certainty of 81.3% for DNB sites and 80.3% for DB sites. Backward stepwise logistic regression was used to exclude the variables that could not add any additional significant prediction value to the whole combination and it was found that cathepsin G and trypsin-like activity were redundant variables ($p>0.05$) (Tables 3.12 and 3.13). The final model included MMP8, elastase and sialidase as significant predictors ($p <0.05$) of treatment outcome, with recording an odds ratio of 0.995, 0.994 and 0.998 in DNB sites and recording an odds ratio (OR) of 0.995, 0.995 and 0.994 in DB sites for MMP8, elastase and sialidase respectively (Table 3.13). On the other hand, all enzyme combinations other than MMP8, elastase and sialidase were assessed to identify the best enzyme profile combination and none of the enzyme profile combinations tested demonstrated prediction values greater than MMP8, elastase and sialidase. Furthermore, it was also found that one enzyme alone was not able to predict treatment outcome at a level greater than the null hypothesis (61% in DNB and 62.5% in DB sites).

Table 3.12. Logistic regression analysis with 2mm PPD improvement (at 6 months) as the dependent variable.

Method	DNB sites	DB sites
	Predictive	Predictive
All variables	81.3%	80.3%
Stepwise (backward conditional)	81.3% (MMP8, elastase, sialidase, trypsin)	80.3% (MMP8, elastase, sialidase, cathepsin g)
	81.3% (MMP8, elastase, sialidase)	**80.3% (MMP8, elastase, sialidase)**
	61% (each single enzyme)	62.5% (each single enzyme)

Table 3.13. Summary of logistic regression for each individual explanatory variable for site response to treatment by the following 6 months.

Disease sampled site	Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR		p value
				LCL	UCL	
DNB	MMP8	-0.005	0.995	0.99	1.3	**0.006**
	Elastase	-0.006	0.994	0.99	1.2	**0.002**
	Cathepsin G	0.0001	1	0.99	1	0.74
	Trypsin like	0.0001	1	1	1	0.67
	Sialidase	-0.002	0.998	0.99	1.2	**0.03**
DB	MMP8	-0.005	0.995	0.99	1.3	**0.007**
	Elastase	-0.005	0.995	0.99	1.3	**0.001**
	Cathepsin G	0.001	1	0.99	1.005	0.38
	Trypsin like	0.0001	1	1	1	0.74
	Sialidase	-0.006	0.994	0.99	1.3	**0.001**

Finally, there was no statistically significant difference in treatment outcome (>2mm improvement in PPD) between DNB and DB sites at the 6 month time point. Logistic regression analysis for both sites was performed as described in section 3.11, regardless of the presence or absence of bleeding at diseased sampled sites at baseline. The analysis showed that combination profiles of all enzymes can predict treatment outcome with certainty of 80.5%. Similarly, cathepsin G and trypsin-like activity could not add

additional significant predictive value to the whole enzyme profile combination (Table 3.14). MMP8, elastase and sialidase again demonstrated a significant contribution to the final model in predicting treatment outcome at the 6 month time point, as shown in Table 3.15.

Table 3.14. Logistic regression analysis with 2mm PPD improvement (at 6 months) as the dependent variable for both DNB and DB sites combined.

Method	**DNB and DB sites**
	Predictive
All variables	80.5%
Stepwise (backward conditional)	80.5%(MMP8, elastase, sialidase, trypsin)
	80.5% (MMP8, elastase, sialidase)
	61% (each single enzyme)

Table 3.15. Summary of logistic regression for each individual explanatory variable for site response to treatment in the following 6 months for both DNB and DB sites combined.

Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR		p value
			LCL	UCL	
MMP8	-0.005	0.995	0.99	1.3	**0.006**
Elastase	-0.006	0.994	0.99	1.3	**0.002**
Cathepsin G	0.0001	1	0.99	1	0.54
Trypsin like	0.0001	1	1	1	0.68
Sialidase	-0.005	0.995	0.99	1.3	**0.005**

In addition, the median levels of each GCF biomarker at baseline for sites that responded by ≥2mm change in PPD compared with sites that responded by less than 2mm change in PPD at the 6 month review, were assessed after two cycles of the treatment and the data are shown in table 3.16. Among the GCF biomarkers, MMP8, elastase and sialidase demonstrated statistically significant differences between the respondent and non-

respondent groups, indicating statistically significantly higher levels of MMP8, elastase and sialidase in sites that responded by less than 2 mm in PPD (Table 3.16). Cathepsin G and trypsin-like activity showed no statistical differences between the two groups. Consequently increased levels of MMP8, elastase and sialidase demonstrated significant ability to predict the outcome of treatment.

Table 3.16. Analysis of baseline median enzyme levels (ng/µl) in respondent (n= 102) versus non-respondent (n= 52) sites.

Disease sampled site	Enzyme (Baseline)	Respondent (median)	Non-Respondent (median)	p value*
DNB sites	MMP8	122	231	**0.003**
	Elastase	68	307	**0.001**
	Cathepsin G	16	15	0.4
	Trypsin like	30	21	0.4
	Sialidase	10	26	**0.001**
DB sites	MMP8	138	352	**0.001**
	Elastase	53	447	**0.001**
	Cathepsin G	16	6.3	0.23
	Trypsin like	16	28	0.11
	Sialidase	4.2	35	**0.001**

* Mann Whitney test

3.12 Effect of antibiotic therapy on periodontal treatment outcome and prediction value of biomarkers

As described in section 3.9, 17 out of 77 patients who completed the study received antibiotics and it was found that the antibiotic prescription improved the sites' response to the non-surgical periodontal treatment. To determine the effect of antibiotics on prediction values, regression analysis was performed for the patients who did not receive an antibiotic (60 patients) and the result showed that when the participants who received an antibiotic were excluded from the analysis the prediction value increased to 88% in DNB and 90% in DB (Table 3.17). Additionally, the effects (β) of MMP8, elastase and

sialidase increased in terms of outcome of the treatment, with sialidase demonstrating the greatest increase, from -0.002 and -0.006 to -0.03 and -0.02 in DNB and DB sites, respectively (Table 3.18). Similarly, cathepsin G and trypsin-like activity added no predictive value to the enzyme profile combination (Table 3.17). Furthermore, as before no single enzyme could alone predict the outcome of treatment at a level higher than the null hypothesis (58% for DNB and 58.3% for DB sites).

Table 3.17. Logistic regression analysis with 2mm PPD improvement (at 6 months) as the dependent variable for patients (n= 60) who did not receive antibiotics.

Method	**DNB sites**	**DB sites**
	Predictive	**Predictive**
All variables	88%	90%
Stepwise (backward conditional)	88% (MMP8, elastase, sialidase, trypsin)	90% (MMP8, elastase, sialidase, cathepsin G)
	88% (MMP8, elastase, sialidase)	**90% (MMP8, elastase, sialidase)**
	58% (each single enzyme)	58.3 % (each single enzyme)

Table 3.18. Summary of logistic regression for each individual explanatory variable for site response to treatment in the following 6 months for patients (n= 60) who did not receive antibiotics.

Disease sampled site	Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR		p value
				LCL	UCL	
DNB	MMP8	-0.004	0.995	0.99	1.3	**0.002**
	Elastase	-0.006	0.994	0.99	1.2	**0.001**
	Cathepsin G	0.0001	1	0.99	1	0.74
	Trypsin like	0.0001	1	1	1	0.67
	Sialidase	-0.03	0.972	0.99	1.3	**0.005**
DB	MMP8	-0.005	0.995	0.99	1.3	**0.001**
	Elastase	-0.004	0.995	0.99	1.3	**0.001**
	Cathepsin G	0.001	1	0.99	1.005	0.38
	Trypsin like	0.0001	1	1	1	0.74
	Sialidase	-0.02	0.985	0.98	1.3	**0.001**

3.13 Translating to chair side test

The feasibility of translating these tests to a chairside test was examined in a series of preliminary experiments as described in chapter 2 section 2.13. The result shows that there was no response before 15 minutes but it was very clear after 15 minutes incubation (Figure 3.19).

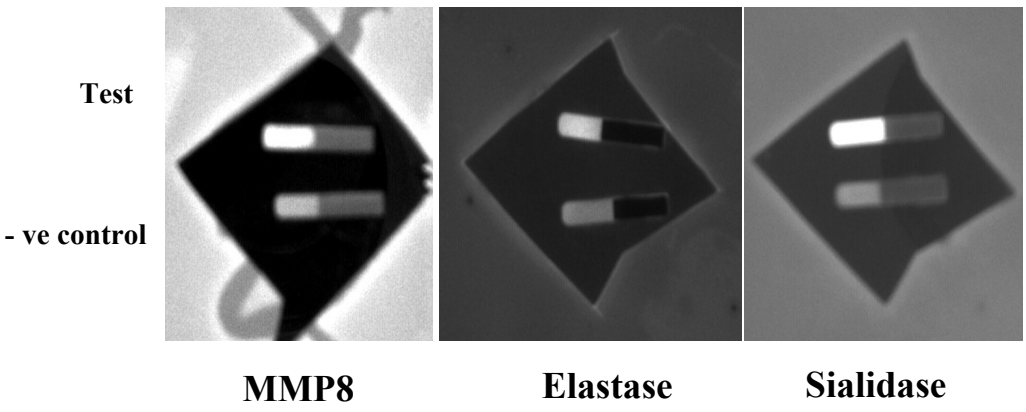


Figure 3.19. Chair side testing for MMP8, sialidase and elastase using Periopaper® strip (15 minutes incubation).

Chapter 4- Discussion

4. Discussion

Traditional methods for detecting and diagnosing periodontal disease almost entirely rely upon the use of clinical measures, which includes measurement of PPD, CAL, BOP and bone loss. These examinations cumulatively demonstrate periodontal tissue breakdown (past periodontal history) rather than the current status of the disease. Furthermore, clinical measures are poor predictors of future disease progression and likely outcome following treatment (Greenstein, 1997). In this regard, clinical measures have been investigated to identify patients with high risk of further disease progression but no correlations were found between them (Halazonetis et al., 1989, Haffajee et al., 1983). However, Haffajee et al. (1991) demonstrated that by combining several clinical measures, including PPD, plaque, BOP, CAL, age, sex, number of missing teeth and so on, it is possible to identify 80% of patients with high risk of further disease deterioration. Due to the realisation that clinical parameters cannot provide us with enough information on the current status of the disease and neither can they predict future disease progression or likely outcome following treatment (Greenstein, 1997), new interest has been focused on diagnostic information that can be achieved through the identification of biomarkers, for example through analysis of constituents in GCF, saliva and the dental biofilm.

The rationale behind this study was based on the premise that periodontal disease has a multifactorial aetiology; therefore, when looking for diagnostic and prognostic biomarkers with the highest levels of accuracy, the researcher has to look beyond the single biomarker and consider combinations of several host and bacterial biomarkers, and in fact no individual biomarker has yet been demonstrated as being acceptably reliable for clinical use (Sorsa et al., 2016, Kinney et al., 2014, Buduneli and Kinane, 2011). The enzymes studied here have been investigated individually and their detection in periodontal disease is well documented (Herrmann et al., 2001, Mailhot et al., 1998, Beighton et al., 1992, Beighton and Life, 1989). In line with this earlier work and data, this study monitored the effect of periodontal therapy on the levels of each enzyme and, based on the level of baseline amounts of some of these enzymes, their ability to predict treatment outcome. The aims of this longitudinal clinical study were therefore, to find a “finger print” enzyme profile that could differentiate healthy and diseased sites and perhaps more importantly, act as a useful prognostic indicator of the outcome of non-surgical treatment in patients with chronic periodontitis.

Chapter 4- Discussion

The principal findings from this study suggest the diagnostic utility (differentiate healthy sites from diseased sites) of three enzymes in GCF, namely MMP8, elastase and sialidase. They showed sensitivity / specificity of 86% / 83%, 78% / 80% and 79% / 79%, respectively, and to some extent, trypsin like enzyme (65% / 69%). Moreover, one of the unique findings of this study is that a combination of GCF enzymes MMP8, elastase and sialidase can give good prediction (≥80.3%) of the outcome of non-surgical periodontal treatment.

Previous studies of biomarkers in periodontitis have largely focused on individual biomarkers (Leppilahti et al., 2015, Herrmann et al., 2001, Mailhot et al., 1998, Beighton et al., 1992), but it is unlikely that a single biomarker would be able to reflect the complex nature of the disease. Consequently, this study investigated several biomarkers simultaneously to try to cover both host and bacterial features of the disease. Second, most previous studies looked at the diagnostic value of their chosen biomarkers, i.e. regarding differentiating health from disease although this can usually be achieved by clinical means. What is missing is the prognostic value of these biomarkers, to assist clinicians make decisions concerning treatment. Taking that into account, in this study we decided to look at both the diagnostic and prognostic value of these biomarkers. Finally, in most of the previous studies the biomarker value correlated with the mean of the clinical measures, whereas in this study the biomarker values were investigated on a site specific basis for both diagnostic and prognostic value, and the biomarker levels were not pooled together as in some studies (Kinney et al., 2014).

An improvement of 2mm in PPD is considered as the primary outcome measure in this study, an improvement of ≥ 2mm which is acceptable as real change by most clinicians that is beyond measurement error. On the other hand, < 2mm is considered as a compromised treatment outcome (Cobb, 1996). This is very important as periodontitis is site specific in nature and for the clinician it is ideal to be able to distinguish between sites that will and will not respond to treatment or those sites, which are at high risk of further disease deterioration. Another advantage of this is ensuring that averaging of biomarker levels and clinical measures does not mask the prognostic value of treatment outcome.

GCF and subgingival biofilm contain substances that play a role in local tissue breakdown (Barros et al., 2016, Curtis et al., 1989) and so they were selected as quick,

site specific, non-invasive sources of a wide range of potential biomarkers of periodontal disease. As explained in chapter 1, most of the candidate host biomarkers selected for study in GCF are enzymes secreted by neutrophils, which are considered the key inflammatory cell contributing to destruction in periodontal disease (Bender et al., 2006). Whereas, bacterial enzymes (trypsin-like and sialidase) are produced by the key periodontal pathogens *P. gingivalis* and *T. forsythia* and *T. denticola* (Fenno et al., 2001) and trypsin like enzyme is considered to be a major etiological factor of periodontal disease.

4.1 Laboratory tests to support the methodology

4.1.1 GCF sampling

Studies on GCF samples have employed various collection methods. Periopaper® strips have now become widely used (Wassall and Preshaw, 2016, Silva and Gomes, 2009, Darany et al., 1992, Lamster et al., 1985), and capillary tubes or similar methods of collecting GCF have also been employed, but both have their advantages and disadvantages (Griffiths, 2003). Capillary tubes can be difficult to place and the opening of the tube is usually blocked by plaque or other substances, which makes the process of collection lengthier, whereas papers strips are easier, quick, minimally invasive and can be performed for individual sites. This method has been reported to be useful for collecting metabolically active components such as proteolytic and glucuronidase enzymes (Waddington et al., 1996, Waddington et al., 1994). The only problem with using Periopaper® strips is that this method might be affected by binding of certain enzymes, which can then be difficult to elute e.g. free elastase (Gustafsson, 1996). However, in this study this issue was addressed via checking different elutant buffers and selecting the one with the highest recovery percent as explained below.

4.1.2 Volume determination

One way of presenting quantitative enzyme data is as a concentration in the GCF (amount/volume). For this purpose, knowing the volume of GCF samples collected is therefore essential. In this study, the GCF volumes have been quantified using the Periotron 8000®, which measures volume on Periopaper® strips by conductance. Human serum was used to calibrate the Periotron as it more closely resembles GCF in

composition (electrolyte and protein) (Lamster, 1997). The maximum volume of fluid that was found to be absorbed by Periopaper was 2.5 µl. However, the Periotron could only differentiate between volumes up to 1.7 µl. This finding is in contrast to that reported by Chapple et al. (1999) who stated that the calibration line plateaus beyond 1.2 µl. In our study, several repetitions using different pipettes were performed and gave very similar data, it is thought that human error in pipetting was not likely to be the cause of the difference. Perhaps more likely is that differences in calibration of the pipette used between the studies and the use of a different model of Periotron might have resulted in a different calibration curve. Humidity and temperature of the room are other factors that may produce differences in Periotron readings but no attempts were made here to standardize them, as this was not feasible in the clinical setting employed.

Another method for measuring GCF volume is by weight. It was found that the weight of each GCF microliter volume, as pipetted from a micropipette, was 1 milligram. However, this method is not as easy as the Periotron volume measure because it needs a sensitive balance, which cannot be placed in the clinic; in addition, Periopaper® strips and the tubes containing elution buffer have to be weighed before sampling, and then after sampling the outer surface of tube has to be dried and then reweighed. This method is useful, though, for samples that have a volume >1.7 µl which could not be measured accurately by the Periotron. In total, 38 clinical GCF samples were collected above the sensitivity of the Periotron instrument and so the volume was determined by weighing.

4.1.3 Enzyme recovery

Following collection and determination of GCF volume, the next step is recovery of desired molecules from the Periopaper® strips. Some molecules can be recovered easily, others less so, and although the exact reason for this is not clear, it seems that the electrostatic charge of the molecule itself plays a role (Gustafsson, 1996). That is why several techniques and elution conditions were tried to maximise recovery, including the use of detergents. Indeed, this may be a source of conflicting results between different groups of researchers. Centrifugal elution has been reported to be the best amongst the physical techniques performed (Griffiths, 2003) and yielded about 90% recovery, which was consistent with previous studies (Nakashima et al., 1994, Griffiths et al., 1988). For the present study, the recovery level was examined for two reasons. Firstly, the recovery percent for Periopaper® has not been examined before, since other workers have used

other types of filter paper to sample and it is possible that the amount of non-specific binding differs from one type of paper strip to another (Wassall and Preshaw, 2016). Secondly, for each of the enzymes investigated here, different elution buffers have been used by previous workers, but each has advantages and disadvantages.

Applying enzymes just in buffer, five commonly used elution buffers were investigated and compared with normal PBS buffer. The recovery percent of enzymes using PBS buffer was very low or even zero for elastase, cathepsin G and MMP8, and this is in agreement with an earlier study (Gustafsson, 1996). It is assumed that this lack of recovery was due to a strong non-specific adsorption process to the Periopaper, which was strong enough to even withstand detergent effects. Each elution buffer produced different percentage recoveries for each enzyme except trypsin, which was recovered at 93% and above even in normal PBS buffer. Whilst the reason for that is not clear, it may be due to trypsin like enzyme being positively charged (its isoelectric point is 10) (Martínez et al., 1988) and thus making its recovery easier. Cetylpyridinium chloride demonstrated the highest percent recovery for all of the enzymes except MMP8. This proved not to be due to poor recovery of the protein but to inhibition of enzyme activity by cetylpyridinium chloride. It is not clear whether Cetylpyridinium chloride denatures the enzyme or whether it acts as an inhibitor.

Since enzymes present in GCF are in a high protein environment, it seemed possible that those proteins could effectively compete for non-specific binding sites on the Periopaper and so improve the elution of GCF enzymes. Indeed, this proved to be the case (Table 3.1) as the recovery percent of all enzymes (except trypsin with PBS, Tween 20 and BSA) was increased with all elution buffers. Overall, BSA provided the best recovery percentage amongst all tested elutant buffers and hence it is used in this study, and this is in accordance with Wassall and Preshaw (2016).

As three of the tested enzymes are excreted by neutrophils, there was concern that the elutant buffers might lyse any neutrophils present in the GCF samples so artificially increasing concentration of enzymes in the free state. The test has shown that BSA solution did not affect neutrophils and therefore we were confident that the enzymes detected in the GCF represent enzymes already present in free form.

One potential problem with assaying a number of enzyme activities in a single sample is ensuring that each enzyme is being assayed at its optimum pH. The optimum pHs for all

five enzymes, except sialidase was either neutral or slightly alkaline, which allowed PBS to be used as the reaction buffer (Uitto et al., 2003, Rawlings and Barrett, 1994, Weiss, 1989). The optimum pH of bacterial sialidase is 5.5 (Thompson et al., 2009), however, it has a fairly broad pH range and so was still highly active at pH 7.2. A second problem was the optimum period of the reaction. From examination of the activity of each enzyme with a standard amount of substrate over a time period it was established that maximum substrate conversion was achieved by a minimum of 4 hours incubation at 37 °C. To ensure maximum substrate conversion by GCF samples the incubation times used 18 hours except for MMP8, which was incubated for 4 hours.

Molecules investigated within GCF samples have been reported using a range of formats, such as total amount per sample, concentration (mg/ml or enzyme units/ml), or with reference to sampling time (Wassall and Preshaw, 2016). In clinical medicine, the data are more frequently expressed as mass per unit volume when analysing body fluids. However, GCF samples present some challenges, principally as the very small sample volume, risk of evaporation and difficulty in extracting the enzyme (Lamster et al., 1988, Lamster et al., 1986). However, it is more convenient and often more clearly understood when data are expressed as a function of volume. Consequently, This study has attempted to address all of the factors that may affect this including using the Periotron to establish the sample volume at the chair side to minimise evaporation of the sample, and supplementing this with weighing to overcome the limitations of the Periotron instrument.

4.2 Clinical data

According to our power calculations, participation by 90 patients was needed to ensure the statistical power of the study, as explained in section 2.14.1. Although 77 patients in a total of 89 subjects completed the whole study period, that does not affect the power of the study as cathepsin G and trypsin like enzymes were considered as two separate factors during the power calculation and they did not add significant prediction value to the whole enzyme combination. On the other hand, the number of patients examined is sufficient to draw conclusions about the value of the biomarkers chosen. Finally, the clinical data for those who dropped out during the study were examined and no statistically significant differences were observed compared to those who completed the study and importantly this ensured that removing those subjects did not affect the final

result of the study. However, 58% (7 out of 12 subjects) of the subjects who dropped out received antibiotic therapy, whereas in subjects who completed the study 22% of them received antibiotics. The reasons for the high drop out rate amongst subjects who received antibiotics are not known but it is tempting to speculate that it is related to the fact that they experienced a good treatment outcome and they felt no further treatment was necessary.

Clinical parameters were used to determine the effect of treatment in patients with periodontal disease. In the current study, besides collecting clinical measures of sampled sites, full mouth clinical measures were collected, all of which showed reductions through the 6 month time period of the study. This approach was important in determining whether the patients gained benefit from the treatment generally. It was also important to justify the selection of the three test sites in terms of their representativeness of similar sites elsewhere in the subject. The DNB sites were selected as representative of non-inflamed sites (as absence of bleeding is felt to be marker of stability) (Lang et al., 1986), whereas DB sites were selected to represent inflamed sites. Of course, if more than three sites had been sampled in each patient this would perhaps have been more representative of the whole mouth of the patient but this would have presented some practical problems. First, it is very difficult to collect samples from many sites in terms of the time in the chair for the patient and, more importantly, as the patient needs to be sampled twice after baseline there is a greater chance that patients will drop out from the study. Secondly, from a statistical point of view, the larger the number of sites sampled, the larger the number of patients that need to be recruited, as the number of sampled sites is also considered as a factor in power calculations. Thirdly, as the plan was to recruit 90 patients, these sites may represent the disease on a population basis rather than an individual basis. Lastly, to overcome the issue of whether these sites represented the whole mouth or not, the full mouth clinical data were recorded at each appointment and analysed to check the effect of treatment on the whole mouth.

The outcomes of the clinical measures (PPD, CAL and BOP) in this study showed improvement at the 3 month time point and further improvements were noticed at 6 month time point, which is in harmony with the data published by (Cobb, 1996) and the systematic review by (Cobb, 2002), and this explains why the baseline enzyme levels were analysed versus the 6 month treatment outcome. The percent of bleeding on probing reduction was 66% (from 22.6 ±16.2 to 7.7± 10.6) and this is in agreement with the

finding of the systematic review by (Cobb, 2002). Furthermore, the mean PPD reduction of sampled sites (PPD≥ 6mm) was 2.29mm for DNB sites and 2.32mm for DB sites, which is in line with the figures reported by Cobb (2002). It is worth mentioning that the degree of response in decreasing PPD is strongly correlated with the initial pocket depth (Cobb, 1996) i.e the deeper the pockets initially the greater changes in PPD. However, sites above 6 mm in PPD tend to respond equally (Cobb, 2002).

Considering 2mm improvement in PPD as the primary outcome measure and taking that into account, one third of both DNB and DB sites did not respond to the treatment. It is difficult to draw comparisons with previous studies, as the other studies did not report individual site variations in their data. However, using a 1.5mm threshold point, a study by Claffey and Egelberg (1995) showed that 22% of sites did not respond to treatment, which is comparable.

Although the aim of this study was not to determine the impact of antibiotics (azithromycin is used in Sheffield) on treatment outcome, it was considered important to compare the effect of antibiotic therapy on treatment outcome as another variable alongside non-surgical periodontal treatment. Of the patients who completed the study 17 received antibiotic therapy at the 3 month time point of the study. As is apparent in Table 3.8, at baseline there were no statistically significant differences between subjects that received an antibiotic and subjects that did not receive an antibiotic in terms of their presenting full mouth and site specific clinical data, except BOP and PPD ≤ 3mm. After one cycle of the treatment and at the 3 month review there are statistically significant differences in all full mouth and site specific clinical data except PI% and PPD in DB sites. Observing this poor response in full mouth clinical measures in these patients led the clinician to prescribe azithromycin. Such antibiotic treatment at this stage led to significant reduction in full mouth and site specific clinical measures at the following review (six month time point). In particular it was noteworthy that at the 6 month appointment, subjects that received antibiotic therapy showed a significant reduction in the proportion of sites with PPD≥ 6mm (from 17± 9 to 1.8±2.9 vs. 14± 8 to 4.02±4.3 for non-antibiotic patients) This is in line with previous studies where the effects of azithromycin were examined (Emingil et al., 2012, Han et al., 2012). In terms of the sites selected for GCF sampling, however, the average levels of PPD in DNB and DB sites were not different in subjects that received antibiotics and those that did not. This can be explained by the fact that antibiotics were prescribed based on full mouth treatment

outcome rather than site specific treatment outcome at 3 month review and averaging PPD might have masked the changes. On a site specific basis, determined using a threshold of 2mm improvement in PPD as a successful treatment outcome, 82% of sites with PPD of ≥6mm demonstrated ≥ 2mm improvement in PPD in subjects that received an antibiotic, whereas 61% of sampled representative sites showed 2mm improvement in PPD. This result is in harmony with the finding in a previous study (Han et al., 2012) that 79% of sites with PPD of ≥ 7mm in subjects that received an antibiotic showed 3mm improvement in PPD and 57% of PPD sites showed 3mm improvement in subjects that did not receive an antibiotic.

It is worth mentioning here that as azithromycin was prescribed based on the basis of full mouth treatment outcome rather than on site specific treatment outcome, it was not possible to make a fair comparison of enzyme levels at sites between patients who received an antibiotic with those that did not receive an antibiotic. The earlier explanation might account for why there were no significant differences in site specific enzyme levels at baseline between those that received antibiotic and those that did not. However, on 6 month review, the levels of MMP8, elastase and sialidase showed significant reduction in subjects that received an antibiotic compared to those that did not, and this is in agreement with data reported on MMP8 (Leppilahti et al., 2015).

4.3 Enzyme levels

The rationale for using enzymes in GCF as potential biomarkers has been described in Section 1.10 and their selection has been generally justified. Our findings are that the average levels of all tested enzymes detected in DNB and DB sites were higher than those found in healthy sites and this is in accordance with previous studies (Kinney et al., 2014, Herrmann et al., 2001, Mailhot et al., 1998, Beighton et al., 1992, Beighton and Life, 1989). However, limited differences were found in the enzyme levels between DNB and DB sites; indeed, some enzyme levels were higher in DNB than DB sites within the same patient. It is worth mentioning that levels of the enzymes are much more significant in the cases of MMP8, elastase and sialidase. The baseline biomarker levels of MMP8, elastase and sialidase were correlated with the initial PPD, which is in agreement with that reported in the literature (Mantyla et al., 2003, Eley and Cox, 1996a, Pederson et al., 1995, Nieminen et al., 1993, Kitawaki, 1983). Furthermore, we have found that the baseline enzyme levels were correlated with treatment outcome and this was highly

significant in the cases of MMP8, elastase and sialidase i.e. the higher the enzyme level at baseline, the less likely to respond to the treatment. This indicates that these enzymes are reflecting the destructive processes occurring in periodontal disease biology and that is consistent with other findings in the literature on MMP8 and elastase (Leppilahti et al., 2015, Eley and Cox, 1996b). However, to the best of our knowledge we are the first to report the indirect correlation of GCF sialidase levels with treatment outcome.

Previous studies have reported that mean levels of these enzymes reduced throughout the study time points (Kinney et al., 2014, Yucekal-Tuncer et al., 2003), which is consistent with the results obtained in this study. However at the individual site level, some sites did not show such decreases and indeed some sites showed further increases in enzyme levels. Interestingly these increases in biomarker levels were mostly associated with sites that had compromised treatment outcome.

MMP8 is present in GCF in easily detectable quantities and has been found to play an important role in destruction of type I collagen in periodontal tissues (Sorsa et al., 2004). The enzyme is unstable though, which is why even the commercial enzyme is only available as a latent form that needs to be activated. One problem is that no specific substrate is available for MMP8 alone and some other members of the MMP family can break down the substrate used, such as MMP-9 (Neumann et al., 2004). It may, therefore, be more correct to refer to the activity detected as MMP8-like, however, while acknowledging the inaccuracy and for the sake of brevity the term MMP8 has been used because the activity in GCF was evaluated using pure MMP8 to create the standard curve. With this limitation in mind, in most of the patients, the amount of MMP8 found in diseased sites was higher than in the healthy sites and this is in agreement with previous studies (Kivela-Rajamaki et al., 2003, Mantyla et al., 2003).

Elastase is one of the main constituents of primary granules of PMNs and its involvement in periodontal tissue destruction is well documented (Jin et al., 2002, Jin et al., 2000). Elastase has the potential to serve as an adjunctive measure in screening of periodontal disease and monitoring treatment outcomes (Ingman et al., 1994). The enzyme can exist in both a free form and a form complexed with its inhibitor, α-2 macroglobulin. The two forms are thought to exist in a stable ratio, so that assaying one form gives an indication of the total enzyme present. Previous work has shown that free elastase binds to paper and is not eluted in PBS while the elastase-α-2-macroglobulin complex is eluted,

however, using detergents the recovery percent increased (Gustafsson, 1996). This is in agreement with our data that the percentage recovery of elastase using PBS was zero while using 1%BSA as elutant buffer provided 93% recovery of free elastase. The study found that the concentration of elastase correlated with the initial PPD and this is consistent with reports by other researchers (Armitage et al., 1994). It has also been demonstrated that cathepsin G plays a role in destruction of periodontal tissues, particularly when its inhibitors are not present (Starkey et al., 1977) and synergistic action between elastase and cathepsin G has also been reported (Boudier et al., 1981).

The findings show that the amounts of elastase and cathepsin G in diseased sites are higher than in healthy sites and this is in accordance with the literature (Pederson et al., 1995, Darany et al., 1992). Interestingly, increased levels of cathepsin G were seen more often compared to elastase. This could be due to the fact that these two enzymes are secreted from the same primary granules of neutrophils. On the other hand, cathepsin G levels were not as high as those of elastase, using our assay, and this may be due to cathepsin G having weak activity against the synthetic substrate employed (Rehault et al., 1999). Consequently it is difficult to determine whether there was truly less cathepsin G present in the samples than elastase or whether the difference was due to differences in efficiency of the assays.

Trypsin-like enzymes have been found to be involved in destruction of periodontal tissue (Travis et al., 1997). However, while host trypsin is present in serum at 248 +/- 94.9 ng/ml (Artigas et al., 1981) much of the trypsin-like activity at diseased sites is thought to be bacterial in origin. Several bacteria have been reported to export trypsin-like protease extracellularly and the BAPNA substrate has been used to measure the activity of trypsin-like enzyme in GCF by several groups. Generally, we found that the amounts of trypsin in diseased sites were higher than in healthy sites and this supports the findings of Eley and Cox (1992a). However, Loesche et al. (1987) reported that there is no trypsin-like activity in GCF. This could be due to the differences in sampling of GCF, as those authors used intra-crevicular washing to collect the samples and employed a different substrate, (BANA).

Several sialidase-producing bacteria are present in the dental biofilm, such as *T. forsythia* and *P. gingivalis* (Corfield, 1992, Holt and Bramanti, 1991) suggesting that this enzyme might be a useful marker of their presence at a periodontal site. The literature on

sialidases and periodontal diseases in very sparse but those reports that have been published support our findings that sialidase activity was found to be higher in diseased sites than in healthy sites. Also, others have reported sialidase to be detected at higher levels in periodontitis subjects than gingivitis subjects and when it is coupled with other enzymes it has been possible to distinguish these two kinds of periodontally diseased patients with 61.3% sensitivity and 91.9% specificity (Beighton et al., 1992, Kitawaki et al., 1983).

4.4 Diagnostic value of the enzymes

The question arises then as to what evidence has been obtained which indicates that knowledge of these enzyme levels helps diagnosis of disease and/or understand the response of sites to conventional clinical treatment. The diagnostic capabilities of these biomarkers were investigated by the ROC curve to determine whether a given enzyme level could act as a threshold point to differentiate health from disease. The sensitivity and specificity for each of the tested biomarkers were MMP8 (86%/83%), elastase (78%/80%) and sialidase (79%/79%) and these had the greatest sensitivity and specificity among all the 5 enzymes (Table 3.11). Other workers have reported that the diagnostic capabilities of MMP8 had 69% sensitivity and 70% specificity in saliva (Kinney et al., 2011) and 89% sensitivity and 87% specificity in GCF (Leppilahti et al., 2014b), and this is in line with our result that MMP8 has diagnostic capability of 86% sensitivity and 83% specificity. Elastase again showed high diagnostic capability to distinguish between healthy and diseased sites and to the best of our knowledge this is the first time that the sensitivity and specificity have been applied to determine the diagnostic capability of elastase. Sialidase showed the next highest diagnostic capability after MMP8 and elastase. Beighton et al. (1992) showed that sialidase could differentiate between gingivitis and periodontitis with 61.3% sensitivity and 91.9%, specificity (Beighton et al., 1992) and our findings provide new evidence for the importance of this enzyme in differentiating healthy from diseased sites.

In contrast, trypsin-like activity and cathepsin G had the lowest sensitivity and specificity amongst the tested biomarkers, and to our knowledge this is the first time this has been applied to their diagnostic and prognostic value. In previous studies cathepsin G and trypsin-like activity has only been evaluated in relation to disease status rather than as biomarkers for treatment outcome (Kunimatsu et al., 1995, Eley and Cox, 1992b). This

could be due either to the fact that they do not have sufficient discriminatory ability individually or that they represent biological functions (e.g. neutrophil activity) that are better assessed by the other three enzymes. For example, cathepsin G was found to enhance tissue destruction by activating pro-MMPs (Kahari and Saarialho-Kere, 1999) as well as working synergistically with elastase (Boudier et al., 1981).

4.5 Prognostic value of the enzymes

The unique feature of the current study is that we looked beyond the diagnostic value of the biomarkers, as the literature indicates that the current means for diagnosing periodontal disease have limitations in terms of predicting the outcome of treatment. Like recent literature in which regression analysis was used to determine the usefulness of combined biomarkers (Gursoy et al., 2010, Ramseier et al., 2009), the current study involved testing these biomarkers to predict the treatment outcome using backward stepwise logistic regression. Only MMP8 and elastase amongst the enzymes tested have previously been examined to predict the outcome of treatment and further disease progression (Kinney et al., 2014, Palcanis et al., 1992). MMP8 has been reported to be a good predictor of treatment outcome (Sorsa et al., 2016, Leppilahti et al., 2015) and when it is combined with other biomarkers this produces a better prognosis of treatment outcome (Kinney et al., 2014). Elastase has been investigated for its ability to predict disease progression using bone loss as an indicator, and showed sensitivity/specificity of 84%/66% in one study (Palcanis et al., 1992) and 77%/61% in another study (Armitage et al., 1994). These findings are in clear support of our results.

Here we demonstrated that the combination of these two enzyme levels can better predict the treatment outcome (2mm improvement in PPD) when it is combined with sialidase. The effect (β in Table 3.13) of these three enzymes was shown to be indirectly correlated with improvement of PPD by at least 2mm (treatment outcome), with an odds ratio of 0.995, 0.994 and 0.995 for MMP8, elastase and sialidase in DNB sites, respectively, and 0.995 (MMP8), 0.995 (elastase) and 0.994 (sialidase) in DB sites. In contrast, cathepsin G and trypsin-like activity did not provide any additional significant predictive value to the three enzymes combination (P value > 0.05 as shown in Table 3.12). In parallel to this, the baseline enzyme levels at responder and non-responder sites were compared and statistically significant differences in MMP8, elastase and sialidase levels were found between sites with successful treatment outcome and those with compromised treatment

outcome (Table 3.16). Higher enzyme levels at baseline again were associated with compromised treatment outcome.

In conclusion, this study has shown that MMP8, elastase and sialidase have the highest diagnostic value among the biomarkers examined. Moreover, among the enzymes tested, these three gave a better prognostic measure for the outcome of treatment. In contrast each of these enzymes alone cannot predict treatment outcome more than the null hypothesis (62.5%). This is the first time that a biomarker 'finger print' has been shown to have potentially useful prognostic value of a site's likely response to non-surgical periodontal treatment.

The primary clinical implications of these biomarkers include prioritizing patients' treatment and arranging recall appointments for those treated patients that are at high risk of progressive disease. It also gives hope that these biomarkers can be used for periodontal screening in epidemiological studies. Furthermore, it will help to provide personalized and site-tailored treatment such as use of adjunctive local or systemic antibiotic therapy, or provision of more advanced treatment means (surgical intervention), and these would all help to minimise unnecessary under or over treatment.

The potential for translating this test (enzyme-substrate reaction) to a chair side test was examined in a very preliminary way here and we were able to show that application of the enzyme-substrate directly to the Periopaper® strips could achieve an easily detected colour/fluorescence change after 15 minutes with the threshold level of enzyme. Further work is required to establish how this could then be translated into an efficient multi-enzyme prognostic test but that is beyond the scope of this thesis. It is envisaged that adjusting the concentration of the substrate will allow recognition of enzymes only at or above the threshold concentration level. Consequently a bespoke Periopaper could be produced which is impregnated with the appropriate level of substrate for each of the three enzymes, and each using a different reporter molecule. This would then be placed in a periodontal pocket and the different reporters determined using a dedicated small reader or mobile phone App.. If further developed and adopted, it would enable clinicians to predict treatment outcome and consequently to consider alternative treatment choices.

4.6 Limitations

We identified some limitations in the current study. For example, it would be better to collect samples from more than two diseased sites per patient, which would allow easier comparison of sites that have the same sort of clinical measures. However, from a statistical point of view, using more sample sites would require more patients to be recruited and so extend considerably the length of the study at a single centre. Further to that, collecting more GCF samples would necessitate patients spending more time receiving their treatment, which of course would make recruitment more difficult.

Further studies should also look at these biomarkers in terms of early indications of disease onset, transition from gingivitis to periodontitis, and differentiating active sites from inactive sites. Furthermore, examination of the biomarker value of these enzymes in saliva would be valuable in predicting treatment outcome at subject level. The biomarker value of these enzymes would be valuable to be tested in patients with potential confounders of periodontal disease (such as diabetes mellitus; smoking) and in subjects with aggressive periodontitis. More work needs to be conducted to enable the chair-side test based on enzyme-substrate reaction to be made available to clinicians. However before putting effort in to developing the test further, it is important to establish that the enzyme thresholds identified here are applicable to another cohort. Consequently a validation study has been conducted in an independent cohort as described Chapter 5.

Chapter 5- Enzyme biomarkers validation in an independent cohort

Chapter 5- Enzyme biomarkers validation

5.1 Introduction

As described earlier in chapter one, there are several steps to finding and proving the usefulness of biomarkers (section 1.7.2 Development of a pathway for diagnostic tests based on host and bacterial biomarkers). It was explained that in the process of diagnostic and prognostic testing based on host and bacterial biomarkers, it is necessary to validate the result based on an independent cohort (Lamster, 1997).

Validation can be defined as the degree of closeness of the reported result to the true result. The process of validation includes providing a number of characteristics of the biomarker, such as its intrinsic quality and its determinants (Bonassi et al., 2001). The aim of validation is to determine the soundness of potential biomarkers versus results in patients in an independent cohort. In this phase, the potential biomarkers would be examined against subject variability that more reasonably represents the variability in the population (De Bock et al., 2010). Consequently, for these biomarkers to be reliable, validation studies are paramount (Li et al., 2005). Validation aims to identify the threshold point of the selected biomarkers with the highest sensitivity and specificity so that ultimately they can be used to detect the disease, status of the disease or treatment outcome.

To determine the threshold point the receiver-operating characteristic (ROC) curve is a useful method to determine the threshold point with the highest sensitivity and specificity. When this test detects the presence of disease this is termed sensitivity (i.e. true positive), whereas specificity is when the test is negative in the absence of disease (i.e. true negative).

For this validation study GCF samples were used that had been obtained previously, from 30 patients with chronic periodontitis as part of an independent cohort study and had aimed to test the same enzymes. The same patient inclusion and exclusion criteria had been used as in the main study above. However, there were differences in the method used to collect GCF samples in this independent cohort study. The samples had been collected using a fine, flexible micropipette tip, while in the main study periopaper® strips were used. Also, in this independent cohort study the samples were stored at -80 °C before analysis, while in the main study they were analysed straight after collection.

Chapter 5- Enzyme biomarkers validation

5.1.1 Aims and objects of this study

The aim of this independent cohort study was to evaluate levels of enzymes in periodontal sites with a view to determining their diagnostic and prognostic value. In the context of this project, the GCF samples provided a data set from an independent patient cohort that were used to validate the threshold levels of biomarkers as diagnostic and prognostic indicators in the main study and vice versa.

The objective was:-

To validate MMP8, elastase, cathepsin G, trypsin-like enzyme activity and sialidase in GCF as putative diagnostic and prognostic markers for periodontal disease in an independent cohort.

Chapter 5- Enzyme biomarkers validation

5.2 Materials and methods

5.2.1 Materials and methods

The materials and methods used are exactly the same as those described in chapter 2, except for the following:

1. The length of this independent cohort study was one year (The study protocol was approved by NRES Committee Yorkshire and Humberside, study number: 10/H1308/45 and granted on 28.10.10) (Appendix 6), registered with Sheffield Teaching Hospital NHS for Research Governance (STH 15611) and the clinical measurements plus GCF samples were collected at baseline, 3 months, 6 months and one year by single clinician (Professor A. Rawlinson). However, for purpose of validation and as most of the changes in clinical measures had happened by the six month time point and there were no statistically significant differences between the 6 month and 12 month time points, baseline enzyme levels were analysed against the six month time point treatment outcome.
2. The GCF samples in the independent cohort study were collected by micropipette, the actual volume collected was determined by weighing (Figure 3.2), whereas in the main study the GCF samples were collected by periopaper® strips and GCF volume was mostly determined using the Periotron® machine.
3. The GCF samples in the independent cohort study were stored and then analysed, while in the main study the GCF samples were analysed immediately after collection.

5.2.2 Statistical analysis

As described in chapter 2 section 2.14.3, ROC curves were used to determine the diagnostic and prognostic threshold points for each enzyme using data from the independent cohort study. For validation the baseline continuous values of biomarkers in the independent cohort study were dichotomised using the preselected threshold point (in the main study) as being below and above their corresponding thresholds and vice versa. ROC curves were reanalysed with binary enzyme values (dichotomised using the diagnostic cutoff point from the main study) to validate the diagnostic capabilities of these biomarkers in differentiating healthy sites from diseased sites. Logistic regression was used with binary baseline enzyme values (dichotomised using prognostic cutoff point

from the main study and vice versa) as predictors against binary outcome data (pocket depth as being improved by 2mm or not) to validate the predictive values of the biomarkers at 6 months.

5.3 Results

5.3.1 Demographic and clinical summary of sample population

Forty seven subjects were screened for their suitability for inclusion into the study. Seventeen of them did not fulfill the inclusion criteria or declined to participate, 30 subjects (14 male and 16 female) were recruited, aged between 40 to 70 years (40% 40-49 years, 30% 50-59 years, 30% >60 years), of whom 4 were smokers. Of these, 28 completed the 3-month appointment, 23 subjects completed the 6-month appointment and 22 completed the full study. The other patients failed to attend further appointments, with the exception of one participant who died during the period of the study.

The severity of disease ranged between localised moderate periodontitis and generalised severe periodontitis (1999 periodontal disease classification system) (Armitage, 1999). Full mouth clinical measures (BOP and PPD) demonstrated statistically significant improvement after the first cycle of the treatment at 3 month review. Further improvements were achieved after the second cycle of the treatment at 6 month review time point. However, no significant further improvements in either full mouth (Table 5.1) or site specific (Figures 5.1, 5.2 and 5.3) clinical measures were noticed at 12 month time point, and for that reason the baseline enzyme levels were analysed against treatment outcome at 6 month time point. The average percentage of sites with PPD of 4-5 mm had reduced from 21 ±10.4 at baseline to 12.63± 5.82 at 3 month time point and demonstrated further reduction to 9.32 ± 3.6 at 6 month review. Meanwhile, the mean percentage of sites with PPD of ≥ 6mm had reduced from 12±7.19 at baseline to 6.68±6.14 at 3 month time point and further decreased at 6 months (2.87±3.31) and 12 months (2.4±3.1) (Table 5.1).

The outcomes of treatment of representative disease sites were assessed by changes in PPD and 2mm improvement was considered as successful treatment outcome. The mean PPDs for DNB and DB sites were 6.86±0.94mm and 7.4±1.4mm, respectively. After the initial treatment phase and at 3 months the mean PPDs for DNB and DB indicated significant reductions to 5.54±1.53mm (p=0.005) and 5.95±1.86mm (p=0.01),

respectively. The second treatment cycle at 3 month time point resulted in further decreases at 6 month review to 4.68±1.88mm (P=0.0001) and 4.27±1.77mm (P=0.0001), respectively. At 12 month review, there were no statistical significant differences in the mean PPD of either DNB (4.5±2.42mm) or DB (3.8±1.91mm) sites when compared to the 6 month time point (DNB p=0.78, DB p=0.41) (Figure 5.1).

However, at site level, 68.1% of DNB sites (15 out 22 sites) showed improvement by at least 2mm, while 6 sites showed improvement by less than 2 mm and 1 site demonstrated further progression. Whereas, 81.8% (18 out of 22 sites) of DB sites showed improvement by ≥ 2mm in PPD, 1 site did not change and 3 sites improved by less than 2mm. Overall, on a site specific basis and considering 2mm improvement in PPD as successful treatment outcome, there were no significant differences in treatment outcome between the 6 month and 12 month time points (Figures 5.2 and 5.3). In terms of patient-based percentages, 71.5% of sites with PPD ≥ 6mm showed 2mm reduction, whilst 22.9% reduced by less than 2mm, 3.7% did not change and the remaining 1.9% showed further progression. Also, the mean percentage of PI and BOP of DB sites showed significant reduction over the study period; however, the DNB sites showed an increase in the mean percentage of BOP over the study period (Table 5.1).

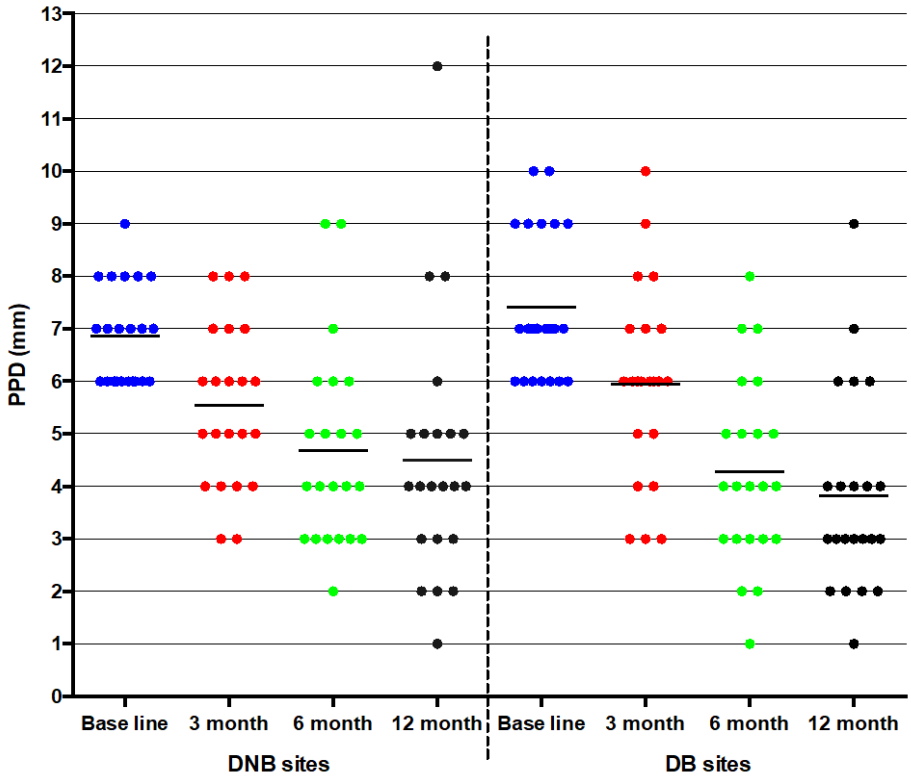


Figure 5.1. Changes in PPD throughout the course of the study in DNB and DB sites.

Table 5.1. Full mouth and representative site-specific clinical data at study time points.

	Variable	Baseline (% sites ± SD)	3 months (% sites ± SD)	P value (baseline vs 3 month)	6 months (% sites ± SD)	P value (baseline vs 6 month)	12 months (% sites ± SD)	P value (baseline vs 12 month)	P value (6 month vs 12 month)
Full mouth clinical data (P value *)	BOP	36.2 ±23.5	17.85 ± 10.4	**0.0001**	10.93 ± 7.78	**0.0001**	10.58 ±8.87	**0.0001**	0.62
	Mean PPD ≤ 3 mm	67±13.5	80.69± 9.32	**0.0001**	87.81± 5.41	**0.0001**	89.04±4.92	**0.0001**	0.71
	Mean PPD 4-5 mm	21 ±10.4	12.63± 5.82	**0.0001**	9.32 ± 3.6	**0.0001**	8.56 ± 5.41	**0.0001**	0.54
	Mean PPD ≥ 6 mm	12 ± 7.19	6.68 ± 6.14	**0.0001**	2.87±3.31	**0.0001**	2.4 ± 3.1	**0.0001**	0.78
Site specific clinical data (P value **)	PI (healthy site)	52	39	**0.01**	39	**0.01**	35	**0.025**	0.48
	PI (DNB site)	91	47	**0.001**	47	**0.001**	42	**0.001**	0.58
	PI (DB site)	91	60	**0.001**	52	**0.001**	45	**0.001**	0.64
	BOP (healthy site)	0	4	NA	0	NA	0	NA	NA
	BOP (DNB site)	0	47	NA	30	NA	33	NA	0.73
	BOP (DB site)	100	52	NA	21	NA	23	NA	0.82

* t-test, ** Chi-squared test, NA= not applicable

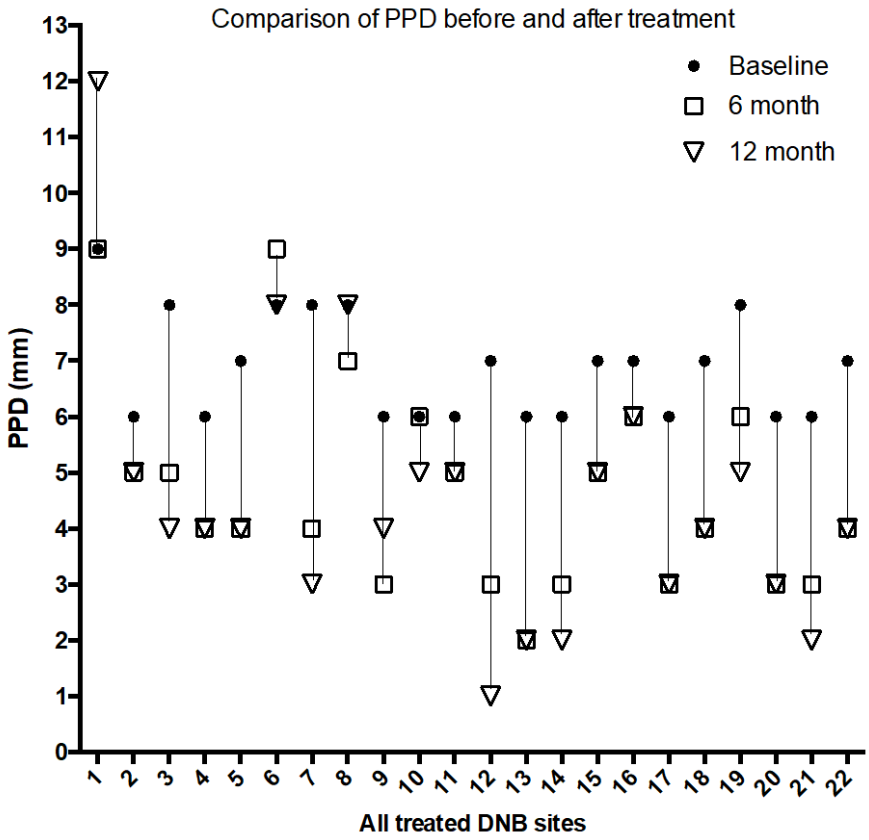


Figure 5.2. Changes in PPD of each DNB from baseline to 6 month and 12 month time points.

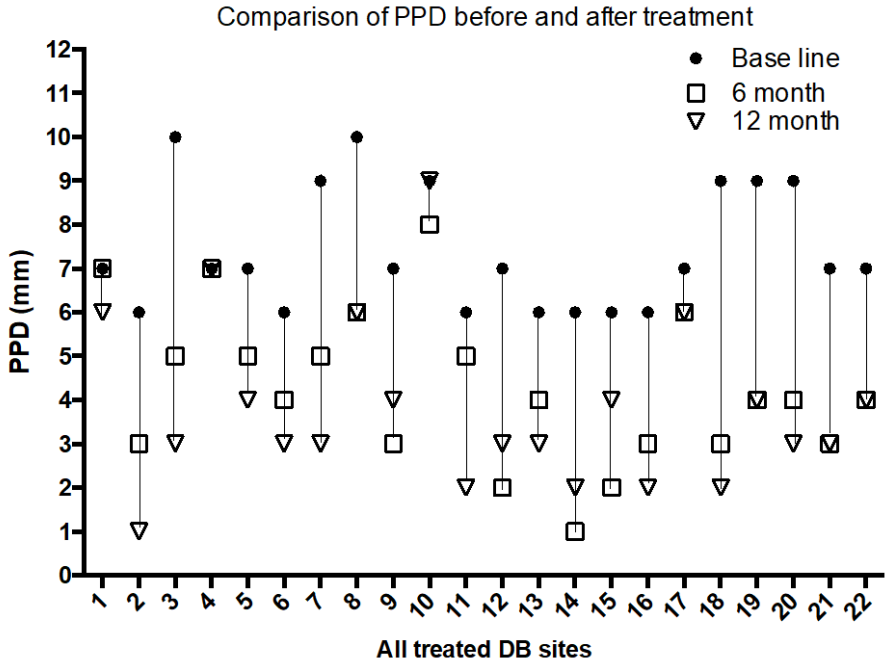


Figure 5.3. Changes in PPD of each DB from baseline to 6 month and 12 month time points.

5.3.2 Enzyme biomarkers

The median levels of all enzyme biomarkers were detected as significantly higher levels in both DNB and DB sites than in healthy sites. The median levels of all biomarkers at diseased sites decreased through the course of the study, however on site a specific basis, there were sites that showed a reasonably high level (Figures 5.4, 5.5, 5.6, 5.7 and 5.8). The correlations between PPD and enzymes levels at baseline were r = 0.8 for MMP8, r = 0.72 for elastase, r = 0.51 for cathepsin G, r = 0.53 for trypsin like and r = 0.64 for sialidase. However, only MMP8, elastase and sialidase showed statistical significant correlation with treatment outcome (r= -0.68, -0.62 and -0.58 respectively).

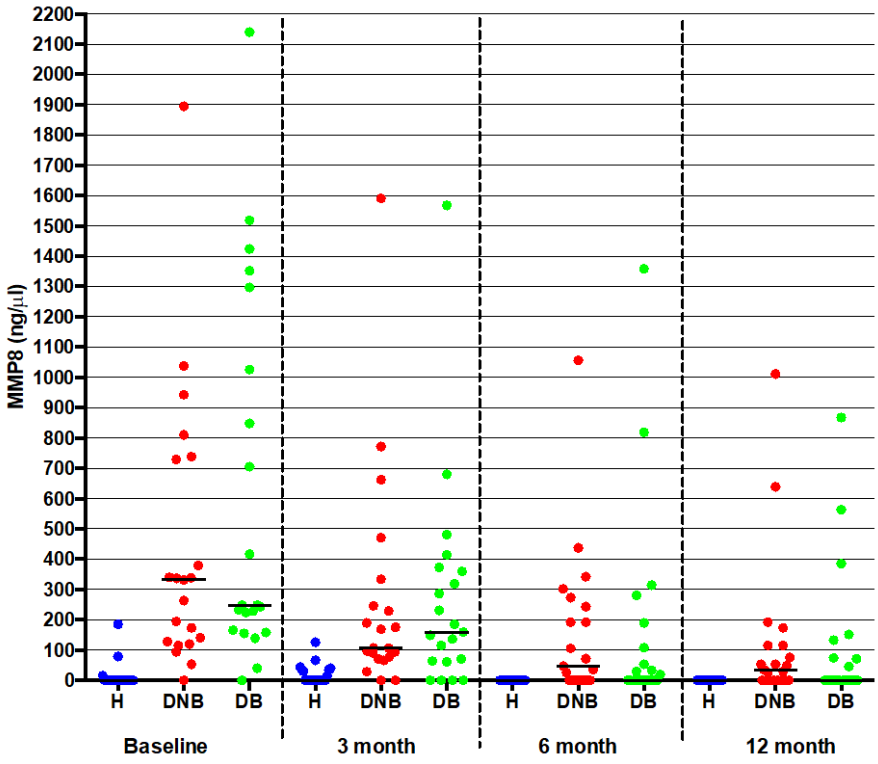


Figure 5.4. Scatter plot of MMP8 in representative sites at different study time points (Horizontal bar= median, H= healthy site, DNB= deep non bleeding and DB= deep bleeding).

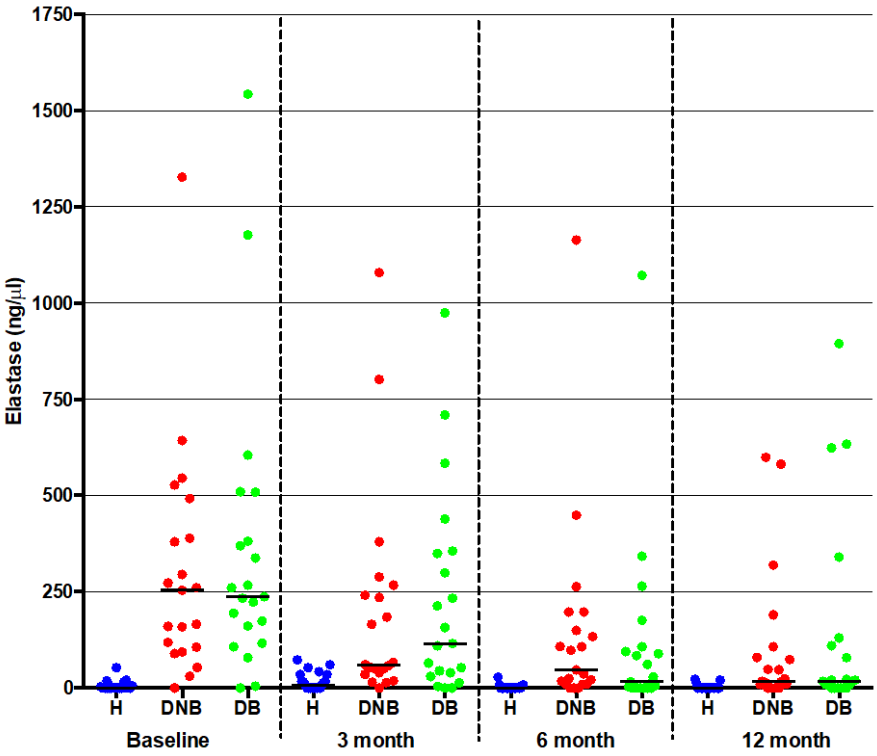


Figure 5.5. Scatter plot of elastase in representative sites at different study time points.

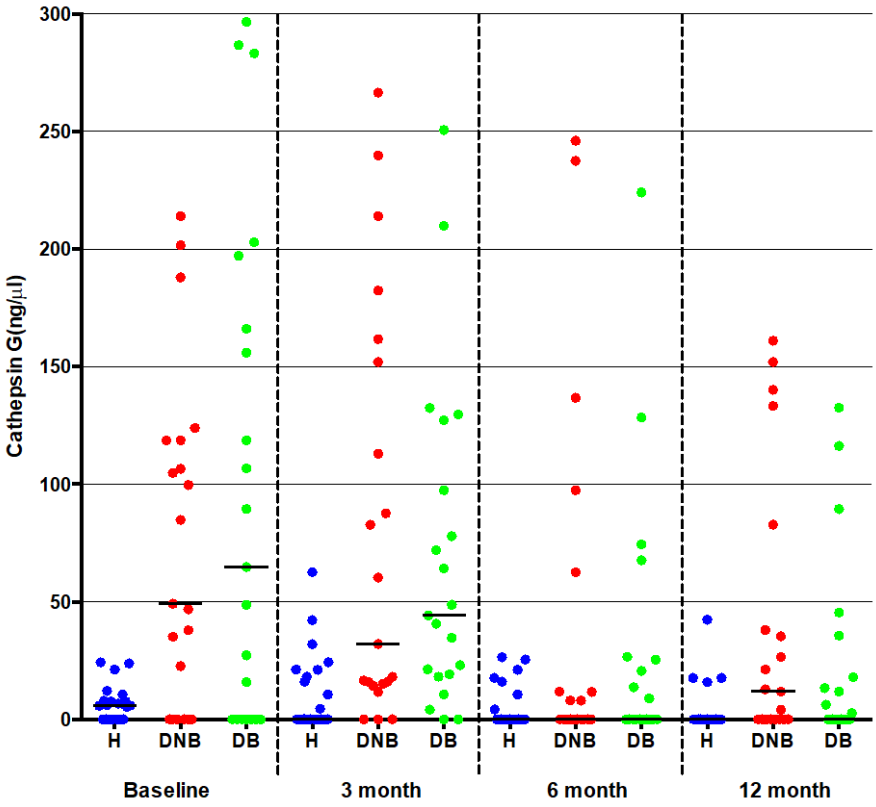


Figure 5.6. Scatter plot of cathepsin G in representative sites at different study time points.

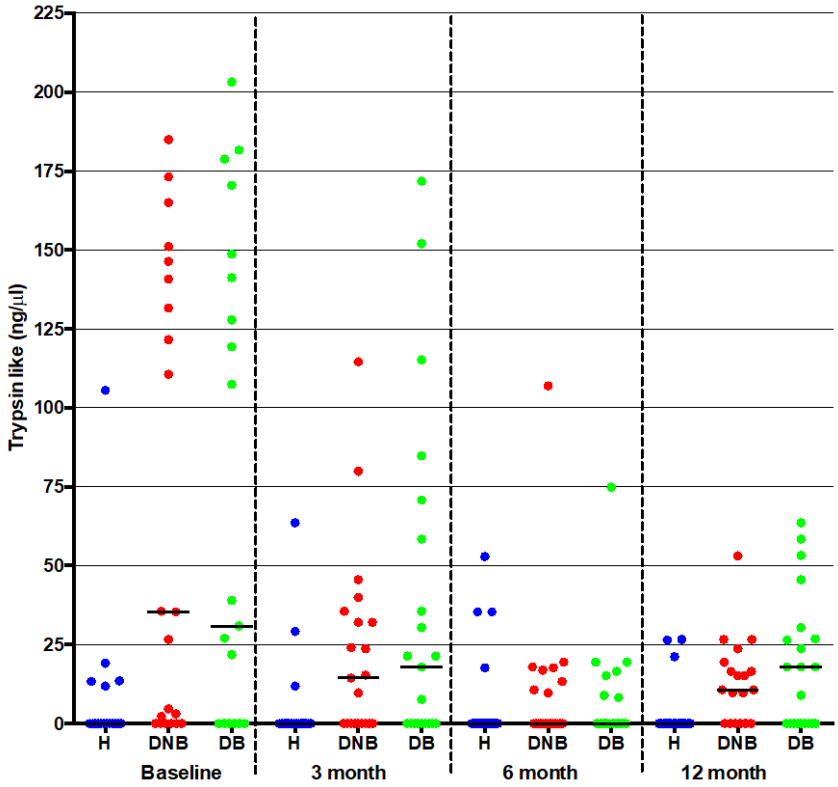


Figure 5.7. Scatter plot of trypsin like enzyme in representative sites at different study time points.

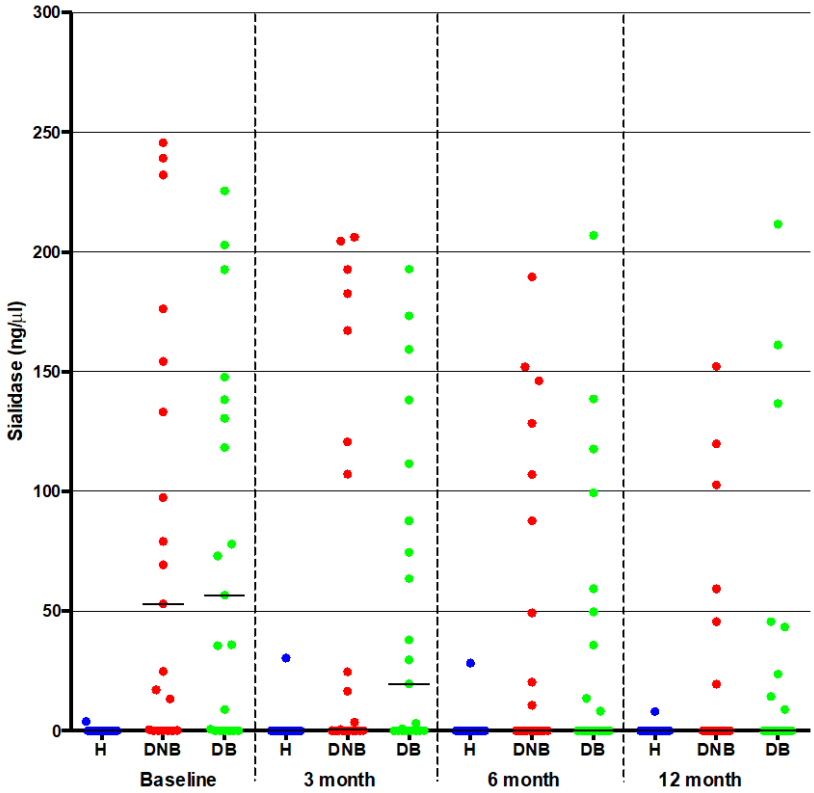


Figure 5.8. Scatter plot of sialidase in representative sites at different study time points.

GCF volumes collected were significantly higher in both DNB (2.42±0.57µl) and DB (2.99±0.66µl) sites than in healthy sites (1.82 ±0.52µl) (p=0.001). The GCF volumes collected in both DNB and DB sites had reduced after the first cycle of the treatment and at the 3 month review to 2.24±0.46µl and 2.21±0.34µl, respectively. Further reductions were noticed at the 6 month (DNB=2.01±0.23µl, DB= 2.15±0.39µl) and 12 month (DNB= 1.93±0.22µl, DB= 1.92±0.29µl) intervals (Figure 5.9).

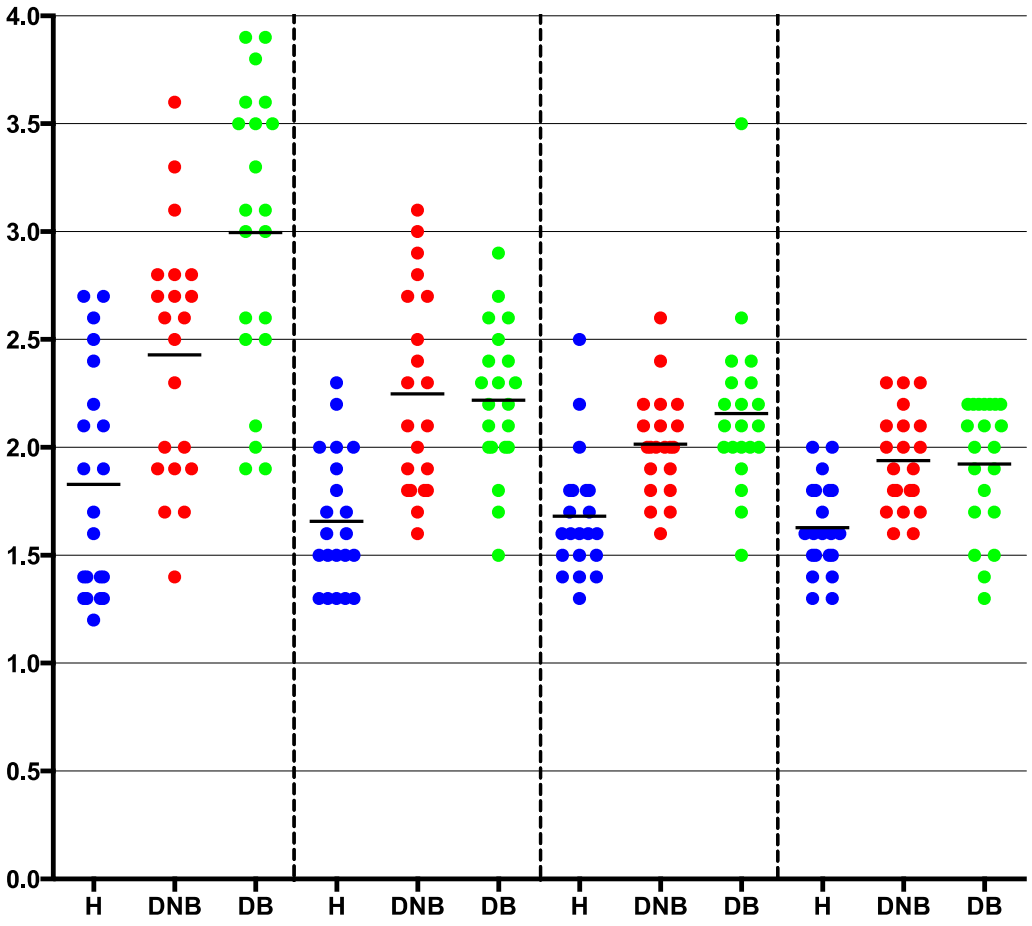


Figure 5.9. Changes in GCF volume throughout the course of the study in sampled sites.

5.3.3 Diagnostic value of the enzymes

ROC curve analysis was used to assess the diagnostic capabilities of these enzymes in differentiating healthy sites from diseased sites. Threshold points with the highest sensitivity and specificity were selected for each enzyme tested at baseline (Figure 5.10).

MMP8, elastase and sialidase showed the greatest sensitivity and specificity and AUCs, i.e. they were more reliable diagnostic biomarkers for identifying healthy and diseased sites. In contrast, cathepsin G and trypsin-like activity showed the lowest sensitivity and specificity (Table 5.2).

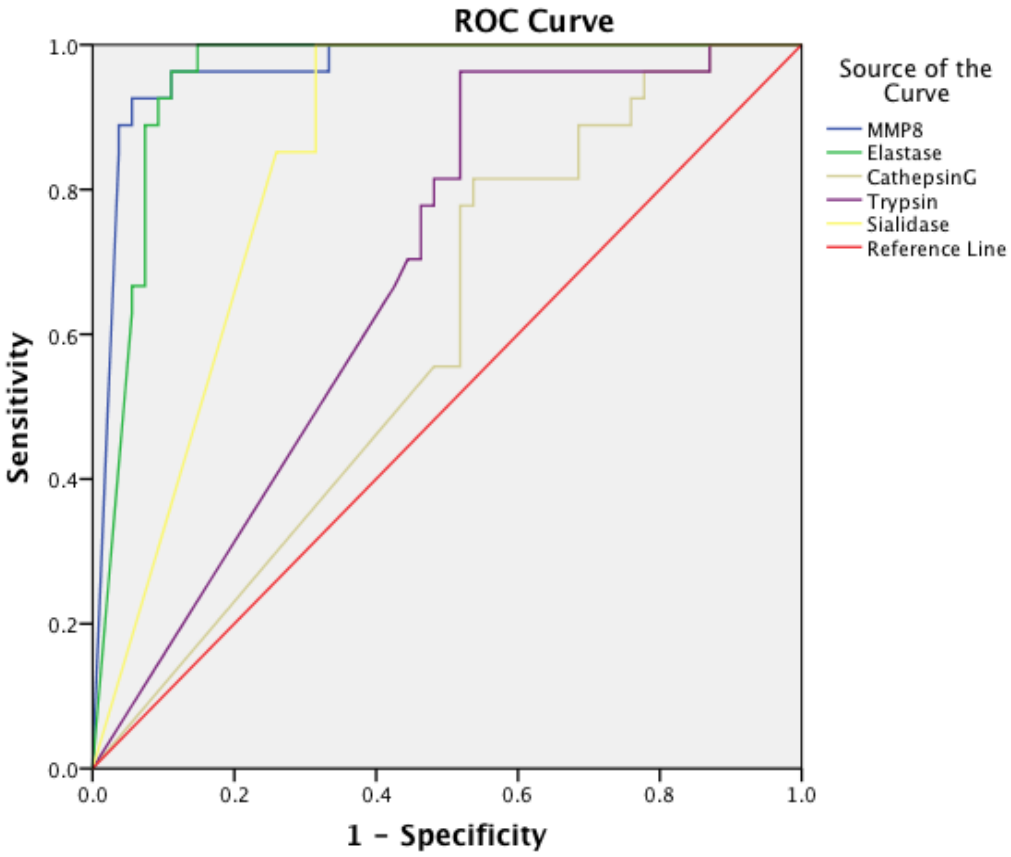


Figure 5.10. ROC for all tested biomarkers at baseline in differentiating between healthy and periodontitis sites.

Table 5.2. Diagnostic properties of specific thresholds of selected GCF enzyme biomarkers.

Variable	Threshold	Sensitivity%/ Specificity%	Area under the curve	95% CI for OR		P value
				LCL	UCL	
MMP8	104	90/90	0.91	0.924	1.21	**0.0001**
Elastase	24	88/90	0.88	0.904	0.908	**0.0001**
Cathepsin G	0.8	55/40	0.63	0.509	0.75	**0.056**
Trypsin like	12	80/55	0.72	0.616	0.836	**0.001**
Sialidase	2.2	86/75	0.88	0.785	1.29	**0.0001**

5.3.4 Usefulness of the enzyme profile as a prognostic tool

The usefulness of these enzymes as prognostic tools in predicting treatment outcome (≥2mm improvement in PPD) was examined using baseline enzyme levels (as independent variables) against the 6 month treatment outcome by logistic regression analysis. It was found that when all the enzymes were combined they predicted 85% of treatment outcome. Similar to the main cohort, redundant variables that could not add any additional prediction value to the enzyme combination were removed by backward stepwise technique and it was found that the combination of MMP8, elastase and sialidase had the highest prediction values for both DNB (88%) and DB (86%) sites (Tables 5.3), with an odds ratio of 0.995 for all three enzymes in both DNB sites and DB sites (Table 5.4). Meanwhile, trypsin-like enzyme and cathepsin G did not add any significant predictive value to the enzyme combination (Table 5.3) and it was found that their effect (β) was not significant (P> 0.5) (Table 5.4). Moreover, all other possible combinations of the tested biomarkers were examined and again the MMP8, elastase and sialidase combination were demonstrated to be the best possible combination in terms of predictive value. Finally, it was found that no enzyme alone could predict treatment outcome at a higher level than the null hypothesis (61.8%) (Table 5.3).

Table 5.3. Logistic regression analysis with 2mm PPD improvement (at 6 months) as the dependent variable.

Method	**DNB sites**	**DB sites**
	Predictive	**Predictive**
All variables	85%	85%
Stepwise (backward conditional)	88%(MMP8, Elastase, Sialidase, Trypsin)	82%(MMP8, Elastase, Sialidase, Trypsin)
	88% (MMP8, Elastase, Sialidase)	**86% (MMP8, Elastase, Sialidase)**
	74% (MMP8, Sialidase)	70 %(MMP8, Elastase)
	61.8% (each single enzyme)	61.8% (each single enzyme)

Table 5.4. Summary of logistic regression for each individual explanatory variable for the response of sites to treatment during the following 6 months.

Disease sampled site	Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR		p value
				LCL	UCL	
DNB	MMP8	-0.005	0.995	0.99	1.21	**0.004**
	Elastase	-0.005	0.995	0.99	1.14	**0.001**
	Cathepsin g	0.0001	1	0.99	1.002	0.76
	Trypsin like	0.001	1.001	0.99	1.007	0.63
	Sialidase	-0.005	0.995	0.99	1.2	**0.01**
DB	MMP8	-0.005	0.995	0.99	1.21	**0.003**
	Elastase	-0.005	0.995	0.99	1.17	**0.002**
	Cathepsin g	0.001	1.001	1	1.002	0.12
	Trypsin like	0.001	0.999	0.99	1.001	0.3
	Sialidase	-0.004	0.996	0.99	1.2	**0.007**

5.3.5 Application of threshold enzyme levels to validate both the diagnostic and prognostic value between two cohorts

To validate the diagnostic and prognostic value of the tested biomarkers, the predetermined threshold points (diagnostic and prognostic threshold point) from the main study data set were used to dichotomise the baseline enzyme levels in this independent cohort study. In addition, the threshold points from the independent cohort study were used to dichotomise baseline enzyme levels in the main study. Both were then reanalysed using the ROC and logistic regression. The sensitivity and specificity for the threshold points are illustrated in Table 5.5. In this table the threshold value for each enzyme obtained from this independent cohort study data set and those obtained from the main study are shown. However, additionally the 'validated' sensitivity and specificity are shown which represents the values obtained when the 'independent cohort data' are analysed using the enzyme thresholds found in the main study and vice versa. As can be seen, the diagnostic threshold points of all biomarkers except cathepsin G are close to each other. After validation, the degree of sensitivities and specificities of cathepsin G were significantly reduced by the predetermined threshold points, whereas, in the cases of

the other enzymes the degree of sensitivity and specificity was reduced slightly but they still retained their diagnostic value, i.e. they were still reliable diagnostic biomarkers.

Table 5.5. Validation of diagnostic value of the enzymes using predetermined threshold points to differentiate health from disease.

Variable	Independent study		Main study		Validation 1*	Validation 2**
	Threshold (ng/µl)	Sensitivity% / Specificity%	Threshold (ng/µl)	Sensitivity% / Specificity%	Sensitivity% / Specificity%	Sensitivity% / Specificity%
MMP8	104	90 / 90	94	86/83	85 / 86	81 / 78
Elastase	24	88 /90	33	78/80	86 / 88	76 / 79
Cathepsin G	0.8	55 /40	10.7	64/60	28 / 20	30 / 22
Trypsin like	12	80 / 55	11.2	65/69	74 / 50	60 / 63
Sialidase	2.2	86 /75	2.3	79/79	86 / 74	79 / 79

* Validation of diagnostic value in independent study using threshold points from main study.

** Validation of diagnostic value in main study using threshold points from independent study.

Furthermore, the threshold points to differentiate sites that improved by ≥2mm in PPD from those that did not were determined in both the independent cohort study and the main cohort study (Table 5.6). Also these threshold points were again used to dichotomise the baseline enzyme levels in the independent cohort study and in the main cohort study and analysed by logistic regression. The analysis revealed that the main study threshold points reduced the predictive value of the three enzyme profile (MMP8, elastase and sialidase) of the independent cohort data but by only 2% in both DNB sites and DB sites, whereas the independent cohort study threshold points reduced the prediction value of the three enzyme profile (MMP8, elastase and sialidase) of the main study by less than 3% in both DNB and DB sites (Table 5.7).

Table 5.6. Threshold points to differentiate sites that improved by ≥2mm in PPD in both the independent cohort and main cohort study

Variable	Independent study		Main study	
	Threshold (ng/µl)	Sensitivity% / Specificity%	Threshold (ng/µl)	Sensitivity% / Specificity%
MMP8	205	65 / 64	192	63/65
Elastase	160	60 /60	171	61/62
Cathepsin G	5.3	45 /40	16	50/49
Trypsin-like	29	40 / 38	24	44/47
Sialidase	23	56/57	23	60/60

Table 5.7. Validation of prognostic value of the enzymes using independent cohort study threshold points to differentiate sites that improved by ≥2mm in PPD.

Method	**DNB sites**	**DB sites**
	Predictive	**Predictive**
MMP8, Elastase and Sialidase (independent study)	**88%**	**86%**
MMP8, Elastase and Sialidase (main cohort study)	**81.3%**	**80.3%**
Validation 1*	**86%**	**84%**
Validation 2**	**80%**	**79%**

* Validation of prognostic value in independent study using threshold points from main study.

** Validation of prognostic value in main study using threshold points from independent study.

5.4 Discussion

The rationale of this independent cohort study was that for a biomarker to be accepted, it needed to keep its biomarker value when applied to an independent cohort. The main aim of this part of the study was to check the validity of the diagnostic and prognostic threshold point for each of the enzymes tested in the independent cohort and main studies and determine how these predefined threshold points affect the diagnostic and prognostic value of these biomarkers in the independent cohort and main studies. As most of the findings (clinical and enzymes findings) of this independent cohort study are very similar to the findings of the main study, these findings are discussed in more detail in the chapter 4. This part of the discussion will focus more on the objectives of this independent cohort study.

The samples for validation from the independent cohort (using same inclusion and exclusion criteria) were the same enzymes as used for testing the diagnostic and prognostic biomarkers in the main study. The length of the independent cohort study was one year (30 patients recruited) and the samples were collected at baseline, 3 months, 6 months and one year. However, as most of the changes in PPD were observed by the 6 month time point, the biomarker values were examined versus the 6 month treatment outcome (23 patients completed 6 month time point). The differences between the main and independent cohort studies have been described in section 5.2.1 above. However, those differences appeared to have little effect on the enzyme profiles of the independent cohort study which also demonstrates the validity of these biomarkers under different conditions. That is one of the important characteristics of a reliable biomarker (Bonassi et al., 2001).

For diagnostic validation, at first glance the threshold points were higher for elastase and cathepsin G and lower for MMP8 and trypsin-like activity in the main study than in this independent cohort study data set. Sialidase has the closest threshold point in both of the studies, whereas the threshold points of cathepsin G showed the greatest discrepancy (Table 5.5). These threshold points in the main study were used to dichotomise baseline enzyme levels in the independent cohort study and vice versa. When ROC curve analysis was repeated, only cathepsin G showed significant reduction in sensitivity and specificity after the validations. Meanwhile, the diagnostic value of the MMP8, elastase and sialidase combination was not significantly affected by applying the ‘predetermined’

threshold enzyme points from either study to the other. Furthermore, when the prognostic capabilities of these biomarkers were examined, again MMP8, elastase and sialidase were confirmed as being useful. Consequently the basic findings from the validation tests appeared to support the initial findings that MMP8, elastase and sialidase are able to differentiate healthy from disease sites and are a good predictor of treatment outcome. This is the first time to the best of our knowledge that these biomarkers have been validated as a combination through applying their threshold points to a separate, independent cohort.

For a biomarker to be accepted, it must retain its diagnostic and prognostic value in the presence of potential confounders such as diabetes mellitus, but this study has not been large enough to test the confounders properly. Nevertheless, the results of this study are reproducible in different experiments and reliable in different sample cohorts.

Chapter 6- Microbiological biomarkers of periodontal disease

Chapter 6 - Microbiological biomarkers

6.1 Introduction

The species that are mostly examined as biomarkers of periodontitis are red complex bacteria (*P. gingivalis, T. forsythia and T. denticola*), *A. actinomycetemcomitans*, *F. nucleatum* and *P. intermedia*. However, the mere presence of these species is not enough to cause or induce further progress of the disease. Microbial biomarkers are of value to identify sites with compromised treatment outcomes.

For the purpose of this study, three species have been investigated as biomarkers of periodontal disease. Two, *P. gingivalis* and *T. forsythia,* belong to the red complex of pathogens (Hajishengallis and Lambris, 2011, Tanner and Izard, 2006), while *F. nucleatum* belongs to the orange complex but acts as an important bridge organism between early and late colonizer species. The following sections focus, therefore, more particularly on these three species, the features they possess that make them more harmful to the host, and their role in the progression of periodontal disease.

6.1.1 *Porphyromonas gingivalis*, *Tannerella forsythia* and *Fusobacterium nucleatum* as biomarkers of periodontal disease

6.1.1.1 *Porphyromonas gingivalis*

P. gingivalis is a Gram-negative, obligatory anaerobic, non-motile, cocco-bacillus that is strongly associated with periodontal disease. Haemin and vitamin k are required for its growth and it is characterised by forming black colonies when cultured on blood agar (Genco, 1995). *P. gingivalis* belongs to the red complex bacteria which are considered to be the main putative periodontal pathogens and lately it has been identified as a putative keystone pathogen of periodontal disease. The latter hypothesis suggests that even when *P. gingivalis* is present in low numbers in dental biofilm, it can shift the diversity of the local microbial population (Hajishengallis et al., 2012).

P. gingivalis is mainly found in the oral cavity, and gingival crevices in particular, but it can also be found in supragingival biofilm, tongue and saliva (Socransky and Haffajee, 1992). *P. gingivalis* has been found in very low abundance in healthy mouths or sites, but it increases in gingivitis and in advanced forms of periodontal disease (Van Winkelhoff et

al., 2002, Forng et al., 2000, Haffajee et al., 1998). Thus, *P. gingivalis* is rarely found in healthy individuals or sites (Socransky and Haffajee, 1992, Moore et al., 1991). Furthermore, the levels of *P. gingivalis* are considered to be higher in active sites than in inactive sites (Tanner, 2014, Walker and Gordon, 1990, Dzink et al., 1988). Also, residual high load of *P. gingivalis* after periodontal therapy has been reported to be associated with further periodontal tissue destruction, suggesting that *P. gingivalis* has a pivotal role in recurrence of the disease (Choil et al., 1990). In addition, evidence from longitudinal studies has shown that *P. gingivalis* levels remain high in sites that respond poorly to such treatment (Choil et al., 1990, Winkelhof et al., 1988). Taken together the evidence suggests that high number of *P. gingivalis* at a site is likely to indicate a poor prognosis.

6.1.1.2 *Tannerella forsythia*

T. forsythia is a Gram-negative, obligatory anaerobic, spindle shaped bacterium that belongs to the red complex group of putative periodontal pathogens (Sakamoto et al., 2005, Socransky and Haffajee, 2005) commonly found in coexistence with *P. gingivalis* (Yang et al., 2004). *T. forsythia* has a growth requirement for N-acetylmuramic acid (Wyss, 1989). *T. forsythia* has been identified in supragingival biofilm of healthy individuals (Gmur and Guggenheim, 1994), however, it has been found in higher loads in association with disease (Van Winkelhoff et al., 2002, Moncla et al., 1991, Lai et al., 1987). Moreover, active diseased sites showed higher levels and increased frequency of *T. forsythia* than quiescent sites (Tanner, 2014, Dzink et al., 1988) and the number of *T. forsythia* correlated with the degree of periodontal tissue breakdown (Lai et al., 1987).

6.1.1.3 *Fusobacterium nucleatum*

Fusobacterium nucleatum comprises a group of anaerobic, Gram-negative, spindle shaped, non-motile bacilli which have recently been divided into 5 subspecies: *nucleatum*, *polymorphum*, *vincentii*, *fusiform* and *necrophorum* (Dzink et al., 1990). *Fusobacterium nucleatum nucleatum* and *Fusobacterium nucleatum polymorphum* are frequently found in the mouth. It is considered as an important bridge organism in establishment of the structure of the oral biofilm (Kolenbrander et al., 2002). *F. nucleatum* is often found in both supra and subgingival biofilm as well as in both healthy and periodontally diseased individuals (Moore and Moore, 1994) but the subspecies

identity was not available at that time. The prevalence of *F. nucleatum* increases with increasing PPD and severity of the disease (Yang et al., 2014, Riep et al., 2009, Moore and Moore, 1994). The number of *F. nucleatum* has been found to be higher in biofilm samples of periodontitis patients than in healthy subjects and it has been detected in saliva in higher abundance in gingivitis and periodontitis patients than in healthy individuals (Ramseier et al., 2009, Van Winkelhoff et al., 2002,). However, evidence for causation rather than association with disease is lacking (Zhou et al., 2015, Saygun et al., 2011).

6.1.1.4 Summary

In summary, although the bacterial aetiology of periodontal disease has yet to be conclusively established, *P. gingivalis* and *T. forsythia* are considered to be the lead candidates for promoting progression of periodontal disease. *P. gingivalis* and *T. forsythia* loads significantly correlate with the status of the disease and the loads of these two species increase as the healthy site progresses to gingivitis, and further increases with progression to mild then severe periodontitis and they are thought to allow differentiation between healthy subjects and periodontitis patients (Kinney et al., 2014, Riep et al., 2009, Ximénez-Fyvie et al., 2000, Socransky et al., 1998, Socransky et al., 1991).

In terms of response to treatment there has been higher detection of *T. forsythia* and *P. gingivalis* in sites that fail to respond to conventional non-surgical periodontal treatment than in sites that do respond (Kook et al., 2005). The presence of and high levels of *T. forsythia* and *P. gingivalis*, have been suggested to be good markers for treatment outcome (Fujise et al., 2002, Machtei et al., 1997).

F. nucleatum co-aggregates with *P. gingivalis* and *T. forsythia* and this process is thought to enhance colonisation of these two bacteria in the subgingival biofilm (Holt and Ebersole, 2005). *F. nucleatum*, *P. gingivalis* and *T. forsythia* together interact synergistically to promote alveolar bone resorption (Settem et al., 2012, Kesavalu et al., 2007, Kuriyama et al., 2000) and recently, it has been found that as periodontal disease progresses the load of these three species increases (Yang et al., 2014).

Combination of these three species with salivary biomarkers has been reported to be useful for distinguishing healthy subjects from patients with periodontal disease (Kinney et al., 2011), however, it is worth mentioning that this finding does not add any additional

information to the current diagnostic method as we can identify healthy and diseased subjects with current methods of diagnosis. Moreover, robustness of these three species plus GCF and salivary biomarkers have been reported as identifying patients at high risk of further disease progression (Kinney et al., 2014), but that finding does not indicate which sites are at high risk. For that reason it is very important when searching for biomarkers of periodontal disease that the site-specific and multifactorial nature of the disease is taken into account. Therefore, when looking for prognostic biomarkers to provide accurate prediction levels we have to look beyond patient-based biomarkers and examine these biomarkers on a site-specific basis. For these reasons the current study combines collections of GCF constituents and putative pathogens as biomarker 'fingerprints' in order to assess the robustness of their predictive values at diseased sites.

6.1.2 Methods of sampling and detecting oral microorganisms

Several methods have been used to obtain samples and detect oral bacteria. Dental curettes and paper points are the techniques most often used to collect periodontal plaque samples (Baehni and Guggenheim, 1996). Bacterial culture is one of the techniques commonly used to identify bacterial species and it has been critical in revealing microbial diversity in the subgingival biofilm. Multiple species have been identified through use of various atmospheric conditions and selective media. However, culture techniques have certain limitations, such as transportation issues, slow growth, long incubation times, and labour intensiveness, and, most importantly, approximately 50% of the subgingival micro flora have not been cultured to date (Wade, 2013).

Immunological techniques have been developed to identify different species, such as ELISA, immunohistochemistry and immunofluorescence (Tanner et al., 1991). These techniques mostly detect bacteria indirectly via interaction of monoclonal or polyclonal antibodies with species-specific antigens that consequently detect targeted bacteria (Baehni and Guggenheim, 1996) and these are effective and sensitive. However, the methods are costly because of the requirement for specific antibodies and they are only available to a small number of species.

Over recent years DNA based methods such as PCR, DNA-DNA hybridization and 16S rRNA analysis have been widely used to investigate the role of bacteria in periodontal diseases. These molecular techniques facilitate detection of many previously known

bacteria and novel phyla, and have contributed knowledge on diversity of the oral microflora, which subsequently opens possibilities for identifying novel pathogens (Baehni and Guggenheim, 1996).

The amplification and sequencing of the 16S rRNA gene brought a major advance in microbiology. The 16S rRNA gene is selected for the following reasons: it is universally distributed, of large enough size to provide information, contains highly conservative regions, which allows genus-level identification and hyper-variable regions that allows species-level differentiation (Janda and Abbott, 2007). Generally, molecular techniques are rapid, more sensitive, more specific, quantitative, highly reproducible and can detect uncultivated species. The main drawback is the requirement for sequence data.

For the above reasons, in this study the 16S rRNA gene has been chosen to identify and quantify the targeted species in clinical samples using qPCR.

6.1.3 Aims and objectives

6.1.3.1 Aims

The aim of this longitudinal clinical study was to determine whether the levels of *Porphyromonas gingivalis*, *Tannerella forsythia* and *Fusobacterium nucleatum* species detectable in subgingival plaque can be used in combination as a "biomarker finger print" for:-

1. Diagnostic biomarkers for the clinical condition.
a. To differentiate between health and disease based on conventional criteria such as PPD.
2. Prognostic biomarkers of the outcome of treatment in patients with chronic periodontitis.
3. Combines collections of GCF enzymes and putative pathogens as biomarker 'fingerprints' in order to assess the robustness of their predictive values at diseased sites.

6.1.3.2 Objectives:

1. Develop a standard curve for each species using qPCR.
2. Collect and analyse samples at each time point.
3. Identify potential biomarkers in subgingival plaque samples.
4. Test the diagnostic utility of these identified biomarkers.
5. Test the prognostic utility of these identified biomarkers.
6. Combine enzyme and bacterial biomarkers to identify robustness of their prediction value.

6.2 Methodology and materials

6.2.1 Bacterial strains and growth conditions

The bacterial strains used to prepare standard curves were *P. gingivalis* (NCTC 11834), *T. forsythia* (ATCC 43037) and *F. nucleatum* (ATCC 25586). These stains were available in the stocks of the oral microbiology laboratory, School of Clinical Dentistry, University of Sheffield. They were cultured on Fastidious Anaerobic agar supplemented with 7.5% v/v horse blood and incubated in an anaerobic cabinet (3-5 days) under an atmosphere of N_2 80%, H_2 10% and CO_2 10% at 37°C. Purity of the cultures was checked by colony characteristics and Gram staining before analysis by qPCR using species-specific primers for the 16S rRNA gene.

6.2.2 Plaque sample collection and storage conditions

Subgingival plaque samples were collected from the same three representative sites from where the GCF was collected at each time point in the subjects studied (as described in section 2.6), with a sterile curette (Figure 6.1). They were placed into 500µl sterile phosphate buffer saline (PBS, pH 7.2) and stored at -80°C until being processed for qPCR. Samples of 50 subjects (450 samples in total) were analysed in this part of the study. Initially attention was given to the enzymes present and it was later decided to study plaque samples by qPCR. The samples from the subjects at the beginning of this study were analysed by culturing, in pursuing the stated study aims. Consequently plaque from these 27 subjects was not available for analysis using qPCR.

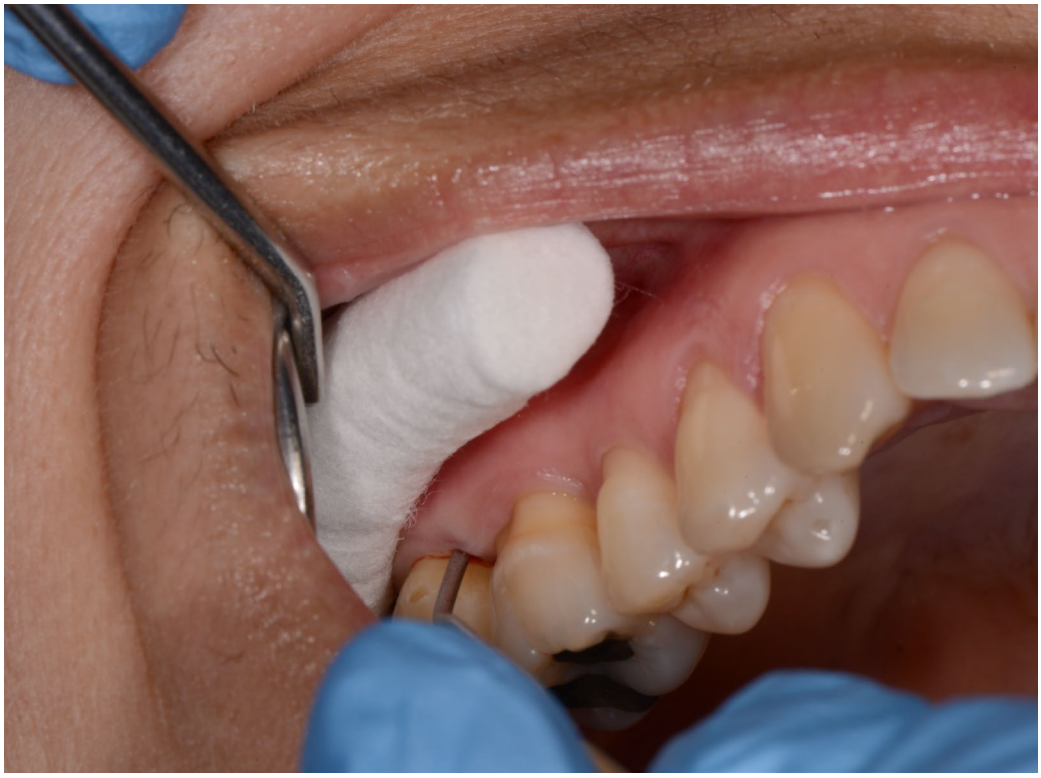


Figure 6.1. Subgingival plaque sampling mesio buccal of tooth 17.

6.2.3 DNA extraction

Each reference strain and each plaque sample was thawed and immediately extracted using QIAamp DNA mini kit (Qiagen) as follows. Plaque samples in sterile PBS were centrifuged at 10000g to pellet the bacterial cells and the volume was adjusted to 300µl as a starting volume for DNA extraction. All samples were analysed within 2 month of collection.

Lysis and extraction was carried out according to the manufacturer's instructions. Briefly this entailed incubation of samples with 90µl of lysozyme (10mg/ml, prepared with Tris-EDTA buffer), 3.6µl mutanolysin (25,000 U/ml, prepared with Tris-EDTA buffer) and 1.8µl lysostaphin (4000 U/ml, prepared with free nuclease water) at 37°C for 1 hour. Then 24µl proteinase K, 4.8µl RNAse A (100mg/ml) and 300µl of kit lysis buffer were added before incubation for 10 minutes at 56°C. After brief centrifugation, 400µl of 100% ethanol were added and the samples were centrifuged briefly.

Next, 700µl of each lysate preparation was applied to a column, washed twice by centrifugation (6000g for 1 minute) sequentially with wash buffers 1 and 2 (500µl), and the DNA eluted into an eppendorf collection tube with 100µl of distilled nuclease free

water.

6.2.4 Primers and probes

All primer sequences used in this study (listed in Table 6.1) targeted the 16S rRNA gene and have been previously published (Ammann et al., 2013, Ramseier et al., 2009). To detect bacterial species in the plaque samples and the reference bacteria (for standard curve), the hyper-variable regions on the 16S rRNA gene were used. Also, to target all bacterial species present in plaque samples, the conserved regions on the 16S rRNA gene were used to design universal primers. This allowed presence of the target species to be assessed as a proportion of the total bacteria present.

The 7900HT Fast Real-Time PCR Detection System (Applied Biosystems) was used to amplify and quantify the target sequences using a 96-well plate format (96 Well PCR Plate, Semi Skirted, FAST). The cycle conditions were as follows:- initial denaturation for 5 minutes at 95°C, followed by 40 cycles of amplification comprising dissociation of DNA at 95°C, annealing of primers (Table 6.1), and extension at 72°C for 1 minute each, and final extension for 7 minutes at 72°C. The PCR reactions for clinical samples and reference bacteria (to construct standard curves) were always conducted in duplicate and triplicate, respectively. Reactions were performed in a total volume of 20µl containing 10µl SYBR® Green PCR Master Mix (Life Technologies), 7µl nuclease free water, 1µl each of forward and reverse primers (5nM) and 1µl DNA template. Data were analysed using Sequence Detection Software (2.4 supplied by Applied Biosystems).

Table 6.1. Primers for qPCR analysis of biofilm bacteria.

Bacterial Species	**Forward Primer**	**Reverse Primer**	**Product length (bases)**	**T_m (°C)**
P. gingivalis	GCGAGAGCCTGAACCAGCCA	ACTCGTATCGCCCGTTATTCCCGTA	90	62°C
T. forsythia	CGATGATACGCGAGGAACCTTACCC	CCGAAGGGAAGAAAGCTCTCACTCT	72	62°C
F. nucleatum	CGCCCGTCACACCACGAGA	ACACCCTCGGAACATCCCTCCTTAC	75	60°C
Universal	CCATGAAGTCGGAATCGCTAG	GCTTGACGGGCGGTGT	86	61°C

6.2.5 qPCR standard curve preparation and quantification of bacterial species in clinical samples

Standard curves for each target bacterial species were prepared using species specific primers and DNA from pure culture. Standard curves for total bacteria using universal primers were generated with DNA purified from *P. gingivalis*. NanoDrop ND-1000 (Thermo-Fisher Scientific) was used to determine DNA concentrations. The DNA concentrations were adjusted to 1ng/µl with nuclease free water and then additional tenfold serial dilutions were made to construct standard curves from (1 to 10^{-5} ng) (Figures 6.2 and 6.3). Each standard curve was generated by plotting the known concentrations of DNA against its crossing point (Ct value) at which all primers showed high linearity (R2>0.99). The efficiency of primers was calculated using standard curve slope values, which were -3.542, -3.4976, -3.82 and -3.39 for *P. gingivalis*, *T. forsythia*, *F. nucleatum* and universal primers respectively. Slope values from -3.32 to -3.6 provide efficiency of 100% to 90%, respectively.

The sensitivity of the assay was determined by lowest Ct value, which should be statistically significantly higher ($P<0.05$, t-test) than the Ct value of the negative control. The DNA concentration of total bacteria and specific bacteria in the clinical samples was determined using their corresponding standard curves. Specific bacterial DNA concentration was divided into the total DNA concentration to find the proportion of each bacterial species in each subgingival clinical sample. There are numerous types of bacteria in the oral cavity and it is impossible to accurately know each of their genome weights and 16s rRNA copy numbers. In the present study, therefore, the total bacterial load in the clinical specimens was calculated on the assumption that the 16s rRNA gene copy numbers of the oral anaerobes were not significantly different from each other (Nadkarni et al., 2002, Griffen et al., 1998).

To calculate numbers of these bacteria, the DNA concentrations obtained from the standard curves were divided into the genomic weight of each species (*P. gingivalis*= 2.58×10^{-6}, *T. forsythia*= 3.73×10^{-6}, *F. nucleatum*= 2.32×10^{-6}) (Ammann et al., 2013). The resulting number of each bacterium is reported without taking the 16srRNA gene copy into account.

Furthermore, the chances of a false negative result in the clinical samples were examined by increasing the volume of clinical sample DNA up to 5µl in the qPCR reaction. It was

noticed that some of the negative result for *P. gingivalis* and *T. forsythia* in the first trial was due to the small volume (adding smaller DNA amount of the specific species) of DNA extracted from the clinical samples. This experiment increased the prevalence of *P. gingivalis* and *T. forsythia* in total, however there were no significant increases in the mean percentages of these two species.

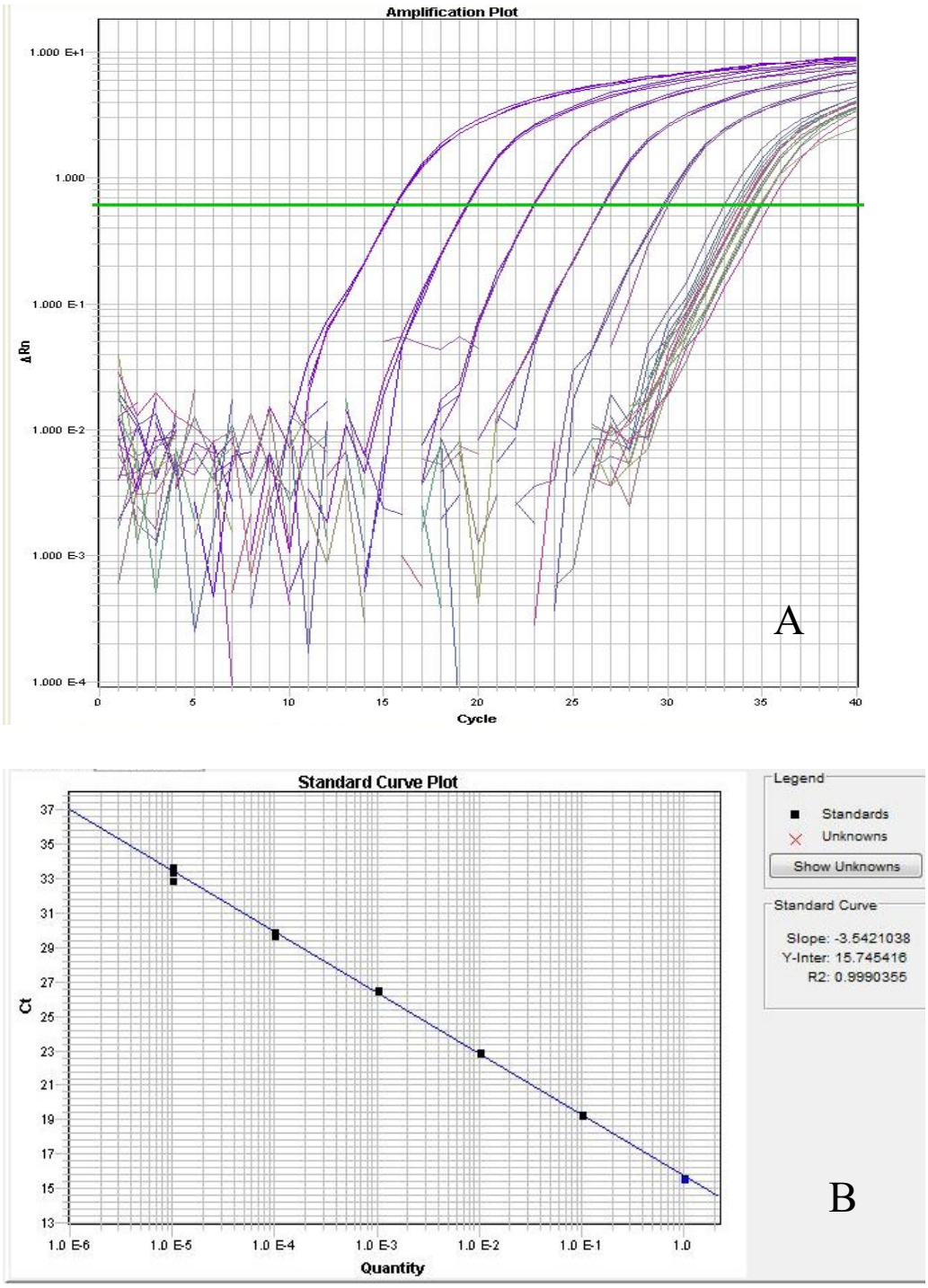


Figure 6.2. Example of amplification (A) and standard curve (B) of the qPCR for *Porphyromonas gingivalis*.

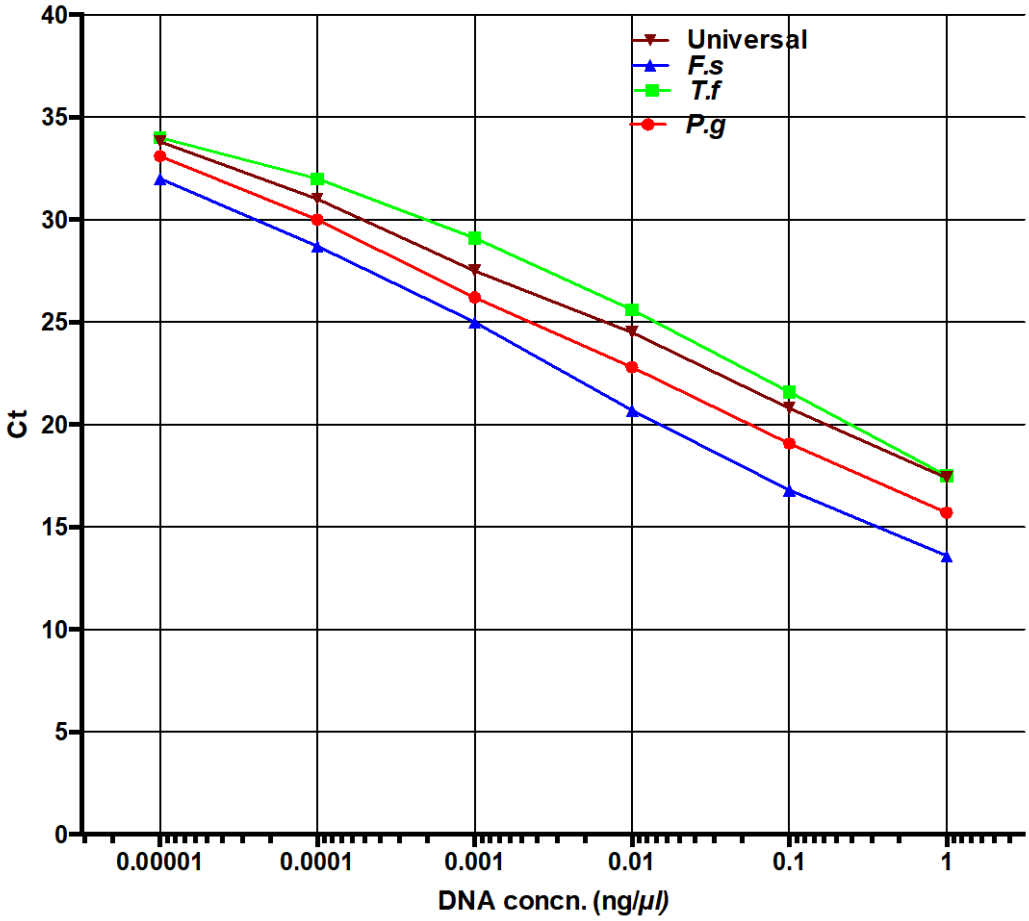
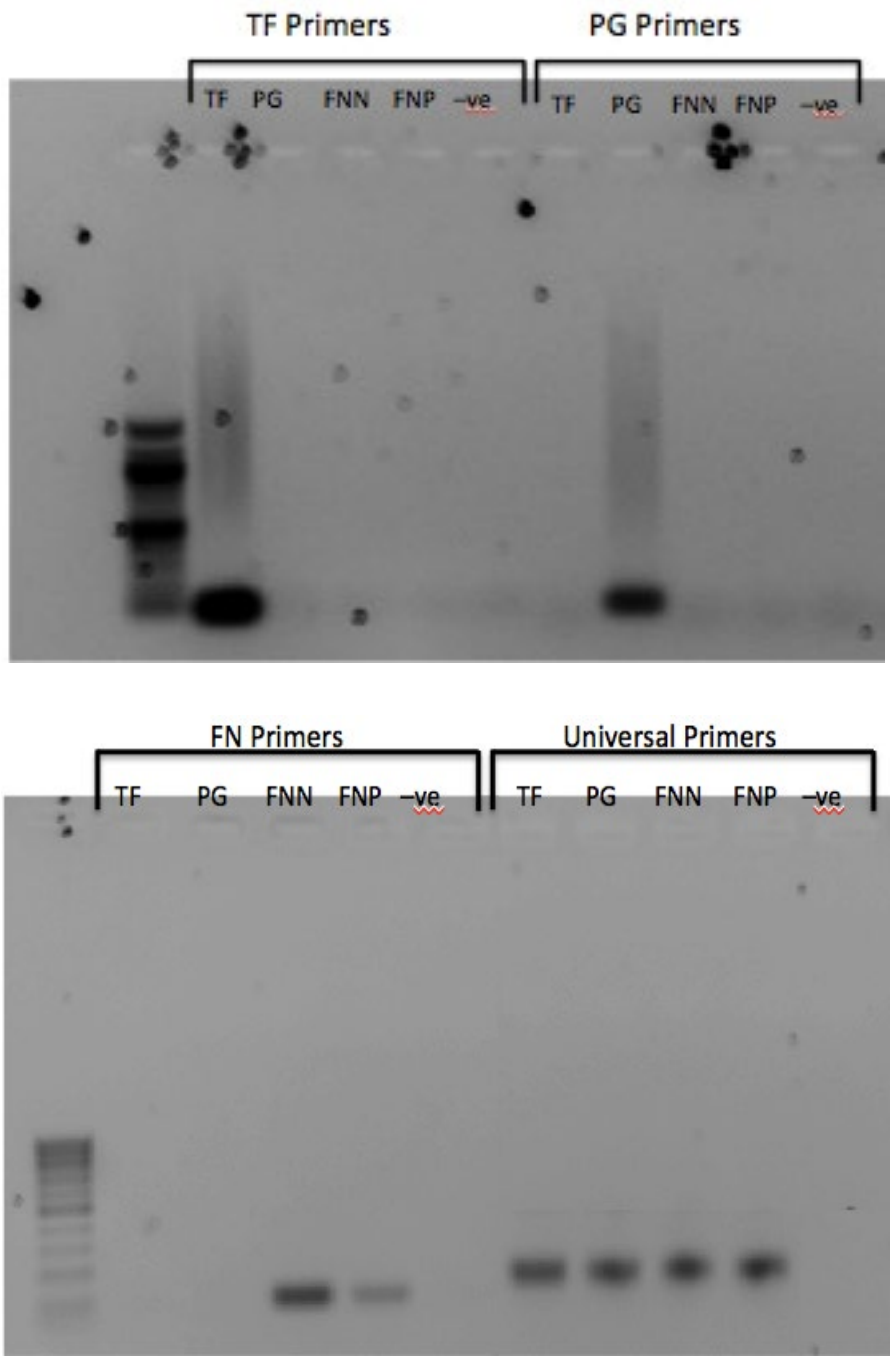


Figure 6.3. Standard curves of the qPCR for all tested primers.

6.2.6 Specificity and cross reactivity of primers

All primers were checked for their specificity by conventional PCR and gel electrophoresis of the amplicons. No nonspecific bands were observed when each specific primer was used against DNA from different bacterial species (Figure 6.4).



PG: P. gingivalis, TF: T. forsythia, FN: F. nucleatum.

Figure 6.4. Specificity of the primers.

The effect of other possible inhibitors or confounder molecules in the plaque samples on the efficiency of each specific primer was evaluated. Portions of each species DNA template was added to DNA from a range of plaque samples and subjected to qPCR. The resultant number of bacteria calculated was compared with that obtained for each plaque sample alone plus the added equivalent number of bacteria (Figure 6.5). No evidence was

found for an effect of plaque constituents on the efficiency of the amplification reaction (Table 6.2).

Table 6.2. Sensitivity of the primers.

Primers	Concentration in plaque (ng/µl)	Concentration in DNA template (ng/µl)	Combined concentration (ng/µl)
P. gingivalis	0.003	0.5	0.498
T. forsythia	0.005	0.3	0.28
F. nucleatum	0.09	0.2	0.283
Universal primers	0.1	0.48	0.58

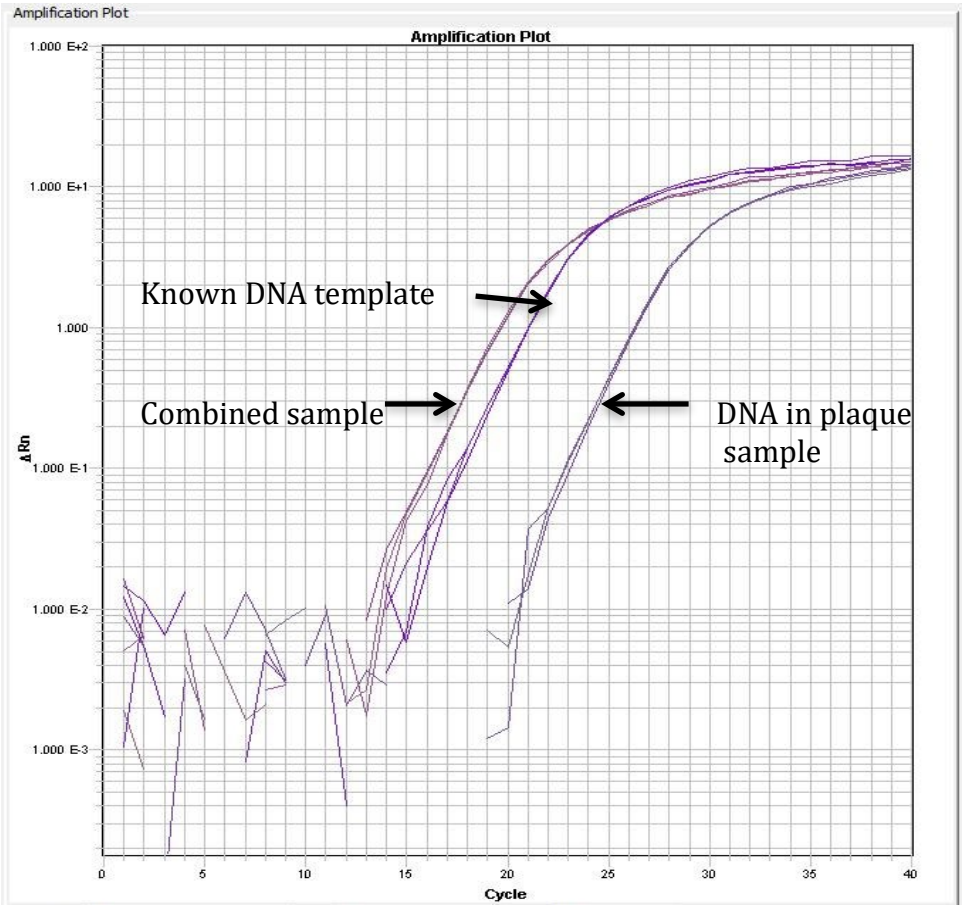


Figure 6.5. Example trace showing the effect of any confounding molecules in plaque on primer efficiency.

6.2.7 Statistical analysis

The statistical analysis of data collected is described in chapter 2, section 2.14. The "continuous" data were tested for normal distribution and thereafter subjected to appropriate parametric/non-parametric testing (Shapiro-Wilk test). The following relationships were investigated and a p-value of <0.05 was taken to be statistically significant. Kruskal Wallis test was used to find the statistically significant differences of biomarker values in three selected sites and biomarker values in each time point. Correlations between clinical measures and biomarkers values were evaluated with Spearman's correlation.

Two-millimeter improvement in PPD was considered to be clinically relevant and was used to dichotomise the outcome variable (pocket depth at 6 months). The areas under the curves of the receiver operating characteristic curve (ROC) were estimated non-parametrically. Threshold points for bacterial levels were selected from the ROC curves as the values with the highest sensitivity and specificity. Baseline continuous values of bacterial biomarkers (as predictors) were analysed by logistic regression against binary outcome measures (pocket depth as being improved by 2mm or not) to find predictive values of the biomarkers (as dependent variables) at 6 months after treatment. Regression analysis with backward stepwise technique was used to exclude redundant biomarkers. All variables included in the final multivariate model were determined to be independent through the assessment of their co-linearity. Odds ratio (OR) estimates and their confidence intervals (CI) were calculated and statistical significance was defined as $P \leq 0.05$. All calculations were performed using the SPSS software package (version 20; SPSS Inc., Chicago IL, USA).

6.3 Results

6.3.1 Demographic and clinical summary of sample population

Four hundred and fifty samples from 50 subjects (23 males and 27 female), who completed the study between 2013 and 2015 were analysed by qPCR. The average age of these subjects, 5 of whom were smokers, was 49.34±8.26 years, ranging from 31 to 68 years of age, with age distribution of 10% aged 30-39, 42% aged 40-49, 32% aged 50-59 and 16 % aged 60-68 years. All samples achieved excellent amplification curves as can be seen below in the representative plot (Figure 6.6).

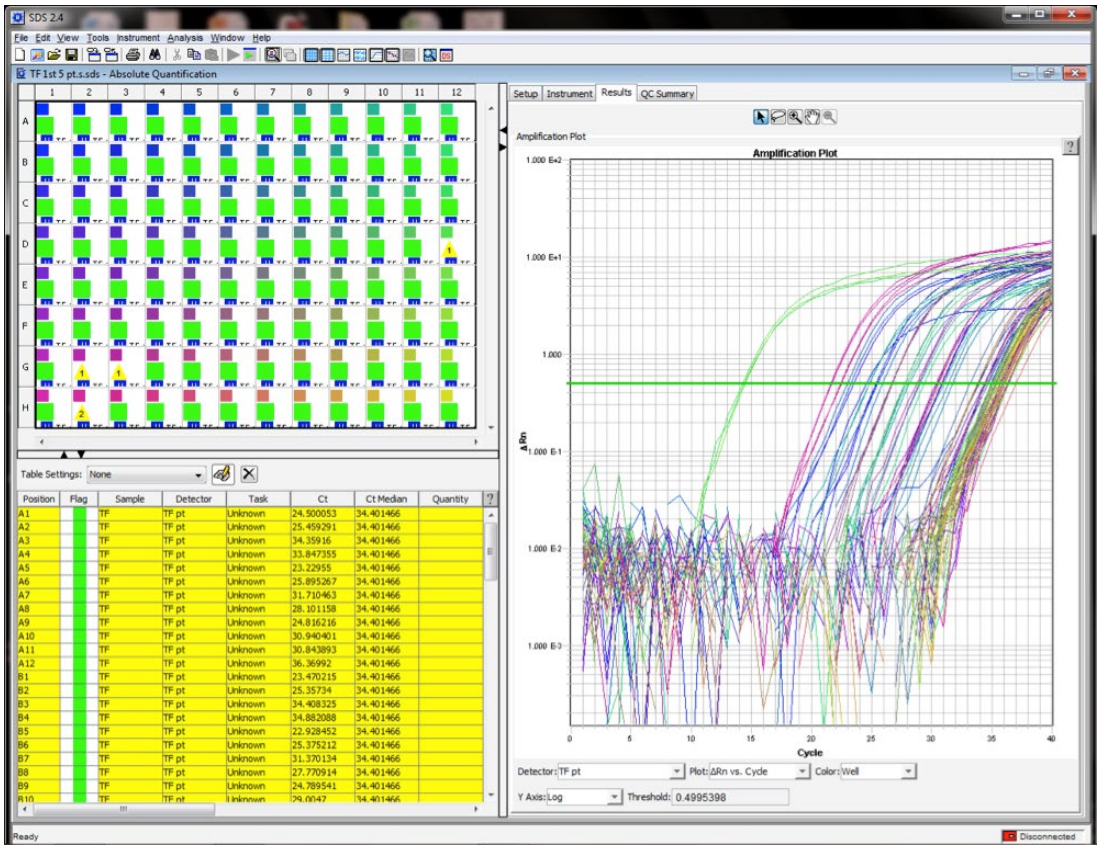


Figure 6.6. A representative print of qPCR amplification plots of clinical samples.

6.3.2 Changes in full mouth and site specific clinical data at study time points

6.3.2.1 Full mouth clinical data

Similar to the GCF data (n= 77), all of the full mouth clinical measures in this study of plaques samples (n=50), except PPD of 4-5mm showed significant improvement following two cycles of the treatment (Table 6.3). For example, after one cycle of the treatment and at the 3 month time point, the average percentages of PI and BOP had decreased from baseline values 65.1± 24.4 and 21.62 ±14.6 to 43.04 ± 24.8 and 16.5 ± 20.4, respectively. Further reductions in the mean percentages of PI and BOP were noticed after a second cycle of the treatment at the 6 month time point (38.54± 26.3, 8.6 ± 12.8, respectively). Similarly, at the 3 month time point a significant improvement in the mean percentage PPD of 4-5mm (16.27 ±9.1 to 20.55±10.85: p = 0.03) and PPD≥ 6mm (from 13.22± 12.51 to 7.04± 7.08%: P= 0.0001) was also found; however, the mean percentage PPD ≤3mm indicated an increase of less than 2%, which was not significant. After the second cycle of the treatment and at 6 month time point, further improvements in the proportion of sites that were PPD≥ 6mm (2.94±3.7%) and sites less than 3 mm in PPD (81.58±11.26%) were achieved. Moreover, at this time point, the percentage of sites that were PPD 4-5mm actually increases at 3 month compared to the baseline this was because a number of sites that were originally deeper clinically improved bringing them in to the 4-5 mm category. This explains why the proportion of sites in the 4-5 mm category that improved at the six month time point was not significant (p = 0.63).

Table 6.3. Full mouth clinical data at study time points (n= 50).

Variable	Baseline (% sites ± SD)	3 months (% sites ± SD)	P value* (baseline vs 3 month)	6 months (% sites ± SD)	P value* (baseline vs 6 month)
PI	65.1± 24.4	43.04 ± 24.8	**0.0001**	38.54± 26.3	**0.0001**
BOP	21.62 ±14.6	16.5 ± 20.4	0.11	8.6 ± 12.8	**0.0001**
Mean PPD ≤ 3mm	70.51 ±16.47	72.41 ± 15.8	0.5	81.58± 11.2	**0.0001**
Mean PPD 4-5 mm	16.27 ±9.1	20.55±10.85	**0.03**	15.48 ± 9.03	0.63
Mean PPD ≥ 6 mm	13.22± 12.51	7.04± 7.08	**0.0001**	2.94±3.7	**0.0001**

* t-test

6.3.2.2 Site specific clinical data

At baseline, supragingival plaque was present adjacent to 86% of DNB and 90% of DB sites. Prevalence of supragingival plaque had significantly reduced to 48%, 60%, respectively, at 3 months, and the second cycle of treatment led to further reduction to 24% for both sites at the end of the study (P= 0.0001) (Table 6.4). On the other hand, plaque was also present in 60% of the healthy sites. Regarding the BOP, the prevalence of bleeding in DB sites showed continuous reduction throughout the course of the study. However, in DNB sites the prevalence increased to 48% at the 3 month time point and then decreased to 36% at the 6 month reexamination (Table 6.4).

Table 6.4. Site specific clinical data at study time points (n= 50).

Variable	Baseline (% sites)	3 months (% sites)	P value* (baseline vs 3 month)	6 months (% sites)	P value* (baseline vs 6 month)
PI (healthy site)	60	32	**0.004**	15	**0.002**
PI (DNB site)	86	48	**0.001**	24	**0.001**
PI (DB site)	90	60	**0.001**	24	**0.001**
BOP (healthy site)	0	12	NA	6	NA
BOP (DNB site)	0	48	NA	36	NA
BOP (DB site)	100	62	NA	46	NA

* Chi-squared test , NA= not applicable

6.3.2.3 Treatment outcomes

Two millimeter improvement in PPD was used as threshold to determine a successful or compromised treatment outcome. At baseline, the mean PPDs for DNB and DB were 6.58±1.02 mm and 6.96±1.45 mm, at 3 months and after the initial treatment phase the mean PPDs for DNB and DB exhibited statistically significant decreases to 5.22±1.64 mm and 5.4±1.26, respectively. The second treatment phase resulted in further decreases by the 6 month time point to 4.3±1.38 mm, 4.42±1.66 mm, respectively (Figure 6.7).

On the other hand, at the individual site level for the sites chosen for study, initial treatment resulted in the proportion of sites reaching the successful treatment endpoint of

"≥2mm improvement of PPD" at the 3 month time point was 42% for DNB sites and 34% for DB sites. At the 6 month time point, 68% of DNB and 66% of DB sites had achieved ≥2mm reduction in PPD. Meanwhile, 6 DNB sites (12%) and 11 DB sites (22%) exhibited improvement of ≤2mm. Moreover, 16% of DNB sites and 8% of DB sites showed no improvement at all and interestingly 4% of DNB and 4% of DB sites demonstrated further disease progression (Figures 6.8 and 6.9). On the other hand, in terms of patient-based outcomes, PPD in 70.8% of sites ≥6mm improved by at least 2mm, 23.4% of sites improved by less than 2mm, while 3.7% showed no improvement and the remaining 2.1% showed further deterioration.

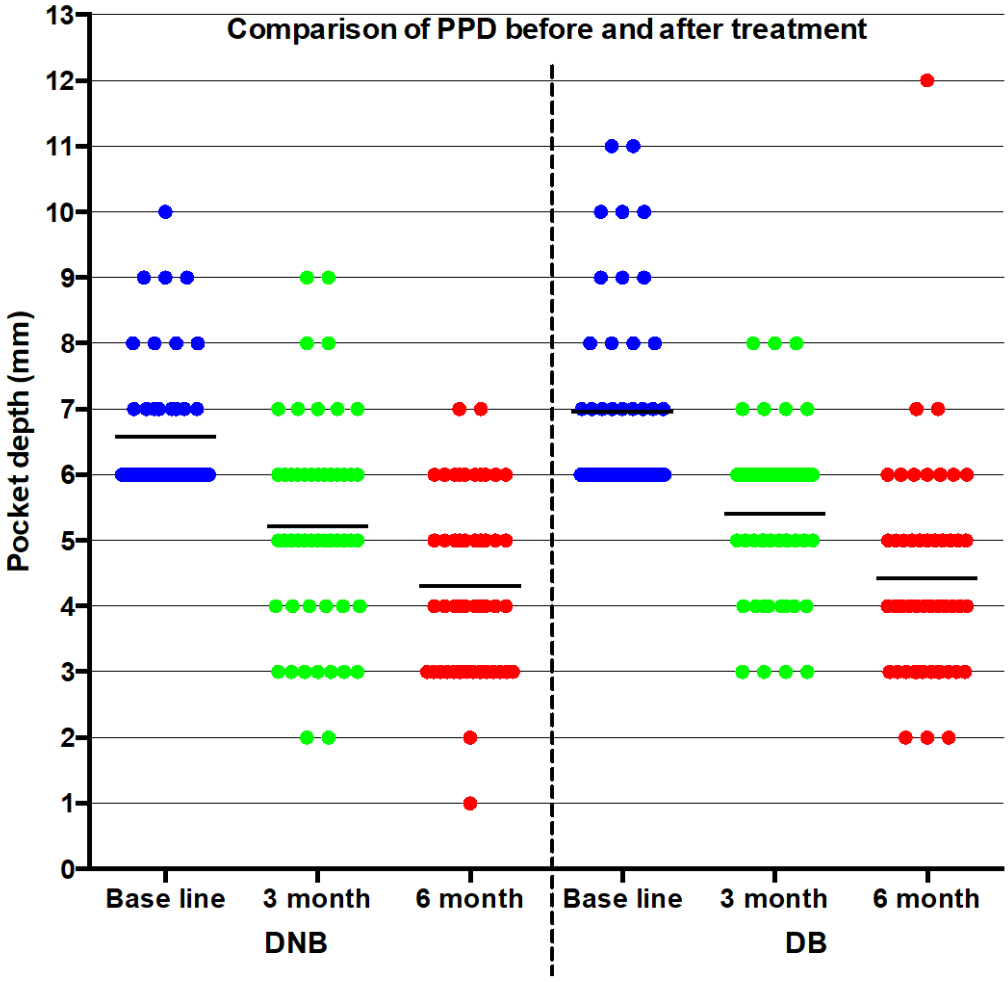


Figure 6.7. Changes in PPD throughout the course of the study in DNB and DB sites (Each point represents a single site).

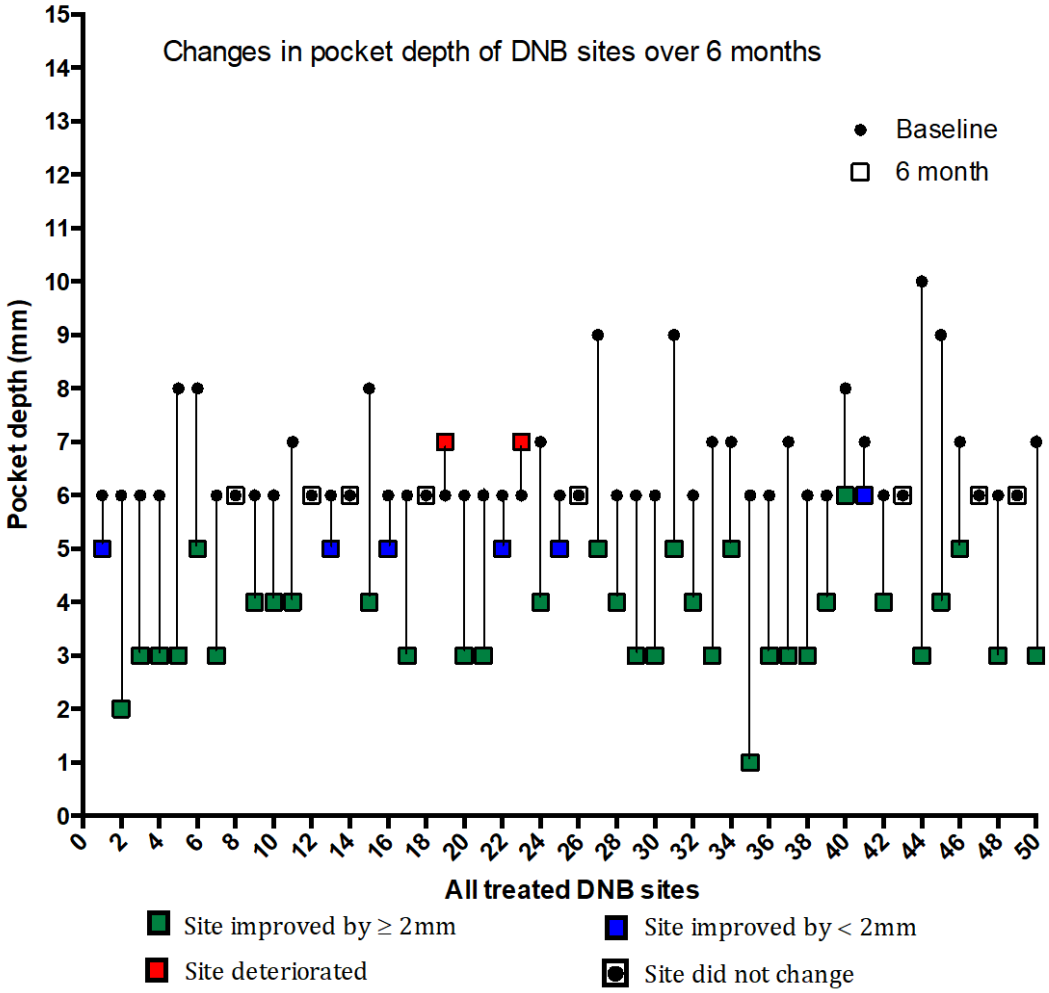


Figure 6.8. Changes in PPD of each DNB from baseline to the 6 month time point.

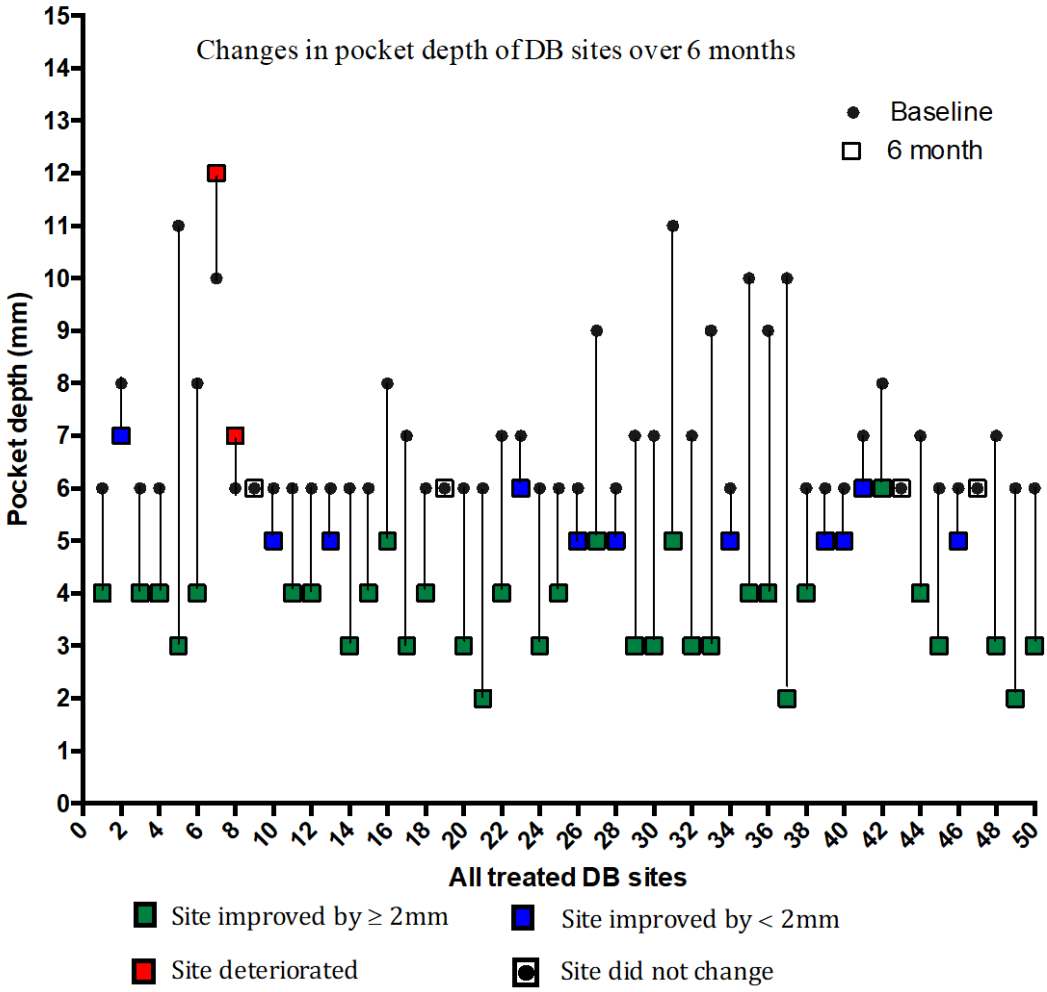


Figure 6.9. Changes in PPD of each DB from baseline to the 6 month time point.

6.3.3 Bacterial data at study time points

These species were detected in the majority of samples (450 samples). The prevalence of *P. gingivalis* at healthy, DNB and DB sites was 40%, 80% and 82%, respectively and similarly *T. forsythia* was detected in 46% of healthy sites, 84% of DNB and 80% of DB sites. Finally, *F. nucleatum* was detected in all healthy and diseased samples sites (DNB and DB). As shown in Figures 6.10, 6.11 and 6.12, Kruskal-Wallis test revealed that the proportions of all examined species were significantly higher in diseased sites (*P. gingivalis* median = 0.99%, 1.4%: *T. forsythia* median = 1.09%, 1.43%: *F. nucleatum* median= 4.04%, 4.82% for DNB and DB sites, respectively) than in healthy sites (*P. gingivalis* median = 0.01%: *T. forsythia* median = 0.07% and *F. nucleatum* median=

2.58%). Generally, among diseased sites the proportion of these species was revealed as again being higher in DB than in DNB sites. However, on an individual basis, some DNB sites had reasonably high levels of these species compared to DB sites. The non-surgical periodontal treatment resulted in significant reduction in percentages of these species at a 3 month time point (Figures 6.10, 6.11 and 6.12). Further reductions in *P. gingivalis*% (median = 0.02 for both DNB and DB sites) and *T. forsythia*% (median= 0.03 for both DNB and DB sites) were noticed after the second cycle of the treatment, at the 6 month time point. However, no significant decrease occurred in the proportion of *F. nucleatum*.

Regarding numbers of these species, numbers of bacterial cells of *P. gingivalis* (median DNB = 1.1 x 10^6, median DB = 1.1 x 10^5) and *T. forsythia* (median DNB = 8.2 x 10^4, median DB = 2.1 x 10^5) were greater at disease sites than healthy sites (*P. gingivalis* median= 2.7 x 10^2, *T. forsythia* median = 3.3 x 10^2). Whereas, only in DB sites were significantly higher numbers of *F. nucleatum* detected (DNB= 4.6 x 10^4, DB= 6.1 x 10^6) compared to healthy sites (7.1 x 10^4) (Figures 6.13, 6.14 and 6.15). Showing a similar pattern to the proportions of these species, the numbers of *P. gingivalis* and *T. forsythia* showed significant reduction at the 3 month and 6 month re-examinations (Figures 6.13 and 6.14). Interestingly, the two phases of the treatment did not lead to a significant decrease in the number of *F. nucleatum* (Figure 6.15). Overall, the proportion and the number of *P. gingivalis* and *T. forsythia* decreased significantly throughout the course of the study.

On the other hand, Spearman correlation coefficients showed no statistically significant correlation between the PPD and the proportions of these species (for *P. gingivalis* r= 0.1 for both DNB and DB; for *T. forsythia* r= 0.1 for DNB and 0.2 for DB and for *F. nucleatum* r= 0.05 for DNB and 0.1 for DB sites). However, levels of *P. gingivalis* and *T. forsythia* at baseline were significantly correlated with treatment outcome at the 6 month time point (2mm improvement in PPD or not). Spearman correlation coefficients of treatment outcome with baseline *P. gingivalis* and *T. forsythia* levels were -0.58, -0.62, respectively, for DNB site and -0.61 and -0.68, respectively, for DB site, while *F. nucleatum* was not significantly correlated with treatment outcome at 6 months (r= 0.09, 0.07 for DNB and DB respectively).

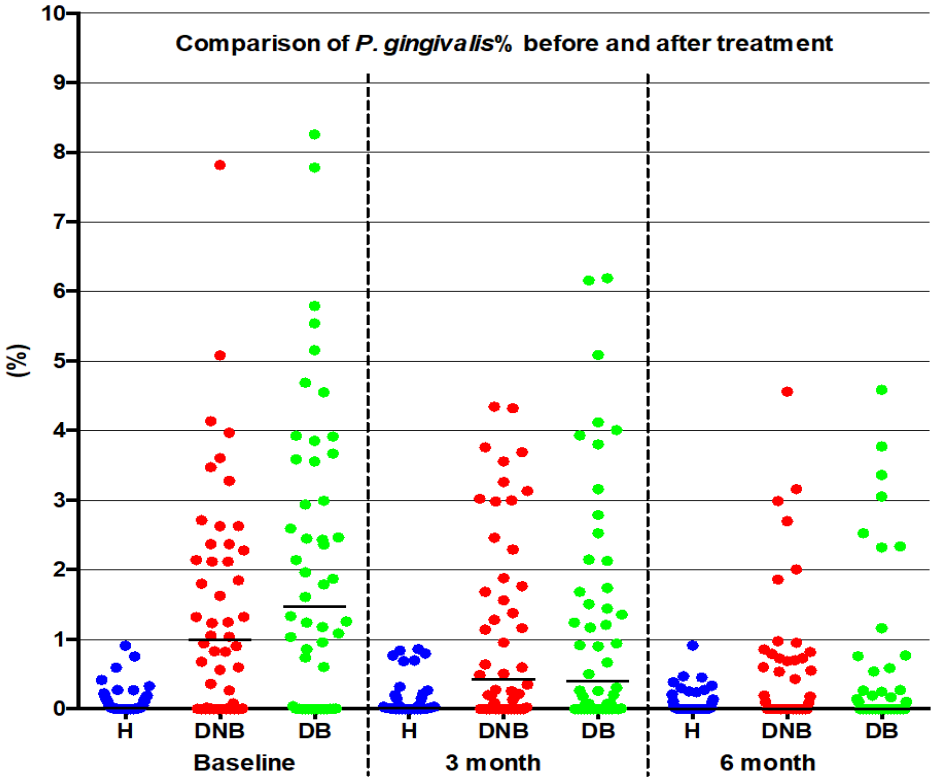


Figure 6.10. Comparison of proportions of *P. gingivalis* at all of the sites at each of the study time points.

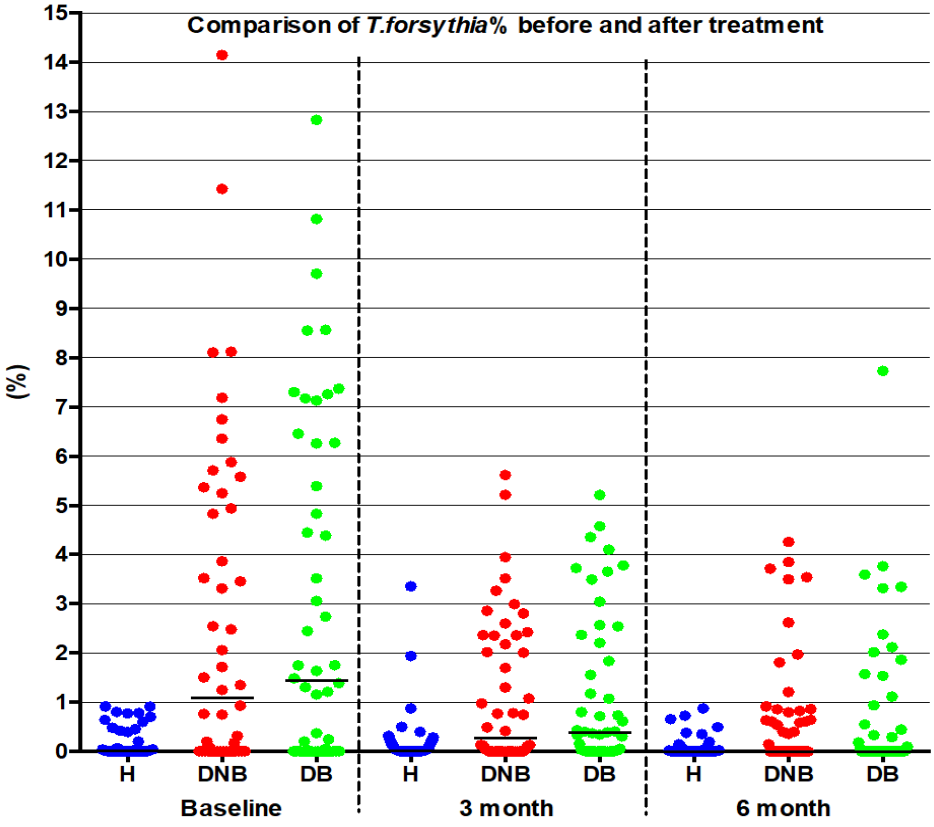


Figure 6.11. Comparison of proportions of *T. forsythia* at all of the sites at each of the study time points.

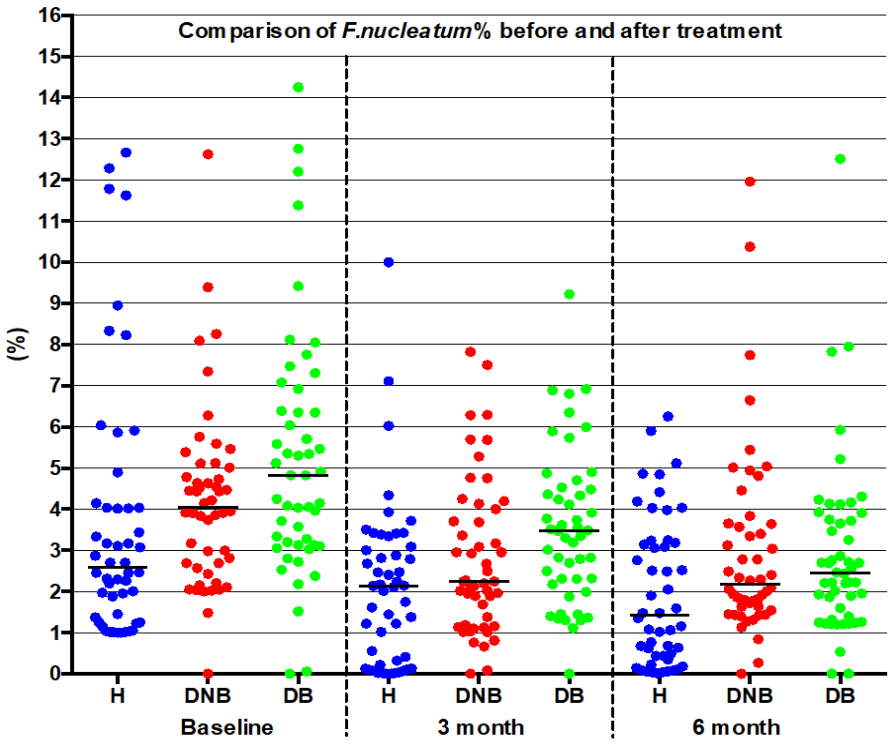


Figure 6.12. Comparison of proportions of *F. nucleatum* at all of the sites at each of the study time points.

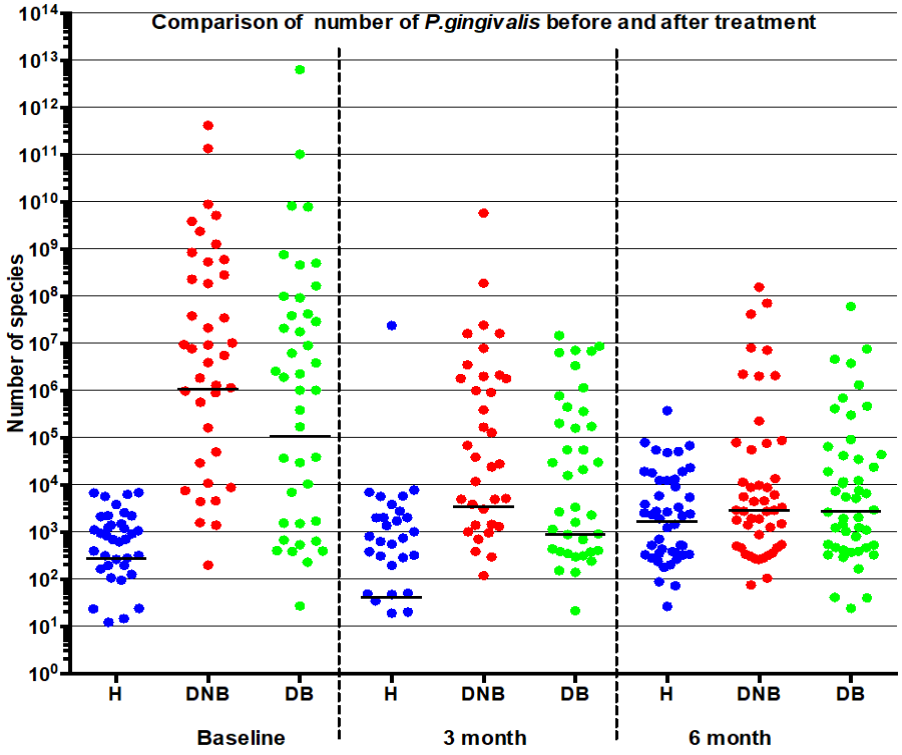


Figure 6.13. Comparison of numbers of *P. gingivalis* at all of the sites at each of the study time points.

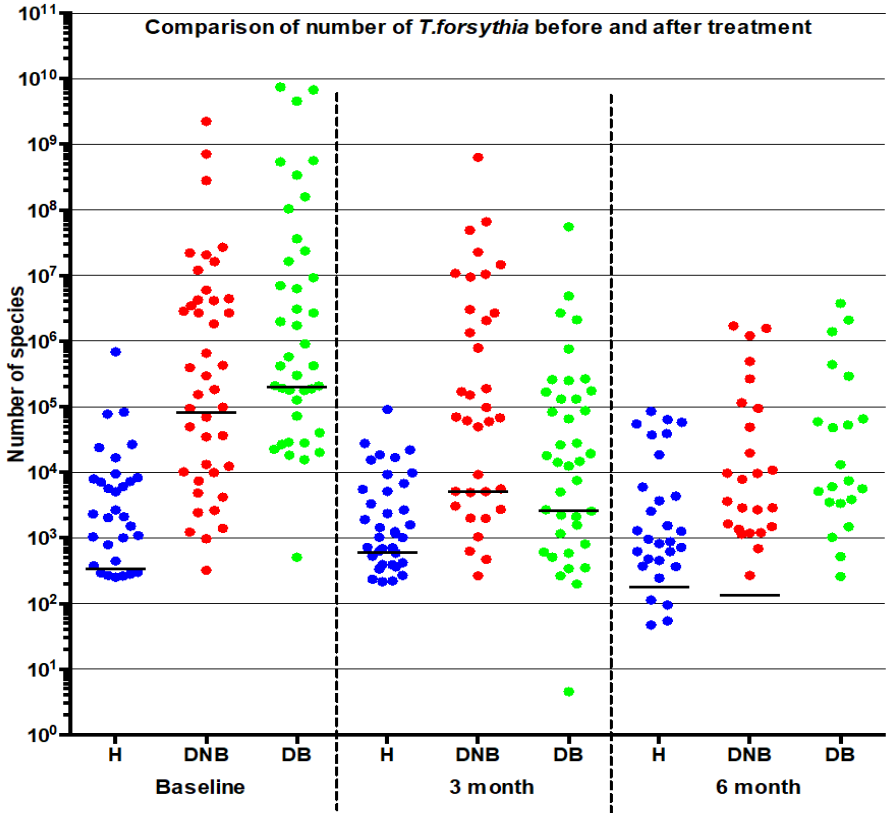


Figure 6.14. Comparison of numbers of *T. forsythia* at all of the sites at each of the study time points.

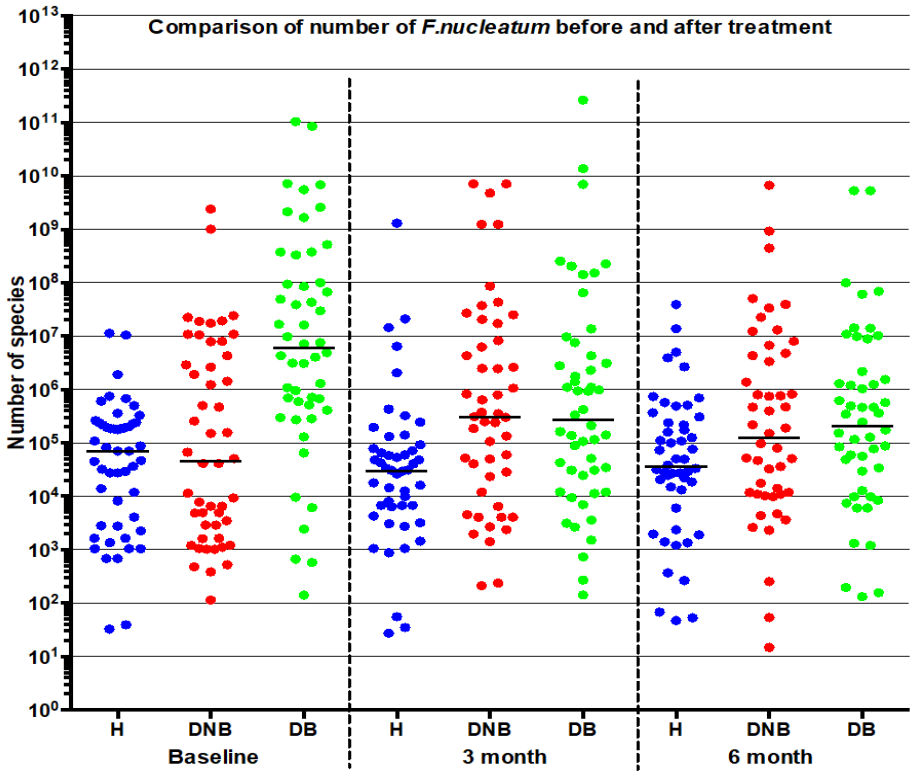


Figure 6.15. Comparison of numbers of *F. nucleatum* at all of the sites at each of the study time points.

6.3.4 Effect of antibiotics on clinical and laboratory measures

Among these 50 patients, antibiotics were prescribed for 12 patients (24%) as adjunct to root surface debridement at three month time point. Analysing these patients separately, at baseline, there was no statistically significant difference in full mouth and site specific clinical and laboratory data between the antibiotic and non-antibiotic group except for the mean PPD ≤ 3mm as shown in Tables 6.5 and 6.6. However, in those not receiving antibiotic initial treatment resulted in significant improvement in all full mouth clinical measures except for the plaque index when compared to antibiotic group (Table 6.5). Furthermore, PPD in DNB sites demonstrated significant reduction (P= 0.04), but not for DB sites. The second cycle of the treatment plus antibiotic therapy (at the 3 month time point) in those patients having a poor response to root surface debridement resulted in further improvement in full mouth clinical measures by the 6 month review. For example, the mean percentage of sites with PPD ≤3mm was increased by 23% (from 56.2±17.7% at 3 months to 79± 14% at 6 month time point). Interestingly, pocket depths of ≥ 6mm appeared to gain more benefit from the antibiotic since the mean percentage of sites with PPD depth ≥ 6 mm was reduced from 14.4±9.2% to 2.1±3.5%. Furthermore, the average PPD of DB sites demonstrated a statistically significant difference at six months between patients who received antibiotics and patients who did not receive antibiotics (Table 6.5). On a site specific basis, the proportion of sites that showed improvements in sites showing in PPD ≥2mm was 83% for DNB and 75% for DB in those prescribed antibiotics compared to 63% for both DNB and DB sites in those not prescribed antibiotics.

Looking at the bacterial levels in sites in patients who received antibiotic and those did not, at baseline there was no statistically significant difference in median levels of any of the bacteria tested. Whereas at the 3 month time point, the root surface debridement resulted in a slight reduction in the median percentage of *P. gingivalis* in both DNB and DB and of *T. forsythia* in DNB sites (Table 6.6) in those who received antibiotics compared to those who had not (statistically significant, Mann Whitney test). The second cycle of the treatment, which included antibiotic therapy in those patients having a poor response to root surface debridement, resulted in much greater reduction in the median percentages of *P. gingivalis* and *T. forsythia* in both DNB and DB sites by 6 months compared to those who had not received antibiotics. There was no significant change in the proportion of *F. nucleatum* present. These percentage differences, however, were not

reflected in significant differences in the actual number of cells of these species when the antibiotic group was compared with the non-antibiotic group at any time point (except *T. forsythia* in DB sites at 3 month time point) (Table 6.6).

Considering the changes in bacterial proportions and number during the 6 month period of the study, data were compared between baseline values, those seen at 3 months and those at 6 months in both groups. There was a significant reduction in proportion and number of all tested species by 3 months in those had not received antibiotic except the number of *T. forsythia* and *F. nucleatum* in DNB sites following conventional non-surgical treatment. In contrast, there was no significant change in any bacterial levels by 3 months in those that went on to receive antibiotic (Table 6.7) but it should be noted that this group had not received their antibiotic therapy at that stage; they did so immediately after the 3 month review. Comparing baseline bacterial levels with those found at 6 months showed that the non-antibiotic group had a significant reduction in the percentage and number of all tested species in both DNB and DB sites, except the percentage of *T. forsythia* and number of *F. nucleatum* in DNB sites, whereas, in the antibiotic group a significant reduction in the proportion of all species (in both DNB and DB sites) and number of *T. forsythia* in both DNB and DB sites were observed. Furthermore, the proportion of all species and number of *T. forsythia* continued to fall between 3 and 6 months in DB and DNB sites in the antibiotic group but there was no further change in bacterial levels (both proportion and number) over that period in the non-antibiotic group (Table 6.7).

Table 6.5. Comparison of full mouth and site specific clinical data between patients receiving antibiotic (n= 12) and not (n= 38) as adjunct to root surface debridement.

	Variable	Baseline		P value* (Antibiotic vs no-antibiotic at baseline)	3 month		P value* (Antibiotic vs no-antibiotic at 6 month)	6 months		P value* (Antibiotic vs no-antibiotic at 6 month)
		Antibiotic	No-antibiotic		Antibiotic	No-antibiotic		Antibiotic	No-antibiotic	
Full mouth data (% sites ± sd)	PI	68.7±22.3	63.9± 25.2	0.5	48.6±27.7	41.2±24.1	0.4	45.5±30.1	36.3±25	0.3
	BOP	25.8 ± 10.1	20.2±15.6	0.1	36.5±32.6	10.1±9.3	**0.01**	4.7 ± 4.1	9.9±13.5	**0.04**
	Mean PPD ≤ 3mm	59.9 ± 17.2	73.8 ±14.9	**0.02**	56.2±17.7	77.6±11.2	**0.001**	79± 14	82±10	0.6
	Mean PPD 4-5 mm	18.7±7.3	15.6 ±9.5	0.2	29.3±12.7	17.7±8.6	**0.01**	17 ± 11	14±8	0.3
	Mean PPD ≥ 6 mm	21.1± 15.8	11.1± 10.3	0.06	14.4±9.2	4.7±4.2	**0.004**	2.1±3.5	3.2±3.7	0.3
Site specific data (mean ± sd)	PPD mm (DNB)	6.5 ± 1	6.6 ± 1.1	0.7	6.1±1.8	4.9±1.5	**0.04**	4±1.1	4.3±1.4	0.4
	PPD mm (DB)	6.9 ± 1.3	6.9 ± 1.5	0.9	5.5±0.8	5.3±1.3	0.6	3.7±0.7	4.6±1.8	**0.02**

- t-test

Table 6.6. Comparison of site specific median bacterial levels between patients receiving antibiotic (n= 12) and patients that did not (n= 38) as adjunct to root surface debridement.

Bacterial levels	Baseline		P value* (Antibiotic vs non-antibiotic at baseline)	3 month		P value* (Antibiotic vs non-antibiotic at 3 month)	6 months		P value* (Antibiotic vs non-antibiotic at 6 month)
	Non antibiotic	Antibiotic		Non antibiotic	Antibiotic		Non antibiotic	Antibiotic	
Pg% (DNB)	0.88	1.27	0.4	0.23	1.4	**0.01**	0.18	0	**0.01**
Pg% (DB)	1.52	1.47	0.5	0.26	1.1	**0.01**	0.15	0	**0.01**
Tf% (DNB)	0.84	1.89	0.5	0.08	1.6	**0.03**	0.39	0	**0.01**
Tf% (DB)	1.51	1.32	0.5	0.34	0.66	0.1	0.02	0	**0.02**
Fn% (DNB)	4.3	3.5	0.4	2.1	3.1	0.1	2.3	1.7	0.1
Fn% (DB)	4.8	4.5	0.4	3.3	3.5	0.6	2.5	1.8	0.1
Pg no. (DNB)	1.2×10^6	3.9×10^5	0.5	1.3×10^3	3.1×10^4	0.2	2.8×10^3	3.2×10^3	0.4
Pg no. (DB)	1.1×10^6	1.8×10^4	0.2	8.9×10^2	9.1×10^2	0.8	6.9×10^3	5.1×10^2	0.2
Tf no. (DNB)	5.3×10^4	1.5×10^6	0.2	4.1×10^3	1.1×10^5	0.1	3.4×10^2	1.3×10^2	0.6
Tf no. (DB)	3.6×10^5	5.1×10^4	0.4	9.8×10^2	1.4×10^5	**0.007**	0	0	0.3
Fn no. (DNB)	4.1×10^4	5.9×10^4	0.8	2.8×10^5	4.7×10^5	0.7	1.2×10^5	1.3×10^5	0.5
Fn no. (DB)	7.3×10^6	2.5×10^6	0.1	1.3×10^5	1.1×10^6	0.2	1.4×10^5	4.1×10^5	0.1

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

- Mann-Whitney u test

Table 6.7. Further comparison of site specific median bacterial levels between patients receiving antibiotic (n= 12) and patients that did not (n= 38) as adjunct to root surface debridement.

Variable	Baseline		3 month		P value* Non antibiotic (baseline vs 3 month)	P value* Antibiotic (baseline vs 3 month)	6 month		P value* Non antibiotic (baseline vs 6 month)	P value* Antibiotic (baseline vs 6 month)	P value* Non antibiotic (3 vs 6 month)	P value* Antibiotic (3 vs 6 month)
	Non antibiotic	Antibiotic	Non antibiotic	Antibiotic			Non antibiotic	Antibiotic				
Pg% (DNB)	0.88	1.27	0.23	1.4	**0.04**	0.9	0.18	0	**0.02**	**0.004**	0.1	**0.004**
Pg% (DB)	1.52	1.47	0.26	1.1	**0.002**	0.5	0.15	0	**0.02**	**0.001**	0.07	**0.001**
Tf% (DNB)	0.84	1.89	0.08	1.6	**0.007**	0.7	0.39	0	0.07	**0.02**	0.7	**0.004**
Tf% (DB)	1.51	1.32	0.34	0.66	**0.007**	0.9	0.02	0	**0.01**	**0.02**	0.1	**0.009**
Fn% (DNB)	4.3	3.5	2.1	3.1	**0.001**	0.5	2.3	1.7	**0.001**	**0.03**	0.3	**0.04**
Fn% (DB)	4.8	4.5	3.3	3.5	**0.003**	0.1	2.5	1.8	**0.001**	**0.004**	0.1	**0.006**
Pg no. (DNB)	1.2×10^6	3.9×10^5	1.3×10^3	3.1×10^4	**0.004**	0.6	2.8×10^3	3.2×10^3	**0.004**	0.7	0.4	0.2
Pg no. (DB)	1.1×10^6	1.8×10^4	8.9×10^2	9.1×10^2	**0.008**	0.3	6.9×10^3	5.1×10^2	**0.01**	0.1	0.09	0.4
Tf no. (DNB)	5.3×10^4	1.5×10^6	4.1×10^3	1.1×10^5	0.08	0.4	3.4×10^2	1.3×10^2	**0.002**	**0.003**	0.07	**0.01**
Tf no. (DB)	3.6×10^5	5.1×10^4	9.8×10^2	1.4×10^5	**0.001**	0.9	0	0	**0.0001**	**0.001**	0.1	**0.001**
Fn no. (DNB)	4.1×10^4	5.9×10^4	2.8×10^5	4.7×10^5	0.1	0.6	1.2×10^5	1.3×10^5	0.2	0.7	0.4	0.4
Fn no. (DB)	7.3×10^6	2.5×10^6	1.3×10^5	1.1×10^6	**0.04**	0.6	1.4×10^5	4.1×10^5	**0.001**	0.6	0.5	0.6

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

* Kruskal-Wallis

6.3.5 Diagnostic value of the bacterial species examined

The diagnostic values of the bacterial species examined were found using ROC curves as described in the statistical methods (section 6.2.7). The threshold point of each species at baseline with highest sensitivity and specificity to differentiate health from disease was selected as shown in Figures 6.16 and 6.17. Generally, *P. gingivalis* and *T. forsythia* when expressed as proportions of the total bacteria detected were shown to be reliable diagnostic biomarkers at threshold levels of 0.23% and 0.35%, respectively (Table 6.8). In addition, using the number of *P. gingivalis* and *T. forsythia* bacterial cells as a criterion the data showed the two species to be reliable diagnostic biomarkers at threshold points of 1.4×10^3 and 7.3×10^3, respectively, but with slightly less differentiation than when their levels were expressed as a proportion of the whole. In either case, *P. gingivalis* and *T. forsythia* have enabled differentiation of healthy from diseased sites, with sensitivity of ≥ 70% and specificity of ≥ 80%, whereas *Fusobacterium* species was not a good a diagnostic biomarker, showing lower sensitivity and specificity percentages (Table 6.8).

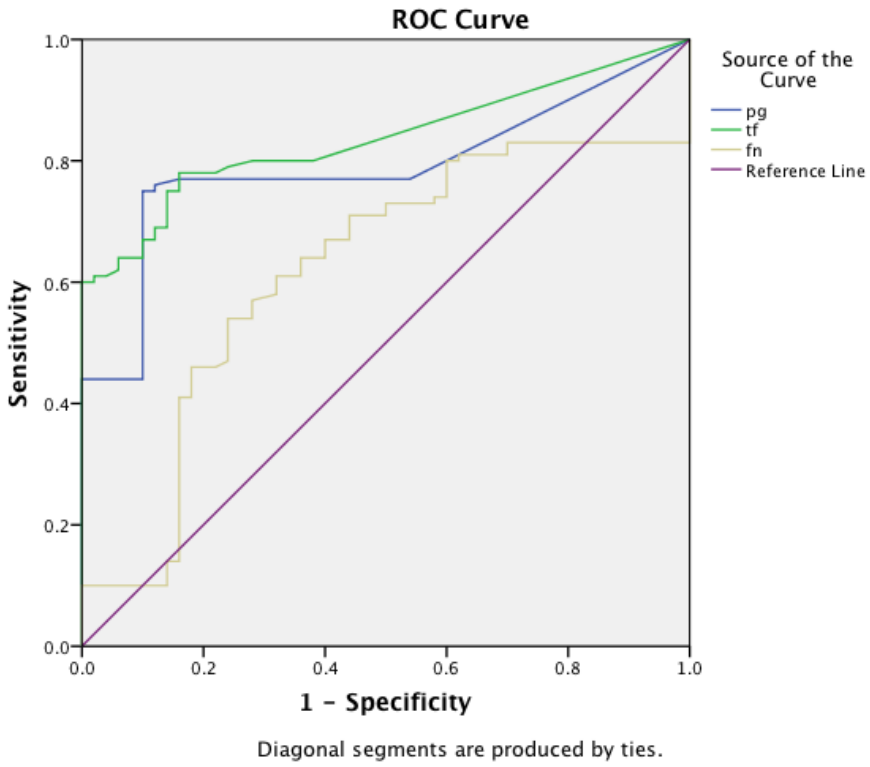


Figure 6.16. ROC curve for the percentage of each of the three bacteria tested as a differentiator of healthy from diseased sites.

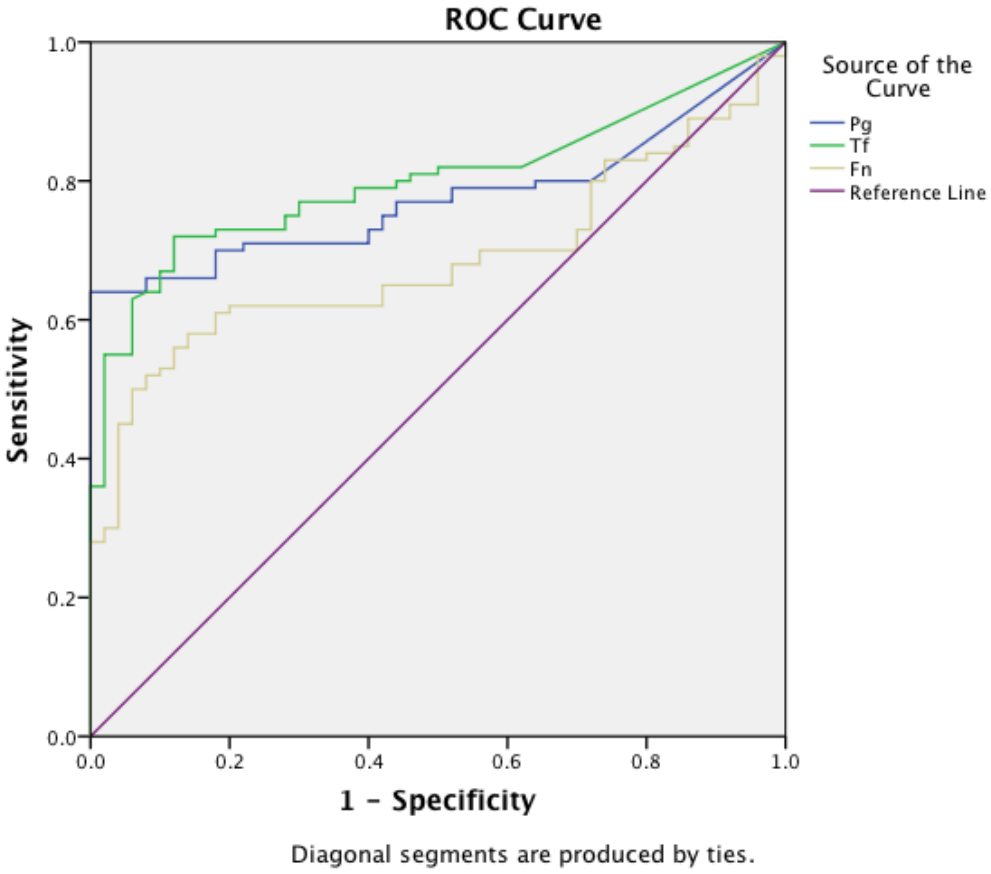


Figure 6.17. ROC curve for the number of each of the three bacteria tested as a differentiator of healthy from diseased sites.

Table 6.8. Threshold points for bacterial levels, showing their sensitivity and specificity in differentiating healthy from diseased sites.

Variable	Threshold	Sensitivity%/ Specificity%	Area under the curve	95% CI for OR		P value
				LCL	UCL	
Pg%	0.23	77/86	0.81	0.71	0.86	**0.0001**
Tf%	0.35	78/84	0.8	0.77	0.89	**0.0001**
Fn%	2.94	65/65	0.62	0.52	0.71	**0.017**
Pg no.	1.4×10^3	70/80	0.78	0.7	0.84	**0.0001**
Tf no.	7.3×10^3	73/80	0.8	0.73	0.87	**0.0001**
Fn no.	2.3×10^5	62/78	0.68	0.6	0.76	**0.001**

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

6.3.6 Value of the bacterial profile as a prognostic tool

The baseline microbial data for sites that recorded successful and compromised treatment outcomes are shown in Table 6.9. Among all the tested variables only the proportion of the bacteria that were *P. gingivalis* and *T. forsythia* at both DNB and DB sites showed statistically significant differences between these two groups with different treatment outcomes (lower in respondent group). Logistic regression analysis was carried out to identify the biomarkers with most notable impact on treatment outcome. First, all variables were put into the analysis and their prediction value plus odds ratio were obtained. At the second stage, stepwise logistic regression analysis was performed to exclude variables with no statistically significant effect on predictive value ($p < 0.05$). This also helped to prevent over fitting the model with redundant variables. The analysis revealed that when the relevant percentages for all the species were included, they could predict the treatment outcome with 76% certainty for both DNB and DB sites, which is statistically significantly higher than the null hypothesis prediction value (61%) (Table 6.10). The stepwise technique excluded the *F. nucleatum*% as its exclusion did not affect the predictive value in either DNB ($p =0.8$) or DB ($p = 0.4$) (Table 6.11), i.e. amongst all three species, statistically significant odds ratios were only found in terms of the proportion of *P. gingivalis* and *T. forsythia* in relation to treatment outcome ($p = 0.01$). The odds of a site responding to non-surgical periodontal treatment within the next 6 months increased with decreasing proportion of *P. gingivalis* and *T. forsythia* in the subgingival plaque samples. Also, the odds ratio for *P. gingivalis*% was higher than for *T. forsythia*% in both the DNB and DB sites (Table 6.11). Interestingly, the number of these three species did not show any association with treatment outcome (Table 6.11) and their prediction value (68% for DNB and 66% for DB) (Table 6.10) was not significantly higher than the null hypothesis (65%).

Finally, to exclude the effect of antibiotic on prognostic value of these bacterial species as biomarkers, logistic regressions were reanalysed only for those patients without antibiotic prescriptions and it was found that the prediction value of combined *P. gingivalis%* and *T. forsythia%* increased to 83% for both DNB and DB sites. Furthermore, the odds ratio for *P. gingivalis%* increased to 1.3 (CI= 0.3-1.4) for DNB and 1.1 (CI= 0.5-1.13) for DB sites. This finding was replicated in the case of *T. forsythia%,* where the odds ratio for DNB site increased to 1.1 (CI= 0.5-1.3) and for DB sites to 1 (CI= 0.5-1.2).

Table 6.9. Analysis of baseline clinical and microbiological variables in respondent versus non-respondent sites.

	Variable (Baseline)	Respondent	Non-Respondent	p value*
DNB sites	*Pg*%	0.58	2.3	**0.007**
	Tf %	0.1	5.64	**0.0001**
	Fn%	4.03	4.19	0.6
	Pg no.	1.6×10^5	1.2×10^6	0.5
	Tf no.	4.9×10^4	2.9×10^5	0.2
	Fn no.	6.5×10^4	1.4×10^5	0.7
DB sites	*Pg*%	0.88	3.58	**0.0001**
	Tf %	0.3	7.13	**0.0001**
	Fn%	4.82	4.14	0.53
	Pg no.	2.9×10^5	9.1×10^6	0.1
	Tf no.	7.2×10^4	5.7×10^5	0.4
	Fn no.	1.3×10^6	4.3×10^7	0.4

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

* Mann-Whitney u test

Table 6.10. Logistic regression showing the predictive value of bacterial proportion and number at baseline for treatment outcome (2mm improvement in pocket depth) at 6 months.

	Method	**DNB**	**DB**
		Predictive	Predictive
Bacterial proportion	All	76% (*Pg%, Tf%, Fn%*)	76% (*Pg%, Tf%, Fn%*)
	Stepwise	76% (*Pg%, Tf%*)	74% (*Pg%, Tf%*)
Bacterial number	All	68% (*Pg, Tf, Fn*)	66% (*Pg, Tf, Fn*)
	Stepwise	66% (*Pg, Tf*)	66% (*Pg, Tf*)

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

Table 6.11. Summary of logistic regression for each individual explanatory variable for site response to treatment in the following 6 months.

Predictor variable	Effects (β)	Odds Ratio (OR)	95% CI for OR		p value
			LCL	UCL	
Pg% (DNB)	-1.2	0.28	0.1	0.7	**0.001**
Pg% (DB)	-0.32	0.68	0.4	1.1	**0.01**
Tf% (DNB)	-0.6	0.53	0.3	0.7	**0.001**
Tf% (DB)	-0.5	0.55	0.3	0.7	**0.001**
Fn% (DNB)	-0.05	0.94	0.5	1.6	0.8
Fn% (DB)	0.12	1.1	0.8	1.5	0.4
Pg no. (DNB)	0.001	1.0	1.0	1.0	0.5
Pg no. (DB)	0.001	1.0	1.0	1.0	0.6
Tf no. (DNB)	0.001	1.0	1.0	1.0	0.3
Tf no. (DB)	0.001	1.0	1.0	1.0	0.7
Fn no.(DNB)	0.001	1.0	1.0	1.0	0.6
Fn no. DB)	0.001	1.0	1.0	1.0	0.4

Pg: P. gingivalis, Tf: T. forsythia, Fn: F. nucleatum.

6.3.7 GCF and plaque biomarkers as a prognostic tool

At this stage, the enzymes (MMP8, elastase and sialidase) and bacterial species (*P. gingivalis%*, *T. forsythia%*) that contributed significantly to predicting treatment outcome were combined. The enzyme profiles alone were able to predict treatment outcome with ≥80% certainty, whereas bacterial species were able to predict treatment outcome with ≥74% certainty. As shown in Table 6.12, combining these two groups of biomarkers demonstrated increased robustness in prediction value of at least 10%. The odds ratio and confidence interval of these biomarkers were previously reported.

Finally, correlations between the tested variables were examined and significant correlation coefficients were detected between *P. gingivalis* and trypsin like enzyme (r= 0.58), *T. forsythia* and sialidase (r=0.66) as well as *P. gingivalis* and *T. forsythia* (r=0.58).

Table 6.12. Logistic regression showing the predictive value of bacterial proportion and GCF enzymes at baseline for treatment outcome (2mm improvement in pocket depth).

Variable	DNB	DB
	Predictive	Predictive
GCF enzyme biomarkers	81.3% (MMP8, elastase and sialidase)	80.3% (MMP8, elastase and sialidase)
Bacterial biomarkers	76% (*P. gingivalis%*, *T. forsythia%*)	74% (*P. gingivalis%*, *T. forsythia%*)
GCF enzyme and bacterial biomarkers	92% (MMP8, elastase, sialidase, *P. gingivalis%*, *T. forsythia%*)	93.3% (MMP8, elastase, sialidase, *P. gingivalis%*, *T. forsythia%*)

6.4 Discussion

Better comprehension of the aetiology and pathogenesis of periodontitis is necessary to develop a diagnostic method with improved diagnostic and prognostic capability (Armitage, 2013). In polymicrobial periodontal infection, determination of bacteria taxa is the first step to understanding interactions between microorganisms, host and the environment. In this part of the study we used this "first step" to define whether combinations of levels of *P. gingivalis*, *T. forsythia* and *F. nucleatum* can differentiate health from disease and whether combining bacterial levels with enzymes in GCF can boost the predictive value close enough to 100%.

The principal findings of this study suggest the diagnostic utility of the percentage of these three species in subgingival plaque. They demonstrated sensitivity/specificity of 77% / 86%, 78% / 84% and 65% / 65%, respectively. It has also been demonstrated that adding the percentage levels of *P.gingivalis* and *T. forsythia* at a site to the levels of the three enzymes can further boost the predictive value (≥92%).

The first two species are considered to be aetiologically important contributors to chronic periodontitis (Hajishengallis and Lambris, 2011, Tanner and Izard, 2006) and have been designated as candidates that lead to further disease progression (Ximénez-Fyvie et al., 2000, Socransky et al., 1991). Furthermore, *F. nucleatum* has been proposed as acting as a scaffold to help colonisation and proliferation of other pathogenic species (Kolenbrander and London, 1993).

The prevalence of *P. gingivalis* and *T. forsythia* was significantly higher in diseased sites (≥80%) than in healthy sites, a finding that is in accordance with previous studies (Torrungruang et al., 2009, Papapanou, 2002, Papapanou et al., 2000). Meanwhile, *F. nucleatum* was detected in the majority of samples regardless of their clinical status and this supports the hypothesis that *F. nucleatum* is a major co-aggregating microorganism in periodontal biofilm (Jakubovics and Kolenbrander, 2010) and corroborates the data reported by Byrne et al., (2009). However, even for *P. gingivalis* and *T. forsythia* the presence and absence of bacterial species was not a sufficient criterion to distinguish healthy sites from diseased sites (Nonnenmacher et al., 2005, Papapanou et al., 2000).

Therefore, quantitative real time qPCR was reasoned to be helpful in adding relevant information regarding abundance of bacteria in relation to periodontal status. The

proportion and number of *P. gingivalis*, *T. forsythia* and *F. nucleatum* (except numbers of *F. nucleatum* in DNB site) were significantly higher in diseased sites than in healthy sites, corroborating the data published in other studies (Lourenço et al., 2014, Ramseier et al., 2009, Tanner et al., 2007). However, these species were not found at the same levels in subgingival plaque samples and, as a rule, sites that experienced a successful treatment outcome over the next six months generally exhibited lower proportions of *P. gingivalis* and *T. forsythia* at baseline compared to sites with compromised treatment outcomes (Table 6.9). It is true that median levels of the two species were reduced significantly after the first and second cycles of treatment, however, on a site-specific basis, some sites still harboured reasonably high levels. However, this was not the case with *F. nucleatum,* a finding which is in agreement with previous studies (Kinney et al., 2011). This study's finding that levels of these bacterial species did not correlate with PPD at baseline is not in line with other studies (Ximénez-Fyvie et al., 2000, Socransky et al., 1991), and this can be explained by the fact that the range of PPD at baseline was not wide.

Among the examined species, both *P. gingivalis* and *T. forsythia* proportions indicated significant diagnostic value (sensitivity/ specificity) to differentiate health from disease, and this corroborates data reported by other studies (Ramseier et al., 2009, Tanner et al., 2007), although, the data suggest that amounts of *P. gingivalis* and *T. forsythia* need to exceed critical threshold points of 0.23% and 0.35%, respectively, to be associated with periodontal disease. In other words, change from health to disease status requires increase in the level and/or frequency of previously present species (Abusleme et al., 2013, Byrne et al., 2009). However, the threshold points for *P. gingivalis* and *T. forsythia* are slightly higher than those reported by previous studies and this could be related to the difference in clinical measures of sampled sites, as in the present study PPDs ≥ 6 were chosen. The percentage of *F. nucleatum* demonstrated the lowest diagnostic value compared to the other two species. Likewise, Ramseier et al. (2009) demonstrated that *F. nucleatum* has lower diagnostic value compared to the other two species. This could be related to the fact that *F. nucleatum* is expected to be present in abundant numbers in both healthy and diseased sites (Jakubovics and Kolenbrander, 2010) because of its central role in plaque formation and integrity. Surprisingly, the actual number of *P. gingivalis* and *T. forsythia* demonstrated slightly lower diagnostic values compared to their proportion of the total bacteria present; conceivably, these discrepancies in results can be explained by lack of

standardized plaque collection, deriving from the present study opting to collect as much sample as possible.

There is a shortage of data on the usefulness of *P. gingivalis*, *T. forsythia* and *F. nucleatum* as a prognostic tool for predicting treatment outcome. The current study contributes to filling this research gap by using stepwise logistic regression to produce findings that demonstrate the usefulness of *P. gingivalis* and *T. forsythia* proportions as prognostic tools to forecast treatment outcome, in agreement with a study by Kinney et al. (2014). The effect of all the species tested was revealed to be inversely correlated with successful treatment outcome (β in Table 6.11); however, only the percentages of *P. gingivalis* and *T. forsythia* were shown to be statistically significantly correlated, with odds ratios of 0.28 and 0.53 in DNB sites and 0.68 and 0.55 for DB sites, respectively. This is also apparent from Table 6.9, which shows that *P. gingivalis*% and *T. forsythia*% are statistically significantly higher in non-respondent sites than in respondent sites. Not surprisingly, the level of *F. nucleatum* did not add significant predictive value to the *P. gingivalis* and *T. forsythia* combination. This result is in accordance with previously published data reporting the usefulness of *P. gingivalis* and *T. forsythia* in predicting disease progression (Papapanou et al., 1997, Grossi et al., 1995). However, in the present study these bacterial species were examined to predict treatment outcome rather than disease progression. However, the number of these species showed no significant prognostic value.

It has been reported that the effects of azithromycin on clinical signs, microbial and GCF biomarkers are very minimal at both patient and site level in patients with generalized aggressive periodontitis and generalized chronic periodontitis. However, there was one exception in that sites with deep PPD were found to obtain more meaningful benefit from azithromycin treatment (Emingil et al., 2012, Han et al., 2012), and this finding is in accordance with the result of the current study (Table 6.5). It was noticed that antibiotic treatment led to an increase in prediction value of the *P. gingivalis%* and *T. forsythia%* combination. This can be explained by the fact that it was assumed that some sites, based on their baseline bacterial levels, would not respond to non-surgical periodontal treatment alone. However, prescribing antibiotics to these patients resulted in reduction of PPD of ≥ 2mm at these sites, and this ultimately resulted in decrease of the prediction value of these two species.

When the GCF biomarkers (MMP8, elastase and sialidase) were combined with *P. gingivalis* and *T. forsythia* levels this boosted the robustness of the overall prediction value by 10%. These data suggest that combination of these two bacterial species with MMP8, elastase and sialidase will help to discriminate sites that will have a successful outcome from sites likely to have a compromised treatment outcome. This small increase in the prediction value could relate to the fact that some of the enzyme biomarkers correlated to each other directly and to others indirectly, as shown in the results section; for example, sialidase and *T. forsythia* were significantly correlated.

We observed certain limitations in this part of the study. First, plaque sampling techniques were not standardized, due to the effort made to collect as much sample as possible, however this means that the amount of plaque present at a site influences the amount of subgingival plaque collected, which renders the comparison between sites based on absolute number of bacteria unfair. To overcome this problem, the proportion of these species was calculated using universal primers to identify the total bacterial DNA concentration in each sample. However, universal primers also have some limitations of possibly not equivalently identifying or amplifying all segments of the bacteria in the sample (Horz et al., 2005), which could affect the calculation of the final proportions of these species; however, within the limitations of the study, and the fact that the efficiency of the primers for *P. gingivalis*, *T. forsythia* and total bacteria were similar, it is suggested that the calculated proportions of the target bacteria were reasonably accurate. Also, as there are fastidious and uncultivable organisms in plaque samples, qPCR currently provides the best estimation of the total number of each species and expression as a proportion of total bacterial cell number appears to be the fairest value for comparative purposes. There are numerous types of bacteria in the oral cavity and it is impossible to accurately determine all of their genome weights and 16s rRNA copy numbers. In the present study, therefore, the total bacterial load in the clinical specimens was calculated on the assumption that the 16s rRNA gene copy numbers of the oral anaerobes did not significantly differ from each other (Nadkarni et al., 2002, Griffen et al., 1998).

Further studies should also look at these biomarkers in terms of early indication of disease onset, transition from gingivitis to periodontitis and differentiation of active sites from inactive sites. Nevertheless, this study demonstrated that there are indeed specific bacterial and enzyme signatures that can differentiate health from disease, as well as predict treatment outcomes. Our findings are supported by publications showing

association between periodontal disease, periodontal pathogens and inflammatory markers (Kinney et al., 2011, Teles et al., 2010). Understanding these relationships would be of value to the clinician and it would have an impact on diagnosis, patient monitoring, controlling disease activity and final treatment plans. Clearly, having diagnostic tools with improved diagnostic and prognostic capabilities would help the clinician to provide site-tailored treatment in a more time-effective manner and avoid wastage of time and money on providing inappropriate treatment. Achieving that result will necessarily involve translating these biomarkers to a chair side test that the clinician can perform in a reasonable period of time.

Chapter 7- Summary, Conclusions and future work

Chapter 7– Summary, Conclusions and future work

7.1 Overall Summary

As described in previous chapters, the principle findings of these studies suggest the diagnostic utility of MMP, elastase and sialidase for differentiating healthy from diseased sites. Furthermore, the combined profile of these three enzymes offers a "finger print" that predicts the treatment outcome of diseased sites. However, cathepsin G and trypsin like enzymes were not demonstrated to have any significant diagnostic or prognostic value. These findings came initially from analysis of GCF samples collected from 77 participants who completed the full length of the study but they were also validated by application to an independent cohort using the same patient inclusion and exclusion criteria.

In an ideal world, a single, simple and inexpensive test for the combination of the three enzymes would be required to translate these findings to the clinic. That requires a test which could be conducted quickly and simply on the GCF sample as it is collected, without requiring complicated addition of enzyme substrate reagents. Although not strictly able to be included in this study properly, we did show that it is feasible to detect the threshold enzyme levels of MMP-8, elastase and sialidase within Periopaper® strips impregnated with substrate.

Not content with three enzymes and sensitivities and specificities in the range of >80% we considered whether detection of key bacterial species might add to the strength of the combined enzymes as diagnostic and prognostic markers. Consequently in a further study, we have shown that when these GCF biomarkers were combined with levels of *P. gingivalis* and *T. forsythia* in subgingival plaque they provided an even more robust system for predicting the treatment outcome.

We acknowledge that there were some limitations in these studies. For example, the sample size of the independent cohort study was small and as plaque samples in the independent cohort study were not collected, the findings of the microbiological study could not be validated in an independent cohort. Finally, the translation of the enzyme assays to a chair side test needs further work.

7.2 Main findings and conclusions

1- Non-surgical periodontal treatment resulted in significant improvement in full mouth clinical measures.

2- However, one third of both DNB and DB sites failed to improve by at least 2mm reduction in PPD when subjected to conventional non-surgical treatment.

3- Average concentrations of all enzymes were higher in diseased sites than healthy sites and their levels generally decreased by 6 months after treatment.

4- Initial MMP8, elastase and sialidase concentrations correlate with PPD.

5- It could be concluded from statistical analysis of the data that MMP8, elastase and sialidase have the highest diagnostic value in terms of differentiating healthy sites from diseased sites.

6- It could be concluded from statistical analysis of the data that a combined profile of MMP8, elastase and sialidase enzyme levels offers a significantly improved indication of a site's likely response to non-surgical periodontal treatment (> 80%).

7- Using an independent cohort and applying the threshold enzyme levels for MMP8, elastase and sialidase to the main cohort study and vice versa, these enzymes were validated as having good diagnostic and prognostic value.

8- Although, the diagnostic value of the trypsin-like enzyme activity detected was not as high as that of MMP8, elastase or sialidase, the diagnostic value of trypsin-like activity proved to have some reliability after validation.

9- In contrast, Cathepsin G showed no real promise as a diagnostic or prognostic biomarker of periodontal disease.

10- Prevalence and levels of *P. gingivalis* and *T. forsythia* were significantly higher in diseased sites than in healthy sites and these levels were generally reduced by 6 months after treatment.

11- *P. gingivalis* and *T. forsythia* had the highest diagnostic value among microbial biomarkers in terms of differentiating healthy sites from diseased sites.

12- When levels of *P. gingivalis* and *T. forsythia* were viewed in combination, this provided a useful indication that a site was not likely to respond to standard non-surgical periodontal treatment alone (≥ 74%).

13- However, combining these bacterial levels (*P. gingivalis* and *T. forsythia)* with MMP8, elastase and sialidase resulted in the highest predictive value for the outcome of treatment i.e. ≥ 92%.

14- Preliminary tests show that it should be possible to devise a chair side test for GCF based on enzyme-substrate reactions directly within a Periopaper. An antibody-based test is likely to be required for assessment of *P. gingivalis* and *T forsythia*, although no attempt was made to develop that in this study.

7.3 Future work

1- Examine the value of these enzymes as biomarkers in saliva and correlate with treatment outcome on a patient basis. Saliva samples would provide data on a patient risk basis, rather than a site risk basis, which has the advantage of being easily collected but the enzyme levels are likely to be lower due to dilution in the mouth. Such a study would require a minimum of 70 patients to provide sufficient statistical power.
2- Examine these biomarkers in terms of early indications of disease onset, transition from gingivitis to periodontitis, and differentiation of active and inactive sites in both saliva and GCF. This would be the ideal study but is likely to be very difficult to perform because a large number of patients would be required and the period of the study would be extensive as not all cases of gingivitis progress to destructive periodontitis.
3- Examine these biomarkers in subjects with potential confounders of periodontal disease such as diabetes mellitus or immunosuppression to determine the systemic influence an altered inflammatory response has on the detection of the three target enzymes and the two bacterial species.
4- Examine these biomarkers in subjects with aggressive periodontitis as a different form of periodontitis.
5- Further development of a rapid chairside test in which enzyme substrates are incorporated into a single Periopaper in such a way that each enzyme gives a separate signal allowing them to be measured independently.

Chapter 8- References

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Aalto, M., Ronnemaa, T. & Kulonen, E. (1974) Effect of neuraminidase on synthesis of collagen and other proteins. *Biochimica Et Biophysica Acta* **342**, 247-253.

Abusleme, L., Dupuy, A. K., Dutzan, N., Silva, N., Burleson, J. A., Strausbaugh, L. D., Gamonal, J. & Diaz, P. I. (2013) The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation. *The ISME journal* **7,** 1016-1025.

Adams, D. (1975) Mucus barrier and absorption through oral mucosa. *Journal of Dental Research* **54**, 19-26.

Al-Shammari, K. F., Giannobile, W. V., Aldredge, W. A., Iacono, V. J., Eber, R. M., Wang, H.-L. & Oringer, R. J. (2001) Effect of non-surgical periodontal therapy on C-telopeptide pyridinoline cross-links (ICTP) and interleukin-1 levels. *Journal of Periodontology* **72**, 1045-1051.

Albandar, J. M. (2002) Global risk factors and risk indicators for periodontal diseases. *Periodontology 2000* **29**, 177-206.

Albandar, J. M., Olsen, I. & Gjermo, P. (1990) Associations between 6 DNA probe-detected periodontal bacteria and alveolar bone loss and other clinical signs of periodontitis. *Acta Odontologica Scandinavica* **48**, 415-423.

Ammann, T., Bostanci, N., Belibasakis, G. & Thurnheer, T. (2013) Validation of a quantitative real-time PCR assay and comparison with fluorescence microscopy and selective agar plate counting for species-specific quantification of an in vitro subgingival biofilm model. *Journal of Periodontal Research* **48,** 517-526.

Apajalahti, S., Sorsa, T., Railavo, S. & Ingman, T. (2003) The in vivo levels of matrix metalloproteinase-1 and -8 in gingival crevicular fluid during initial orthodontic tooth movement. *Journal Of Dental Research* **82**, 1018-1022.

Armitage, G. C. (1999) Development of a classification system for periodontal diseases and conditions. *Annals of periodontology* **4,** 1-6.

Armitage, G. C. (2002) Classifying periodontal diseases- A long-standing dilemma. *Periodontology 2000* **30**, 9-23.

Armitage, G. C. (2004a) Analysis of gingival crevice fluid and risk of progression of periodontitis. *Periodontology 2000* **34**, 109-119.

Armitage, G. C. (2004b) Diagnosis of periodontal diseases. *Journal Of Periodontology* **75**, 779-779.

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Armitage, G. C. (2013) Learned and unlearned concepts in periodontal diagnostics: a 50-year perspective. *Periodontology 2000* **62,** 20-36.

Armitage, G. C., Jeffcoat, M. K., Chadwick, D. E., Taggart, E. J., Numabe, Y., Landis, J. R., Weaver, S. L. & Sharp, T. J. (1994) Longitudinal evaluation of elastase as a marker for the progression of periodontitis. *Journal of Periodontology* **65**, 120-128.

Artigas, J. M., Garcia, M. E., Faure, M. R. & Gimeno, A. M. (1981) Serum trypsin levels in acute pancreatic and non-pancreatic abdominal conditions. *Postgraduate Medical Journal* **57,** 219-222.

Aruni, W., Vanterpool, E., Osbourne, D., Roy, F., Muthiah, A., Dou, Y. & Fletcher, H. M. (2011) Sialidase and sialoglycoproteases can modulate virulence in *Porphyromonas gingivalis*. *Infection and Immunity* **79**, 2779-2791.

Badersten, A., Nilvéaus, R. & Egelberg, J. (1984) Reproducibility of probing attachment level measurements. *Journal of clinical periodontology* **11,** 475-485.

Baehni, P. & Guggenheim, B. (1996) Potential of diagnostic microbiology for treatment and prognosis of dental caries and periodontal diseases. *Critical Reviews in Oral Biology & Medicine* **7,** 259-277.

Baelum, V., Chen, X., Manji, F., Luan, W. M. & Fejerskov, O. (1996) Profiles of destructive periodontal disease in different populations. *Journal of Periodontal Research* **31**, 17-26.

Baggiolini, M., Walz, A. & Kunkel, S. L. (1989) Neutrophil-activating peptide-1 interleukin-8, a novel cytokine that activates neutrophils. *Journal of Clinical Investigation* **84**, 1045-1049.

Baharin, B., Palmer, R. M., Coward, P. & Wilson, R. F. (2006) Investigation of periodontal destruction patterns in smokers and non-smokers. *Journal of Clinical Periodontology* **33**, 485-490.

Baici, A., Knopfel, M. & Fehr, K. (1982a) Cleavage of the 4 human-IgG subclasses with cathepsin G. *Scandinavian Journal of Immunology* **16**, 487-498.

Baici, A., Knopfel, M. & Fehr, K. (1982b) Cathepsin G from human polymorphonuclear leukocytes cleaves human-IgM. *Molecular Immunology* **19**, 719-727.

Bairoch, A. & Apweiler, R. (2000) The swiss-prot protein sequence database and its supplement trembl in 2000. *Nucleic Acids Research* **28**, 45-48.

Bakaletz, L. O. (2004) Developing animal models for polymicrobial diseases. *Nature Reviews Microbiology* **2,** 552-568.

Chapter 8 – References

Baltacioglu, E., Akalin, F. A., Alver, A., Balaban, F., Unsal, M. & Karabulut, E. (2006) Total antioxidant capacity and superoxide dismutase activity levels in serum and gingival crevicular fluid in post-menopausal women with chronic periodontitis. *Journal of Clinical Periodontology* **33**, 385-392.

Bangalore, N., Travis, J., Onunka, V. C., Pohl, J. & Shafer, W. M. (1990) Identification of the primary antimicrobial domains in human neutrophil cathepsin G. *Journal Of Biological Chemistry* **265**, 13584-13588.

Bank, U. & Ansorge, S. (2001) More than destructive: neutrophil-derived serine proteases in cytokine bioactivity control. *Journal of Leukocyte Biology* **69**, 197-206.

Bank, U., Reinhold, D., Schneemilch, C., Kunz, D., Synowitz, H. J. & Ansorge, S. (1999) Selective proteolytic cleavage of IL-2 receptor and IL-6 receptor ligand binding chains by neutrophil-derived serine proteases at foci of inflammation. *Journal of Interferon and Cytokine Research* **19**, 1277-1287.

Barros, S. P., Williams, R., Offenbacher, S. & Morelli, T. (2016) Gingival crevicular fluid as a source of biomarkers for periodontitis. *Periodontology 2000* **70,** 53-64.

Beighton, D. & Life, J. S. C. (1989) Trypsin-like, chymotrypsin-like and glycylprolyl dipeptidase activities in gingival crevicular fluid from human periodontal sites with gingivitis. *Archives of Oral Biology* **34**, 843-846.

Beighton, D., Radford, J. R. & Naylor, M. N. (1992) Glycosidase activities in gingival crevicular fluid in subjects with adult periodontitis or gingivitis. *Archives of Oral Biology* **37**, 343-348.

Beighton, D. & Whiley, R. A. (1990) Sialidase activity of the *streptococcus milleri* group and other viridans group streptococci. *Journal of Clinical Microbiology* **28**, 1431-1433.

Beklen, A., Tuter, G., Sorsa, T., Hanemaaijer, R., Virtanen, I., Tervahartiala, T. & Konttinen, Y. T. (2006) Gingival tissue and crevicular fluid co-operation in adult periodontitis. *Journal of Dental Research* **85**, 59-63.

Bender, J. S., Thang, H. & Glogauer, M. (2006) Novel rinse assay for the quantification of oral neutrophils and the monitoring of chronic periodontal disease. *Journal of periodontal research* **41,** 214-220.

Berthold, P. & Listgarten, M. A. (1986) Distribution of *Actinobacillus actinomycetemcomitans* in localized juvenile periodontitis plaque: an electron immunocytochemical study. *Journal of Periodontal Research* **21,** 473-485.

Chapter 8 – References

Bhatia, A., Singh, M. P., Chopra, R., Bansal, P. & Dhuria, S. (2011) Recent chairside diagnostic kits used in periodontics. *Journal of Stomatognathic Sciences* **2**, 25-27.

Bickel, M. (1993) The role of interleukin-8 in inflammation and mechanisms of regulation. *Journal of Periodontology* **64**, 456-460.

Bieth, J., Spiess, B. & Wermuth, C. G. (1974) Synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochemical Medicine* **11**, 350-357.

Birkedalhansen, H. (1993a) Role of cytokines and inflammatory mediators in tissue destruction. *Journal of Periodontal Research* **28**, 500-510.

Birkedalhansen, H. (1993b) Role of matrix metalloproteinases in human periodontal-diseases. *Journal of Periodontology* **64**, 474-484.

Birkedalhansen, H., Moore, W. G. I., Bodden, M. K., Windsor, L. J., Birkedalhansen, B., Decarlo, A. & Engler, J. A. (1993) Matrix metalloproteinases- a review. critical reviews. *Oral Biology & Medicine* **4**, 197-250.

Bizios, R., Lai, L., Fenton, J. W. & Malik, A. B. (1986) Thrombin-induced chemotaxis and aggregation of neutrophils. *Journal Of Cellular Physiology* **128**, 485-490.

Bode, W., Wei, A. Z., Huber, R., Meyer, E., Travis, J. & Neumann, S. (1986) X-ray crystal-structure of the complex of human-leukocyte elastase (PMN elastase) and the 3rd domain of the turkey ovomucoid inhibitor. *EMBO Journal* **5**, 2453-2458.

Bonassi, S., Neri, M. & Puntoni, R. (2001) Validation of biomarkers as early predictors of disease. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **480,** 349-358.

Bosshardt, D. D. & Lang, N. P. (2005) The junctional epithelium: from health to disease. *Journal of Dental Research* **84**, 9-20.

Boudier, C., Holle, C. & Bieth, J. G. (1981) Stimulation of the elastolytic activity of leukocyte elastase by leukocyte cathepsin G. *Journal of Biological Chemistry* **256**, 256-258.

Bourdillon, M. C., Brechemier, D., Blaes, N., Derouette, J. C., Hornebeck, W. & Robert, L. (1980) Elastase-like enzymes in skin fibroblasts and rat aorta smooth-muscle cells. *Cell Biology International Reports* **4**, 313-320.

Brandtzaeg, P. (1965) Immunochemical comparison of proteins in human gingival pocket fluid, serum and saliva. *Archives of Oral Biology* **10**, 795-803.

Chapter 8 – References

Bretz, W. A., Lopatin, D. E. & Loesche, W. J. (1990) Benzoyl-Arginine Naphthylamide (BANA) hydrolysis by *Treponema-denticola* and or *Bacteroides gingivalis* in periodontal plaques. *Oral Microbiology and Immunology* **5**, 275-279.

Brew, K., Dinakarpandian, D. & Nagase, H. (2000) Tissue inhibitors of metalloproteinases: evolution, structure and function. *Biochimica Et Biophysica Acta-Protein Structure And Molecular Enzymology* **1477**, 267-283.

Brill, N. (1960) Gingival conditions related to flow of tissue fluid into gingival pockets. *Acta Odontologica* **18**, 421-446.

Brill, N. (1962) The gingival pocket fluid: studies of its occurrence, composition, and effect. Royal Dental College, Copenhagen.

Buduneli, N. & Kinane, D. F. (2011) Host-derived diagnostic markers related to soft tissue destruction and bone degradation in periodontitis. *Journal of Clinical Periodontology* **38**, 85-105.

Byrne, S., Dashper, S., Darby, I., Adams, G., Hoffmann, B. & Reynolds, E. (2009) Progression of chronic periodontitis can be predicted by the levels of *Porphyromonas gingivalis* and *Treponema denticola* in subgingival plaque. *Oral Microbiology and Immunology* **24,** 469-477.

Calkins, C. C., Platt, K., Potempa, J. & Travis, J. (1998) Inactivation of tumor necrosis factor-alpha by proteinases (Gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*- implications of immune evasion. *Journal of Biological Chemistry* **273**, 6611-6614.

Calvin, J. & Price, C. P. (1986) Measurement of serum alpha-1-Antichymotrypsin by immunoturbidimetry. *Annals Of Clinical Biochemistry* **23**, 206-209.

Campbell, E. J., Silverman, E. K. & Campbell, M. A. (1989) Elastase and cathepsin G of human monocytes quantification of cellular content, release in response to stimuli, and heterogeneity in elastase-mediated proteolytic activity. *Journal of Immunology* **143**, 2961-2968.

Campbell, E. L., Serhan, C. N. & Colgan, S. P. (2011) Antimicrobial aspects of inflammatory resolution in the mucosa: A role for proresolving mediators. *Journal of Immunology* **187**, 3475-3481.

Cao, C. & Smith, Q. (1989) Crevicular fluid myeloperoxidase at healthy, gingivitis and periodontitis sites. *Journal of Clinical Periodontology* **16**, 17-20.

Chapter 8 – References

Carlsson, J., Herrmann, B. F., Hofling, J. F. & Sundqvist, G. K. (1984) Degradation of the human proteinase-inhibitors alpha-1-antitrypsin and alpha-2-macroglobulin by *Bacteroides gingivalis*. *Infection and Immunity* **43**, 644-648.

Chalmers, N. I., Palmer, R. J., Cisar, J. O. & Kolenbrander, P. E. (2008) Characterization of a *Streptococcus* sp. -*Veillonella* sp. community micromanipulated from dental plaque. *Journal of Bacteriology* **190,** 8145-8154.

Chambers, D. A., Imrey, P. B., Cohen, R. L., Crawford, J. M., Alves, M. & Mcswiggin, T. A. (1991) A longitudinal-study of Aspartate aminotransferase in human gingival crevicular fluid. *Journal of Periodontal Research* **26**, 65-74.

Champagne, C. M., Buchanan, W., Reddy, M. S., Preisser, J. S., Beck, J. D. & Offenbacher, S. (2003) Potential for gingival crevice fluid measures as predictors of risk for periodontal diseases. *Periodontology 2000* **31**, 167-180.

Chapple, I. L. (2009) Periodontal diagnosis and treatment–where does the future lie? *Periodontology 2000* **51**, 9-24.

Chapple, I. L. C. (1997) Periodontal disease diagnosis: current status and future developments. *Journal of Dentistry* **25**, 3-15.

Chapple, I., Landini, G., Griffiths, G., Patel, N. & Ward, R. (1999) Calibration of the Periotron 8000® and 6000® by polynomial regression. *Journal of Periodontal Research* **34,** 79-86.

Choil, J. I., Nakagawa, T., Yamada, S., Takazoe, I. & Okuda, K. (1990) Clinical, microbiological and immunological studies on recurrent periodontal disease. *Journal of Clinical Periodontology* **17,** 426-434.

Cimasoni, G. (1983) Crevicular fluid updated. Monographs In Oral Science **12**, Iii-Vii, 1-152.

Claffey, N. & Egelberg, J. (1995) Clinical indicators of probing attachment loss following initial periodontal treatment in advanced periodontitis patients. *Journal of clinical periodontology* **22,** 690-696.

Cobb, C. M. (1996) Non-surgical pocket therapy: mechanical. *Annals of Periodontology* **1,** 443-90.

Cobb, C. M. (2002) Clinical significance of non surgical periodontal therapy: An evidence-based perspective of scaling and root planing. *Journal of Clinical Periodontology* **29,** 22-32.

Corfield, A. & Schauer, R. (1982) Sialic acids: chemistry, metabolism and function, Vienna, Springer-Verlag.

Chapter 8 – References

Corfield, T. (1992) Bacterial sialidases-roles in pathogenicity and nutrition. *Glycobiology* **2**, 509-521.

Cox, S. W. & Eley, B. M. (1989) Detection of cathepsin B peptidase and cathepsin-l peptidase, elastase peptidase, tryptase peptidase, trypsin peptidase, and dipeptidyl peptidase iv-like activities in crevicular fluid from gingivitis and periodontitis patients with peptidyl derivatives of 7-Amino-4-Trifluoromethyl coumarin. *Journal of Periodontal Research* **24**, 353-361.

Cox, S. W. & Eley, B. M. (1992) Cathepsin-b/l-like, elastase-like, tryptase-like, trypsin-like and dipeptidyl peptidase-iv-like activities in gingival crevicular fluid - a comparison of levels before and after basic periodontal treatment of chronic periodontitis patients. *Journal of Clinical Periodontology* **19**, 333-339.

Curtis, M. A., Griffiths, G. S., Price, S. J., Coulthurst, S. K. & Johnson, N. W. (1988) The total protein-concentration of gingival crevicular fluid variation with sampling time and gingival inflammation. *Journal of Clinical Periodontology* **15**, 628-632.

Curtis, M. A., Gillett, I. R., Griffiths, G. S., Maiden, M. F. J., Sterne, J. A. C., Wilson, D. T., Wilton, J. M. A. & Johnson, N. W. (1989) Detection of high-risk groups and individuals for periodontal diseases - laboratory markers from analysis of gingival crevicular fluid. *Journal of Clinical Periodontology* **16,** 1-11.

Dahlén, G. (1993) Role of suspected periodontopathogens in microbiological monitoring of periodontitis. *Advances in dental research* **7,** 163-174.

Dale, B. A. (2002) Periodontal epithelium: a newly recognized role in health and disease. *Periodontology 2000* **30**, 70-78.

Dallegri, F. & Ottonello, L. (1997) Tissue injury in neutrophilic inflammation. *Inflammation Research* **46**, 382-391.

Darany, D. G., Beck, F. M. & Walters, J. D. (1992) The relationship of gingival fluid leukocyte elastase activity to gingival fluid flow rate. *Journal of Periodontology* **63**, 743-747.

Darveau, R. P., Tanner, A. & Page, R. C. (1997) The microbial challenge in periodontitis. *Periodontology 2000* **14**, 12-32.

De Bock, M., De Seny, D., Meuwis, M. A., Chapelle, J. P., Louis, E., Malaise, M., Merville, M. P. & Fillet, M. (2010) Challenges for biomarker discovery in body fluids using SELDI-TOF-MS. *BioMed Research International* **2010**, 1-15.

Chapter 8 – References

Decarlo, A. A., Windsor, L. J., Bodden, M. K., Harber, G. J., Birkedalhansen, B. & Birkedalhansen, H. (1997) Activation and novel processing of matrix metalloproteinases by a thiol proteinase from the oral anaerobe *Porphyromonas gingivalis*. *Journal of Dental Research* **76**, 1260-1270.

Demichele, M. A. A., Moon, D. G., Fenton, J. W., 2nd & Minnear, F. L. (1990) Thrombin's enzymatic activity increases permeability of endothelial cell monolayers. *Journal of Applied Physiology* **69**, 1599-1606.

Dibdin, G. & Shellis, R. (1988) Physical and biochemical studies of *Streptococcus mutans* sediments suggest new factors linking the cariogenicity of plaque with its extracellular polysaccharide content. *Journal of Dental Research* **67,** 890-895.

Ding, Y., Haapasalo, M., Kerosuo, E., Lounatmaa, K., Kotiranta, A. & Sorsa, T. (1997) Release and activation of human neutrophil matrix metallo- and serine proteinases during phagocytosis of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Treponema denticola*. *Journal of Clinical Periodontology* **24**, 237-248.

Ding, Y., Uitto, V. J., Haapasalo, M., Lounatmaa, K., Konttinen, Y. T., Salo, T., Grenier, D. & Sorsa, T. (1996) Membrane components of *Treponema denticola* trigger proteinase release from human polymorphonuclear leukocytes. *Journal of Dental Research* **75**, 1986-1993.

Doumas, S., Kolokotronis, A. & Stefanopoulos, P. (2005) Anti-inflammatory and antimicrobial roles of secretory leukocyte protease inhibitor. *Infection and Immunity* **73**, 1271-1274.

Drzeniek, R. (1972) Viral and bacterial neuraminidases. *Current Topics In Microbiology and Immunology* **59**, 35-74.

Ducy, P., Geoffroy, V. & Karsenty, G. (1996) Study of osteoblast-specific expression of one mouse osteocalcin gene: characterization of the factor binding to Ose2. *Connective Tissue Research* **35**, 7-14.

Dwarakanath, A. D., Tsai, H. H., Sunderland, D., Hart, C. A., Figura, N., Crabtree, J. E. & Rhodes, J. M. (1995) The production of neuraminidase and fucosidase by *Helicobacter pylori* - their possible relationship to pathogenicity. *Fems Immunology and Medical Microbiology* **12**, 213-216.

Chapter 8 – References

Dzink, J., Socransky, S. & Haffajee, A. (1988) The predominant cultivable microbiota of active and inactive lesions of destructive periodontal diseases. *Journal of Clinical Periodontology* **15,** 316-323.

Dzink, J. L., Sheenan, M. T. & Socransky, S. S. (1990) Proposal of Three subspecies of *Fusobacterium nucleatum* Knorr 1922: *Fusobacterium nucleatum* subsp. nucleatum subsp. nov., comb. nov.; *Fusobacterium nucleatum* subsp. *polymorphum* subsp. nov., nom. rev., comb. nov.; and *Fusobacterium nucleatum* subsp. vincentii subsp. nov., nom. rev., comb. nov. *International journal of systematic bacteriology* **40,** 74-78.

Eckle, I., Kolb, G. & Haveman, K. (1992) Regulation of neutrophil functions by elastase-generated IgG fragments. *Archivum Immunologiae Et Therapiae Experimentalis* **40**, 43-47.

Edgar, W. M. (1992) Saliva: its secretion, composition and functions. *British Dental Journal* **172**, 305-312.

Egelberg, J. (1966) The blood vessels of the dento-gingival junction. *Journal Of Periodontal Research* **1**, 163-179.

Eley, B. & Cox, S. (1996a) Correlation between Gingivain/Gingipain and bacterial dipeptidyl peptidase activity in gingival crevicular fluid and periodontal attachment loss in chronic periodontitis patients. A 2-year longitudinal study. J*ournal of Periodontology* **67**, 703-716.

Eley, B. & Cox, S. (1998) Advances in periodontal diagnosis. 5. potential inflammatory and immune markers. *British Dental Journal* **184**, 220-223.

Eley, B. M. & Cox, S. W. (1992a) Cathepsin B/L-like, elastase-like, tryptase-like, trypsin-like and dipeptidyl peptidase iv-like activities in gingival crevicular fluid - correlation with clinical-parameters in untreated chronic periodontitis patients. *Journal of Periodontal Research* **27**, 62-69.

Eley, B. M. & Cox, S. W. (1992b) Cathepsin-b/l-like, elastase-like, tryptase-like, trypsin-like and dipeptidyl peptidase-iv-like activities in gingival crevicular fluid - A comparison of levels before and after periodontal surgery in chronic periodontitis patients. *Journal of Periodontology* **63**, 412-417.

Eley, B. M. & Cox, S. W. (1996b) A 2-year longitudinal study of elastase in human gingival crevicular fluid and periodontal attachment loss. *Journal of Clinical Periodontology* **23**, 681-692.

Eley, B. M. & Cox, S. W. (1996c) The relationship between gingival crevicular fluid cathepsin b activity and periodontal attachment loss in chronic periodontitis patients: A 2-year longitudinal study. *Journal of Periodontal Research* **31**, 381-392.

Eley, B. M. & Cox, S. W. (2003) Proteolytic and hydrolytic enzymes from putative periodontal pathogens: characterization, molecular genetics, effects on host defenses and tissues and detection in gingival crevice fluid. *Periodontology 2000* **31**, 105-124.

Embery, G., Waddington, R. J., Hall, R. C. & Last, K. S. (2000) Connective tissue elements as diagnostic aids in periodontology. *Periodontology 2000* **24**, 193-214.

Emingil, G., Han, B., Özdemir, G., Tervahartiala, T., Vural, C., Atilla, G., Baylas, H. & Sorsa, T. (2012) Effect of azithromycin, as an adjunct to nonsurgical periodontal treatment, on microbiological parameters and gingival crevicular fluid biomarkers in generalized aggressive periodontitis. *Journal of periodontal research* **47,** 729-739.

Epstein, F. H. & Weiss, S. J. (1989) Tissue destruction by neutrophils. *The New England Journal of Medicine* **320**, 365-376.

Feng, J. T., Shang, S. & Beretta, L. (2006) Proteomics for the early detection and treatment of hepatocellular carcinoma. *Oncogene* **25**, 3810-3817.

Fenno, J. C., Lee, S. Y., Bayer, C. H. & Ning, Y. (2001) The opdB locus encodes the trypsin-like peptidase activity of *Treponema denticola*. *Infection and immunity* **69,** 6193-6200.

Figueredo, C., Ribeiro, M., Fischer, R. & Gustafsson, A. (1999) Increased interleukin-1β concentration in gingival crevicular fluid as a characteristic of periodontitis. *Journal of Periodontology* **70**, 1457-1463.

Fine, D. H. (1992) Incorporating new technologies in periodontal diagnosis into training programs and patient-care - A critical-assessment and a plan for the future. *Journal Of Periodontology* **63**, 383-393.

Flemmig, T. F. (1999) Periodontitis. *Annals of Periodontology* **4**, 32-8.

Fletcher, J., Nair, S., Poole, S., Henderson, B. & Wilson, M. (1998) Cytokine degradation by biofilms of *Porphyromonas gingivalis*. *Current Microbiology* **36**, 216-219.

Chapter 8 – References

Forng, R. Y., Champagne, C., Simpson, W. & Genco, C. (2000) Oral Microbiology: Environmental cues and gene expression in *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans*. *Oral Diseases* **6,** 351-365.

Fujise, O., Hamachi, T., Inoue, K., Miura, M. & Maeda, K. (2002) Microbiological markers for prediction and assessment of treatment outcome following non-surgical periodontal therapy. *Journal of Periodontology* **73,** 1253-1259.

Fukui, K., Fukui, Y. & Moriyama, T. (1971) Neuraminidase activity in some bacteria from human mouth. *Archives Of Oral Biology* **16**, 1361-&.

Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S. & Kaper, J. B. (1992) Role of vibrio-cholerae neuraminidase in the function of cholera-toxin. *Infection and Immunity* **60**, 406-415.

Gazi, M. I., Cox, S. W., Clark, D. T. & Eley, B. M. (1997) Characterization of protease activities In *Capnocytophaga spp*., *Porphyromonas gingivalis*, *Prevotella spp*., *Treponema denticola* and *Actinobacillus actinomycetemcomitans*. *Oral Microbiology and Immunology* **12**, 240-248.

Genco, C. (1995) Regulation of hemin and iron transport in *Porphyromonas gingivalis*. *Advances in dental research* **9,** 41-47.

Giannopoulou, C., Andersen, E., Demeurisse, C. & Cimasoni, G. (1992) Neutrophil elastase and its inhibitors in human gingival crevicular fluid during experimental gingivitis. *Journal of Dental Research* **71**, 359-363.

Gmur, R. & Guggenheim, B. (1994) Interdental supragingival plaque—a natural habitat of *Actinobacillus actinomycetemcomitans*, *Bacteroides forsythus*, *Campylobacter rectus*, and *Prevotella nigrescens*. *Journal of Dental Research* **73,** 1421-1428.

Godoy, V. G., Dallas, M. M., Russo, T. A. & Malamy, M. H. (1993) A role for *Bacteroides fragilis* neuraminidase in bacterial-growth in 2 model systems. *Infection And Immunity* **61**, 4415-4426.

Golub, L. M. & Kleinberg, I. (1976) Gingival crevicular fluid a new diagnostic aid in managing the periodontal patient. *Oral Sciences Reviews* **8**, 49-61.

Golub, L. M., Lee, H. M., Greenwald, R. A., Ryan, M. E., Sorsa, T., Salo, T. & Giannobile, W. V. (1997) A matrix metalloproteinase inhibitor reduces bone-type collagen degradation fragments and specific collagenases in gingival crevicular fluid during adult periodontitis. *Inflammation Research* **46**, 310-319.

Chapter 8 – References

Golub, L. M., Sorsa, T., Lee, H. M., Ciancio, S., Sorbi, D., Ramamurthy, N. S., Gruber, B., Salo, T. & Konttinen, Y. T. (1995) Doxycycline inhibits neutrophil (PMN)-type matrix metalloproteinases in human adult periodontitis gingiva. *Journal of Clinical Periodontology* **22**, 100-109.

Goodson, J. M. (1992) Diagnosis of periodontitis by physical measurement-interpretation from episodic disease hypothesis. *Journal of Periodontology* **63**, 373-382.

Goodson, J. M., Tanner, A. C. R., Haffajee, A. D., Sornberger, G. C. & Socransky, S. S. (1982) Patterns of progression and regression of advanced destructive periodontal disease. *Journal of Clinical Periodontology* **9**, 472-481.

Gramse, M., Bingenheimer, C. & Havemann, K. (1980) Degradation of human-fibrinogen by chymotrypsin-like neutral protease from human granulocytes. *Thrombosis Research* **19**, 201-209.

Grayson, R., Douglas, C. W. I., Heath, J., Rawlinson, A. & Evans, G. S. (2003) Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and *Porphyromonas gingivalis*. *Journal of Clinical Periodontology* **30**, 542-550.

Grbic, J. T., Lamster, I. B., Fine, J. B., Lam, K. S., Celenti, R. S., Herrera-Abreu, M. & Singer, R. E. (1999) Changes in gingival crevicular fluid levels of immunoglobulin a following therapy: association with attachment loss. *Journal of Periodontology* **70**, 1221-1227.

Greenstein, G. (1997) Contemporary interpretation of probing depth assessments: diagnostic and therapeutic implications. a literature review. *Journal of Periodontology* **68**, 1194-1205.

Gregory, R. L., Kim, D. E., Kindle, J. C., Hobbs, L. C. & Lloyd, D. R. (1992) Immunoglobulin degrading enzymes in localized juvenile periodontitis. *Journal of Periodontal Research* **27**, 176-183.

Grenier, D. (1995) Characterization of the trypsin-like activity of *Bacteroides forsythus*. *Microbiology* **141**, 921-926.

Grenier, D. & Mayrand, D. (1987) Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. *Journal of Clinical Microbiology* **25**, 738-740.

Griffen, A. L., Becker, M. R., Lyons, S. R., Moeschberger, M. L. & Leys, E. J. (1998) Prevalence of *Porphyromonas gingivalis* and Periodontal Health Status. *Journal of clinical microbiology* **36,** 3239-3242.

Chapter 8 – References

Griffiths, G., Curtis, M. & Wilton, J. (1988) Selection of a filter paper with optimum properties for the collection of gingival crevicular fluid. *Journal of Periodontal Research* **23,** 33-38.

Griffiths, G. S. (2003) Formation, collection and significance of gingival crevice fluid. *Periodontology 2000* **31**, 32-42.

Grossi, S. G., Genco, R. J., Machtei, E. E., Ho, A. W., Koch, G., Dunford, R., Zambon, J. J. & Hausmann, E. (1995) Assessment of risk for periodontal-disease .2. Risk indicators for alveolar bone loss. *Journal of Periodontology* **66,** 23-29.

Grossi, S. G., Zambon, J. J., Ho, A. W., Koch, G., Dunford, R. G., Machtei, E. E., Norderyd, O. M. & Genco, R. J. (1994) Assessment of risk for periodontal-disease .1. risk indicators for attachment loss. *Journal of Periodontology* **65**, 260-267.

Grover, V., Kapoor, A., Malhotra, R. & Kaur, G. (2014) Clinical relevance of the advanced microbiologic and biochemical investigations in periodontal diagnosis: a critical analysis. *Journal of Oral Diseases* **2014**, 1-11

Gueders, M. M., Balbin, M., Rocks, N., Foidart, J.-M., Gosset, P., Louis, R., Shapiro, S., Lopez-Otin, C., Noël, A. & Cataldo, D. D. (2005) Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *The Journal of Immunology* **175**, 2589-2597.

Guillot, J. L., Pollock, S. M. & Johnson, R. B. (1995) Gingival interleukin-6 concentration following phase I therapy. *Journal Of Periodontology* **66**, 667-672.

Gursoy, U. K., Kononen, E., Pradhan-Palikhe, P., Tervahartiala, T., Pussinen, P. J., Suominen-Taipale, L. & Sorsa, T. (2010) Salivary MMP-8, TIMP-1, and ICTP as markers of advanced periodontitis. *Journal of Clinical Periodontology* **37**, 487-493.

Gusberti, F. A., Syed, S.A., Hofmannt, T., Lang, N. P. (1986) Diagnostic methods for the assessment of potential periodontal disease activity: enzymatic activities of bacterial plaque and their relationship to clinical parameters,. In: Lehner, T., Cimasoni G. (Ed.) The borderland between caries and periodontal disease Iii. 1st Ed. Orlando, Fla.: Grune & Stratton, Inc.

Gustafsson, A., Asman, B. & Bergstrom, K. (1994) Elastase and lactoferrin in gingival crevicular fluid - possible indicators of a granulocyte-associated specific host response. *Journal of Periodontal Research* **29**, 276-282.

Chapter 8 – References

Gustafsson, A. (1996) Methodological considerations in GCF sampling with paper strips: poor recovery of uncomplexed elastase. *Journal of Clinical Periodontology* **23,** 432-436.

Guzman, C. A., Plate, M. & Pruzzo, C. (1990) Role of neuraminidase-dependent adherence in bacteroides-fragilis attachment to human epithelial cells. *Fems Microbiology Letters* **71**, 187-192.

Haffajee, A., Cugini, M., Tanner, A., Pollack, R., Smith, C., Kent, R. & Socransky, S. (1998) Subgingival microbiota in healthy, well maintained elder and periodontitis subjects. *Journal of Clinical Periodontology* **25**, 346-353.

Haffajee, A. D. & Socransky, S. S. (1994) Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000* **5**, 78-111.

Haffajee, A. D., Socransky, S. S. & Goodson, J. M. (1983) Clinical parameters as predictors of destructive periodontal disease activity. *Journal of Clinical Periodontology* **10**, 257-265.

Haffajee, A. D., Socransky, S. S., Lindhe, J., Kent, R. L., Okamoto, H. & Yoneyama, T. (1991) Clinical risk indicators for periodontal attachment loss. *Journal of Clinical Periodontology* **18**, 117-125.

Hajishengallis, G., Darveau, R. P. & Curtis, M. A. (2012) The keystone pathogen hypothesis. *Nature Reviews Microbiology* **10,** 717-725.

Hajishengallis, G. & Lambris, J. D. (2011) Microbial manipulation of receptor crosstalk in innate immunity. *Nature Reviews Immunology* **11,** 187-200.

Halazonetis, T. D., Haffajee, A. D. & Socransky, S. S. (1989) Relationship of clinical parameters to attachment loss in subsets of subjects with destructive periodontal diseases. *Journal of clinical periodontology* **16,** 563-568.

Han, B., Emingil, G., Özdemir, G., Tervahartiala, T., Vural, C., Atilla, G., Baylas, H. & Sorsa, T. (2012) Azithromycin as an adjunctive treatment of generalized severe chronic periodontitis: clinical, microbiologic and biochemical parameters. *Journal of periodontology* **83,** 1480-1491.

Hanemaaijer, R., Sorsa, T., Konttinen, Y. T., Ding, Y. L., Sutinen, M., Visser, H., Vanhinsbergh, V. W. M., Helaakoski, T., Kainulainen, T., Ronka, H., Tschesche, H. & Salo, T. (1997) Matrix metalloproteinase-8 is expressed in rheumatoid synovial fibroblasts and endothelial cells-regulation by tumor necrosis factor-alpha and doxycycline. *Journal of Biological Chemistry* **272**, 31504-31509.

Chapter 8 – References

Haverback, B. J., Dyce, B., Bundy, H. & Edmondson, H. A. (1960) Trypsin, trypsinogen and trypsin inhibitor in human pancreatic juice. *The American Journal of Medicine* **29**, 421-33.

Herrmann, J. M., Gonzáles, J. R., Boedeker, R. H., Vonholdt, J. & Meyle, J. (2001) Microassay for the detection of elastase activity in the gingival crevice. *Journal of Clinical Periodontology* **28,** 31-37.

Hinode, D., Nagata, A., Ichimiya, S., Hayashi, H., Morioka, M. & Nakamura, R. (1992) Generation of plasma kinin by 3 types of protease isolated from *Porphyromonas gingivalis*-381. *Archives of Oral Biology* **37**, 859-861.

Hirai, K., Hoshino, S., Shibata, Y., Fujimura, S. & Nakamura, T. (1997) Isolation and partial characterization of neuraminidase of *Treponema denticola* strain Tn-2. *Dental foundation Medical Association Journal* **39**, 72-75.

Hoffmann, O., Klaushofer, K., Koller, K., Peterlik, M., Mavreas, T. & Stern, P. (1986) Indomethacin inhibits Thrombin-stimulated, but not Thyroxine-stimulated resorption of fetal-rat limb bones. *Prostaglandins* **31**, 601-608.

Hojo, K., Nagaoka, S., Ohshima, T. & Maeda, N. (2009) Bacterial interactions in dental biofilm development. *Journal of Dental Research* **88,** 982-990.

Holt, S. C. & Bramanti, T. E. (1991) Factors in virulence expression and their role in periodontal disease pathogenesis. *Critical Reviews In Oral Biology and Medicine* **2**, 177-281.

Holt, S. C. & Ebersole, J. L. (2005) *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the 'red complex', a prototype polybacterial pathogenic consortium in periodontitis. *Periodontology 2000* **38,** 72-122.

Honma, K., Mishima, E. & Sharma, A. (2011) Role of *Tannerella forsythia* Nanh sialidase in epithelial cell attachment. *Infection And Immunity* **79**, 393-401.

Horz, H. P., Vianna, M. E., Gomes, B. & Conrads, G. (2005) Evaluation of universal probes and primer sets for assessing total bacterial load in clinical samples: general implications and practical use in endodontic antimicrobial therapy. *Journal of clinical microbiology* **43,** 5332-5337.

Huang, R., Li, M. & Gregory, R. L. (2011) Bacterial interactions in dental biofilm. *Virulence* **2,** 435-444.

Imamura, T. (2003) The role of Gingipains in the pathogenesis of periodontal disease. *Journal of Periodontology* **74**, 111-118.

Imamura, T., Banbula, A., Pereira, P. J. B., Travis, J. & Potempa, J. (2001b) Activation of human prothrombin by Arginine-specific cysteine proteinases (Gingipains R) from *Porphyromonas gingivalis*. *Journal of Biological Chemistry* **276**, 18984-18991.

Imamura, T., Potempa, J., Pike, R. N. & Travis, J. (1995) Dependence of vascular-permeability enhancement on cysteine proteinases in vesicles of *Porphyromonas gingivalis*. *Infection and Immunity* **63**, 1999-2003.

Imamura, T., Potempa, J., Tanase, S. & Travis, J. (1997) Activation of blood coagulation factor X by Arginine-specific cysteine proteinases (Gingipain-Rs) from *Porphyromonas gingivalis*. *Journal of Biological Chemistry* **272**, 16062-16067.

Imamura, T., Tanase, S., Hamamoto, T., Potempa, J. & Travis, J. (2001a) Activation of blood coagulation factor IX by Gingipains R, Arginine-Specific cysteine proteinases from *Porphyromonas gingivalis*. *Biochemical Journal* **353**, 325-331.

Ingman, T., Kononen, M., Konttinen, Y. T., Siirila, H. S., Suomalainen, K. & Sorsa, T. (1994) Collagenase, gelatinase and elastase activities in sulcular fluid of osseointegrated implants and natural teeth. *Journal of Clinical Periodontology* **21**, 301-307.

Ingman, T., Tervahartiala, T., Ding, Y. L., Tschesche, H., Haerian, A., Kinane, D. F., Konttinen, Y. T. & Sorsa, T. (1996) Matrix metalloproteinases and their inhibitors in gingival crevicular fluid and saliva of periodontitis patients. *Journal of Clinical Periodontology* **23**, 1127-1132.

Ishikura, H., Arakawa, S., Nakajima, T., Tsuchida, N. & Ishikawa, I. (2003) Cloning of the *Tannerella forsythensis* (*Bacteroides forsythus*) siahi gene and purification of the sialidase Enzyme. *Journal of Medical Microbiology* **52**, 1101-1107.

Ito, F., Yamada, S., Omori, S., Yamada, H. & Nakashizuka, T. (1975) The relationship between the sialic acid contents in saliva and the clinical findings (Author's Transl). *Nihon Shishubyo Gakkai Kaishi* **17**, 110-4.

Jaedicke, K. M., Preshaw, P. M. & Taylor, J. J. (2016) Salivary cytokines as biomarkers of periodontal diseases. *Periodontology 2000* **70,** 164-183.

Jain, N., Jain, G. K., Javed, S., Iqbal, Z., Talegaonkar, S., Ahmad, F. J. & Khar, R. K. (2008) Recent approaches for the treatment of periodontitis. *Drug Discovery Today* **13**, 932-943.

Chapter 8 – References

Jakubovics, N. S. & Kolenbrander, P. E. (2010) The road to ruin: the formation of disease-associated oral biofilms. *Oral diseases* **16,** 729-739.

Janciauskiene, S. (2001) Conformational properties of serine proteinase inhibitors (serpins) confer multiple pathophysiological roles. *Biochimica Et Biophysica Acta-Molecular Basis of Disease* **1535**, 221-235.

Janda, J. M. & Abbott, S. L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology* **45,** 2761-2764.

Janoff, A. (1985a) Elastase in tissue injury. *Annual Review of Medicine*. **36**, 207-216.

Janoff, A. (1985b) Elastase In tissue-injury. *Annual Review Of Medicine* **36**, 207-216.

Jin, L., Leung, W., Corbet, E. & Söder, B. (2002) Relationship of changes in interleukin-8 levels and granulocyte elastase activity in gingival crevicular fluid to subgingival periodontopathogens following non-surgical periodontal therapy in subjects with chronic periodontitis. *Journal of Clinical Periodontology* **29**, 604-614.

Jin, L. J., Soder, P. O., Asman, B. & Bergstrom, K. (1995) Granulocyte elastase in gingival crevicular fluid - improved monitoring of the site-specific response to treatment in patients with destructive periodontitis. *Journal of Clinical Periodontology* **22**, 240-246.

Jin, L., Söder, B. & Corbet, E. F. (2000) Interleukin-8 and granulocyte elastase in gingival crevicular fluid in relation to periodontopathogens in untreated adult periodontitis. *Journal of Periodontology* **71,** 929-939.

Johansson, A., Claesson, R., Hanstrom, L., Sandstrom, G. & Kalfas, S. (2000) Polymorphonuclear leukocyte degranulation induced by leukotoxin from *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research* **35**, 85-92.

Jones, A. & Geczy, C. L. (1990) Thrombin and factor-Xa enhance the production of interleukin-1. *Immunology* **71**, 236-241.

Kahari, V. M. & Saarialho-Kere, U. (1999) Matrix Metalloproteinases and their inhibitors in tumour growth and invasion. *Annals Of Medicine* **31**, 34-45.

Kaminishi, H., Cho, T., Itoh, T., Iwata, A., Kawasaki, K., Hagihara, Y. & Maeda, H. (1993) Vascular-permeability enhancing activity of *Porphyromonas gingivalis* protease in guinea-pigs. *Fems Microbiology Letters* **114**, 109-114.

Chapter 8 – References

Kawabata, K., Hagio, T. & Matsuoka, S. (2002) The Role of neutrophil elastase in acute lung injury. *European Journal of Pharmacology* **451**, 1-10.

Kennett, C. N., Cox, S. W. & Eley, B. M. (1995) Localization of active and inactive elastase, alpha-1-proteinase inhibitor, and alpha-2-macroglobulin in human gingiva. *Journal of Dental Research* **74**, 667-674.

Kennett, C. N., Cox, S. W. & Eley, B. M. (1997) Investigations into the cellular contribution to host tissue proteases and inhibitors in gingival crevicular fluid. *Journal of Clinical Periodontology* **24**, 424-431.

Kesavalu, L., Holt, S. C. & Ebersole, J. L. (1996) Trypsin-like protease activity of *Porphyromonas gingivalis* as a potential virulence factor in a murine lesion model. *Microbial Pathogenesis* **20**, 1-10.

Kesavalu, L., Sathishkumar, S., Bakthavatchalu, V., Matthews, C., Dawson, D., Steffen, M. & Ebersole, J. L. (2007) Rat model of polymicrobial infection, immunity and alveolar bone resorption in periodontal disease. *Infection and Immunity* **75,** 1704-1712.

Kiili, M., Cox, S. W., Chen, H. W., Wahlgren, J., Maisi, P., Eley, B. M., Salo, T. & Sorsa, T. (2002) Collagenase-2 (MMP8) and collagenase-3 (MMP-13) in adult periodontitis: molecular forms and levels in gingival crevicular fluid and immunolocalisation in gingival tissue. *Journal of Clinical Periodontology* **29**, 224-232.

Kilian, M. (1981) Degradation of immunoglobulins A1, A2 and G by suspected principal periodontal pathogens. *Infection and Immunity* **34**, 757-765.

Kinane, D. F. (2000) Regulators of tissue destruction and homeostasis as diagnostic aids in periodontology. *Periodontology 2000* **24**, 215-225.

Kinane, D. F., Darby, I. B., Said, S., Luoto, H., Sorsa, T., Tikanoja, S. & Mantyla, P. (2003) Changes in gingival crevicular fluid matrix metalloproteinase-8 levels during periodontal treatment and maintenance. *Journal of Periodontal Research* **38**, 400-404.

Kinane, D. F., Mooney, J. & Ebersole, J. L. (1999) Humoral immune response to *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in periodontal disease. *Periodontology 2000* **20**, 289-340.

Kinney, J. S., Morelli, T., Oh, M., Braun, T. M., Ramseier, C. A., Sugai, J. V. & Giannobile, W. V. (2014) Crevicular fluid biomarkers and periodontal disease progression. *Journal of Clinical Periodontology* **41**, 113-120.

Kinney, J. S., Morelli, T., Braun, T., Ramseier, C. A., Herr, A. E., Sugai, J. V., Shelburne, C. E., Rayburn, L. A., Singh, A. K. & Giannobile, W. V. (2011) Saliva/pathogen biomarker signatures and periodontal disease progression. *Journal of dental research* **90,** 752-758.

Kinney, J. S., Ramseier, C. A. & Giannobile, W. V. (2007a) Oral fluid-based biomarkers of alveolar bone loss in periodontitis. *Ann N Y Acad Sci* **1098,** 230–251.

Kinney, J. S., Ramseier, C. A. & Giannobile, W. V. (2007b) Oral Fluid–based biomarkers of alveolar bone loss in periodontitis. *Annals of The New York Academy Of Sciences* **1098**, 230-251.

Kitawaki, M. (1983) Neuraminidase activity in gingival crevicular fluid. Nihon Shishubyo Gakkai Kaishi **25**, 513-25.

Kitawaki, M., Iijima, K. I., Nakashizuka, T. & Hayakawa, T. (1983) Neuraminidase activity in human crevicular fluid. *Journal of Periodontal Research* **18**, 318-320.

Kivela-Rajamaki, M. F., Teronen, O. P., Maisi, P., Husa, V., Tervahartiala, T. I., Pirila, E. M., Salo, T. A., Mellanen, L. & Sorsa, T. A. (2003) Laminin-5 gamma 2-chain and collagenase-2 (MMP8) in human peri-implant sulcular fluid. *Clinical Oral Implants Research* **14**, 158-165.

Knaeuper, V., Wilhelm, S. M., Seperack, P. K., Declerck, Y. A., Langley, K. E., Osthues, A. & Tschesche, H. (1993) Direct activation of human neutrophil procollagenase by recombinant stromelysin. *Biochemical Journal* **295**, 581-586.

Koj, A., Regoeczi, E., Toews, C. J., Leveille, R. & Gauldie, J. (1978) Synthesis of anti-thrombin III and alpha-1-antitrypsin by perfused rat-liver. *Biochimica Et Biophysica Acta* **539**, 496-504.

Kolenbrander, P. E., Andersen, R. N., Blehert, D. S., Egland, P. G., Foster, J. S. & Palmer, R. J. (2002) Communication among oral bacteria. *Microbiology and molecular biology reviews* **66,** 486-505.

Kolenbrander, P. E. & london, J. (1993) Adhere today, here tomorrow: oral bacterial adherence. *Journal of bacteriology,* **175,** 3247.

Kook, J. K., Sakamoto, T., Nishi, K., Kim, M. K., Seong, J. H., Son, Y. N. & Kim, D. K. (2005) Detection of *Tannerella forsythia* and/or *Prevotella intermedia* might be useful for microbial predictive markers for the outcome of initial periodontal treatment in Koreans. *Microbiology and immunology* **49,** 9-16.

Chapter 8 – References

Korkmaz, B., Horwitz, M. S., Jenne, D. E. & Gauthier, F. (2010) Neutrophil elastase, proteinase 3 and cathepsin G as therapeutic targets in human diseases. *Pharmacological Reviews* **62**, 726-759.

Kornman, K. S. (2008) Mapping the pathogenesis of periodontitis: a new look. *Journal of Periodontology* **79**, 1560-1568.

Kornman, K. S., Crane, A., Wang, H. Y., Giovlne, F. S. D., Newman, M. G., Pirk, F. W., Wilson, T. G., Higginbottom, F. L. & Duff, G. W. (1997) The interleukin-1 genotype as a severity factor in adult periodontal disease. *Journal of Clinical Periodontology* **24**, 72-77.

Korte, D. L. & Kinney, J. (2016) Personalized medicine: an update of salivary biomarkers for periodontal diseases. *Periodontology 2000* **70,** 26-37.

Koshikawa, N., Yasumitsu, H., Umeda, M. & Miyazaki, K. (1992) Multiple secretion of matrix serine proteinases by human gastric-carcinoma cell-lines. *Cancer Research* **52**, 5046-5053.

Kunimatsu, K., Mine, N., Kato, I., Hase, T., Aoki, Y. & Yamamoto, K. (1993) Possible functions of human neutrophil serine proteinases, medullasin and cathepsin-G, in periodontal tissue breakdown. *Journal of Periodontal Research* **28**, 547-549.

Kunimatsu, K., Mine, N., Muraoka, Y., Kato, I., Hase, T., Aoki, Y. & Yamamoto, K. (1995) Identification and possible function of cathepsin G in gingival crevicular fluid from chronic adult periodontitis patients and from experimental gingivitis subjects. *Journal of Periodontal Research* **30**, 51-57.

Kunimatsu, K., Ozaki, Y., Hara, Y., Aoki, Y., Yamamoto, K. & Kato, I. (1997) Immunohistochemical study of cathepsin g and medullasin in inflamed gingival tissues from periodontal patients. *Journal of Periodontal Research* **32**, 264-270.

Kuriyama, T., Nakagawa, K., Kawashiri, S., Yamamoto, E., Nakamura, S. & Karasawa, T. (2000) The virulence of mixed infection with Streptococcus constellatus and Fusobacterium nucleatum in a murine orofacial infection model. *Microbes and Infection* **2,** 1425-1430.

Kuula, H., Salo, T., Pirila, E., Tuomainen, A. M., Jauhiainen, M., Uitto, V.-J., Tjaderhane, L., Pussinen, P. J. & Sorsa, T. (2009) Local and systemic responses in matrix metalloproteinase 8-deficient mice during *Porphyromonas gingivalis*-induced periodontitis. *Infection and Immunity* **77**, 850-859.

Chapter 8 – References

Lai, C. H., Listgarten, M., Shirakawa, M. & Slots, J. (1987) *Bacteroides forsythus* in adult gingivitis and periodontitis. *Oral Microbiology and Immunology* **2,** 152-157.

Lamont, R. & Rosan, B. (1990) Adherence of mutans streptococci to other oral bacteria. *Infection and Immunity* **58,** 1738-1743.

Lamster, I. (1990) Evaluation of the host response in crevicular fluid, saliva, and blood: implications and applications for the diagnosis of periodontal disease. *Periodontal Case Reports*: **12**, 6-9.

Lamster, I. B. (1997) Evaluation of components of gingival crevicular fluid as diagnostic tests. *Annals of Periodontology* **2**, 123-37.

Lamster, I. B., Hartley, L. J. & Vogel, R. I. (1985) Development of a biochemical profile for gingival crevicular fluid: methodological considerations and evaluation of collagen-degrading and ground substance-degrading enzyme activity during experimental gingivitis. *Journal of Periodontology* **56**, 13-21.

Lamster, I. B., Oshrain, R. L., Celenti, R., Levine, K. & Fine, J. B. (1991a) Correlation analysis for clinical and gingival crevicular fluid parameters at anatomically related gingival sites. *Journal of Clinical Periodontology* **18**, 272-277.

Lamster, I. B., Oshrain, R. L., Celenti, R. S., Fine, J. B. & Grbic, J. T. (1991b) Indicators of the acute inflammatory and humoral immune-responses in gingival crevicular fluid - relationship to active periodontal disease. *Journal of Periodontal Research* **26**, 261-263.

Lamster, I. B., Oshrain, R. L., Harper, D. S., Celenti, R. S., Hovliaras, C. A. & Gordon, J. M. (1988) Enzyme-activity in crevicular fluid for detection and prediction of clinical attachment loss in patients with chronic adult periodontitis - 6 month results. *Journal of Periodontology* **59**, 516-523.

Lamster, I. B., Oshrain, R. L. & Gordon, J. M. (1986) Enzyme activity in human gingival crevicular fluid: considerations in data reporting based on analysis of individual crevicular sites. *Journal of Clinical Periodontology* **13,** 799-804.

Lang, N. P. (2014) Commentary: bacteria play a critical role in the etiology of periodontal disease. *Journal of Periodontology* **85**, 211-213.

Lang, N. P., Joss, A., Orsanic, T., Gusberti, F. A. & Siegrist, B. E. (1986) Bleeding on probing. A predictor for the progression of periodontal disease? *Journal of Clinical Periodontology* **13,** 590-596.

Chapter 8 – References

Lantz, M. S., Allen, R. D., Vail, T. A., Switalski, L. M. & Hook, M. (1991) Specific cell components of bacteroides-gingivalis mediate binding and degradation of human fibrinogen. *Journal of Bacteriology* **173**, 495-504.

Laughon, B. E., Syed, S. A. & Loesche, W. J. (1982a) Rapid identification of *Bacteroides gingivalis*. *Journal of Clinical Microbiology* **15**, 345-346.

Laughon, B. E., Syed, S. A. & Loesche, W. J. (1982b) API-ZYM system for identification of *Bacteroides* spp, *Capnocytophaga* spp, and *Spirochetes* of oral origin. *Journal of Clinical Microbiology* **15**, 97-102.

Leach, S. A. (1963) Release and breakdown of sialic acid from human salivary mucin and its role in formation of dental plaque. *Nature* **199**, 486-7.

Lee, A. J., Walsh, T. F., Hodges, S. J. & Rawlinson, A. (1999) Gingival crevicular fluid osteocalcin in adult periodontitis. *Journal of Clinical Periodontology* **26**, 252-256.

Lee, W., Aitken, S., Kulkarni, G., Birek, P., Overall, C. M., Sodek, J. & Mcculloch, C. A. G. (1991) Collagenase activity in recurrent periodontitis - relationship to disease progression and doxycycline therapy. *Journal of Periodontal Research* **26**, 479-485.

Lee, W., Aitken, S., Sodek, J. & Mcculloch, C. A. G. (1995) Evidence of a direct relationship between neutrophil collagenase activity and periodontal tissue destruction in-vivo role of active enzyme in human periodontitis. *Journal of Periodontal Research* **30**, 23-33.

Lein, M., Nowak, L., Jung, K., Koenig, F., Lichtinghagen, R., Schnorr, D. & Loening, S. A. (1997) Analytical aspects regarding the measurement of metalloproteinases and their inhibitors in blood. *Clinical Biochemistry* **30**, 491-496.

Lemoine, O., Devaster, J. M., Deviere, J., Thiry, P., Cremer, M. & Ooms, H. A. (1994) Trypsin Activity - A new marker of acute alcoholic pancreatitis. *Digestive Diseases And Sciences* **39**, 2634-2638.

Lemos, J. A., Abranches, J. & Burne, R. A. (2005) Responses of cariogenic streptococci to environmental stresses. *Current issues in molecular biology* **7,** 95-108.

Leppilahti, J. M., Ahonen, M. M., Hernandez, M., Munjal, S., Netuschil, L., Uitto, V. J., Sorsa, T. & Mantyla, P. (2011) Oral rinse MMP8 point of care immuno test identifies patients with strong periodontal inflammatory burden. *Oral Diseases* **17**, 115-122.

Chapter 8 – References

Leppilahti, J. M., Hernández-Ríos, P. A., Gamonal, J. A., Tervahartiala, T., Brignardello-Petersen, R., Mantyla, P., Sorsa, T. & Hernández, M. (2014a) Matrix metalloproteinases and myeloperoxidase in gingival crevicular fluid provide site-specific diagnostic value for chronic periodontitis. *Journal of Clinical Periodontology* **41**, 348-356.

Leppilahti, J. M., Kallio, M. A., Tervahartiala, T., Sorsa, T. & Mäntylä, P. (2014b) Gingival crevicular fluid matrix metalloproteinase-8 levels predict treatment outcome among smokers with chronic periodontitis. *Journal of Periodontology* **85**, 250-260.

Leppilahti, J. M., Sorsa, T., Kallio, M. A., Tervahartiala, T., Emingil, G., Han, B. & Mäntylä, P. (2015) The utility of gingival crevicular fluid matrix metalloproteinase-8 response patterns in prediction of site level clinical treatment outcome. *Journal of Periodontology* **86**, 777-787.

Li, C., Kurniyati, Hu, B., Bian, J., Sun, J., Zhang, W., Liu, J., Pan, Y. & Li, C. (2012) Abrogation of neuraminidase reduces biofilm formation, capsule biosynthesis, and virulence of *Porphyromonas gingivalis*. *Infection and Immunity* **80**, 3-13.

Li, J., Helmerhorst, E., Leone, C., Troxler, R., Yaskell, T., Haffajee, A., Socransky, S. & Oppenheim, F. (2004) Identification of early microbial colonizers in human dental biofilm. *Journal of Applied Microbiology* **97,** 1311-1318.

Li, J., Orlandi, R., White, C. N., Rosenzweig, J., Zhao, J., Seregni, E., Morelli, D., Yu, Y., Meng, X.-Y. & Zhang, Z. (2005) Independent validation of candidate breast cancer serum biomarkers identified by mass spectrometry. *Clinical chemistry,* **51,** 2229-2235.

Lindhe, J., Lang, N. P. & Karring, T. (2008) Clinical periodontology and implant dentistry, Uk, Blackwell Publishing Ltd.

Listgarten, M. A. (1980) Periodontal probing - what does it mean. *Journal of Clinical Periodontology* **7**, 165-176.

Listgarten, M. A. & Loomer, P. M. (2003) Microbial identification in the management of periodontal diseases. a systematic review. *Annals of Periodontology* **8**, 182-192.

Llano, E., Pendas, A. M., Knauper, V., Sorsa, T., Salo, T., Salido, E., Murphy, G., Simmer, J. P., Bartlett, J. D. & Lopezotin, C. (1997) Identification and structural and functional characterization of human enamelysin (MMP20). *Biochemistry* **36**, 15101-15108.

Chapter 8 – References

Loe, H. & Holm-Pedersen, P. (1965) Absence and presence of fluid from normal and inflamed gingivae. *Periodontics* **149**, 171-177

Loe, H., Theilade, E. & Jensen, S. B. (1965) Experimental gingivitis in man. *Journal of Periodontology* **36**, 177-187.

Loesche, W. (1975) Chemotherapy of dental plaque infections. *Oral Sciences Reviews* **9,** 65-107.

Loesche, W., Lopatin, D., Stoll, J., Van Poperin, N. & Hujoel, P. P. (1992) Comparison of various detection methods for periodontopathic bacteria: can culture be considered the primary reference standard? *Journal of Clinical Microbiology* **30,** 418-426.

Loesche, W. J., Bretz, W. A., Kerschensteiner, D., Stoll, J., Socransky, S. S., Hujoel, P. & Lopatin, D. E. (1990) Development of a diagnostic-test for anaerobic periodontal infections based on plaque hydrolysis of Benzoyl-dl-Arginine-Naphthylamide. *Journal of Clinical Microbiology* **28**, 1551-1559.

Loesche, W. J., Syed, S. A. & Stoll, J. (1987) Trypsin-like activity in subgingival plaque - a diagnostic marker for *spirochetes* and periodontal-disease. *Journal of Periodontology* **58**, 266-273.

Lomas, D. A., Stone, S. R., Llewellynjones, C., Keogan, M. T., Wang, Z. M., Rubin, H., Carrell, R. W. & Stockley, R. A. (1995) The control of neutrophil chemotaxis by inhibitors of cathepsin G and chymotrypsin. *Journal of Biological Chemistry* 270, 23437-23443.

Loos, B. G. & Tjoa, S. (2005) Host-derived diagnostic markers for periodontitis: do they exist in gingival crevice fluid?. *Periodontology 2000* **39**, 53-72.

Louis, G. B. (2006) Principles for evaluating health risks in children associated with exposure to chemicals. WHO.

Lourenço, T. G. B., Heller, D., Silva-Boghossian, C. M., Cotton, S. L., Paster, B. J. & Colombo, A. P. V. (2014) Microbial signature profiles of periodontally healthy and diseased patients. *Journal of clinical periodontology* **41,** 1027-1036.

Lura, H. E. (1961) An investigation into the relation between the sialic acid of saliva and dental caries. *Archives of Oral Biology* **4**, 141-146.

Machtei, E. E., Dunford, R., Hausmann, E., Grossi, S. G., Powell, J., Cummins, D., Zambon, J. J. & Genco, R. J. (1997) Longitudinal study of prognostic factors in established periodontitis patients. *Journal of Clinical Periodontology* **24,** 102-109.

Chapter 8 – References

MacIvor, D. M., Shapiro, S. D., Pham, C. T. N., Belaaouaj, A., Abraham, S. N. & Ley, T. J. (1999) Normal neutrophil function in cathepsin G-deficient mice. *Blood* **94**, 4282-4293.

Madianos, P. N., Papapanou, P. N. & Sandros, J. (1997) *Porphyromonas gingivalis* infection of oral epithelium inhibits neutrophil transepithelial migration. *Infection and Immunity* **65**, 3983-3990.

Mailhot, J., Potempa, J., Stein, S., Travis, J., Sterrett, J., Hanes, P. & Russell, C. (1998) A relationship between proteinase activity and clinical parameters in the treatment of periodontal disease. *Journal of Clinical Periodontology,* **25,** 578-584.

Malagi, S. (2012) Chairside diagnostic test kits in periodontics- A review. *International Arab Journal of Dentistry*, **3**. 99-103.

Mantyla, P., Stenman, M., Kinane, D. F., Tikanoja, S., Luoto, H., Salo, T. & Sorsa, T. (2003) Gingival crevicular fluid collagenase-2 (MMP8) test stick for chair-side monitoring of periodontitis. *Journal of Periodontal Research* **38**, 436-439.

Mantyla, P., Stenman, M., Paldanius, M., Saikku, P., Sorsa, T. & Meurman, J. H. (2004) Chlamydia pneumoniae together with collagenase-2 (MMP8) in periodontal lesions. *oral diseases* **10**, 32-35.

Marsh, P. (1994) Microbial ecology of dental plaque and its significance in health and disease. *Advances in dental research* **8,** 263-271.

Marsh, P. D. (2003a) Dental plaque as a microbial biofilm. *Caries research* **38,** 204-211.

Marsh, P. (2003b) Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub-and supragingival environment. *Oral Diseases* **9,** 16-22.

Marsh, P. D. (1989) Host defenses and microbial homeostasis-role of microbial interactions. *Journal of Dental Research* **68,** 1567-1575.

Marsh, P. D., Martin, M. V., Lewis, M. A. & Williams, D. (2009) *Oral microbiology*. Elsevier Health Sciences.

Martins, F. T., Assis, D. M., Dos Santos, M. H., Camps, I., Veloso, M. P., Juliano, M. A., Alves, L. C. & Doriguetto, A. C. (2009) Natural polyprenylated benzophenones inhibiting cysteine and serine proteases. *European Journal of Medicinal Chemistry* **44**, 1230-1239.

Chapter 8 – References

Martínez, A., Olsen, R. L. & Serra, J. L. (1988) Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy Engraulis encrasicholus. *Comparative Biochemistry and Physiology* **91,** 677-684.

Masada, M. P., Persson, R., Kenney, J. S., Lee, S. W., Page, R. C. & Allison, A. C. (1990) Measurement of interleukin-1-alpha and interleukin-1-beta in gingival crevicular fluid - implications for the pathogenesis of periodontal disease. *Journal of Periodontal Research* **25**, 156-163.

McBride, B. & Gisslow, M. (1977) Role of sialic acid in saliva-induced aggregation of Streptococcus sanguis. *Infection and Immunity* **18,** 35-40.

Mccauley, L. K. & Nohutcu, R. M. (2002) Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy. *Journal of Periodontology* **73**, 1377-1391.

McCulloch, C. A. G. (1994) Host enzymes in gingival crevicular fluid as diagnostic indicators of periodontitis. *Journal Of Clinical Periodontology* **21**, 497-506.

McGee, J. M., Tucci, M. A., Edmundson, T. P., Serio, C. L. & Johnson, R. B. (1998) The relationship between concentrations of proinflammatory cytokines within gingiva and the adjacent sulcular depth. *Journal of Periodontology* **69**, 865-871.

Mealey, B. L. & Oates, T. W. (2006) Diabetes mellitus and periodontal diseases. *Journal of Periodontology* **77**, 1289-1303.

Michalowicz, B. S., Aeppli, D., Virag, J. G., Klump, D. G., Hinrichs, J. E., Segal, N. L., Bouchard, T. J. & Pihlstrom, B. L. (1991) Periodontal findings in adult twins. *Journal of Periodontology* **62**, 293-299.

Miyauchi, M., Sato, S., Kitagawa, S., Hiraoka, M., Kudo, Y., Ogawa, I., Zhao, M. & Takata, T. (2001) Cytokine expression in rat molar gingival periodontal tissues after topical application of lipopolysaccharide. *Histochemistry And Cell Biology* **116**, 57-62.

Modeer, T. & Twetman, S. (1979) Lysozyme activity in saliva from children with various degree of gingivitis. *Swedish Dental Journal* **3**, 63-67.

Moilanen, M., Pirila, E., Grenman, R., Sorsa, T. & Salo, T. (2002) Expression and regulation of collagenase-2 (MMP8) in head and neck squamous cell carcinomas. *Journal of Pathology* **197**, 72-81.

Moilanen, M., Sorsa, T., Stenman, M., Nyberg, P., Lindy, O., Vesterinen, J., Paju, A., Konttinen, Y. T., Stenman, U. H. & Salo, T. (2003) Tumor-associated trypsinogen-2 (trypsinogen-2) activates procollagenases (MMP-1,-8,-13) and

stromelysin-1 (MMP-3) and degrades type I collagen. *Biochemistry* **42**, 5414-5420.

Moore, L., Moore, W., Cato, E., Smibert, R., Burmeister, J., Best, A. & Ranney, R. (1987) Bacteriology of human gingivitis. *Journal of Dental Research* **66,** 989-995.

Moore, W., Moore, L., Ranney, R., Smibert, R., Burmeister, J. & Schenkein, H. (1991) The microflora of periodontal sites showing active destructive progression. *Journal of Clinical Periodontology* **18,** 729-739.

Moore, W. & Moore, L. V. (1994) The bacteria of periodontal diseases. *Periodontology 2000* **5,** 66-77.

Moncla, B., Motley, S., Braham, P., Ewing, L., Adams, T. & Vermeulen, N. (1991) Use of synthetic oligonucleotide DNA probes for identification and direct detection of *Bacteroides forsythus* in plaque samples. *Journal of Clinical Microbiology* **29,** 2158-2162.

Monti, E., Bassi, M. T., Bresciani, R., Civini, S., Croci, G. L., Papini, N., Riboni, M., Zanchetti, G., Ballabio, A., Preti, A., Tettamanti, G., Venerando, B. & Borsani, G. (2004) Molecular cloning and characterization of Neu4, the fourth member of the human sialidase gene family. *Genomics* **83**, 445-453.

Monti, E., Bassi, M. T., Papini, N., Riboni, M., Manzoni, M., Venerando, B., Croci, G., Preti, A., Ballabio, A., Tettamanti, G. & Borsani, G. (2000) Identification and expression of Neu3, a novel human sialidase associated to the plasma membrane. *Biochemical Journal* **349**, 343-351.

Monti, E., Preti, A., Nesti, C., Ballabio, A. & Borsani, G. (1999) Expression of a novel human sialidase encoded by the Neu2 gene. *Glycobiology* **9**, 1313-1321.

Moreno, M., Romero, P., Nieves, B., Salazar, M. & Burguera, L. (1999) Microbiological characteristics of adult periodontitis associated with anaerobic bacteria. *Anaerobe* **5**, 261-262.

Muller, H. E. (1974) Pathogenic role of microbial neuroaminidase. *Deutsche Medizinische Wochenschrift* **99**, 1933-1940.

Muller, H. E. (1976) Neuraminidase as a factor of pathogenicity in microbial infections. *Zentralblatt Fur Bakteriologie Mikrobiologie Und Hygiene Series A-Medical Microbiology Infectious Diseases Virology Parasitology* **235**, 106-110.

Chapter 8 – References

Nadkarni, M. A., Martin, F. E., Jacques, N. A. & Hunter, N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. *Microbiology* **148**, 257-266.

Nakamura, R., Hinode, D., Terai, T. & Morioka, M. (1991) Extracellular enzymes of Porphyromonas gingivalis equals Bacteroides gingivalis in relation to periodontal destruction. In: Periodontal Disease: Pathogens and Host Immune Responses (Hamada, S., Holt, S.C. and McGhee, J.R., Eds.), pp. 129-141, Quintessence Publishing Co., Tokyo.

Nakashima, K., Demeurisse, C. & Cimasoni, G. (1994) The recovery efficiency of various materials for sampling enzymes and polymorphonuclear leukocytes from gingival crevices. *Journal of Clinical Periodontology* **21,** 479-483.

Neumann, U., Kubota, H., Frei, K., Ganu, V. & Leppert, D. (2004) Characterization of Mca-Lys-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2,, a fluorogenic substrate with increased specificity constants for collagenases and tumor necrosis factor converting enzyme. *Analytical biochemistry* **328,** 166-173.

Newman, M. G., Takei, H., Klokkevold, P. R. & Carranza, F. A. (2012) Carranza's clinical periodontology, St. Louis, Missouri, Elsevier Inc.

Nieminen, A., Nordlund, L. & Uitto, V. J. (1993) The effect of treatment of the activity of salivary proteases and glycosidases in adults with advanced periodontitis. *Journal of Periodontology* **64**, 297-301.

Nomura, Y., Shimada, Y., Hanada, N., Numabe, Y., Kamoi, K., Sato, T., Gomi, K., Arai, T., Inagaki, K. & Fukuda, M. (2012) Salivary biomarkers for predicting the progression of chronic periodontitis. *Archives of Oral Biology* **57,** 413-420.

Nonnenmacher, C., Dalpke, A., Rochon, J., Flores-De-Jacoby, L., Mutters, R. & Heeg, K. (2005) Real-time polymerase chain reaction for detection and quantification of bacteria in periodontal patients. *Journal of periodontology* **76,** 1542-1549.

Nord, C. E., Modéer, T., Söder, P. Ö. & Bergström, J. (1971) Enzyme activities in experimental gingivitis in man. *European Journal Of Oral Sciences* **79**, 510-514.

Offenbacher, S. (1996) Periodontal diseases: pathogenesis. *Annals Of Periodontology* **1**, 821-878.

Offenbacher, S., Heasman, P. A. & Collins, J. G. (1993) Modulation of host PGE2 secretion as a determinant of periodontal disease expression. *Journal of Periodontology* **64**, 432-444.

Chapter 8 – References

Offenbacher, S., Odle, B. M. & Vandyke, T. E. (1986) The use of crevicular fluid prostaglandin-E2 levels as a predictor of periodontal attachment loss. *Journal of Periodontal Research* **21**, 101-112.

Ohlsson, K. (1988) Acute pancreatitis biochemical, pathophysiological and therapeutics aspects. *Acta Gastro-Enterologica Belgica* **51**, 3-12.

Ohta, K., Makinen, K. K. & Loesche, W. J. (1986) Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. *Infection and Immunity* **53**, 213-220.

Okada, Y. & Nakanishi, I. (1989) Activation of matrix metalloproteinase-3 (stromelysin) and matrix metalloproteinase-2 (gelatinase) by human neutrophil elastase and cathepsin G. *Febs Letters* **249**, 353-356.

Okamoto, K., Nakayama, K., Kadowaki, T., Abe, N., Ratnayake, D. B. & Yamamoto, K. (1998) Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. *Journal of Biological Chemistry* **273**, 21225-21231.

Okamoto, T., Akaike, T., Suga, M., Tanase, S., Horie, H., Miyajima, S., Ando, M., Ichinose, Y. & Maeda, H. (1997) Activation of human matrix metalloproteinases by various bacterial proteinases. *Journal of Biological Chemistry* **272**, 6059-6066.

Oppenheim, F. (1970) Preliminary observations on the presence and origin of serum albumin in human saliva. *Helvetica Odontologica Acta* **14**, 10-17.

Overall, C. M. & Sodek, J. (1987) Initial characterization of a neutral metalloproteinase, active on native 3/4-collagen fragments, synthesized by ros 17/2-8 osteoblastic cells, periodontal fibroblasts, and identified in gingival crevicular fluid. *Journal of Dental Research* **66**, 1271-1282.

Owen, C. A. & Campbell, E. J. (1999a) The cell biology of leukocyte-mediated proteolysis. *Journal of Leukocyte Biology* **65**, 137-150.

Owen, C. A. & Campbell, E. J. (1999b) Extracellular proteolysis: new paradigms for an old paradox. *The Journal Of Laboratory and Clinical Medicine* **134**, 341-351.

Owen, C. A., Campbell, M. A., Boukedes, S. S. & Campbell, E. J. (1997) Cytokines regulate membrane-bound leukocyte elastase on neutrophils: a novel mechanism for effector activity. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **272**, 385-393.

Chapter 8 – References

Owen, C. A., Campbell, M. A., Sannes, P. L., Boukedes, S. S. & Campbell, E. J. (1995) Cell surface-bound elastase and cathepsin G on human neutrophils - A novel, nonoxidative mechanism by which neutrophils focus and preserve catalytic activity of serine proteinases. *Journal of Cell Biology* **131**, 775-789.

Ozmeric, N. (2004) Advances in periodontal disease markers. *Clinica Chimica Acta* **343**, 1-16.

Page, R. C. & Schroeder, H. E. (1976) Pathogenesis of inflammatory periodontal disease - summary of current work. *Laboratory Investigation* **34**, 235-249.

Palcanis, K. G., Larjava, I. K., Wells, B. R., Suggs, K. A., Landis, J. R., Chadwick, D. E. & Jeffcoat, M. K. (1992) Elastase as an indicator of periodontal disease progression. *Journal of Periodontology* **63**, 237-242.

Papapanou, P. N., Baelum, V., Luan, W.-M., Madianos, P. N., Chen, X., Fejerskov, O. & Dahlén, G. (1997) Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression. *Journal of periodontology* **68,** 651-666.

Papapanou, P. N. (2002) Population studies of microbial ecology in periodontal health and disease. *Annals of periodontology* **7**, 54-61.

Papapanou, P. N., Neiderud, A.-M., Papadimitriou, A., Sandros, J. & Dahlén, G. (2000) " Checkerboard" assessments of periodontal microbiota and serum antibody responses: A case-control study. *Journal of periodontology* **71**, 885-897.

Partridge, N. C., Hillyard, C. J., Nolan, R. D. & Martin, T. J. (1985) Regulation of prostaglandin production by osteoblast-rich calvarial cells. *Prostaglandins* **3**0, 527-539.

Pashley, D. (1976) A mechanistic analysis of gingival fluid production. *Journal Of Periodontal Research* **11**, 121-134.

Pederson, E. D., Stanke, S. R., Whitener, S. J., Sebastiani, P. T., Lamberts, B. L. & Turner, D. W. (1995) Salivary levels of alpha(2)-macroglobulin, alpha(1)-antitrypsin, C-reactive protein, cathepsin G and elastase in humans with or without destructive periodontal disease. *Archives of Oral Biology* **40**, 1151-1155.

Perlitsh, M. J. & Glickman, I. (1967) Salivary neuraminidase 3. its relation to oral disease. *Journal of Periodontology* **38**, 189-&.

Persson, G. R., Rouen, T. A. & Page, R. C. (1990) Relationship between gingival crevicular fluid levels of Aspartate aminotransferase and active tissue

destruction in treated chronic periodontitis patients. *Journal Of Periodontal Research* **25**, 81-87.

Pham, C. T. N. (2006) Neutrophil serine proteases: specific regulators of inflammation. *Nature Reviews Immunology* **6**, 541-550.

Polanowska, J., Krokoszynska, I., Czapinska, H., Watorek, W., Dadlez, M. & Otlewski, J. (1998) Specificity of human cathepsin G. *Biochimica Et Biophysica Acta-Protein Structure And Molecular Enzymology* **1386**, 189-198.

Pollanen, M. T., Salonen, J. I. & Uitto, V. J. (2003) Structure and function of the tooth-epithelial interface in health and disease. *Periodontology 2000* **31**, 12-31.

Potempa, J., Banbula, A. & Travis, J. (2000) Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontology 2000* **24**, 153-192.

Potempa, J., Pike, R. & Travis, J. (1995a) The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-Gingipain or Lys-Gingipain. *Infection and Immunity* **63**, 1176-1182.

Potempa, J., Pavloff, N. & Travis, J. (1995b) *Porphyromonas gingivalis* - a proteinase gene accounting audit. *Trends in Microbiology* **3**, 430-434.

Potempa, J., Pike, R. & Travis, J. (1997) Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. *Biological Chemistry* **378**, 223-230.

Pozo, P., Valenzuela, M. A., Melej, C., Zaldivar, M., Puente, J., Martinez, B. & Gamonal, J. (2005) Longitudinal analysis of metalloproteinases, tissue inhibitors of metalloproteinases and clinical parameters in gingival crevicular fluid from periodontitis-affects patients. *Journal of Periodontal Research* **40**, 199-207.

Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S. P., Elsliger, M. A., Qu, J. Y., Leclerc, D., Gravel, R., Dallaire, L. & Potier, M. (1997) Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nature Genetics* **15**, 316-320.

Radford, J. R., Naylor, M. N. & Beighton, D. (1992) Effects of scaling on gingival crevicular fluid gcf enzyme activities. *Journal Of Dental Research* **71**, 621-626.

Rahman, S., Bunning, R. A. D., Dobson, P. R. M., Evans, D. B., Chapman, K., Jones, T. H., Brown, B. L. & Russell, R. G. G. (1992) Bradykinin stimulates the production of prostaglandin-E2 and interleukin-6 in human osteoblast-like cells. *Biochimica Et Biophysica Acta* **1135**, 97-102.

Chapter 8 – References

Ramseier, C. A., Kinney, J. S., Herr, A. E., Braun, T., Sugai, J. V., Shelburne, C. A., Rayburn, L. A., Tran, H. M., Singh, A. K. & Giannobile, W. V. (2009) Identification of pathogen and host-response markers correlated with periodontal disease. *Journal of periodontology* **80,** 436-446.

Ransjo, M., Marklund, M., Persson, M. & Lerner, U. H. (1998) Synergistic interactions of bradykinin, thrombin, interleukin 1 and tumor necrosis factor on prostanoid biosynthesis in human periodontal-ligament cells. *Archives of Oral Biology* **43**, 253-260.

Rawlings, N. D. & Barrett, A. J. (1994) Families of serine peptidases. *Methods in enzymology* **244**, 19-61.

Reddy, S., Kaul, S., Mgs, P., Agnihotri, J., Asutkar, H. G. & Bhowmik, N. (2011) Biomarkers in periodontal diagnosis:"what the future holds...". *International Journal of Clinical Dental Science* **2**. 76-83.

Rehault, S., Brillard-Bourdet, M., Juliano, M. A., Juliano, L., Gauthier, F. & Moreau, T. (1999) New, sensitive fluorogenic substrates for human cathepsin G based on the sequence of serpin-reactive site loops. *Journal of Biological Chemistry* **274,** 13810-13817.

Reilly, C. F., Fukunaga, Y., Powers, J. C. & Travis, J. (1984) Effect of neutrophil cathepsin G on elastin degradation by neutrophil elastase. *Hoppe-Seylers Zeitschrift Fur Physiologische Chemie* **365**, 1131-1135.

Reilly, C. F., Tewksbury, D. A., Schechter, N. M. & Travis, J. (1982) Rapid conversion of Angiotensin-I to Angiotensin-II by neutrophil and mast cell proteinases. *Journal of Biological Chemistry* **257**, 8619-8622.

Reinhardt, R. A., Stoner, J. A., Golub, L. M., Lee, H.-M., Nummikoski, P. V., Sorsa, T. & Payne, J. B. (2010) Association of gingival crevicular fluid biomarkers during periodontal maintenance with subsequent progressive periodontitis. *Journal of Periodontology* **81**, 251-259.

Reynolds, J. J. & Meikle, M. C. (1997) Mechanisms of connective tissue matrix destruction in periodontitis. *Periodontology 2000* **14**, 144-157.

Rice, A. & Banda, M. J. (1995) Neutrophil elastase processing of gelatinase-A is mediated by extracellular matrix. *Biochemistry* **34**, 9249-9256.

Riep, B., Edesi-Neuß, L., Claessen, F., Skarabis, H., Ehmke, B., Flemmig, T. F., Bernimoulin, J.-P., Göbel, U. B. & Moter, A. (2009) Are putative periodontal

pathogens reliable diagnostic markers? *Journal of Clinical Microbiology* **47,** 1705-1711.

Risteli, J., Elomaa, I., Niemi, S., Novamo, A. & Risteli, L. (1993) Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation. *Clinical Chemistry* **39**, 635-640.

Roberts, F. A., Houston, L. S., Lukehart, S. A., Mancl, L. A., Persson, G. R. & Page, R. C. (2004) Periodontitis vaccine decreases local prostaglandin E-2 levels in a primate model. *Infection and Immunity* **72**, 1166-1168.

Rogers, R., Newbrun, E. & Tatevossian, A. (1979) Neuraminidase activity in human dental plaque fluid. *Archives of Oral Biology* **24**, 703-705.

Roggentin, P., Rothe, B., Kaper, J. B., Galen, J., Lawrisuk, L., Vimr, E. R. & Schauer, R. (1989) Conserved sequences in bacterial and viral sialidases. *Glycoconjugate Journal* **6**, 349-353.

Romanelli, R., Mancini, S., Laschinger, C., Overall, C. M., Sodek, J. & Mcculloch, C. A. G. (1999) Activation of neutrophil collagenase in periodontitis. *Infection and Immunity* **67**, 2319-2326.

Roy, S., Honma, K., Douglas, C. W. I., Sharma, A. & Stafford, G. P. (2011) Role of sialidase in glycoprotein utilization by *Tannerella forsythia*. *Microbiology* **157**, 3195-3202.

Rozkiewicz, D., Daniluk, T., Zaremba, M., Cylwik-Rokicka, D., Luczaj-Cepowicz, E., Milewska, R., Marczuk-Kolada, G. & Stokowska, W. (2005) Bacterial composition in the supragingival plaques of children with and without dental caries. *Advances in medical sciences* **51,** 182-186.

Ryan, M. E. & Golub, L. M. (2000) Modulation of matrix metalloproteinase activities in periodontitis as a treatment strategy. *Periodontology 2000* **24**, 226-238.

Ryan, M. E., Ramamurthy, S. & Golub, L. M. (1996) Matrix metalloproteinases and their inhibition in periodontal treatment. *Current Opinion In Periodontology* **3**, 85-96.

Sakamoto, M., Umeda, M. & Benno, Y. (2005) Molecular analysis of human oral microbiota. *Journal of Periodontal Research* **40,** 277-285.

Samaranayke, L. P. (2002) Essential microbiology for dentistry, Edinburgh, Churchill Livingstone.

Chapter 8 – References

Sanz, M., Lau, L., Herrera, D., Morillo, J. M. & Silva, A. (2004) Methods of detection of *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *Journal of Clinical Periodontology* **31**, 1034-1047.

Saygun, I., Nizam, N., Keskiner, I., Bal, V., Kubar, A., Açıkel, C., Serdar, M. & Slots, J. (2011) Salivary infectious agents and periodontal disease status. *Journal of Periodontal Research* **46,** 235-239.

Schauer, R. (1982) Chemistry, metabolism, and biological functions of sialic acids. *Advances In Carbohydrate Chemistry And Biochemistry* **40**, 131-234.

Schauer, R. (1985) Sialic acids and their role as biological masks. *Trends In Biochemical Sciences* **10**, 357-360.

Schenkein, H. A. (2006) Host responses in maintaining periodontal health and determining periodontal disease. *Periodontology 2000* **40,** 77-93.

Schenkein, H. A. (1991) Complement factor dbar-like activity of *Porphyromonas gingivalis* W83. *Oral Microbiology and Immunology* **6**, 216-220.

Schroeder, H. E. & Listgarten, M. A. (1997) The gingival tissues: the architecture of peridontal protection. *Periodontology* 2000 **13**, 91-120.

Senior, R. M. & Campbell, E. J. (1983) Neutral proteinases from human inflammatory cells - a critical-review of their role in extracellular-matrix degradation. *Clinics In Laboratory Medicine* **3**, 645-666.

Seppa, H. E. J. & Jarvinen, M. (1979) Main neutral protease of rat skin is a mast cell enzyme - immunohistochemical localization of the enzyme in rat skin with the peroxidase-antiperoxidase (pap) complex method. *Acta Histochemica* **64**, 174-178.

Settem, R. P., El-Hassan, A. T., Honma, K., Stafford, G. P. & Sharma, A. (2012) *Fusobacterium nucleatum* and *Tannerella forsythia* induce synergistic alveolar bone loss in a mouse periodontitis model. *Infection and Immunity* **80,** 2436-2443.

Severi, E., Hood, D. W. & Thomas, G. H. (2007) Sialic acid utilization by bacterial pathogens. *Microbiology* **153**, 2817-2822.

Shinohara, M., Ohura, K., Ogata, K., Inoue, H., Miyata, T. & Yoshioka, M. (1994) The relationship between the sialic acid concentrations in the serum and whole saliva

in rats with naturally-occurring gingivitis. *Japanese Journal of Pharmacology* **64**, 61-63.

Signoretto, C., Burlacchini, G., Bianchi, F., Cavalleri, G. & Canepari, P. (2006) Differences in microbiological composition of saliva and dental plaque in subjects with different drinking habits. *The new microbiologica* **29,** 293-302.

Silva, F. G. D. A. & Gomes, S. C. (2009) Validation of an alternative absorbent paper for collecting gingival crevicular fluid. *Periodontia* **19,** 85-90.

Simonson, L. G., Robinson, P. J., Pranger, R. J., Cohen, M. E. & Morton, H. E. (1992) *Treponema denticola* and *Porphyromonas gingivalis* as prognostic markers following periodontal treatment. *Journal of Periodontology* **63,** 270-273.

Sissons, C., Wong, L. & Cutress, T. (1995) Patterns and rates of growth of microcosm dental plaque biofilms. *Oral Microbiology and Immunology* **10,** 160-167.

Si-Tahar, M., Renesto, P., Balloy, V. & Chignard, M. (1994) Synergism between interleukin-8 and tumor necrosis factor-alpha for neutrophil-mediated platelet activation. *European Cytokine Network* **5**, 455-460.

Skaleric, U., Zajsek, P., Cvetko, E., Lah, T. & Babnik, J. (1986) Alpha-2 - macroglobulin in gingival fluid - correlation with alveolar bone loss in periodontal-disease. *Journal of Clinical Periodontology* **13**, 833-836.

Slots, J. (1981) Enzymatic characterization of some oral and non-oral gram-negative bacteria with the API ZYM system. *Journal of Clinical Microbiology* **14**, 288-294.

Slots, J. & Ting, M. (1999) *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* in human periodontal disease: occurrence and treatment. *Periodontology 2000* **20,** 82-121.

Smith, A. J., Alexander, M., Mackenzie, D., Lennon, A., Riggio, M. P. & Macfarlane, T. W. (1998) Microbial factors and gingival crevicular fluid Aspartate aminotransferase levels - a cross-sectional study. *Journal of Clinical Periodontology* **25**, 334-339.

Smith, Q. T., Harriman, L., Au, G. S., Stoltenberg, J. L., Osborn, J. B., Aeppli, D. M. & Fischer, G. (1995) Neutrophil elastase in crevicular fluid - comparison of a middle aged general population with healthy and periodontitis groups. *Journal of Clinical Periodontology* **22**, 935-941.

Chapter 8 – References

Socransky, S. & Haffajee, A. (1991) Microbial mechanisms in the pathogenesis of destructive periodontal diseases: a critical assessment. *Journal of Periodontal Research* **26**, 195-212.

Socransky, S. S. & Haffajee, A. D. (1992) The bacterial etiology of destructive periodontal disease: current concepts. *Journal of Periodontology* **63**, 322-331.

Socransky, S. S. & Haffajee, A. D. (2002) Dental biofilms: difficult therapeutic targets. *Periodontology 2000* **28**, 12-55.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C. & Kent, R. L. (1998) Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology* **25**, 134-144.

Socransky, S., Haffajee, A., Smith, C. & Dibart, S. (1991) Relation of counts of microbial species to clinical status at the sampled site. *Journal of Clinical Periodontology* **18,** 766-775.

Socransky, S. S. & Haffajee, A. D. (1990) Microbiological risk factors for destructive periodontal diseases. *Risk assessment in dentistry,* In: Bader J. D., ed. *Risk Assessment* in *Dentistry*. Chapel Hill : University of North Carolina *Dental* Ecology 79-90.

Socransky, S. S. & Haffajee, A. D. (1993) Effect of therapy on periodontal infections. *Journal of Periodontology* **64,** 754-759.

Socransky, S. S. & Haffajee, A. D. (2005) Periodontal microbial ecology. *Periodontology 2000* **38,** 135-187.

Soell, M., Elkaim, R. & Tenenbaum, H. (2002) Cathepsin C, matrix metalloproteinases, and their tissue inhibitors in gingiva and gingival crevicular fluid from periodontitis affected patients. *Journal of Dental Research* **81**, 174-178.

Sorsa, T., Ding, Y. L., Salo, T., Lauhio, A., Teronen, O., Ingman, T., Ohtani, H., Andoh, N., Takeha, S. & Konttinen, Y. T. (1994) Effects of tetracyclines on neutrophil, gingival, and salivary collagenases - a functional and western-blot assessment with special reference to their cellular sources in periodontal-diseases. In: Greenwald, R. A. & Golub, L. M. (Eds.) Inhibition of matrix metalloproteinases: therapeutic potential. p. 112-131.

Sorsa, T., Gursoy, U. K., Nwhator, S., Hernandez, M., Tervahartiala, T., Leppilahti, J., Gursoy, M., Könönen, E., Emingil, G. & Pussinen, P. J. (2016) Analysis of matrix metalloproteinases, especially MMP8, in gingival creviclular fluid,

mouthrinse and saliva for monitoring periodontal diseases. *Periodontology 2000* **70,** 142-163.

Sorsa, T., Hernandez, M., Leppilahti, J., Munjal, S., Netuschil, L. & Mantyla, P. (2010) Detection of gingival crevicular fluid MMP8 levels with different laboratory and chair-side methods. *Oral Diseases* **16**, 39-45.

Sorsa, T., Ingman, T., Suomalainen, K., Haapasalo, M., Konttinen, Y. T., Lindy, O., Saari, H. & Uitto, V. J. (1992) Identification of proteases from periodontopathogenic bacteria as activators of latent human neutrophil and fibroblast-type interstitial collagenases. *Infection and Immunity* **60**, 4491-4495.

Sorsa, T., Mantyla, P., Ronka, H., Kallio, P., Kallis, G. B., Lundqvist, C., Kinane, D. F., Salo, T., Golub, L. M., Teronen, O. & Tikanoja, S. (1999) Scientific basis of a matrix metalloproteinase-8 specific chair-side test for monitoring periodontal and peri-implant health and disease. *Ann N Y Acad Sci* **878**, 130-140.

Sorsa, T., Salo, T., Koivunen, E., Tyynela, J., Konttinen, Y. T., Bergmann, U., Tuuttila, A., Niemi, E., Teronen, O., Heikkila, P., Tschesche, H., Leinonen, J., Osman, S. & Stenman, U. H. (1997) Activation of type iv procollagenases by human tumor-associated trypsin-2. *Journal of Biological Chemistry* **272**, 21067-21074.

Sorsa, T., Tervahartiala, T., Leppilahti, J., Hernandez, M., Gamonal, j., Tuomainen, A. M., Lauhio, A., Pussinen, P. J. & Mantyla, P. (2011) Collagenase-2 (MMP8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. therapeutic response to non-antimicrobial properties of tetracyclines. *Pharmacological Research* **63**, 108-113.

Sorsa, T., Tjaderhane, L., Konttinen, Y. T., Lauhio, A., Salo, T., Lee, H.-M., Golub, L. M., Brown, D. L. & Mantyla, P. (2006) Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Annals of Medicine* **38**, 306-321.

Sorsa, T., Tjaderhane, L. & Salo, T. (2004) Matrix metalloproteinases (MMPs) in oral diseases. *Oral Diseases* **10**, 311-318.

Sorsa, T., Uitto, V. J., Suomalainen, K., Turto, H. & Lindy, S. (1987) A trypsin-like protease from *Bacteroides gingivalis* - partial-purification and characterization. *Journal of Periodontal Research* **22**, 375-380.

Starkey, P. M. & Barrett, A. J. (1976) Neutral proteinases of human spleen - purification and criteria for homogeneity of elastase and cathepsin G. *Biochemical Journal* **155**, 255-263.

Starkey, P. M., Barrett, A. J. & Burleigh, M. C. (1977) The degradation of articular collagen by neutrophil proteinases Ec-3.4.21. *Biochimica Et Biophysica Acta* **483**, 366-397.

Stenman, M., Ainola, M., Valmu, L., Bjartell, A., Ma, G. F., Stenman, U. H., Sorsa, T., Luukkainen, R. & Konttinen, Y. T. (2005) Trypsin-2 degrades human type II collagen and is expressed and activated in mesenchymally transformed rheumatoid arthritis synovitis tissue. *American Journal of Pathology* **167**, 1119-1124.

Sugawara, S., Nemoto, E., Tada, H., Miyake, K., Imamura, T. & Takada, H. (2000) Proteolysis of human monocyte CD14 by cysteine proteinases from *Porphyromonas gingivalis* leading to LPs-hypo responsiveness. *Journal of Endotoxin Research* **6**, 163-163.

Suido, H., Nakamura, M., Mashimo, P. A., Zambon, J. J. & Genco, R. J. (1986) Arylaminopeptidase activities of oral bacteria. *Journal of Dental Research* **65**, 1335-1340.

Sundqvist, G., Bengtson, A. & Carlsson, J. (1988) Generation and degradation of the complement fragment C5a in human-serum by *Bacteroides gingivalis*. *Oral Microbiology and Immunology* **3**, 103-107.

Sundqvist, G., Carlsson, J. & Hanstrom, L. (1987) Collagenolytic activity of black-pigmented bacteroides species. *Journal Of Periodontal Research* **22**, 300-306.

Suomalainen, K. (1992) Relationship of collagenase and cathepsin G activity in gingival crevicular fluid. *Scandinavian Journal of Dental Research* **100**, 216-221.

Syed, S. A., Gusberti, F. A., Loesche, W. J. & Lang, N. P. (1984) Diagnostic potential of chromogenic substrates for rapid detection of bacterial enzymatic-activity in health and disease associated periodontal plaques. *Journal of Periodontal Research* **19**, 618-621.

Taba Jr, M., Kinney, J., Kim, A. S. & Giannobile, W. V. (2005) Diagnostic biomarkers for oral and periodontal diseases. *Dental Clinics Of North America* **49**, 551-571.

Takada, K. & Hirasawa, M. (2000) Expression of trypsin-like activity by the genera corynebacterium and actinomyces in canine periodontitis. *Journal of Medical Microbiology* **49**, 621-625.

Talonpoika, J. T. & Hämäläinen, M. M. (1994) Type I collagen carboxyterminal telopeptide in human gingival crevicular fluid in different clinical conditions and after periodontal treatment. *Journal of Clinical Periodontology* **21**, 320-326.

Tang, H. B., Dimango, E., Bryan, R., Gambello, M., Iglewski, B. H., Goldberg, J. B. & Prince, A. (1996) Contribution of specific pseudomonas aeruginosa virulence factors to pathogenesis of pneumonia in a neonatal mouse model of infection. *Infection and Immunity* **64**, 37-43.

Tanner, A., Hamada, S., Holt, S. & Mcghee, J. (1991) Microbial succession in the development of periodontal disease. *Periodontal Disease: Pathogens and Host Immune Responses. Tokyo: Quintessence* **22,** 13-25.

Tanner, A., Kent, R., Maiden, M. & Taubman, M. (1996) Clinical, microbiological and immunological profile of healthy, gingivitis and putative active periodontal subjects. *Journal of Periodontal Research* **31,** 195-204.

Tanner, A. C. R., Kent, R., Kanasi, E., Lu, S. C., Paster, B. J., Sonis, S. T., Murray, L. A. & Van Dyke, T. E. (2007) Clinical characteristics and microbiota of progressing slight chronic periodontitis in adults. *Journal of clinical periodontology* **34,** 917-930.

Tanner, A. C. (2014) Anaerobic culture to detect periodontal and caries pathogens. *Journal of Oral Biosciences* **57**, 18-26.

Tanner, A. C. & Izard, J. (2006) *Tannerella forsythia*, a periodontal pathogen entering the genomic era. *Periodontology 2000* **42,** 88-113.

Taylor, J. J. & Preshaw, P. M. (2016) Gingival crevicular fluid and saliva. *Periodontology 2000* **70,** 7-10.

Teles, R., Sakellari, D., Teles, F., Konstantinidis, A., Kent, R., Socransky, S. & Haffajee, A. (2010) Relationships among gingival crevicular fluid biomarkers, clinical parameters of periodontal disease, and the subgingival microbiota. *Journal of periodontology* **81,** 89-98.

Tervahartiala, T., Konttinen, Y. T., Ingman, T., Hayrinenimmonen, R., Ding, Y. & Sorsa, T. (1996) Cathepsin G in gingival tissue and crevicular fluid in adult periodontitis. *Journal of Clinical Periodontology* **23**, 68-75.

Tervahartiala, T., Pirila, E., Ceponis, A., Maisi, P., Salo, T., Tuter, G., Kallio, P., Tornwall, J., Srinivas, R., Konttinen, Y. T. & Sorsa, T. (2000) The in vivo expression of the collagenolytic matrix metalloproteinases (MMP-2,-8,-13, and-14) and matrilysin (MMP-7) in adult and localized juvenile periodontitis. *Journal of Dental Research* **79**, 1969-1977.

Theilade, E. (1986) The non-specific theory in microbial etiology of inflammatory periodontal diseases. *Journal of Clinical Periodontology* **13,** 905-911.

Chapter 8 – References

Thompson, H., Homer, K. A., Rao, S., Booth, V. & Hosie, A. H. F. (2009) An orthologue of *Bacteroides fragilis* Nanh is the principal sialidase in *Tannerella forsythia*. *Journal of Bacteriology* **191**, 3623-3628.

Tkalcevic, J., Novelli, M., Phylactides, M., Iredale, J. P., Segal, A. W. & Roes, J. (2000) Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity* **12**, 201-210.

Tonetti, M. S. (1997) Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. *Journal of Periodontal Research* **32**, 104-109.

Tonetti, M. S., Imboden, M. A. & Lang, N. P. (1998) Neutrophil migration into the gingival sulcus is associated with transepithelial gradients of interleukin-8 and icam-1. *Journal of Periodontology* **69**, 1139-1147.

Tong, H. H., Blue, L. E., James, M. A. & Demaria, T. F. (2000) Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infection and Immunity* **68**, 921-924.

Torrungruang, K., Bandhaya, P., Likittanasombat, K. & Grittayaphong, C. (2009) Relationship between the presence of certain bacterial pathogens and periodontal status of urban Thai adults. *Journal of periodontology* **80,** 122-129.

Travis, J. (1988) Structure, function, and control of neutrophil proteinases. *American Journal of Medicine* **84**, 37-42.

Travis, J., Pike, R., Imamura, T. & Potempa, J. (1997) *Porphyromonas gingivalis* proteinases as virulence factors in the development of periodontitis. *Journal of Periodontal Research* **32**, 120-125.

Travis, J. & Salvesen, G. S. (1983) Human-plasma proteinase-inhibitors. *Annual Review of Biochemistry* **52**, 655-709.

Tsutsui, H., Kinouchi, T., Wakano, Y. & Ohnishi, Y. (1987) Purification and characterization of a protease from *Bacteroides gingivalis*-381. *Infection and Immunity* **55**, 420-427.

Tuyau, J. E. & Sims, W. (1975) Occurrence of haemophili in dental plaque and their association with neuraminidase activity. *Journal of Dental Research* **54**, 737-739.

Chapter 8 – References

Uitto, V. J. (1987) Human gingival proteases .1. extraction and preliminary characterization of trypsin-like and elastase-like enzymes. *Journal of Periodontal Research* **22**, 58-63.

Uitto, V. J., Larjava, H., Heino, J. & Sorsa, T. (1989) A protease of *Bacteroides gingivalis* degrades cell-surface and matrix glycoproteins of cultured gingival fibroblasts and induces secretion of collagenase and plasminogen-activator. *Infection and Immunity* **57**, 213-218.

Uitto, V. J., Overall, C. M. & Mcculloch, C. (2003) Proteolytic host cell enzymes in gingival crevice fluid. *Periodontology 2000* **31**, 77-104.

Van Der Velden, U. (2005) Purpose and problems of periodontal disease classification. *Periodontology 2000* **39**, 13-21.

Van Lint, P. & Libert, C. (2006) Matrix metalloproteinase-8: cleavage can be decisive. *Cytokine & Growth Factor Reviews* **17**, 217-223.

Van Palenstein Helderman, W. (1976) Lysozyme concentrations in the gingival crevice and at other oral sites in human subjects with and without gingivitis. *Archives of Oral Biology* **21**, 251-255.

Van Winkelhoff, A., Loos, B., Van Der Reijden, W. & Van Der Velden, U. (2002) *Porphyromonas gingivalis*, *Bacteroides forsythus* and other putative periodontal pathogens in subjects with and without periodontal destruction. *Journal of Clinical Periodontology* **29,** 1023-1028.

Waddington, R., Embery, G. & Samuels, R. (1994) Characterization of proteoglycan metabolites in human gingival crevicular fluid during orthodontic tooth movement. *Archives of Oral Biology* **39**, 361-368.

Waddington, R., Langley, M., Guida, L., Luorio, G., Labella, R., Embery, G. & Caruso, F. (1996) Relationship of sulphated glycosaminoglycans in human gingival crevicular fluid with active periodontal disease. *Journal of Periodontal Research* **31**, 168-170.

Wade, W. G. (2013) The oral microbiome in health and disease. *Pharmacological Research* **69,** 137-143.

Walker, C. & Gordon, J. (1990) The effect of clindamycin on the microbiota associated with refractory periodontitis. *Journal of Periodontology* **61,** 692-698.

Wang, P. L., Shirasu, S., Shinohara, M., Daito, M., Fujii, T., Kowashi, Y. & Ohura, K. (1999) Purification and characterization of a trypsin-like protease from the

culture supernatant of *Actinobacillus actinomycetemcomitans* Y4. *European Journal of Oral Sciences* **107**, 147-153.

Wassall, R. R. & Preshaw, P. M. (2016) Clinical and technical considerations in the analysis of gingival crevicular fluid. *Periodontology 2000* **70,** 65-79.

Watanabe, H., Hattori, S., Katsuda, S., Nakanishi, I. & Nagai, Y. (1990) Human neutrophil elastase - degradation of basement-membrane components and immunolocalization in the tissue. *Journal of Biochemistry* **108**, 753-759.

Watorek, W., Farley, D., Salvesen, G. & Travis, J. (1988) Neutrophil elastase and cathepsin G: structure, function, and biological control. *Advances In Experimental Medicine and Biology* **240**, 23-31.

Weiss, S. J. (1989) Tissue destruction by neutrophils. *New England Journal of Medicine* **320**, 365-376.

Weiss, S. J. & Reddy, V. Y. (1989) Tissue destruction by neutrophils-reply. *New England Journal of Medicine* **321**, 329-329.

Wewers, M. D., Herzyk, D. J. & Gadek, J. E. (1988) Alveolar fluid neutrophil elastase activity in the adult respiratory-distress syndrome is complexed to alpha-2-macroglobulin. *Journal of Clinical Investigation* **82**, 1260-1267.

Wingrove, J. A., Discipio, R. G., Chen, Z. X., Potempa, J., Travis, J. & Hugli, T. E. (1992) Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas* (*Bacteroides*) *gingivalis*. *Journal of Biological Chemistry* **267**, 18902-18907.

Winkelhof, A., Velden, U. & Graaff, J. (1988) Microbial succession in recolonizing deep periodontal pockets after a single course of supra-and subgingival debridement. *Journal of Clinical Periodontology* **15,** 116-122.

Wolff, L. F., Koller, N. J., Smith, Q. T., Mathur, A. & Aeppli, D. (1997) Subgingival temperature: relation to gingival crevicular fluid enzymes, cytokines, and subgingival plaque micro-organisms. *Journal of Clinical Periodontology* **24,** 900-906.

Wong, D. T. & Segal, A. (2008) Salivary diagnostics: enhancing disease detection and making medicine better. *European Journal Of Dental Education* **12**, 22-29.

Wyss, C. (1989) Dependence of proliferation of *Bacteroides forsythus* on exogenous N-acetylmuramic acid. *Infection and Immunity* **57,** 1757-1759.

Chapter 8 – References

Xiang, X., Sowa, M. G., Iacopino, A. M., Maev, R. G., Hewko, M. D., Man, A. & Liu, K.-Z. (2010) An update on novel non-invasive approaches for periodontal diagnosis. *Journal of Periodontology* **81**, 186-198.

Ximénez-Fyvie, L. A., Haffajee, A. D. & Socransky, S. S. (2000) Comparison of the microbiota of supra-and subgingival plaque in health and periodontitis. *Journal of Clinical Periodontology* **27,** 648-657.

Yakob, M., Kari, K., Tervahartiala, T., Sorsa, T., Soder, P. O., Meurman, J. H. & Soder, B. (2012) Associations of periodontal microorganisms with salivary proteins and MMP8 in gingival crevicular fluid. *Journal of Clinical Periodontology* **39**, 256-263.

Yang, H.-W., Huang, Y. F. & Chou, M. Y. (2004) Occurrence of *Porphyromonas gingivalis* and *Tannerella forsythensis* in periodontally diseased and healthy subjects. *Journal of Periodontology* **75,** 1077-1083.

Yang, N. Y., Zhang, Q., Li, J. L., Yang, S. H. & Shi, Q. (2014) Progression of periodontal inflammation in adolescents is associated with increased number of *Porphyromonas gingivalis*, *Prevotella intermedi*a, *Tannerella forsythensis* and *Fusobacterium nucleatum*. *International Journal of Paediatric Dentistry* **24,** 226-233.

Yoshida, N. (1993) Purification of human neutrophil collagenase, establishment of its monoclonal antibodies and application to gingival crevicular neutrophils. Kokubyo Gakkai Zasshi. *The Journal Of The Stomatological Society*, Japan **60**, 121-30.

Yu, Q. & Stamenkovic, I. (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes & Development* **14**, 163-176.

Yucekal-Tuncer, B., Uygur, C. & Firatli, E. (2003) Gingival crevicular fluid levels of aspartate amino transferase, sulfide ions and N-Benzoyl-dl-Arginine-2-Naphthylamide in diabetic patients with chronic periodontitis. *Journal of Clinical Periodontology* **30**, 1053-1060.

Yun, P. L. W., Decarlo, A. A. & Hunter, N. (1999) Modulation of major histocompatibility complex protein expression by human gamma interferon mediated by cysteine proteinase-adhesin polyproteins of *Porphyromonas gingivalis*. *Infection and Immunity* **67**, 2986-2995.

Chapter 8 – References

Zafiropoulos, G. G. K., Floresdejacoby, L., Todt, G., Kolb, G., Havemann, K. & Tatakis, D. N. (1991) Gingival crevicular fluid elastase-inhibitor complex - correlation with clinical indexes and subgingival flora. *Journal of Periodontal Research* **26**, 24-32.

Zambon, J. J. (1996) Periodontal diseases: microbial factors. *Annals of periodontology* **1,** 879-925.

Zambon, J. J., Nakamura, M. & Slots, J. (1985) Effect of periodontal therapy on salivary enzymatic-activity. *Journal Of Periodontal Research* **20**, 652-659.

Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395.

Zhou, X., Liu, X., Li, J., Aprecio, R. M., Zhang, W. & Li, Y. (2015) Real-time PCR quantification of six periodontal pathogens in saliva samples from healthy young adults. *Clinical oral investigations* **19**, 937-946..

Zia, A., Khan, S., Bey, A., Gupta, N. & Mukhtar-Un-Nisar, S. (2011) Oral biomarkers in the diagnosis and progression of periodontal diseases. *Biology and Medicine* **3**, 45-52.

Appendices

Appendix 1

Patient Information Sheet
Version 3. 31st May 2013

A Pilot Study Biomarkers in Patients with Periodontal Disease

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

Periodontal (gum) diseases are caused by bacteria found around the teeth. A number of enzymes called proteases have been associated with tissue damage in these conditions. We wish to test the bacteria present, the presence of specific bacterial DNA and the presence of these enzymes in the fluid that forms between the gum and tooth surface as a marker of periodontal disease. The results will be compared with the clinical measurements normally recorded in the clinic for a correlation between the biomarkers and your gum condition. To do this, we would like to investigate the contents in the fluid found in diseased pockets around the teeth. In addition, we would also like to investigate the components of the fluid found and the bacteria present between healthy gums and teeth, to act as controls for comparison with areas having gum disease. A sample of saliva will be taken to compare with the other fluid samples and for this you will be asked you to spit into a small plastic tube.

Why have I been chosen?

You have been chosen because you have a gum condition. If you agree to decide to take part, you will be one of several similar patients participating in this study.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen if I agree to take part in the study?

Clinical measures of gum health (crevice depth) will be entered on a special form. Samples of fluid will be collected from a small number of crevices using a small paper stip and plaque samples will be taken using a small piece of blotting paper or a curette (small scraper). This will be undertaken at the start of the study, and at two other time points, 3 month and at 6 months after treatment. In addition a sample of saliva will be taken at the start, at your 3 months and 6 months review interval following treatment.

What do I have to do?

The measurements and samples will be collected at a routine dental appointment. The additional time involved will be approximately 15 minutes or less. The teeth being sampled will be isolated with cotton wool rolls and a saliva ejector, and samples of the fluid between your gum and tooth (3 teeth) will be taken using a small paper strip and should not cause any additional discomfort. Measurements of the depth between your gum and tooth, whether or not the area bleeds, tooth mobility, plaque score and a measurement of the loss of tooth support will also be recorded at this time.

If you wish to withdraw from the study at a later date, the samples taken will be destroyed. Any unused samples will also be destroyed at the end of the study.

What are the possible side effects if I take part?
There are no known side effects of taking part.

What are the possible disadvantages and risks of taking part?
The procedures of sampling the fluid and plaque may take up to an additional 15 minutes but they are not associated with any further discomfort.

What are the possible benefits of taking part?
There are no direct benefits to you of taking part. It is hoped that the information we get from this study may help other people with gum disease in the future.

What if something goes wrong?
If you are harmed by your participation in this study there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action. In addition, this study is registered with the University of Sheffield for research trials insurance.

Will my taking part in the study be kept confidential?
All information which is collected about you during the course of the research will be kept strictly confidential. Information collected will be entered onto a special form and kept in a file in a locked office. The information collected will be made anonymous before being analysed on a personal computer.

What will happen to the results of the research study?
The results will be written up and submitted for publication in scientific journals. No names or personal details will be mentioned in any reports of the study and care will be taken so that individuals cannot be identified from reports of the study.

Who is organising & hosting the research?
The study is organised by Professor A Rawlinson, Academic Unit of Restorative Dentistry, and is hosted by the University of Sheffield. The study is funded as part of a postgraduate students PhD enhanced fees.

What if I wish to complain about the way in which this study has been conducted?
If you have *any* cause to complain about *any* aspect of the way in which you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms are available to you and you are not compromised in any way because you have taken part in a research study.

If you have any complaints or concerns please contact the project co-ordinator: Professor A Rawlinson: Telephone 0114 271 7911

Otherwise you can use the normal hospital complaints procedure and contact the following person: Dr David Throssell Medical Director, Sheffield Teaching Hospitals Trust, 8 Beech Hill Road, Sheffield, S10 2SB. Telephone 0114 271 2178.
In addition, you may also contact The Patient Services Team in the following ways:

* Telephone on 0114 271 2400,
* Via email on PST@sth.nhs.uk
* In person in the Patient Partnership Department on B Floor, RHH and the Huntsman main entrance on C Floor, NGH.

Appendix 2

Centre Number: N/A

Study Number: STH 17158

Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **A Pilot Study of Biomarkers in Patients with Periodontal Disease**

Name of Researcher: **Professor A Rawlinson**

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated **31st May 2013** (version **3**) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature

Consent form date of issue: 19th **September 2014**
Consent form version number: **3**

Appendix 3

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - South Yorkshire
North East REC Centre
Unit 002, TEDCO Business Centre
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Telephone: 0191 428 3387
Facsimile: 0191 428 3432

14 May 2013

Prof. Andrew Rawlinson
Head of Academic Unit of Restorative Dentistry
University of Sheffield
School of Clinical Dentistry
Claremont Crescent
Sheffield
S10 2TA

Dear Prof. Rawlinson

Study title:	**A Pilot Study of Biomarkers in Patients with Periodontal Disease**
REC reference:	**13/YH/0114**
Protocol number:	**STH17158**
IRAS project ID:	**126263**

Thank you for your email of 07 May 2013. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 03 May 2013

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Other: Response to Conditions of the Favourable Opinion	Andrew Rawlinson	07 May 2013
Participant Consent Form	2	07 May 2013
Participant Information Sheet	2	07 May 2013

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	*Version*	*Date*
Covering Letter	Professor A Rawlinson	03 April 2013
Investigator CV	Professor A Rawlinson	06 February 2013
Investigator CV	C W Ian Douglas	
Investigator CV	Graham Stafford	06 February 2013
Investigator CV	Sarhang Sarwat Hama Gul	06 February 2013
Investigator CV	Nivan Al-Hammouri	06 February 2013

Other: Response to Conditions of the Favourable Opinion	Andrew Rawlinson	07 May 2013
Participant Consent Form	2	07 May 2013
Participant Information Sheet	2	07 May 2013
Protocol	1	06 February 2013
REC application	IRAS Version 3.5, 126263/432579/1/252	25 April 2013
Referees or other scientific critique report		22 February 2013

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

13/YH/0114 **Please quote this number on all correspondence**

Yours sincerely

[redacted]

Miss Sarah Grimshaw
Committee Co-ordinator

E-mail: nrescommittee.yorkandhumber-southyorks@nhs.net

Copy to: *Dr Nana Theodorou, Sheffield Teaching Hospitals NHS Foundation Trust*

NHS
Health Research Authority

NRES Committee Yorkshire & The Humber - South Yorkshire
North East REC Centre
Unit 002, TEDCO Business Centre
Rolling Mill Road
Jarrow
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NE32 3DT

Telephone: 0191 428 3387
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03 May 2013

Prof. Andrew Rawlinson
Head of Academic Unit of Restorative Dentistry
University of Sheffield
School of Clinical Dentistry
Claremont Crescent
Sheffield
S10 2TA

Dear Prof. Rawlinson

Study title:	**A Pilot Study of Biomarkers in Patients with Periodontal Disease**
REC reference:	**13/YH/0114**
Protocol number:	**STH17158**
IRAS project ID:	**126263**

The Research Ethics Committee reviewed the above application at the meeting held on 25 April 2013.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Miss Sarah Grimshaw, nrescommittee.yorkandhumber-southyorks@nhs.net.

Ethical opinion

The Coordinator, Miss Sarah Grimshaw, welcomed Professor Ian Douglas (Academic Supervisor) to the meeting via teleconference and thanked him for attending.

The Committee noted that the researchers would be taking measurements, gingival crevicular fluids (GCF) and plaque samples but requested clarification as to whether this was extra to routine clinical care.

Professor Douglas clarified that this would be routine care and that patients would normally be treated on a three-monthly basis.

Members queried whether the researchers required 90 participants for the statistical analyses.

Professor Douglas confirmed that that was the case.

The Committee therefore questioned whether a sample size of 120-150 participants would be

more realistic in case of withdrawals.

Professor Douglas agreed as the target number of patients was 90 but they were unsure how recruitment would go.

The REC commented that if the researchers requested to recruit more than 90 participants there was room for a drop-out rate.

Members requested clarification as to how and by whom participants would be recruited.

Professor Douglas responded that participants would go to hospital for their referral and would be assessed by you who would inform them of the study. If they were interested you would give them a copy of the Participant Information Sheet and Consent Form which they could take away to consider participation; Professor Douglas commented that you would be able to tell if participants were likely to say yes or no at this stage. If interested the participants would be booked to the research hygienist clinic where they would receive normal care.

The Committee commented that participants usually receive a minimum of 24 hours to consent to participation and queried whether participants would receive a stamped addressed envelope to return the signed consent form in.

Professor Douglas replied that this could be provided.

Members requested clarification that if patients did not wish to participate they would receive the same treatment.

Professor Douglas confirmed that this was the case.

The REC noted that an internal pilot would be conducted after three months and queried whether this was an interim analysis.

Professor Douglas confirmed that this was the case; it would be used to check if they were detecting all the enzymes and DNA they were testing for. If they were not then these tests would be removed from the protocol.

The Committee noted that the information would be stored on a laptop and questioned what security arrangements it had in place.

Professor Douglas confirmed that it would be stored in a locked office and was encrypted and password protected.

Members queried why the researchers would not be informing the participants' dentists of their involvement in the research.

Professor Douglas replied that this was because there was no impact on the treatment or the outcome of the treatment. There was also no benefit to the patient for their dentist to be contacted.

The teleconference ended.

The Committee discussed the responses.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

1. Removal of the point in the Consent Form about contacting participants' dentists.

2. Amendment of the three places in the Participant Information Sheet where the text is justified, centre-aligned and bold to be unjustified, left-aligned and unbold.

3. Confirmation that a stamped addressed envelope will be provided to participants for return of the signed Consent Form.

4. Members advise that the number of participants to recruit is increased to 120 in order to adjust in case of withdrawals. If enough participants are recruited for statistically significant results before this number is reached there would be no need to recruit more participants (This is a suggestion not a stipulation).

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter	Professor A Rawlinson	03 April 2013
Investigator CV	Professor A Rawlinson	06 February 2013

Investigator CV	C W Ian Douglas	
Investigator CV	Graham Stafford	06 February 2013
Investigator CV	Sarhang Sarwat Hama Gul	06 February 2013
Investigator CV	Nivan Al-Hammouri	06 February 2013
Participant Consent Form	1	06 February 2013
Participant Information Sheet	1	06 February 2013
Protocol	1	06 February 2013
REC application	IRAS Version 3.5, 126263/432579/1/252	25 April 2013
Referees or other scientific critique report		22 February 2013

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

13/YH/0114	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely

[redacted]

pp
Ms Jo Abbott
Chair

Email: nrescommittee.yorkandhumber-southyorks@nhs.net

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers" SL-AR2

Copy to: *Dr Nana Theodorou, Sheffield Teaching Hospitals NHS Foundation Trust*

NHS
Health Research Authority
NRES Committee Yorkshire & The Humber - South Yorkshire
Unit 002, JARROW Business Centre
Rolling Mill Road
Jarrow
Tyne and Wear
NE32 3DT

Tel: 0191 428 3561

24 September 2014

Prof. Andrew Rawlinson
Head of Academic Unit of Restorative Dentistry
School of Clinical Dentistry/ University of Sheffield
Claremont Crescent
Sheffield
S10 2TA

Dear Prof. Rawlinson

Study title:	**A Pilot Study of Biomarkers in Patients with Periodontal Disease**
REC reference:	**13/YH/0114**
Protocol number:	**STH17158**
Amendment number:	**Minor Amendment 4 - change to consent form**
Amendment date:	**23 September 2014**
IRAS project ID:	**126263**

Thank you for your letter of 23 September 2014, notifying the Committee of the above amendment.

The Committee does not consider this to be a "substantial amendment" as defined in the Standard Operating Procedures for Research Ethics Committees. The amendment does not therefore require an ethical opinion from the Committee and may be implemented immediately, provided that it does not affect the approval for the research given by the R&D office for the relevant NHS care organisation.

Documents received

The documents received were as follows:

Document	*Version*	*Date*
Covering letter on headed paper	Email from Andrew Rawlinson	19 September 2014
Notice of Minor Amendment	Minor Amendment 4 - change to consent form	23 September 2014
Participant consent form		19 September 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

13/YH/0114:	**Please quote this number on all correspondence**

Yours sincerely

[redacted]

Miss Kerry Dunbar
REC Assistant

E-mail: nrescommittee.yorkandhumber-southyorks@nhs.net

Copy to: *Dr Nana Theodorou, Sheffield Teaching Hospitals NHS FT*

Ref: STH17158

Sheffield Teaching Hospitals NHS
NHS Foundation Trust

25th September 2014

Andrew Rawlinson
Head of Academic Unit of Restorative Dentistry
School of Clinical Dentistry/ University of Sheffield
Claremont Crescent
Sheffield
S10 2TA

Dear Andrew,

Minor Amendment

Letter of Continued NHS Permission

STH ref:	STH17158
NIHR CSP ref:	N/A
REC ref:	13/YH/0114
MHRA ref:	N/A **EudraCT no.:** N/A
Study title:	Enzyme Biomarkers in Periodontal Disease
Chief Investigator:	A Rawlinson
Principal Investigator:	A Rawlinson
Sponsor:	Sheffield Teaching Hospitals NHS Foundation Trust
Funder:	PhD/ Directorate
Amendment Ref:	Minor Amendment 4 - change to consent form

Thank you for submitting the following documents:

Document	*Version/date*
Consent form	Version 3, 19 Sep 14
Amendment 4 approval letter from REC	24 Aug 14

These have been reviewed by the Research Department who have no objection to the amendment and can confirm continued NHS permission for the study at STH.

Yours sincerely

[redacted]

Professor S Heller
Director of R&D, Sheffield Teaching Hospitals NHS Foundation Trust
Telephone +44 (0) 114 22 65934
Fax +44 (0) 114 22 65937

Appendix 4

The University Of Sheffield.

Finance and Commercial

To	Prof. Andrew Rawlinson
Your ref	STH17158
Date Issued	7.3.13

Certificate of Insurances (non clinical trial)

Trial Number	NCT 12/28
Department	Clinical Dentistry
Principal Investigator	Prof. Andrew Rawlinson

Title of Trial

A Pilot Study of Biomarkers in Patients with Periodontal Disease

Name of Investigators	As stated
Commencement Date	01/04/2013

The University has in place insurance against liabilities for which it may be legally liable and this cover includes any such liabilities arising out of the above research project/study

[redacted]

Colin Rose MA ACII
Risk2Value Ltd, Insurance Adviser to the University of Sheffield

M:\fi_share\Insurance\Insurances\CTRIALS\NCT TRIALS\nct certificate.docx

Appendix 5

Data Capture Form

Patient's Study number:

Age: **Gender:**

Smoking Status: **Yes** **No**
If yes
Number of cigarettes per day: **Duration:**

First Visit: **Date:**

Saliva sample No:
Mean PI%: **Mean BI%:**
Mean PPD%: 1- (≤3mm) **2- (3-4mm)** **3- (≥6mm)**

Clinical Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
Plaque index			
Bleeding index			
Pocket depth			
Clinical attachment loss			
Bone loss			

Laboratory Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
GCF sample No.			
Plaque sample No.			
GCF volume (µl)			
MMP8 (ng/µl) ,ng/sample			
Elastase (ng/µl) ,ng/sample			
Cathepsin G (ng/µl) ,ng/sample			
Trypsin like (ng/µl) ,ng/sample			
Sialidase (ng/µl) ,ng/sample			

Antibiotics prescription:

Second Visit: Date:

Saliva sample No:
Mean PI%: Mean BI%:
Mean PPD%: 1- (≤3mm) 2- (3-4mm) 3- (≥6mm)

Clinical Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
Plaque index			
Bleeding index			
Pocket depth			
Clinical attachment loss			
Bone loss			

Laboratory Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
GCF sample No.			
Plaque sample No.			
GCF volume (µl)			
MMP8 (ng/µl) ,ng/sample			
Elastase (ng/µl) ,ng/sample			
Cathepsin G (ng/µl) ,ng/sample			
Trypsin like (ng/µl) ,ng/sample			
Sialidase (ng/µl) ,ng/sample			

Type of Treatment:

Antibiotics prescription:

Third Visit: **Date:**

Saliva sample No:

Mean PI%: **Mean BI%:**

Mean PPD%: 1- (≤3mm) **2- (3-4mm)** **3- (≥6mm)**

Clinical Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
Plaque index			
Bleeding index			
Pocket depth			
Clinical attachment loss			
Bone loss			

Laboratory Measures	Site 1 (Healthy site)	Site 2 (Deep non-bleeding site)	Site 3 (Deep bleeding site)
GCF sample No.			
Plaque sample No.			
GCF volume (µl)			
MMP8 (ng/µl)/, ng/sample			
Elastase (ng/µl) , ng/sample			
Cathepsin G (ng/µl) , ng/sample			
Trypsin like (ng/µl) , ng/sample			
Sialidase (ng/µl) , ng/sample			

Type of Treatment:

Antibiotics prescription:

Appendix 6

NHS

National Research Ethics Service

Sheffield Research Ethics Committee

Yorkshire and Humber REC Office
First Floor, Millside
Mill Pond Lane
Meanwood
Leeds
LS6 4RA

Telephone: 0113 3050160

28 October 2010

Prof Andrew Rawlinson
Head of Academic Unit of Restorative Dentistry
University of Sheffield
School of Clinical Dentistry
Claremont Cres
Sheffield
S10 2TA

Dear Prof Rawlinson

Study Title:	**Prototype Sensor for Periodontal Disease Monitoring**
REC reference number:	**10/H1308/45**
Protocol number:	**STH15611**

Thank you for your letter of 12 October 2010, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair.

The Vice-Chair noted your query regarding whether results should be passed to the patient's dental practitioner. It was noted that the Patient Information Sheet does not reference this. The Vice-Chair agreed with your rationale, that there would be no useful purpose in doing this.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to

the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where the only involvement of the NHS organisation is as a Participant Identification Centre (PIC), management permission for research is not required but the R&D office should be notified of the study and agree to the organisation's involvement. Guidance on procedures for PICs is available in IRAS. Further advice should be sought from the R&D office where necessary.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Investigator CV		
Protocol	2	08 June 2010
REC application		12 May 2010
Covering Letter		23 April 2010
Advertisement	1	24 April 2010
Letter of invitation to participant	1	24 April 2010
GP/Consultant Information Sheets	1	24 April 2010
Participant Information Sheet	2	08 June 2010
Response to Request for Further Information		12 October 2010
Participant Consent Form	2	08 June 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments

- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

10/H1308/45	**Please quote this number on all correspondence**

Yours sincerely

[redacted]

Neil Sykes
Vice-Chair

Email: john.robinson@leedspft.nhs.uk

Enclosures: "After ethical review – guidance for researchers" *SL- AR2*

Copy to: *Ramila Patel, 1st Floor, 11 Broomfield Road, Sheffield, S10 2SE*