Gingival crevicular matrix metalloproteinases and their inhibitor in health, disease and treated periodontitis

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To God be all the glory

If only there were occasion for repose

If only this long road had an end

And in the track of a hundred thousand years, out of the heart of dust

Hope sprang again, like greenness.

*Khayyam**

* Persian poet and astronomer (1048-1131)

The Ruba'yyat of Khayyam. Translated by P. Avery & J. Heath-Stubbs, Penguin Books, London, England, 1979.

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ABBREVIATIONS

α1-AT:	α1-antitrypsin
α2-M:	α2-macroglobulin
AL:	attachment level
ANUG:	acute necrotizing ulcerative gingivitis
AP:	adult periodontitis
BOP:	bleeding on probing
BRS:	best responding site
BSA:	bovine serum albumin
CB:	coating buffer
CEJ:	cementoenamel junction
CV:	coefficient of variation
ELAM-1:	endothelial leucocyte adhesion molecule 1
ELISA:	enzyme linked immunosorbent assay
FIB-CL:	fibroblast collagenase
GBI:	gingival bleeding index
GCF:	gingival crevicular fluid
GI:	gingival index
HRP:	horseradish peroxidase
IB:	incubation buffer
ICAM-1:	intercellular adhesion molecule 1
IL-1α:	interleukin-1α
IL-1ß:	interleukin-1ß
INF-γ:	interferon-γ
JP:	juvenile periodontitis
LF:	lactoferrin
LPS:	lipopolysacharide

MANOVA: multivariate analysis of variance

MGI: modified gingival index

MMP: matrix metalloproteinases

OD: optical density

OHI: oral hygiene index

OHI-S: simplified oral hygiene index

PBI: papillary bleeding index

PBS: phosphate buffered saline

PBST: phosphate buffered saline plus 0.05% Tween 20

PD: probing/pocket depth

PDI: periodontal disease index

PI: periodontal index

PlI: plaque index

PMN: polymorphonuclear leucocyte

PMN-CL: polymorphonuclear leucocyte collagenase

PP: prepubertal periodontitis

RCE: relative coefficient of excretion

RP: refractory periodontitis

RPP: rapidly progressive periodontitis

SBI: sulcular bleeding index

SL: stromelysin

Su: suppuration

SD: standard deviation

SEM: standard error of the mean

TGF-ß: transforming growth factor ß

TIMP: tissue inhibitor of metalloproteinases

TNF-α: tumour necrosis factor α

WRS: worst responding site

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DECLARATION

This thesis is the original work of the author.

SUMMARY

Proteinases have long been implicated in the pathogenesis of periodontal disease. Matrix metalloproteinases (MMPs) are a family of enzymes thought to be involved in periodontal tissue breakdown as well as tissue remodelling. MMPs are capable of degrading a variety of extracellular matrix components. Among these enzymes polymorphonuclear leucocyte derived collagenase (PMN-CL) and fibroblast derived collagenases (FIB-CL) have the unique ability to degrade type I, II and III collagens across the triple helix of the collagen fibre. Tissue inhibitor of metalloproteinases (TIMP), which are locally produced by host cells, inhibit these proteinases. TIMP appears to preferentially inhibit FIB-CL and stromelysin (SL). Most of the cells which produced FIB-CL and SL, also produce TIMP. No studies have so far been conducted on fibroblast derived MMPs and TIMP in gingival crevicular fluid (GCF). These experiments were carried out to investigate the GCF levels of FIB-CL, SL and TIMP in relation to periodontal disease status.

GCF samples were collected by means of sterile paper strips inserted into the crevice until mild resistance was felt and left for 30 seconds. GCF volume was assessed using the Periotron 6000. The enzymes and inhibitor levels were assayed using modifications of sandwich ELISAs described by

Cooksley et al. (1990). Antibodies detected both active and latent MMPs and the free form of TIMP. The results were expressed as absolute amounts i.e. pg/30 seconds of sample collection. Using these methods, it was possible to assess all three GCF proteins in the same sample (paper strip).

The clinical studies conducted in this thesis are: a) a cross-sectional study of matrix metalloproteinases and their inhibitor and b) a longitudinal study of matrix metalloproteinases and their inhibitor before and after treatment.

The cross-sectional study was carried out to test the ability of GCF levels of FIB-CL, SL and TIMP to distinguish healthy, gingivitis and periodontitis sites. GCF samples were collected from forty patients, each provided three samples from healthy, gingivitis and periodontitis sites. The mean GCF levels (pg/30s) of SL and TIMP were significantly higher in diseased (gingivitis and periodontitis) when compared to healthy sites. Both the enzyme and inhibitor failed to differentiate gingivitis from periodontitis. SL and TIMP demonstrated moderate correlation with clinical parameters when pooled data from three categories of sites were used. FIB-CL reached the detectable level of the assay in only 20.8% of the sites and did not demonstrate association with disease status.

The aim of the longitudinal study was to investigate the effects of periodontal therapy on the GCF levels of FIB-CL, SL and TIMP as well as the ability of the baseline levels of these proteins to predict the outcome of treatment. Twenty one patients with advanced periodontal disease, each providing 8 GCF samples, comprised the study population. GCF sampling and clinical recordings were performed at three time points namely, baseline (before treatment), reassessment (after hygiene phase therapy (HPT)) and at the follow-up (after additional therapy) examinations. Attachment level (AL) and pocket depth (PD) were measured using the "Florida probe". All patients received oral hygiene instruction, scaling and root planing. Further treatment needs were determined based on the pocket depths and the state of bleeding on probing (BOP) six weeks after HPT, at the reassessment visit. Follow-up examination was performed at least six weeks after additional therapy.

Clinical parameters were reduced at both post therapy visits, while the mean level of SL decreased after HPT, TIMP level increased at this visit. Both proteins showed significant reduction at the follow-up visit. When sites were grouped according to their response to treatment, using different criteria i.e. PD, BOP and AL, this pattern of change was observed in all groups of sites which did or did not respond to treatment. The percentage of sites with detectable amounts of FIB-CL was 26.8%, 17.4% and 12% at three successive visits respectively. Baseline GCF levels

of MMPs and TIMP failed to predict the outcome of treatment. The association between clinical and biochemical parameters were investigated in several situations. Of these, SL and TIMP demonstrated significant and positive correlation with GCF volume only in the group of sites which gained attachment after therapy. As FIB-CL was detected in very small number of sites, no attempt was made to correlate its level with the clinical parameters.

The failure of SL and TIMP to distinguish gingivitis from periodontitis sites could be due to the fact that probing depths served as a differentiating factor between these groups of sites, keeping in mind that probing depth measurements taken at one point in time do not necessarily reflect active periodontal destruction. Moreover, the fact that our antibodies detected total enzyme together with the observation that periodontitis sites had more active enzyme whereas gingivitis sites harboured more latent enzyme (Kryshtalskyj et al. 1986) could also explain the inability of SL in discriminating gingivitis from periodontitis sites.

The failure of enzymes, at baseline, to predict the outcome of treatment could have resulted from different destructive potential (different proportion of active and latent enzyme) in different sites with comparable pocket depths. The increase of TIMP after HPT may reflect its involvement in the healing process whereas its decrease at the follow-

up visit may be due to the further improvement in clinical conditions. However, in this study, the GCF levels of SL and TIMP in sites which did or did not respond to treatment demonstrated the same pattern of change at both post therapy visits. This could be explained by the followings: a) overall improvement in the clinical conditions of the entire dentition; b) the possibility that the level of active enzyme has been lower in sites which responded to treatment; and that the overall improved clinical condition, in an individual, may exert an indirect influence on the levels of MMPs and TIMP in sites which did not respond to treatment. Association between GCF constituents and clinical parameters has been established in some previous studies while this has not been the case in others. This discrepancy may be due to the different methods of GCF collection and reporting data as well as various laboratory techniques. Moreover, as different results were obtained when association between clinical and biochemical parameters were investigated in different groups of sites in theses studies, it seems that identical clinical conditions are also necessary for reproducibility of relationships between clinical and biochemical parameter.

CHAPTER 1

INTRODUCTION

1.1 The anatomy of the periodontium

The periodontium consists of supporting tissues of the tooth, including gingiva, periodontal ligament, root cementum and alveolar bone. The main functions of the periodontium, also called the attachment apparatus, are to attach the teeth to the jaw bones and to resolve the forces generated by the mastication (Lindhe & Karring, 1989).

1.1.1 The oral mucosa

The oral mucosa can be divided into three zones; a) the masticatory mucosa i.e. the gingiva and the covering of the hard palate, b) the specialised mucosa i.e. the mucosa covering the dorsum of the tongue, and c) the oral mucous membrane, lining the remaining of the oral cavity.

1.1.2 The gingiva

The gingiva is the part of the oral mucosa that covers the alveolar processes of the jaws and the cervical portions of the teeth. The gingiva is anatomically divided into marginal, attached, and interdental areas. The marginal gingiva is demarcated from the adjacent attached gingiva by a shallow linear depression, the free gingival groove, in about 50% of cases (Ainamo & Löe,1966). The free gingival groove is positioned at a level corresponding to the level

of cemento-enamel junction (CEJ). In fully erupted teeth the free gingival margin is located on the enamel approximately 0.5 to 2mm coronal to the CEJ.

In human teeth the gingival margin, also known as marginal gingiva and free gingiva) seldom forms a knife-edged termination against the tooth, but it is rounded, this is the orifice of the gingival sulcus. The gingival sulcus is a shallow crevice around the tooth bounded by the tooth surface on one side and the epithelium lining of the marginal gingiva on the other side. In the presence of absolute normal conditions, the depth of gingival sulcus is 0 or about 0 (Gottlieb & Orban, 1933). In fully developed teeth the coronal aspect of gingival sulcus is lined with sulcular epithelium, the nonkeratinised extension of the oral epithelium, and the bottom of the sulcus is formed by the coronal surface of the junctional epithelium. The junctional epithelium unites the gingival connective tissue with the enamel surface of the tooth, its length ranges from 0.25 to 1.35mm. The attached gingiva continues with the marginal gingiva and extends in apical direction to the mucogingival junction, where it becomes continuous with the, relatively loose and movable, alveolar mucosa. It has a firm texture and pink colour which is tightly bound to the underlying alveolar bone and therefore, is comparatively immobile in relation to the underlying tissue. The width of the attached gingiva differs in the different areas of the mouth (Bowers, 1963). It is greater

in the incisors region and less in the posterior segments; attached gingiva in the first premolar area shows the least width (Ainamo & Löe, 1966). The interdental gingiva occupies the interproximal space beneath the areas of tooth contact. The interdental gingiva can be 'pyramidal' or a 'col' shape. In the pyramidal form there is one papilla with its tip immediately beneath the contact point, whereas the col shape presents a valley-like depression which connects a facial and lingual papilla and conforms to the shape of interproximal contact (Cohen, 1959).

1.1.2.1 Gingival epithelium

The gingiva is covered by three areas of epithelium. the oral epithelium which faces the oral cavity, the sulcular epithelium which faces the tooth without any contact with its surface, and the junctional epithelium which is in contact with tooth surface. The principal cell type of oral and gingival epithelium is the keratinocyte. Other cell types found in the epithelium are non-keratiocytes (clear cells), including the Langerhans cells which are believed to play a role in the defence mechanism of oral mucosa (DiFranco et al., 1985); Merkel cells which are terminals of nerve fibres (Ness, Morton & Dale, 1987) and melanocytes which synthesize melanin (Schroeder, 1969). The keratinocytes constitute about more than 90% of gingival epithelium. The process of keratinisation involves a sequence of biochemical and morphologic events that occur

in the cell as it migrates from the basal layer towards the surface. Gingival epithelium can express three types of surface differentiation; a) keratinisation in which the cells on the surface form scales of keratin and lose their nuclei; b) parakeratinisation in which the cells of the superficial layers show some signs of keratinisation and retain their nuclei, although pyknotic; the granular layer is absent; c) non-keratinisation in which the cells of the surface layers have their nuclei and no signs of keratinisation are present.

1.1.2.2 The oral epithelium

The oral epithelium covers the crest and outer surface of the marginal gingiva as well as the surface of the attached gingiva. The stratified squamous epithelium covering these areas is keratinized or parakeratinised or a combination of both and is joined to the underlying connective tissue by a basal lamina (Stern, 1965). The border between oral epithelium an underlying connective tissue is uneven and characterised by deep epithelial ridges (rete pegs) that surround finger-like connective tissue papillae.

1.1.2.3 The sulcular epithelium

The sulcular epithelium which lines the gingival sulcus, is a thin, nonkeratinised stratified squamous epithelium. It extends from the coronal limit of the junctional epithelium

to the crest of the gingival margin and like the oral epithelium possesses the rete pegs. The sulcular epithelium has the potential to keratinize if it is reflected and exposed to the oral cavity (Caffesse, Karring & Nasjleti, 1977) or if bacterial flora of the sulcus is totally eliminated (Caffesse, Kornman & Nasjleti, 1980).

1.1.2.4 The junctional epithelium

The junctional epithelium, also known as dento-gingival epithelium, consists of a collar-like band of stratified squamous nonkeratinised epithelium. It is three to four layers thick in early life, but the number of layers increases with age to 10 or even 20 and its length ranges from 0.25 to 1.35mm. The reduced enamel epithelium (the remnants of enamel producing apparatus which covers the crown of the unerupted tooth) is gradually transformed during tooth eruption into the junctional epithelium (Listgarten, 1966a). The junctional epithelium is attached to the tooth surface (epithelial attachment) by means of a basal lamina (Listgarten, 1966b). The basal lamina consists of a lamina densa (adjacent to the tooth surface) and a lamina lucida to which hemidesmosomes are attached. Unlike the oral and sulcular epithelium, junctional epithelium in normal, non-inflamed gingiva lacks rete pegs and connective tissue papillae at its boundary with underlying connective tissue (Lindhe & Karring, 1989).

1.1.2.5 The gingival connective tissue

The connective tissue of the gingiva, which is known as lamina propria, consists of cells, fibres, nerve processes and blood vessels embedded in a dense collagenous environment. This densely collagenous connective tissue contains a prominent system of collagen fibre bundles, the gingival fibres, which are mostly arranged in four groups; circular, dentogingival, dentoperiosteal and transseptal (Arnim & Hagerman, 1953, Lindhe & Karring, 1989). The main cell is the fibroblast (65% of the total population), which synthesises and secretes the basic elements of the connective tissue, the collagen fibres. There are other cells including mast cells (Shelton & Hall, 1968), undifferentiated mesenchymal cells, and macrophages.

Leucocytes have been found in clinically healthy gingival sulci in human and experimental animals. The main leucocytes found are the PMNs which appear in small numbers in the extravascular area in the connective tissue adjacent to the bottom of the sulcus. In a recent study, it was shown that 18% of the connective tissue adjacent to the apical portion of the junctional epithelium were PMNs after 6 months of optimal plaque control (Brecx *et al.*, 1987). It is from there that they travel across the epithelium (Cattoni, 1951; Grant & Orban, 1960) to the gingival sulcus where they accumulate and are eventually lost to the oral cavity.

1.1.3 The periodontal ligament

Periodontal ligaments are connective tissue structures which surround the root and connect it with the alveolar bone. The most important part of the periodontal ligament is the principal fibres, which were first described by Black in 1887. These are collagenous fibres, arranged in bundles, and follow a wavy course with their terminal portions inserted into the root cementum and alveolar bone (Sharpey's fibres). The collagen of the periodontal ligament is mostly of Type I, though some Type III collagen is present as well (Butler *et al.*, 1975). The turnover rate of periodontal ligament collagen is estimated to be two to three times faster than of gingival collagen (Rippin, 1978). The principal fibres are arranged in the following groups: a) transseptal group which extend interproximally over the alveolar crest inserting into the cementum of adjacent teeth; b) alveolar crest group which extend obliquely from the cementum, just beneath the junctional epithelium, to the alveolar crest; c) horizontal group which extend at right angle to the long axis of the tooth from the cementum to the alveolar bone; d) oblique group which are the largest group in the periodontal ligament and extend from the cementum in a coronal direction obliquely to the bone; and finally e) apical group which radiate from cementum to the bone at the fundus of the socket. Other fibres in periodontal ligament are the relatively few elastic fibres (Thomas,1927); and the

oxytalan fibres (Goggins, 1966) which are distributed around the blood vessels.

1.1.4 The cementum

Cementum is a specialised calcified tissue of mesodermal origin which forms the outer covering of the anatomic root and, occasionally, small portions of the crown of the teeth. Cementum does not undergo physiological resorption and remodelling. Two different types of cementum exist, acellular (primary) and cellular (secondary), both consist of a calcified interfibrillar matrix and collagen fibres. There are two sources of collagen fibres in cementum: a) Sharpey's fibres (extrinsic fibre system) which are the embedded portion of the principal fibres of the periodontal ligament (Romaniuk, 1967); and b) another group of fibres (intrinsic fibre system), belonging to the cementum matrix *per se*, produced by cementoblasts (Selvig, 1965). The intrinsic fibre system is composed of fibres oriented more or less parallel to the long axis of the root which cross the extrinsic fibres (Sharpey' fibre) at right angles (Lindhe & Karring, 1989). The cemento-enamel junction (CEJ) is the point at which enamel (outer layer of the anatomic crown) meets the cementum. Three types of relationship, at the CEJ, exist between enamel and cementum. Cementum overlaps the enamel in 60% of the cases; there is an edge to edge joint in 30% of the cases; and in 10% the enamel and the cementum fail to meet.

1.1.5 The alveolar bone

The alveolar process is the part of maxilla and mandible that forms and supports the tooth sockets (alveoli). It develops when the tooth development and eruption takes place and gradually disappears when the tooth is lost. The alveolar process consists of a) the alveolar bone proper, a thin and compact layer of bone also called the cribriform plate, where the Sharpy's fibres are inserted into the bone (Selvig, 1965); b) the supporting alveolar bone including cancellous trabeculae, the pattern of which is affected by occlusal forces (Parfitt, 1962); and c) the facial and lingual/palatal plates of compact bone. The interdental septum consists of cancellous bone enclosed within a compact border. The periodontal ligament and the root cementum together with the alveolar bone form the attachment apparatus for the teeth. The fundamental function of this apparatus is to distribute and resorb forces applied to the teeth by mastication and other tooth contacts (Lindhe & Karring, 1989).

1.1.5.1 Alveolar bone remodelling

Alveolar bone remodelling is an ongoing process in which two types of cells are involved; a) osteoblasts which produce osteoid consisting of collagen fibres and matrix. The latter undergoes calcification by the deposition of the minerals which are subsequently transformed to

hydroxyapatite; and b) osteoclasts which are responsible for osteolysis by resorbing the mineral as well as organic bone materials (Lindhe & Karring, 1989).

1.2 Definitions

1.2.1 Clinical and histological appearance of 'normal' periodontium

In the normal periodontium, the gingiva is clinically characterised by its pale pink colour, firm consistency, scalloped outline, which does not bleed on probing and fills the entire space between adjacent teeth (Ainamo & Löe, 1966, Wennström, 1988); the gingival sulcus is rarely present; histologically, no inflammatory exudate is present and only a few polymorphonuclear leucocytes are seen traversing the connective tissue and junctional epithelium; (Lindhe, 1989); alveolar bone is located 1mm apical to the cemento-enamel junction (Eliasson *et al.*, 1986). Normal gingiva is, therefore, free from inflammation at both the clinical and microscopical level which can be found in humans who, daily, exercise meticulous supervised plaque control for several weeks. These conditions are, however, rarely met in average human subjects (Taichman & Lindhe, 1989). In the light of this fact and the findings of (Seymour, Powell & Aitken, 1983; Brecx *et al.*, 1987) that an inflammatory infiltrate, comprising of polymorphonuclear leucocytes and small round mononuclear cells is always

present in gingival biopsies from clinically healthy gingivae, it seems that normal gingiva should be distinguished from clinically healthy gingiva.

1.2.2 Definition and classification of periodontal disease

The periodontium comprises diverse tissues of different origin; therefore, the term periodontal disease, if used in the general sense, would include a range of diseases affecting various elements of the periodontium (Kinane & Davies, 1990). However, in this thesis, the term periodontal disease will refer to gingivitis and periodontitis, the inflammatory processes associated with the presence of dental plaque.

In the World Workshop in Clinical Periodontics (1989) different forms of periodontitis were classified as follows:

I. Adult periodontitis
II. Early-onset periodontitis
    A. Prepubertal periodontitis (localised, generalised)
    B. Juvenile periodontitis (localised, generalised)
    C. Rapidly progressive periodontitis
III. Periodontitis associated with systemic disease
IV. Necrotizing ulcerative periodontitis
V. Refractory periodontitis

However, there is a considerable overlapping between the different categories.

1.2.2.1 Clinical features of gingivitis and periodontitis

In gingivitis, which is the most common form of gingival disease, pathological changes are confined to the gingiva. Gingivitis is clinically manifested by gingival redness and swelling and increased tendency of soft tissue to bleed on gentle probing (Taichman & Lindhe, 1989). In periodontitis, the deeper structures such as alveolar bone, periodontal ligament and root cementum are affected by pathological changes. In general, periodontitis is clinically manifested by pocket formation, open interdental contacts, and loosening and migration of the teeth.

Prepubertal periodontitis (PP) is a very rare condition with its onset during or immediately following eruption of the primary teeth. In the generalized form all the primary teeth are affected and severe bone loss is evident radiographically, whereas in the localized form some of the teeth are involved to a lesser extent (Page & Schroeder, 1982).

Juvenile periodontitis (JP), with its onset thought to be at puberty, may be diagnosed at any age beyond puberty. JP is characterized by deep pockets and bone loss associated

with the permanent first molars and/or incisors, with usually a symmetrical distribution of lesions (Baer *et al.*, 1971).

Rapidly progressive periodontitis's (RPP) onset occurs between puberty and 30-35 years of age (Page *et al.*, 1983) and is characterized by severe bone loss affecting most teeth, without any consistent pattern of distribution. In both JP and RPP the amount of bone loss is not commensurate with the amount of plaque (Page & Schroeder, 1982). Early onset forms of periodontitis in general and prepubertal periodontitis in particular are rare and most, if not all, of the cases have a genetic basis.

Adult periodontitis (AP) is the most common form of the disease, with its onset beyond the age of 30-35 years. The lesions are not confined to any certain teeth and the amount of microbial deposits (dental plaque) are consistent with the severity of lesions (Page *et al.*, 1983).

Periodontitis associated with systemic disease. Different forms of periodontitis described above exhibit pathologic changes in the periodontium without any accompanying or underlying systemic disease, whereas systemic conditions may modify the normal defence, thus predisposing individual to specific forms of periodontitis. Reduction in number or function of PMNs results in increase rate and severity of periodontal breakdown (Kinane & Davies, 1990). In these

cases periodontitis could be considered as a symptom of the underlying disorder.

Acute necrotizing ulcerative gingivitis (ANUG) is a rapidly progressive inflammation of the gingiva which may enter a chronic stage. ANUG rarely occurs in a generalized form and may be very advanced in anterior teeth whereas the rest of the teeth may not be involved. Disease is caused by a fusospirochetal complex of micro-organisms in the presence of a number of predisposing factors such as poor oral hygiene, smoking and stress (Sabiston 1986). When the infection reaches the alveolar bone and periodontal fibres, the disease is termed *Necrotizing ulcerative periodontitis* (Proceeding of the World Workshop in Clinical Periodontology, 1989).

Refractory periodontitis (RP) is seen as an enigma in clinical periodontology. According to the World Workshop in Clinical Periodontics (1989), this condition has been described as cases with multiple sites which continue to lose attachment despite appropriate management. However, whether a particular site is truly refractory or whether refractory periodontitis is a separate disease entity is a critical question. It is quite clear that several forms of periodontal disease can become resistant to treatment. Therefore it seems more realistic to refer the term *refractory* to sites which do not respond to treatment rather than the diagnosis of periodontitis (Adams, 1992).

1.3 Epidemiology of periodontal disease

Epidemiological studies performed over the past half a century have employed the radiographic evidence of alveolar bone resorption, periodontal pocket depth /attachment level and the degree of gingival inflammation to assess the prevalence of periodontal disease (Marshall-Day & Shourie, 1949; Schour & Massler, 1947; Russell, 1956; Ramfjord, 1959). The prevalence of gingivitis, in early epidemiological studies, approached approximately 100% for children and young adolescents. Marshall-Day and Shourie (1949) reported that 73% to 97% of Indian children aged 9-17 and 81.2% of those aged 5-10 years had gingivitis. Similarly, a prevalence of 99% was reported for 13-year old children in postwar Italy by Schour & Massler (1947). Marshall-Day, Stephen & Quigley (1955) reported that with increasing age, a concomitant decrease in the prevalence of gingivitis with no bone involvement and an increase in prevalence of periodontitis, as indicated by radiographic bone loss, was evident; and at the age of 40 years, approximately 100% of the subjects were affected by destructive periodontitis. Using the Periodontal Index (PI) of Russell (1956), extensive data has been collected from different populations all around the world. Analyses of these observations have revealed that variation in prevalence and severity of periodontal disease is associated with race, geographic area, sex, socioeconomic status and education level. Using a better known Oral

Hygiene Index (OHI) of Greene and Vermillion (1960), it has been shown that the evident racial, geographic, socioeconomic, gender and level of education disappear when disease severity is directly related to the oral hygiene level (Schrep, 1964). This point has been suggested by Russell (1963) that more than 90% of the variance of the periodontal disease experience in the population can be explained by age and oral hygiene. Although, different indices and various thresholds for detection of gingivitis and periodontitis have been employed, the results of these studies led to the concept that: a) periodontitis is a global disease affecting high proportions of the population; b) the disease starts as gingivitis at an early age which if left untreated will develop to periodontitis; c) most of the variance of the periodontal disease in the population can be explained by age and oral hygiene. These concepts dominated periodontal literature until recently when a more appropriate analyses of epidemiological data was made. Data collected by the PI of Russell (1956) and the Periodontal Disease Index (PDI) of Ramfjord (1959) have been expressed as mean values for gingivitis scores as well as pocket depths and attachment loss. However, it has been recognised that reporting data as mean values could result in data smoothing which may obscure the differences within and between individuals with respect to extent and severity of periodontal disease (Papapanou, Wennström & Gröndahl, 1989). When the extent and severity of periodontal involvement were taken into consideration a different

overall picture of the prevalence and progression of periodontal disease became evident. Recent cross-sectional studies have shown that although a periodontium of reduced height is common with increasing age, relatively few individuals in each age group suffer from advanced periodontal destruction. These subjects account for most of the sites which express severe periodontal breakdown. Papapanou, Wennström & Grödahl (1988) reported that 23% of subjects accounted for 75% of the total number of the sites showing advanced bone loss (≥6mm). Jenkins and Kinane (1989) pointed out that 14.4% of patient who had been referred to the Glasgow Dental Hospital for a variety of dental complaints, showed advanced bone loss. Longitudinal studies have in addition shown that few sites undergo extensive periodontal destruction within a given observation period. Lindhe, Haffajee and Socransky (1983) reported that during a six year follow up of Swedish periodontally affected subjects, who were not receiving active treatment, only 3.9% and 11.6% of their sites had lost attachment >2mm at 3 and 6 years respectively. Similar percentage (3.2%) of sites exhibiting attachment loss were reported in the same study for a group of American who were followed for one year. However, a higher percentage (7%) of sites which lost attachment over a 1 year period has been reported by Haffajee, Socransky and Goodson (1983a). Löe *et al.* (1986), who conducted a 15 year long study of the natural history of periodontal disease in Sri Lanka, reported that only 8% of the total

sample showed rapid progression of periodontal disease, as defined by tooth mortality rates and interproximal attachment levels, whereas in 11% of the subjects gingivitis never converted to periodontitis. Jenkins, MacFarlane and Gilmour (1988) reported that in a one year longitudinal study of untreated periodontitis patients only 1.9% of sites exhibited attachment loss of more than 2mm. In a two year follow up of a group of untreated subjects, it was shown that only 0.7% of sites had lost attachment of >2mm, however, 70% of the sites that experienced further attachment loss belonged to 12% of the subjects (Lindhe *et al.*, 1989). In a 10-year retrospective study, Papapanou *et al.* (1989) demonstrated that only 7% of subjects experienced bone loss of ≥3mm and 10 sites or more with bone loss of ≥6mm were found in 4% of the subjects over a 10 year period.

The current view, therefore, is that: a) periodontal disease is subject related; b) a small subset of individuals within a given population suffer from advanced periodontal destruction; c) relatively few subjects and sites undergo active periodontal destruction within a given period of time; and d) in the absence of treatment, gingival inflammatory lesion does not necessarily convert to destructive periodontal disease (Papapanou, 1989).

1.4 Gingival crevicular fluid

1.4.1 Production

Gingival crevicular fluid is an inflammatory exudate or a pre-inflammatory transudate present in the gingival crevice which contains several components of different origin. GCF components are mainly derived from: a) interstitial fluid and locally produced factors from migrating or resident host cells; b) serum; c) tissue degradation/turnover products; and d) microbial sources (Cimasoni, 1983). Production of GCF in the presence of inflammation was shown to be due to an increase in the vascular permeability of the subepithelial vasculature in inflamed gingiva (Brill & Björn, 1959; Egelberg 1966). Either chemical (topical application of histamine) or mechanical (massage of the marginal gingiva with a ball burnisher or scraping with a blunt probe) stimuli to the gingiva resulted in an increased vascular permeability which in turn increased the GCF flow (Egelberg, 1966). Löe and Holm-Pedersen (1965) reported an increasing level of GCF as the severity of gingival inflammation increased. However, they failed to show the presence of GCF in healthy gingiva with or without the mechanical stimulation. Although, when paper strips were deeply inserted into the crevice substantial amounts of GCF were collected. Brill (1960) in his clinical and histological study observed that sites with histological signs of inflammation had, significantly more GCF than

sites without inflammation at a histological level. It seems that GCF in clinically healthy gingiva is produced under different mechanism. In his hypothesis, Alfano (1970) suggested that pre-inflamed flow of GCF may be osmotically mediated in the following way: In clinically healthy gingiva during initial plaque accumulation macromolecules derived from subgingival plaque can diffuse intercellularly to the basement membrane, where due to its relative impermeability in health, they will accumulate. An osmotic gradient can then be created and a flow of GCF generated. Pashley (1976) described a model which can be applied to the situation in both health and disease. He suggested that GCF production is governed by relative differences in: a) passage of fluid from capillaries into the tissues (capillary filtrate); b) removal of this fluid by lymphatics of the gingiva (lymphatic uptake) and c) filtration through the junctional epithelium into the gingival crevice (GCF production). When the rate of capillary filtrates exceeds that of lymphatic uptake, fluid will leave the area. Factors modulating these processes include the filtration coefficient of the capillary and lymphatic endothelium as well as the osmotic pressure within the different compartments. Thus, even in health, if the osmotic pressure of the sulcular compartment, due to the plaque derived products, exceeds that of tissue fluid, there will be a net increase in the flow of GCF. In an attempt to verify the model described by Alfano (1970), Bickle, Cimasoni and Anderson (1986) studied the

contribution of serum in GCF production during early (pre-inflammatory) plaque accumulation by measuring albumin concentration in GCF and its ratio to that of the serum. They recovered no fluid from healthy gingiva and low concentrations of albumin during pre-inflammatory plaque accumulation. These observations supported the osmotic gradient model of Alfano (1970). With increasing plaque accumulation, both the GCF albumin concentration and its ratio to that of the serum increased. Chronically inflamed sites showed a GCF:serum albumin ratio of approximately 1. However, Stoller, Karras and Johnson (1990) concluded that the increase of GCF volume in the presence of dental plaque is due to the presence of significant quantities of water in dental plaque. They have not discussed the other possibilities, such as osmotic pressure, in GCF production. As the GCF traverses the inflamed tissue, on its way, picks up enzymes and other molecules that participate in the destruction process, as well as products of cell and tissue degradation. Therefore, GCF offers a great potential as a source of factors that may be associated with active tissue destruction (Page, 1992).

1.4.2 Methods of collection and volume determination

A variety of methods have been used for the collection of GCF which can reflect the type of analysis to be performed on the samples and the amount of fluid required. They include, absorbent precut paper strips/paper points placed

at the orifice, partly into or to the bottom of the pocket or sulcus, the capillary tubes or micropipettes and the gingival washing (Golub *et al.*, 1976; Villela *et al.*, 1987; Adonogianaki, Moughal & Kinane, 1993). When large volumes of GCF are required capillary tubes (Ishikawa *et al.*, 1972; Larivèe, Sodek & Ferrier, 1986) or micropipettes (Eisenhauer *et al.*, 1983) are used. When cell types and numbers in the fluid are required gingival crevicular washings (Salonen & Paunio, 1991; Adonogianaki *et al.*, 1993) are employed.

Exact placement of the sampling device is important. It has been shown (Mann, 1963; Löe & Holm-Pedersen, 1965) that the insertion of the paper strips in the crevice will irritate the gingival tissue and induce vascular permeability which in turn gives cause to increased GCF production.

The effects of repeated sampling on GCF flow when using paper strips have been the focus of several studies which have shown that GCF volume tends to decrease by repeated sampling (Binder, Goodson & Socransky, 1987; Curtis *et al.*, 1988; Lamster *et al.*, 1989; Persson & Page, 1990). Although in most cases repeated sampling resulted in a reduction in total amounts of GCF components (Lamster *et al.*, 1989; Persson & Page, 1990) some investigators reported an increase or steady flow rate (Binder *et al.*, 1987; Villela *et al.*, 1987). This discrepancy could have

resulted from lengthy sampling periods (Villela *et al.*, 1987) or deep intercrevicular sampling (Binder *et al.*, 1987). Lamster *et al.* (1989) employed repeated sampling for 30 seconds and observed that GCF constituents did not return to baseline level even after 60 minutes. They also found that the fluid volume in the crevice recovers more rapidly than constituents derived from host cells.

Sampling time is also of great importance. Persson and Page (1990) employed four sampling times (5, 10, 20 and 30 seconds) and four different sampling sequences, alternating the order by which samples of different time periods were taken. They suggested that 5s or 10s first time sampling of GCF is adequate whereas lengthier sampling periods (20s or 30s) tend to dilute aspartate aminotransferase activity. However, Cao and Smith (1989) have reported that myeloperoxidase activity is higher in 30s compared to 5s samples from the same site when the samples are taken one week apart.

In general, it can be concluded that while the same sampling technique, sampling time and sampling sequence may not be the best for all studies the procedure of choice should have the following features: a) causes the least interference with gingival tissues; b) is the shortest in time and provides enough sample for subsequent analysis; and c) collects fluid present at the site prior to sampling. Thus, it seems that sampling for 30s or less

with a paper strip would be ideal as it provides a sample of GCF of sufficient size for subsequent analysis while minimising irritation of the gingival tissues.

GCF volume collected on paper strips has been measured by assessing the wetted area of the strip, by weighing of paper strip before and after sample collection or by the use of the Periotron (Harco Electronics, Winnipeg, Manitoba, Canada). The wetted area of the strip can be assessed directly using a microscope fitted with a graticule (Egelberg & Attström, 1973) or after staining with ninhydrin (Egelberg, 1964). Although staining facilitates viewing and assessment of the wetted area, the sample is not of any use for GCF analysis afterwards. Weighing prior to and after GCF sampling of paper strips has been performed for assessing the volumes beyond the measuring range of the Periotron (Cimasoni & Giannopoulou, 1988). Assessing the GCF volume by weighing of the strip and the use of the Periotron make the subsequent analysis of the biochemical components of GCF possible. The Periotron 6000 has been extensively used by investigators in periodontal field and it has proven satisfactory for the assessment of GCF volume (Cimasoni, 1983).

However, assessing minute amounts of GCF by any of the above methods (volumes of 0.01-0.2 µl) is demanding and could be subject to uncontrollable errors (Lamster *et al.*, 1988).

1.5 Clinical assessment of the periodontium

1.5.1 Introduction

The periodontium is clinically evaluated by means of different indices which distinguish between healthy tissues, gingival inflammation and destructive periodontal disease. These clinical indices are based on visual observation, palpation/percussion of the teeth, the use of the periodontal probe and radiographic examination. The use of GCF flow has also been suggested to supplement other clinical findings. Subject's oral hygiene performance and plaque accumulation are assessed using plaque indices. Generally accepted criteria for an index include simplicity, validity, sensitivity, reproducibility and amenability to statistical analysis (Hazen, 1974).

1.5.2 Assessment of oral hygiene

The assessment of oral hygiene dates back to the 1930s (Ainamo & Bay 1975). Oral hygiene indices are based on the measurement of the tooth surface area covered by dental plaque and/or thickness of the material in the gingival margin. In 1959 Ramfjord introduced his Periodontal Disease Index (PDI) with different components for assessing the gingival condition and plaque accumulation on the tooth surfaces as well as an index for calculus assessment. Quigley and Hein (1962) published their plaque index which

is graded according to the coronal extension of plaque on the tooth surface. In 1964 Greene and Vermillion introduced their Simplified Oral Hygiene Index (OHI-S) including calculus index and debris index of which dental plaque is one component. Since then numerous indices have been developed to assess plaque accumulation (reviewed by Fischman, 1986). In 1964 Silness and Löe described their plaque index which is one of the most widely used plaque indices in periodontal trials. This index was fully described by Löe (1967) and scores plaque accumulation from 0 to 3. This plaque index is based on the different amount of plaque present on the tooth surface/gingival margin and can be used to score individual sites, as single site score, teeth as average of four sites per tooth (buccal, lingual and interproximal) and individuals or groups of teeth. The last two scores are based on site scores divided by the number of sites assessed. To this date oral hygiene indices have been calculated by adding up the plaque and calculus scores. Silness and Löe (1964) stated that the presence and amount of plaque alone gives an adequate expression of the state of oral hygiene. They also stated that running an explorer along the tooth surface both supra and subgingivally gives better results than the use of disclosing solutions. The plaque index (Silness and Löe, 1964) remains one of the most widely used indices although it has been criticised for its subjective nature and disruptive character when distinguishing between scores 0 and 1, especially when it is used for within and

between examiner calibration. The plaque index has the advantage of having the ability to assess very subtle changes in plaque accumulation (Lindhe, 1989). It has also been suggested that the plaque index is suitable when the plaque/gingivitis cause and effect relationship is to be considered (Fischman, 1988).

1.5.3 Assessment of gingival inflammation

Assessment of gingival inflammation is performed by the use of gingivitis indices as well as GCF flow. These indices rely on one or more of the following parameters: gingival colour, gingival contour, gingival bleeding and the extent of gingival involvement. Such indices were initially developed for use in epidemiological surveys (Ciancio, 1986). One of the first gingival indices (PMA) was described by Schour and Massler (1947) which evaluates the condition of papillary (P), marginal (M) and attached (A) gingiva with varying degrees of inflammation. Later, Russel (1956) introduced the Periodontitis Index (PI) and Ramfjord (1959) published the Periodontal Disease Index (PDI). Both indices as well as the Navy Periodontal Disease Index (Elliott *et al.*, 1972) incorporated the assessment of gingival inflammation at the lower parts of their scales, which reflects the notion dominated at the time, that gingivitis precedes periodontal destruction. However, Löe (1967) has given two reasons for not including a gingival index into a complex system, which also assesses the amount

of periodontal breakdown. The first reason is, that gingival inflammation and periodontal breakdown are two different incomparable parameters and should not be put into one index; the second reason is, that there is no need for transforming pocket depth or attachment loss as based on measurement in millimetre to a different system of figures (index). He, therefore, suggested that the quality of gingiva should be scored according to the Gingival Index (Löe & Silness, 1963) and the quantity of periodontal destruction measured in millimetre. More recent indices for the assessment of gingival inflammation are based on visual changes and/or bleeding on probing of the gingival unit. Among these are: the Gingival Index (GI)(Löe & Silness, 1963; Löe, 1967), the Sulcular Bleeding Index (SBI)(Muhlemann & Mazor, 1958; Muhlemann & Son, 1971), the Gingival Bleeding Index (GBI)(Ainamo & Bay, 1975) and the Papillary Bleeding Index (PBI)(Muhlemann, 1977; Barnett, Ciancio & Mather, 1980). The Gingival Index of Löe and Silness (1963), has been widely accepted and provided a conceptual change in the assessment of gingival inflammation as it scores both its severity and location. The GI scores inflammation from 0 to 3. Scores 2 and 3 incorporate the bleeding on gentle pressure component which was later modified to bleeding on probing (Löe, 1967). The invasive nature of GI is a disadvantage for this index when it is used in longitudinal studies of gingivitis where trauma to the gingival tissues could yield an altered response. In addition, a non-invasive index facilitates

the use of other clinical examinations during the same appointment such as GCF sampling which is influenced by the irritation of the gingival tissues. To address this problem Lobene and co-workers (1986) made some modifications to the GI of Löe and Silness (1963) by eliminating its bleeding on probing/pressure component yielding a completely non-invasive index. They also expanded the lower part of the scales resulting in a scoring system of 0-4 which gives the index greater sensitivity to the earliest changes of gingival tissues. Although, the Modified Gingival Index (MGI) (Lobene, *et al.*, 1986) lacks the bleeding component it showed a great correlation with the Gingival Index of Löe and Silness (1963) and could be equally used in clinical trials. However, it should be kept in mind that the bleeding component in the GI is only present in the higher part of the scale (GI=2 or 3) and, therefore, the differentiation between clinically healthy gingiva and mild inflammation (GI= 0 and 1, respectively) is still dependent on visual criteria alone (Lobene *et al.*, 1989).

By incorporating the bleeding on probing component in some indices, they have the ability to assess the presence of inflammation at the depth of the pocket in case of periodontitis. Therefore, the use of another index, where it is desired, to determine the presence or absence of bleeding is recommended (Lobene *et al.*, 1989). When bleeding upon probing is assessed the use of pressure

sensitive probes is recommended as they allow a standardised force to be applied in order to test for bleeding.

As late as 1960, it has been suggested that GCF flow could offer some abilities in the assessment of the gingival inflammation (Brill, 1960). Since then both cross-sectional (Mann, 1963; Egelberg, 1964; Löe and Holm-Pedersen, 1965; Borden, Golub & Kleinberg,1977) and experimental gingivitis studies (Löe & Holm-Pedersen, 1965; Egelberg and Attström, 1973) have been conducted in order to assess the correlation between GCF flow and gingival inflammation, and a positive relationship has been obtained. The same kind of trials have also been conducted to establish the relationship between Periotron units and gingival inflammation (Suppipat, Johansen & Gjermo, 1977; Garnick, Pearson & Harrell, 1979) from which positive associations with clinical signs of gingival inflammation were reported. The Periotron was initially introduced, for chairside use, for exactly this purpose. The manufacturer assigned different degrees of disease severity (from health or mild gingivitis to periodontitis) to increasing Periotron units which could be called the GCF flow index. In conclusion, it is the type and purpose of study which determines the type of indices to be used (Ainamo & Bay, 1975; Ciancio, 1986; Fischman, 1986).

1.5.4 Assessment of probing pocket depth and attachment level

Over the years periodontal probing has been and continues to be one of the more useful diagnostic tools to determine the presence and severity of periodontal disease; it also serves to evaluate the outcome of periodontal therapy; and more recently, is being used to record the disease activity/progression via changes occurring in attachment level (Armitage, Svanberg and Löe, 1977; Van der Velden, 1978; Listgarten, 1980; Fowler *et al.*, 1982, Haffajee *et al.*, 1983a). These measurements are based on the identification of the most apical extent of the periodontal pocket, which is measured either from the gingival margin (pocket depth) or a fixed reference point, usually the cementoenamel junction (attachment level). There are several problems associated with pocket depth/attachment level measurement which could give cause to erroneous results. These problems include: a) variations in probing force; b) variations in the diameter of the probe tip; c) errors in the graduation of the probes; d) errors in probe placement; e) errors in reading the probe and data recording and f) errors in the precision of measurement (Jeffcoat *et al.*, 1986). Additional to these physical factors influencing the measurement, biological variations such as transient fluctuation in gingival margin location over time, could further complicate the pocket depth measurement. The location of cementoenamel junction, when

used as a fixed point in the measurement of attachment level, may introduce another source of error (Badersten, Nilvèus & Egelberg, 1984; Clark *et al.*, 1987). It has been reported (Van der Velden, 1979) that variations of the probing force as well as variations in the probe's tip diameter will influence the penetration of the probe through the junctional epithelium and gingival tissues. By using higher forces deeper probing depth recordings were obtained and a plateau was reached at a probing force of 1.25 N, using a probe with a tip diameter of 0.63mm. Freed, Gapper and Kalkwarf (1983) have also reported that probing force applied by different operators could vary from 5g to 135g. They also found that, as a mean, probing forces applied in the posterior areas were significantly higher than probing force used in anterior segments. Van der Velden (1978) pointed out that variation in the graduation of the probe may also be a source of error. It has been shown (Armitage *et al.*, 1977; Caton, Greenstein & Polson, 1981) that deeper probe penetration was obtained in areas demonstrating signs of increased inflammation, indicating that probe penetration could be influenced by the degree of inflammation of the area. In the measurement of attachment level at single rooted teeth, using a conventional periodontal probe and an occlusal stent, inter and intra examiner agreement was within 1mm at approximately 90% of the duplicate measurements, while it was found to be the case in 73% to 84% of duplicate measurements when cementoenamel junction served as a

reference point (Badersten *et al.*, 1984). Clark *et al.* (1987) reported that the cementoenamel junction was found to be masked by its subgingival location or the presence of the calculus in 17% of the sites. In an attempt to control probing force, pressure control probes were introduced (Van der Velden & de Vries, 1978; Polson *et al.*, 1980), and in order to reduce errors originating from location and relocation of the probe as well as identification of the cementoenamel junction occlusal stents have been used (Isidor, Karring & Attström, 1984; Badersten *et al.*, 1984; Clark *et al.*, 1987). In recent years, by advancing technology in construction of pressure sensitive probes some other aspects of the methodological problems associated with measurement of probing depth and attachment level have been addressed. A new pressure sensitive probe which automatically detects the cementoenamel junction was introduced by Jeffcoat *et al.* (1986). An electronic pressure sensitive probe, the Florida Probe, which measures pocket depth and attachment level to the nearest 0.2mm was developed by Gibbs *et al.* (1988). The Florida Probe is equipped with different handpieces which allow pocket depth measurement as well as the measurement of the attachment level both from custom-made stent and the occlusal surface (Magnusson *et al.*, 1988; Osborn *et al.*, 1990). Another electronic pressure sensitive probe, first described by Birek, McGulloch and Hardly (1987) and was later modified by Karim, Birek and Mcgulloch (1990), the Toronto Probe, measures attachment level to the nearest 0.1mm under

controlled angulation.

However, the attention has recently been given to the pressure sensitive probes over their relatively lack of tactile sensation when facing subgingival deposits during the probing procedure (Griffiths *et al.*, 1988). Mullally and Linden (1994) reported a significant bias towards shallower pocket depths with electronic pressure sensitive probe compared to hand probing.

1.5.4.1 Detection of attachment level change

The response to the treatment is conventionally evaluated by change in pocket depth and the state of bleeding on probing. The change in pocket depth reflects attachment gain, shrinkage in inflamed marginal gingiva as well as recession resulting from instrumentation. In order to assess the response to treatment at a specific site, particularly in a study setting, longitudinal measurements of attachment level taken before and after treatment, would therefore provide a more accurate measure. The variable of interest is then the change in attachment level which has occurred between two time points. It is of great importance, therefore, to employ methods that allow clinician/ researcher to define the change in attachment level that safely exceeds measurement error and could provide a threshold for disease activity. Several studies have been carried out, in an attempt to define measurement

error when using a conventional periodontal probe to measure probing attachment level with or without an occlusal stent (Haffajee *et al.*, 1983b; Badersten *et al.*, 1984; Isdor *et al.*, 1984; Clark *et al.*, 1987; Best *et al.*, 1990). In order to reduce the number of false positively detected sites as loosing attachment, Haffajee *et al.* (1983b) suggested a criterion of 3 standard deviations of the differences of replicate measurements. This figure in their study was 2.46mm (SD:0.82mm), which was further rounded up to 3mm. The 3mm threshold was subsequently applied in several studies. Fleiss *et al.* (1991) pointed out the variances for replicate measurements differed from patient to patient and even from site to site and, therefore criticised the application of a threshold derived from one population to another. Using pressure sensitive probes with high resolution provides a more accurate and reproducible assessment of probing attachment level than manual conventional probing (Jeffcoat & Reddy, 1991b; Mullally & Linden, 1994). This would reduce variability between replicate or sequential measurements resulting in smaller threshold for attachment level changes to be declared. Three more sophisticated methods for detecting significant attachment level changes namely: regression, the running medians and the tolerance method have been described by Haffajee *et al.* (1983b). In the regression method the slope and y intercept of a regression line are computed from a linear least squares fit of attachment level measurements as a function of time and the slope is

tested for a significant departure from 0. A threshold of projected attachment level change per unit time has also to be exceeded. In the running medians method attachment level measurements are plotted against time and systematic variations can be differentiated from minor changes by considering the medians of three successive data points and setting an arbitrary threshold based on the variability of replicate measurements. Finally, the tolerance method is based on standard deviations of replicates, usually 2, measurements at each time point. For a pair of attachment level measurements to be considered different from the preceding pair, attachment level change has to exceed three different thresholds which are derived from population, subject and site standard deviations of replicate measurements. Badersten *et al.* (1987) used the regression analysis for the identification of attachment level change. She also developed an end point analysis method, in order to compensate for the insensitivity of the former to detect abrupt changes at the early/late phases of the study. In the end-point analysis method, the deeper of the pair of the initial measurements is compared against the shallower of the final pair of the measurements. When the change exceeds a specified threshold, attachment level change is declared. Another method has been developed on the cumulative sum of successive measurements (cumsum)(Aeppli & Pihlstrom, 1989; Jeffcoat & Reddy, 1991a). In this method differences from baseline are computed for each observation, which are subsequently added to obtain a

sequence of sums which increase or decrease with the number of positive and negative changes. If the rate of change exceeds a predetermined critical value, a significant change in attachment is declared.

The regression and the tolerance methods are the most commonly used methods for detecting the attachment changes in clinical studies. The regression method is most suitable for assessing gradual linear changes in attachment over a long period of time. The tolerance method requires measurements at as few as two points in time, therefore is more suitable for detecting abrupt differences in attachment level in short periods in time and has the advantage of considering patient and population variances in addition to site variance (Haffajee *et al.*, 1983b; Aeppli & Pihlstrom, 1989) as it has been reported by Fleiss *et al.* (1991) that variances for replicate measurements differs from patient to patient and even from site to site.

1.5.4.2 Identification of active and inactive sites based on change in attachment levels

Detection of periodontally active sites/patient by the use of clinical indices, introduced in previous sections, which are conventionally performed in almost all of the periodontal clinics, seems to be of little value. Gingival and bleeding indices as well as GCF flow represent measures

of transient inflammatory status of the superficial structures of the periodontium, whereas plaque and calculus indices reflect the oral hygiene status of the sites/ patients and can also provide a measure for the assessment of patient cooperation and motivation. Recording pocket depth alone does not take into account the presence of gingival enlargement or recession, while attachment level measurement, by using a fixed landmark on the tooth (cementoenamel junction), overcomes this problem. But, at best, probing depth and attachment level measurements, at any given time, could provide a history of past disease experience in a site/patient rather than disease activity (Griffiths *et al.*, 1988).

1.5.4.3 The use of clinical parameters in prediction of disease behaviour and response to treatment: Evaluation by change in attachment levels

Distinguishing between disease-active and disease-inactive sites is a central problem to periodontal treatment planing as well as evaluating the efficacy of the therapy. At present, the presence of the active sites is determined by longitudinal observations of attachment level or alveolar bone status. Several attempts have been made to correlate clinical parameters, traditionally used in clinical diagnosis of periodontitis, with periodontal disease activity as assessed by longitudinal probing attachment level measurements (Greenstein & Caton, 1990). Several

studies have shown poor correlations between plaque accumulation and destructive periodontal disease in untreated as well as patients on maintenance care, which could be attributed to the transient nature of this parameter (Haffajee *et al.*, 1983a; Badersten *et al.*, 1985; Jenkins *et al.*, 1988; Lindhe *et al.*, 1989; Badersten *et al.*, 1990; Kaldahl *et al.*, 1990). In one of the earliest reports on the ability of clinical parameters in predicting periodontal disease activity, Haffajee *et al.* (1983a) reported a specificity of 0.71 and a sensitivity of 0.42 for presence of plaque, just prior to the monitoring session, at specific sites which had lost attachment. Badersten *et al.* (1985) in a two year monitoring of periodontally treated patients at three monthly intervals, demonstrated that dental plaque, when present in all nine occasions , has a predictability of 25% for attachment loss at a threshold of ≥1.5mm. When dental plaque was present in 8 and 6 examination times, its predictability for attachment loss dropped to 12% and 4% respectively. Jenkins and coworkers (1988) attempted to investigate the use of clinical and microbiological parameters as predictors of periodontal destruction and pointed out that presence of dental plaque cannot predict attachment loss. Badersten *et al.* (1990) pointed out that, during a 6 year monitoring period, only 30% of the sites which exhibited plaque in ≥75% of occasions they were examined actually demonstrated attachment loss. In another attempt, during a one year study on untreated patients, Haffajee *et al.*

(1991) reported positive associations between percentage of sites with visible plaque accumulation and the number of subjects exhibiting attachment loss over 3mm. Thus, the higher the percentage of sites harbouring plaque within a subject the more likely the subsequent attachment loss. The authors however suggested that the association observed between plaque and further attachment loss could have been due to the strong association of plaque and attachment level at baseline which in turn was strongly related to subsequent attachment loss. Bleeding on probing has long been considered as an important part of diagnostic procedure in periodontal practice. Some studies have evaluated the possible correlation between bleeding upon probing and gingival inflammation with active tissue destruction in periodontal disease. Badersten *et al.* (1985), monitoring periodontally treated patients for a 24 months period, reported 17% predictability for bleeding on probing in all the three monthly intervals to be associated with attachment loss as defined by ≥1.5mm change in attachment level. Lang *et al.* (1986) and Badersten *et al.* (1990) reported a diagnostic predictability for accumulated bleeding scores of 30% and 23% respectively. Jenkins *et al.* (1988) from their one year study of untreated periodontal patients reported that gingival inflammation cannot be used as a predictor of further attachment loss. Lang *et al.* (1990) suggested that absence of bleeding on probing is a good indicator for the maintenance of periodontal health while, its presence could not be used as

an effective indicator/predictor of periodontal destruction. When the study (Joss, Adler & Lang, 1994) continued for another 2 years, the authors concluded that patients with higher prevalences of bleeding on probing are at higher risk of experiencing further attachment loss. However it has also been demonstrated that there is a positive association between percentage of sites within a patient that demonstrate bleeding on probing at baseline, and risk of further attachment loss (Haffajee *et al.*, 1991). As it was described for plaque scores, the authors, however, suggested that association of bleeding on probing with subjects' baseline attachment level may, in large part, explain its association with subsequent attachment loss. It was revealed, in the same study (Haffajee *et al.*, 1991), that there was no significant difference in additional attachment loss between groups of patients with different percentages of sites exhibiting overt gingivitis at baseline.

Suppuration has also shown to be a rather poor predictor of periodontal disease activity. Its predictive value ranged from 17% to 30% for single and accumulated suppuration observations respectively (Haffajee *et al.*, 1983a; Badersten *et al.*, 1985; Haffajee *et al.*, 1991).

Deeper probing depths at specific sites cannot necessarily diagnose/predict ongoing periodontal disease activity (Haffajee *et al.*, 1983a; Jenkins *et al.*, 1988). Residual

probing depths ≥7mm showed 20%, 27%, 40% and 52% diagnostic predictability respectively in a 1, 2, 4 and 5 year longitudinal study of periodontitis patients on maintenance care (Badersten *et al.*, 1985; Badersten *et al.*, 1990). These results indicate that long monitoring periods may be required to improve the diagnostic value of clinical parameters at specific sites. Increasing individual mean levels of pocket depth or attachment level above certain thresholds as well as increasing percentages of sites within a patient demonstrating probing depth or attachment level measurement >3mm were, however, risk indicators for further attachment loss suggesting that previous periodontal destruction predisposes the site/patient to future periodontal breakdown (Haffajee *et al.*, 1991).

The predictive value of a multitude of subjective clinical observations, employed by experienced clinicians, for predicting the response to treatment was evaluated by Vanooteghem *et al.* (1990). Three independent clinicians examined patients with adult periodontitis, prior to the commencement of treatment, and identified sites that in their opinion would continue to lose attachment after initial nonsurgical therapy. The patients were monitored for a period ranging between 24 and 36 months. The results demonstrated a limited agreement between probing attachment loss determined by linear regression and the clinicians' prediction of attachment loss.

Although ,in the above studies, different methods have been employed in order to detect attachment level changes, their results showed that clinical assessment of the periodontium, particularly in one single visit, cannot predict disease behaviour and response to the treatment, or diagnose active periodontal destruction. However, this is not surprising as the parameters involved in the assessment of the periodontal disease are *static* measurements of a *dynamic* process. This information could quite rightly confuse the clinician who had long learnt to rely on clinical diagnostic criteria in prognosis of periodontal disease. Pathogenesis of periodontal disease has received great attention; and numerous studies aiming to elucidate possible mechanisms of periodontal destruction have been conducted in recent years. These efforts could perhaps lead to the development of laboratory based diagnostic tests which are hopped to provide more sensitive diagnostic/prognostic tools to supplement, currently used, clinical parameters.

1.6 Aetiology and pathogenesis of periodontal disease

Destructive periodontal disease, as evidenced by bone loss, has afflicted mankind since the beginning of history. In the early writings, the relationship of calculus to periodontal disease has been considered, a notion dominating the history of dentistry until the present

century. John W. Riggs (1811-1885) believed when tooth is covered by hard deposits (calculus) it will be considered as a foreign body and an inflammatory reaction is followed. John Hunter (1728-1793), offered a clear illustrations of the anatomy of the teeth and their supporting structures (periodontium). Before the concept of periodontium (the relationship between soft and hard supporting tissues of the teeth) was evolved, periodontal disease was believed to affect either the gums or the alveolar bone, but not both structures. Since then the understanding of periodontal disease has greatly developed. Much of this progress has been achieved during the past three decades. New findings answer some questions and bring up other questions in the investigators' mind, which lead them to undertake more sophisticated studies and explore in the new fields. In this way the knowledge of periodontology makes its way toward further refinement and deeper understanding of the field, although still many questions concerning the aetiology, pathogenesis and progression of periodontal disease remain to be answered.

Chronic inflammation is the backbone of periodontal disease and it is well established that accumulation of dental plaque (bacteria) is the aetiological agent responsible for initiation and maintenance of the inflammatory process (Löe, Theilade & Jensen, 1965; Theilade *et al.*, 1966; Zachrisson, 1968; Hellden & Lindhe, 1973; Axelsson & Lindhe 1978). The clinical trials by Löe *et al.* (1965) and

Theilade *et al.* (1966), entitled "Experimental gingivitis in man" clearly demonstrated that there is a causal relationship between dental plaque formation and development of inflammation in the gingival tissues. In these studies, after an initial baseline examination subjects were instructed to refrain from all oral hygiene measures for 21 days. All the subjects participated in these studies developed gingivitis in the course of no brushing ranging from 9 to 21 days. When oral hygiene procedures were reinstituted gingival inflammation resolved, in all subjects, within a period varying from seven to eleven days. The histological changes in gingival tissues during experimental gingivitis have also been studied by Zachrisson (1968). After 15-17 days of withdrawal of oral hygiene , when clinical gingivitis developed, histological examination revealed inflammatory infiltrates confined to the connective tissue adjacent to the pocket epithelium. The predominating cells in the infiltrate were medium-large and small lymphocytes, macrophages and fibroblasts. The experimental design of these studies could, however, be criticised, as subjects stopped oral hygiene procedures for different time periods and therefore no statistical analysis of the data could be performed. In addition, some of participants in the Löe *et al.* (1965) and Zachrisson (1968) studies exhibited an initial degree of gingival inflammation and therefore baseline gingival conditions were not standardised. This point was, however, taken into consideration by Theilade

et al. (1966). These investigators were the first to unequivocally show that plaque accumulation causes gingival inflammation.

In order to have a better understanding of histopathologic changes during the development of gingival and periodontal inflammation, Page and Schroeder (1976) have described the progression of gingival and periodontal lesion into four phases namely: *initial, early, established* and *advanced.* The *initial* and *early* lesions represent rather 'acute' stages of gingivitis whereas the *established* lesion depicts more 'chronic' gingivitis. The *advanced* lesion describes the progression of gingivitis into periodontitis.

The *initial* lesion is localised around the gingival sulcus and usually develops after 2-4 days of plaque accumulation, but the vascular changes beneath the junctional epithelium can be seen within 24 hours of plaque accumulation. The increased vascular permeability leads to the migration of polymorphonuclear leucocytes (PMN) from the vessels into the connective tissue and eventually into the gingival crevice. Destruction of perivascular collagen is seen at this stage.

The *early* lesion develops within 4-7 days of plaque accumulation. At this stage, the vessels of the coronal portion of the dentogingival plexus remain dilated while the number of functioning vessels increase. With

additional plaque accumulation there is more pronounced infiltration of PMNs and monocytes/ macrophages within the dentogingival epithelium as well as loss of perivascular collagen. Some fibroblasts within the inflammatory cell infiltrate exhibit signs of degeneration and a slight lateral proliferation of the junctional epithelium at its coronal portion is evident at this stage.

For the *established* lesion, according to this model, to occur two to three weeks of plaque accumulation are required. Large numbers of mature plasma cells are seen at this stage of inflammatory process. The shift to plasma cell dominated lesion is the pathognomonic feature of the *established* lesion. As gingivitis develops severe degeneration of gingival fibroblasts is maintained, collagen continues to be lost in the perivascular areas, vascular permeability and exudation increase and proliferation of the junctional epithelium, now referred to as pocket epithelium, continues with rete pegs extending into the connective tissue infiltrate. The pocket epithelium is not attached to the tooth surface. At approximately one month of plaque accumulation, the cellular as well as permeability response appear to plateau. It seems that a balance has been achieved which may last for extended periods of time without the *established* lesion progressing into the *advanced* lesion. The last stage of the inflammatory process is known as the *advanced* lesion. As plaque continues its apical downgrowth

along the crown and cementum of the root surface there is a deepening of the periodontal pocket which is accompanied by the proliferation of junctional epithelium over the detached root surface. The characteristic features of advanced stage are the loss of connective tissue attachment (Sharpey's fibres) as well as alveolar bone loss. Ulceration of the pocket epithelium may also be present and osteoclasts can be found in the areas of bone resorption. The *advanced* lesion is synonymous with periodontitis.

Although this model has mainly evolved through early animal studies (Garant & Mulvihil, 1972; Attström, Graf-de Beerand & Schroeder, 1975; Schroeder & Lindhe, 1975), it has provided a temporal framework for both clinicians and scientists and is helpful for descriptive purposes. Since the above model was first introduced it has dominated descriptions of gingival and periodontal pathology. It should always be remembered that there is no clear cut division in different phases of inflammatory process, when it is considered *in vivo*, and all the above changes could occur simultaneously, with the more chronic features being superimposed on the more acute. Moreover, it has been reported that a much longer period than two or three weeks is required for a plasma cell dominated lesion to appear (Brecx *et al.*, 1988). These authors conducted a six month experimental gingivitis in human in which they observed a continuous increase in the plasma cell fraction of the inflammatory infiltrate with time; but, even after 4-6

months of no oral hygiene, the gingival lesions were not dominated by plasma cells. Thus, the investigators concluded that a period of neglected oral hygiene of more than 6 months may be required to lead to a plasma cell dominated lesion. In their study, PMNs and lymphocytes were the dominant cells in the connective tissue infiltrate even 6 months after oral hygiene measures were abolished. Furthermore, there are always some PMNs and lymphoid cells in gingival connective tissue infiltrate even after extended periods of supervised oral hygiene leading to clinically healthy gingivae (Brecx *et al.*, 1987) suggesting that this may be necessary for the maintenance of gingival health. However, the percentage of PMNs in the gingival connective tissue, in their study, was higher than what was reported by Page and Schroeder (1976) in clinically healthy conditions. Thus, the author did not rule out the possibility that this may be due to continuous mechanical injury caused by meticulous oral hygiene practices.

1.7 Pathogenesis of periodontal disease

Microorganisms that colonize the cementum/enamel or epithelial walls of sulcus/pocket could cause injury to the periodontal tissues in both direct and indirect ways.

1.7.1 Direct injury

In a classical infection, damage to the host is initiated

by microorganisms adhering and invading the involved tissues. This phenomenon has been shown in cases of acute ulcerative gingivitis where the presence of spirochetes has been documented in the dentogingival epithelium (Listgarten, 1965). The bacterial invasion of gingival tissues in some cases of advanced periodontitis has been reported (Frank, 1980; Saglie *et al.*, 1982). It has recently been shown that *Porphyromonas gingivalis* is able to invade epithelial cell layers and multiply intercellularly in an *in vitro* system (Papapanou *et al.*, 1994). However, the presence of bacteria within the gingival tissues has been challenged by other investigators (Liakoni, Barber & Newman, 1987; Newman 1990), as whether this indicates active invasion of the tissues by the microorganisms or represent, their passive translocation during the late stages of disease. Further studies are needed to resolve this controversial issue. Even if true invasion of gingival tissues by microorganisms does not occur, they may still cause tissue damages in two different ways (Birkedal-Hansen, 1993); a) periodontal pathogens elaborate proteolytic enzymes capable of degrading stromal structures (Robertson *et al.*, 1982; Birkedal-Hansen *et al.*, 1988); b) microorganisms produce a variety of substances which may directly trigger resident and immigrant cells for the release or secretion of degenerative enzymes. Such substances include the leucotoxin produced by *Actinobacillus actinomycetemcomitans* which can cause release of collagenase from PMNs (Baehni *et al.*, 1979).

Proteinases of *Porphyromonas gingivalis* can cause tissue destruction by inducing expression of matrix metalloproteinases (MMPs) by mucosal keratinocytes and fibroblasts (Birkedal-Hansen *et al.*, 1984).

1.7.2 Indirect injury

Antigenic, chemotactic and toxic factors, derived from dental plaque, indirectly damage the periodontal tissues. It has been reported that bacterial endotoxin can penetrate into the gingival tissues in dogs through intact crevicular epithelium (Schwartz, Stinson & Parker, 1972). Indirect injury to the host tissues occurs via provocation of an inflammatory response that induces cytokine release from host cells, which in turn activate one or more of the following degradative pathways (Birkedal-Hansen, 1993).

Plasminogen-dependent pathway- Plasmin (Pln) is a serine proteinase that is converted from its inactive circulating precursor form, plasminogen (Plg), by specific activating enzymes, plasminogen activators. The activating proteinases are expressed by a wide range of stromal, epithelial and endothelial cell types in response to growth factors and cytokines. The activated enzyme, plasmin, cleaves a range of extracellular matrix substrates, but not interstitial collagen fibres (Birkedal-Hansen *et al.*, 1993).

PMN-serine proteinase pathway- PMN leucocytes, when are triggered, may mediate the degradation of extracellular matrix macromolecules by releasing two serine proteinases, neutrophil elastase and cathepsin G. These enzymes have some level of activity against several extracellular matrix proteins but do not cleave interstitial collagens (Birkedal-Hansen *et al.*, 1993).

Phagocytic pathway- Fibroblasts, macrophages and epithelial cells, under certain circumstances, possess the capability of phagocytosing fragments of collagen fibrils which are subsequently degraded in their phagosomes. This process is performed by thiol proteinases. However, it is difficult to reconcile the intracellular and extracellular pathways. One possibility is that the fragments of fibrils may be excised by a collagenase-dependent reaction and are subsequently internalised by the cells for digestion in phagosomes (Birkedal-Hansen *et al.*, 1993).

Osteoclastic bone resorption- The enzymatic mechanisms of organic matrix removal from bones and teeth is not completely understood. However, according to a proposed hypothesis, osteoblasts initiate the resorptive process by dissolution of the layer of osteoid using a collagenase-dependent proteolytic process which leads to the exposure of the of the underlying mineralized bone surface. The vacating osteoblasts or the dissolving bone surface release some signals which, perhaps, attract osteoclast precursors

to the denuded mineral surface, where they differentiate to osteoclasts. It is thought that osteoclasts release acidic thiol-proteinases into the sealed microenvironment between the undulating osteoclast plasma membrane and the bone surface which are capable of degrading the collagenous matrix at low pH (Birkedal-Hansen *et al.*, 1993).

Matrix metalloproteinases pathway- Matrix metalloproteinases (MMPs) are a family of 9 or more metal-dependent homologous enzymes which are able to degrade almost all extracellular matrix macromolecules. MMPs are involved in the extracellular matrix destruction and remodelling in both physiological and pathological conditions. These proteinases are released/produced by immigrant/resident host cells (Birkedal-Hansen *et al.*, 1993; Birkedal-Hansen, 1993). The role of MMPs in the pathogenesis of periodontal disease, their structure, cellular sources, regulation and ways of activation will be explained in section 1.7.6.1.

Apart from the above pathways, depression of the host immune response during the initial stages of exposure to dental plaque has been suggested to lead to periodontal destruction (Shenker, 1987). Autoimmunity to collagen components has also been suggested to contribute to the pathogenesis of periodontal disease (Hirsch, 1988). Anusaksathien and Dolby (1991) have suggested that autoantibodies detected in periodontal disease are derived

from natural antibodies and their role is the elimination of dead cells and damaged tissue constituents which appear in the disease process. However, they did not rule out the possibility that this procedure which deals with the consequences of tissue damage, may in certain circumstances become excessive and contribute to the progress of disease.

1.7.3 The role of inflammatory mediators in the pathogenesis of periodontal disease (indirect injury by bacteria)

As has been discussed in the previous section, bacteria induce periodontal tissue destruction indirectly by activating host defense cells. The activated cells including infiltrated leucocytes and plasma cells, resident fibroblasts and other connective tissue cells, in turn produce or release mediators that stimulate the effectors of connective tissue breakdown. Interleukin 1 (IL-1) and tumour-necrosis factor α (TNF-α) have an important role in this process (Birkedal-Hansen *et al.*, 1993). Two distinct forms of IL-1 exist designated as IL-1α and IL-1ß. This cytokine is produced predominantly by macrophages, lymphocytes and PMNs following activation by lipopolysaccharide (LPS); it can also be released from fibroblasts, keratinocytes and endothelial cells. IL-1 has a variety of activities including: a) inducing formation of new osteoclasts and causing bone resorption by activating osteoclasts; b) inducing fibroblasts and macrophages for

production of MMPs and urokinase type plasminogen activator; c) induction of prostaglandin E_2 (PGE_2) synthesis by macrophages and fibroblasts; and d) attraction of the inflammatory cells into the sites of inflammation (reviewed by Page, 1991). IL-1α and IL-1ß has been detected in GCF from patients with periodontitis (Masada *et al.*, 1990). Moreover, IL-1 activity in GCF, measured by a bioassay in an experimental gingivitis study, peaked prior to the development of clinically detectable inflammation (Kinane *et al.*, 1992). The levels of IL-1α and IL-1ß were found to be significantly higher in periodontitis sites than in clinically healthy sites (Stashenko, *et al.*, 1991). Comparing GCF levels of IL-1ß from healthy, gingivitis and periodontitis sites within and between different groups of subjects, it has been shown that the level of IL-1ß was significantly higher in diseased sites than healthy sites. However, there was no significant difference between GCF levels of IL-1ß from gingivitis and periodontitis sites (Ebersole *et al.*, 1993). TNF-α is produced by macrophages activated by LPS. Although not as potent as IL-1, TNF-α shares its properties. Stashenko *et al.* (1991) reported that periodontally affected sites demonstrated higher levels of TNF-α compared to healthy sites. Prostaglandins, the metabolites of arachidonic acid, are also implicated in the inflammatory process. The concentration of PGE_2 in GCF above certain thresholds may be used as an indicator of increased patient susceptibility to future periodontal breakdown (Offenbacher *et al.*, 1986). It has also been

shown that topical application of PGE_2 to the gingival sulcus in rats resulted in increased bone resorption by activation and increase of osteoclasts (Miyauchi *et al.*, 1992). It has recently been reported that PGE_2 levels were significantly higher in GCF samples from gingivitis and periodontitis sites than those from healthy sites (Ebersole *et al.*, 1993). The role of these mediators in regulation of MMPs and TIMP will be discussed in sections 1.7.6.4 and 1.7.7.4 respectively.

However, the exact mechanisms leading to periodontal destruction, are interwoven, extremely complex and in a constant state of reevaluation.

1.7.4 The role of polymorphonuclear leucocytes in the pathogenesis of periodontal disease

The polymorphonuclear leucocyte (PMN) is a short lived white blood cell with a lobulated nucleus and numerous granules within its cytoplasm. PMN granules mainly contain microbicidal enzymes, lactoferrin, collagenase, gelatinase, neutral proteases and lysozyme (Bainton, Ullyot & Farquhar, 1971).

The PMNs are the predominant leucocytes within the gingival crevice in both healthy and diseased sites which are attracted, from circulation, to the area via chemotactic stimuli elicited from dental plaque (Hellden & Lindhe,

1973). They can also be seen traversing the inflamed gingival connective tissue (Seymour *et al.*, 1983; Brecx *et al.*, 1988; Moughal *et al.*, 1992). In an experiment on dogs, Attström & Egelberg (1970) have shown that carbon labelled peripheral blood neutrophils from the circulation migrate into the gingival crevice and their migration rate appears to be higher in inflamed crevices. Using experimental gingivitis model, it has been reported that the PMN numbers increase in the gingival crevice with the development of experimental gingivitis (Kowashi, Jaccard & Cimasoni,1980; Thurre *et al.*, 1984). The number of PMNs was found to be significantly higher in periodontitis sites than in control sites (healthy and gingivitis), although their viability and phagocytosis ability were lower in the periodontitis sites (Thurre *et al.*, 1984). Adhesion molecules on leucocytes are essential in their migration from the vessels into the gingival tissue, and through the junctional epithelium into the gingival crevice. Moughal *et al.* (1992), in an experimental gingivitis study, showed that the connective tissue vessels express ELAM-1 and ICAM-1 in clinically healthy as well as in inflamed gingivae and PMNs were found in greater concentration in areas expressing intense ELAM-1 and ICAM-1 staining. Furthermore, junctional epithelium and its adjacent connective tissue as well as sulcular epithelium stained strongly positive for ICAM-1, suggesting the importance of this adhesion molecule in PMN migration into the gingival crevice.

PMNs play an important role in host defence and form the first line of defence in the gingival sulcus against periodontal bacteria. These are evidenced by the observations that quantitative (neutropenia) and qualitative (chemotactic or phagocytic) PMN deficiencies result in severe periodontal destruction (Wilton *et al.*, 1988; Kinane & Davies, 1990). There are also evidences suggesting that PMNs may damage periodontal tissues by releasing their lysosomal contents into the extracellular environment. Several factors, including bacterial toxins, regurgitation during feeding and phagocytosis, attachment to surface-bound immune complexes, adherence to the surfaces during *in vivo* and *in vitro* migration and cell death and subsequent lysis, could stimulate PMN leading to its degranulation (Wilton, 1986). PMNs, therefore, release the contents of their granules as they emigrate towards the gingival crevice as well as during phagocytosis or encounter of antigen antibody complexes and bacterial toxins. Henson (1971a), in an *in vitro* study, reported that neutrophils when adhered to antigen-antibody complexes or phagocytosed particles coated with antibody or complement released their granular contents. In a successive report, Henson (1971b) has described the mechanisms by which the release of PMN granular constituent occurred. The author has stated that neutrophils adherent to immune complexes released their enzymes by direct extrusion, whereas in the case of PMNs phagocytosing particles, degranulation was into the phagocytic vacuole

which was subsequently opened to the outside of the cells. It has been shown that PMNs interact with oral bacteria and release their lysosomal contents into the extracellular milieu (Taichman *et al.*, 1977; Tsai *et al.*, 1978). It has been reported that PMNs incubated with serum have increased the lysosomal release indicating that bacterial opsonization by complement components and immunoglobulins could enhance the process (Baehni *et al.*, 1977; Taichman *et al.*, 1977; Baehni *et al.*, 1978). It appears, therefore, that although PMNs play a protective role in the gingival crevice, the interplay between microbial plaque components, complement components, immune complexes and PMNs, initiates the release of PMN granular contents which could cause damage to the periodontal tissues (Ishikawa *et al.*, 1982; Wilton, 1986; Deguchi *et al.*, 1990; Altman *et al.*, 1992). Thus, the PMN appears to have both a protective and potentially destructive role in the pathogenesis of periodontal disease and should be viewed as a double edged sword. Two models have been suggested to explain this rather contradictory issue.

The first model, in chronological order, proposed by Page and Schroeder (1982) involves gingival connective tissue PMNs. In this model two compartments have been suggested for PMN function, namely the gingival connective tissue and the crevice/pocket. Under normal conditions, a constant stream of PMNs leaving the gingival blood vessels is guided across the connective tissue and junctional epithelium into

the gingival crevice by a gradient of chemotactic agents produced by most of the bacteria present in dental plaque. In a clinically healthy situation, the transforming neutrophils leave no trace of their passage and cause no tissue damage. These PMNs form the first line of defence against dental plaque around the teeth. If the epithelial barrier is disrupted, perhaps by the increasing numbers of PMNs passing through the junctional and pocket epithelium as a result of subgingival plaque extension, then an open entrance for bacterial component and metabolites which flood the connective tissue is established and the chemotactic gradient, driving neutrophils towards the gingival crevice/pocket, is disrupted. As a consequence, PMNs consequently remain in connective tissue and randomly move, where they encounter bacteria and their substances which flooded the connective tissue because of the rupture of the epithelial barrier. Thus, PMNs become activated inside the connective tissue and undertake phagocytosis, resulting in release of lysosomal contents which together with other activated systems and host cells cause extensive tissue damage. Once the epithelial barrier and the chemotactic gradient are re-established the destructive process subsides and PMNs are again guided towards the sulcus/pocket. The second model suggested by Wilton (1986), involves crevicular PMN function which can be viewed as a two phase system: fluid and surface. In the fluid phase, PMNs phagocytose plaque bacteria, while their released enzymes can inhibit bacterial attachment and/or

remove attached bacteria. Enzymes released during this process can be neutralised by protease inhibitors present in the area. Contrary to the fluid phase, in the surface phase PMNs attach to the plaque surface or the sulcular and junctional epithelium, where they release their enzymes and oxygen metabolites. Since, this process takes place in a closed environment, inaccessible to protease inhibitors (Wright & Silverstein, 1984), could result in damage to the epithelium and connective tissue. However, both the above models are over simplifications of the processes leading to periodontal destruction, but they still provide both the clinician and researcher with some valuable information as how the PMNs contribute to the pathogenesis of periodontal disease.

1.7.5 Proteinases in pathogenesis of periodontal disease

Proteinases (endopeptdases) have been extensively investigated regarding their possible role in the pathogenesis of periodontal disease. These proteinases are believed to be effective in periodontal destruction. PMNs and monocyte/macrophages are the main source of these enzymes, although other host cells such as fibroblasts, keratinocytes, epithelial and endothelial cells have some contribution.

Over the past two decades various studies including cross-sectional, experimental gingivitis and longitudinal studies before and after periodontal treatment, have been conducted in order to assess proteinases activity/amount in GCF. In most cases proteinases activity has been assessed by using either native proteins or chromogenic and fluorogenic substrates, whereas immunoassays have been used for the assessment of the amount of the enzymes.

Proteinases of bacterial origin have also been implicated in the pathogenesis of periodontal disease. Several putative periodontal pathogens including *P. gingivalis* and *A. actinomycetemcomitans* demonstrate proteolytic activity (Robertson *et al.*, 1982 and Birkedal-Hansen *et al.*, 1988). However, it has been questioned whether these enzymes could penetrate the periodontal tissues and maintain sufficient concentrations to destroy periodontal tissues (Kelstrup & Theilade, 1974; Birkedal-Hansen *et al.*, 1988). Furthermore, it has been shown that the predominant collagenase in GCF are of host origin as they cleave the collagen molecule into three and one quarter fragments, which is characteristic of vertebrate collagenase function (Villela *et al.*, 1987; Sorsa *et al.*, 1990).

As mentioned briefly in section 1.7.2, connective tissue degradation in destructive periodontal disease is partially caused by matrix metalloproteinases, which will be reviewed in detail in the following section.

1.7.6 Proteinases: Matrix metalloproteinases (MMPs)

1.7.6.1 Definition, characteristics and nomenclature

MMPs are members of a family of homologous zinc contained proteinases of animal origin which act outside the cell on components of the extracellular matrix (Nagase, Barreett & Woessner, 1992a).

Woessner (1991) outlined the main characteristics of MMPs as follows: a) their catalytic mechanism depends on zinc at the active centre; b) the proteinases are secreted/released in zymogen form; c) the pro-enzyme can be activated by proteinases or by organomercurials; d) their activation is accompanied or followed by a loss of M_r of about 10,000; e) their cDNA sequences all show similarity to that of collagenase; f) the enzymes cleave one or more components of the extracellular matrix; g) the enzymes' activity is inhibited by tissue inhibitors of metalloproteinases (TIMPs).

The term "matrixin" has been suggested to designate this family of enzymes. "Collagenase gene family" is another term proposed for this group of proteinases. It seems that these terms have not been accepted as the majority of investigators in the field use the term "matrix metalloproteinases" to name the family of enzymes. Individual MMPs have been named after their substrates in

Table 1.1 Matrix metalloproteinase (MMPs)

Enzyme	Abbre.	MMP#	M_r	Substrate
Fibroblast collagenase	FIB-CL	MMP1	57	Collagen I, II,III, (III>>I), VII, VIII, X, gelatin
M_r 72K gelatinase type IV collagenase	M_r 72K GL	MMP2	72	Gelatin, collagen IV, V, VII, X, XI elastin, fibronectin
Stromelysin-1	SL-1	MMP3	60 55	Fibronectin, laminin, collagen IV, V, IX, X, elastin pro-collagenase
Putative metallo-proteinase-1	PUMP-1	MMP7	28	Fibronectin, laminin, collagen IV, gelatin, pro-collagenase
PMN-type collagenase	PMN-CL	MMP8	75	Same as FIB-CL (I>>III)
M_r 92K gelatinase type IV collagenase	M_r 92K GL	MMP9	92	Gelatin, collagen IV, V, elastin
Stromelysin-2	SL-2	MMP10	60 55	Same as SL-1
Stromelysin-3	SL-3	MMP11	*	*

*= not determined
M_r= molecular weight (kD)

Modified from Birkedal-Hansen (1993) and Birkedal Hansen *et al.* (1993).

the extracellular matrix, mostly different types of collagen. Stromelysin has been named after its origin, the stromal cell (Chin, Murphy & Werb, 1985). Some authors have suggested that the members of MMPs be designated by a sequence of numbers, therefore the enzymes' names would be: matrix metalloproteinase 1, which is abbreviated MMP-1 (Nagase *et al.*, 1992a). However, at present both systems are used by workers. Table 1.1 shows the names of the MMPs as well as their correspondent numbers.

1.7.6.2 Sources of matrix metalloproteinase

Some MMPs are produces by resident cells such as fibroblasts from different sources, keratinocytes, endothelial cells, chondrocytes and osteoblasts. These enzymes are synthesized on demand by initiating transcription of the corresponding genes. This process causes a lag period of 6 to 12 hours before enzymes can be detected in the extracellular environment, but once that starts, production and secretion can be sustained for several days. PMNs are the other source of MMPs. The enzymes are synthesized during the development of a PMN and stored in its specific granules. The proteases are rapidly released when the cell is triggered. The PMN responds in full force but the destructive activity can not be sustained beyond minutes. A prolonged response is achieved, when needed, by continuous recruitment of new cells (reviewed by Birkedal-Hansen *et al.*, 1993).

1.7.6.3 Modular structure of matrix metalloproteinases

All MMP are regarded as derivatives of the five-domain modular structure, characteristic of collagenases and stromelysins, formed either by addition or deletion of domains (Fig. 1.1). Among these domains are the catalytic domain that contains the Zn^{++} binding site and a COOH terminal domain which plays a role in determining substrate specificity (reviewed by Birkedal-Hansen, 1993; and Birkedal-Hansen *et al.*, 1993).

1.7.6.4 Regulation of matrix metalloproteinases

The activity of MMPs in the extracellular environment is regulated in four different ways namely: a) transcriptional regulation of MMP genes; b) precursor activation; c) differences in substrate specificity; and d) MMP inhibitors.

Transcriptional regulation. Cytokines and growth factors play an important role in the regulation of most of MMPs at transcriptional level. Thus, stimulation or repression of the MMP genes by these mediators gives cause to 20 to 50 fold change in mRNA and protein levels. Interleukin-1α and ß (IL1-α and ß) and tumour necrosis factor-α (TNF-α) increase the enzyme production (Meikle *et al.*, 1989), while interferon-γ (IFN-γ) down-regulates MMPs expression. Transforming growth factor-ß (TGF-ß) decreases some MMPs'

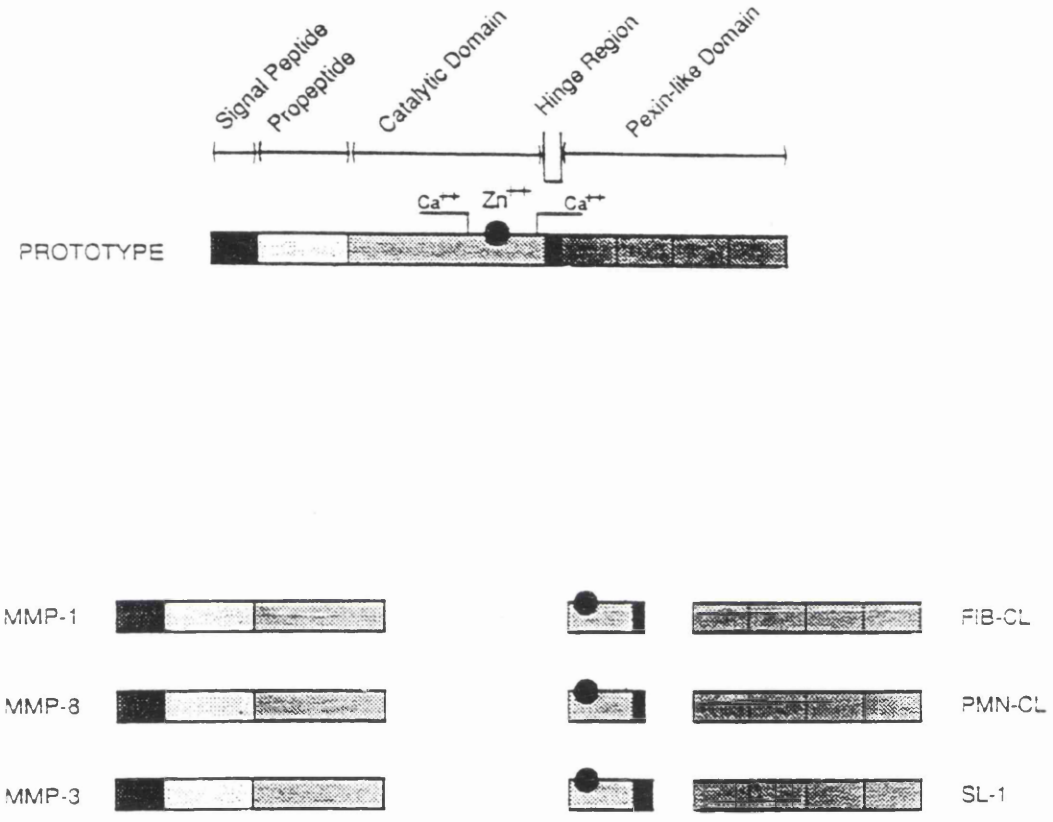

Figure 1.1 Domain structure of matrix metalloproteinases Modified from Birkeda-Hansen et al. (1993).

production and increases others (Birkedal-Hansen, 1993). TGF-ß1 has been shown to reduce enzymes synthesis (Overall, Wrana & Sodek, 1991).

Activation of precursor. MMPs are secreted/released in a latent form. The activation process of the precursors in the extracellular space, therefore, is another step in the regulation of MMPs (Nagase *et al.*, 1992b). The mechanism of activation of MMP precursors is reviewed in section 1.7.6.5

Substrate specificity. Despite somewhat overlapping substrate specificities, the MMP activity is regulated, to some extent, at the substrate level. The ability of FIB-CL and PMN-CL in dissolving type I, II and III collagens is not shared by other MMPs (Birkedal-Hansen *et al.*, 1993).

Inhibition. The activity of MMPs is regulated by protease inhibitors. α2-macroglobulin (α2-M) plays an important role in the regulation of MMP activity by the rapid capture of the enzymes (Birkedal-Hansen, 1993). Tissue inhibitors of metalloproteinases (TIMPs) act in the very local area around the cell from which metalloproteinases are secreted (Cawston, McLaughlin & Hazelmanet, 1987) and thus, regulate the activity of MMPs.

1.7.6.5 Activation of matrix metalloproteinase

As mentioned in section 1.7.6.1. MMPs are synthesized as proenzymes and are presumably activated by certain physiological agents after secretion into the extracellular environment. Organomercurials, trypsin and plasmin have been shown to activate pro-MMPs *in vitro* (Grant *et al.*, 1992; Nagase *et al.*, 1992b). Although MMPs can readily be activated in vitro, the biologic activation mechanisms are still poorly understood. Since organomercurials and trypsin are not present in the tissues, it is likely that *in vivo* activation is caused by plasmin. Plasminogen may enter the tissues from the circulation, and the mesenchymal cells can produce plasminogen activator (Woessner, 1991). Murphy *et al.* (1992) have reported that the presence of both plasminogen and cell layers was necessary for MMP activation indicating a plasmin dependent mechanism.

Activation of FIB-CL with incubation by organomercurials or serine proteinases (trypsin and plasmin) results in a partially active enzyme (Mr 43000) which, when was further incubated with SL converted to a fully active enzyme (Mr 41000). The incubation of FIB-CL with SL alone for a long time (96 hours) results in fully active enzyme (Nagase *et al.*, 1992b). However, Woesssner (1991) has questioned the *in vivo* validity of these observations. Furthermore, Sorsa *et al.* (1992a) have shown that proteases produced by periodontopathogenic bacteria such as *P. gingivalis* can

activate latent FIB-CL and to a lesser extent PMN-CL.

1.7.6.6 The role of matrix metalloproteinases in the pathogenesis of periodontal disease

Soon after the discovery of collagenase in the involuting tadpole tail in the early 60s, which was followed by the identification of a similar enzyme in a mammalian tissue (Birkedal-Hansen, 1988), a new line of investigation was introduced in the periodontal field by the pioneer work of Fullmer and co-workers (1966) who reported collagenase activity in cultures of human diseased gingival tissues. Later, Fullmer *et al.* (1969a) reported work in gingival tissues which indicated that both epithelial cells and inflamed gingival connective tissue are capable of producing collagenase in tissue culture. Collagenase production has been reported to be higher in inflamed gingival specimens than those obtained from clinically healthy gingivae (Fullmer *et al.*, 1969b). Ohlsson and co-workers (1973) have reported that collagenase activity was found to be 7 times higher in material collected from inflamed gingiva compared to those from healthy gingiva. The enzyme was of PMN origin. However, in this experiment two groups of healthy and inflamed samples were taken from two different groups of individuals thus, not allowing the host response to be taken into account. Presence of collagenase has been demonstrated in homogenates of gingiva obtained from periodontitis patients by measuring

collagenase activity (Uitto, Turto & Saxen, 1978). Immunolocalization of collagenase demonstrated that gingival biopsies taken from patients with periodontal disease showed some immunoreactive enzyme, although relatively little in most of the samples, whereas gingival specimens obtained from treated subjects expressed no significant immunoreactive enzyme (Woolley & Davies, 1981). Heath *et al.* (1982) using radio-labelled substrates demonstrated that explants of gingival tissue from periodontally affected patients were able to synthesize three MMPs, collagenase, gelatinase and stromelysin in culture. The relationship between collagenase in GCF and disease activity has been investigated by evaluating the changes in collagenase activity before and after treatment in a group of patients with localized juvenile periodontitis (LJP)(Larivèe, Sodek & Ferrier, 1986). The LJP patients received periodontal treatment including scaling and root planing, antibiotics (tetracycline for 21 days) and flap surgery. The enzyme activity for active collagenase showed a significant reduction 5 weeks after periodontal surgery, whereas the total enzyme activity remained high after surgical treatment (5 weeks) and then dropped significantly, at the recall visit, 6 months after the last surgery. In another attempt, the association between GCF collagenolytic activity and disease severity has been investigated in four groups of subjects with gingivitis, adult periodontitis, localized juvenile periodontitis as well as periodontally healthy controls

(Villela *et al.*, 1987). This study showed that collagenolytic activity in GCF increases with the severity of disease in the order: healthy < gingivitis < periodontitis. Collagenase and gelatinase activity in mouthrinse samples has also been assessed from patients with AP and LJP as well as healthy controls, before and after periodontal therapy. A number of edentulous subjects were also sampled as an additional control to rule out the possibility of enzymes being derived from any source other than gingival crevice (Gangbar *et al.*, 1990). AP patients received oral hygiene, scaling and root planing whereas subjects with LJP received antibiotics (tetracycline) as well. Some of the patients in both groups underwent surgical therapy. The authors reported that periodontitis patients had significantly higher active enzyme activity than healthy subjects whereas edentulous individuals showed neither active nor latent enzyme activity. Both collagenase and gelatinase activity were reported to be reduced following treatment which was significant only for active collagenase after scaling and antibiotic therapy in LJP patients. From an attempt to correlate GCF collagenase activity with disease progression in a group of patients with recurrent periodontal disease, Lee *et al.* (1991) reported that the finding of active collagenase at a site is associated with recent disease activity. Uitto, Suomalainen and Sorsa, (1990) conducted an experiment on salivary collagenase which was observed to be mainly of PMN origin. They also reported that in healthy individuals

collagenase was partially latent, whereas in saliva obtained from periodontitis patients it was mostly in an active form and collagenase activity was found to be higher in saliva from diseased than from healthy subjects. The collagenase activity reduced significantly after treatment. Collagenase in GCF from adult periodontitis patients has been reported to be PMN derived while, GCF collagenase in subjects suffering from juvenile periodontitis seems to be of fibroblast origin (Suomalainen *et al.*, 1991, 1992). Sorsa and coworkers (1992b) have also suggested that GCF collagenase in LJP patients is fibroblast derived while in subjects suffering from diabetes mellitus, the enzyme is mostly from PMN origin. These authors state that the collagenase in LJP patients is of fibroblast-type because of the reported impaired PMN function. This seems questionable as the dysfunction of PMNs in LJP patients is in dispute (Kinane *et al.*, 1989a, b); and it has been found that collagenase in patients suffering from diabetes is of PMN origin (Sorsa *et al.*, 1992) whilst this group of patients are reported to have impaired PMN function (Manouchehr-Pour *et al.*, 1981a, b). Scaling and root planing has been demonstrated to reduce collagenase activity and the proportion of active enzyme in GCF (Suomalainen, 1992). By the use of reverse transcription polymerase chain reaction (RT-PCR), Nomura *et al.* (1993) reported a higher FIB-CL mRNA levels in gingivae from periodontitis patients than those from healthy subjects. In a recent study, using mouthrinses, Ingman *et al.* (1993)

investigated the origin of the salivary collagenase and the enzyme activity in patients with adult and juvenile periodontitis as well as healthy subjects. These workers found the salivary collagenase activity in LJP patients was comparable to collagenase activity detected in healthy subjects, whereas it was significantly higher in AP patients. They also suggested that the enzyme was of fibroblast origin in LJP patients. Another recent study carried out by the same group (Ingman *et al.*, 1994), revealed that both saliva and GCF collected from different groups of subjects contain gelatinase of PMN as well as fibroblast origin. Moreover, immunolocalization of both fibroblast and PMN collagenases and stromelysin has shown that PMNs in inflamed connective tissue and gingival granulation tissue specimen obtained from AP patients contain PMN-CL, whereas FIB-CL and SL were detected in fibroblasts and macrophages from granulation tissues only. However, control specimens showed no immunoreactivity for either of the enzymes (Ingman, 1994). Moreover, Meikle *et al.* (1994), using the same technique, reported that in gingival biopsies obtained from periodontally affected patients fibroblasts, macrophages and epithelial cells contained fibroblast type collagenase and gelatinase as well as SL. A significant reduction in collagenase activity in saliva after periodontal therapy has been reported by Hayakawa *et al.* (1994). These workers have also reported that most of the total collagenase in saliva of healthy subjects was in latent form, whereas mainly

active collagenase was present in saliva from periodontally affected patients.

However, care should be exercised when different studies on MMPs in which, subjects with distinct forms of disease participated and different proteins have been analyzed and various methods of GCF collection, laboratory techniques, expression of results have been employed, are being compared.

1.7.7 Inhibition of matrix metalloproteinases

The control of extracellular proteolysis is of great importance which is accomplished by protease inhibitors either locally produced or circulating in plasma. Tissue inhibitors of metalloproteinases (TIMPs) form the first line of defence against MMPs in a local area whereas the second line of defence is found in the serum by α2-macroglobulin (α2-M)(Woessner, 1991). This protease inhibitor is an excellent substrate for FIB-CL which inactivates the enzyme rapidly. SL also binds to α2-M, but at a much slower rate than collagenase (Enghild *et al.*, 1989). It has been suggested that α2-M is very effective where the local supply of TIMP is exhausted and so can act as a back-up control mechanism to prevent connective tissue breakdown for a prolonged period of time (Cawston *et al.*, 1987).

1.7.7.1 Tissue inhibitors of metalloproteinase (TIMPs)

Tissues contain members of TIMP family which form non-covalent bonds with active forms of MMPs and, in some instances, with latent enzymes as well (Birkedal-Hansen, 1993). These family of inhibitors have low molecular weight (28.5 kDa for TIMP1 and 21 kDa for TIMP2)(Banda *et al.*, 1992) and are produced by most of the cells capable of secreting FIB-CL and SL (MacNaul *et al.*, 1990). TIMP1 appears to preferentially inhibit FIB-CL and SL (Banda *et al.*, 1992), whereas TIMP2 is more effective against gelatinases (Birkedal-Hansen, 1993). A new inhibitor named large inhibitor of metalloproteinases (LIMP) with a molecular weight of 76 kD has been purified from fibroblast culture medium which inhibits fibroblast-type collagenase and gelatinase as well as SL and is distinct from TIMP (Cawston *et al.*, 1990). The role played by TIMPs in regulating matrix degradation is exerted by proteinase elimination as well as by blockage of MMP activation (Birkedal-Hansen *et al.*, 1993).

TIMP production by fibroblasts is upregulated by interleukin-1ß (IL-1ß) as well as transforming growth factor ß1 (TGF-ß1), whereas it is suppressed in the presence of interferon-γ (IFN-γ) in conjunction with IL-1ß; however, IFN-γ alone has no effect on TIMP expression (Unemori *et al.*, 1991).

Therefore the interaction of cytokines and growth factors with resident cells of periodontium in regulation of MMPs (reviewed in section 1.7.5.5) and TIMP, which seems very complicated, may determine whether repair or destruction of periodontium will occur and why gingival inflammation may or may not turn to periodontitis.

1.7.7.2 Tissue inhibitor of metalloproteinases in periodontal disease

Little attention has been paid to TIMP in the periodontal field than other proteinase inhibitors. In an early *in vitro* study Heath and co-workers (1982) showed that human diseased gingival tissues (fibroblasts) could produce TIMP. Larivèe *et al.* (1986) reported that GCF collected from healthy subjects and healthy sites within LJP patients had significantly higher inhibitor than diseased sites in LJP patients and there was a significant increase in inhibitor activity of diseased sites after treatment. The authors stated that they excluded blood contaminated samples in order to analyze TIMP activity which is produced locally. However, considering the method they used for GCF collection, the chance that samples being contaminated with serum can not be ruled out thus, resulting in the analysis of other inhibitors as well. These results are in agreement with the findings of Hayakawa *et al.* (1994) who measured the concentration of TIMP in saliva. Using reverse transcription polymerase chain reaction (RT-PCR),

Nomura *et al.* (1993) reported a higher mRNA levels for TIMP1 in gingivae from periodontitis patients than those from healthy subjects, while there was no difference in the level of TIMP2 mRNA between healthy and periodontitis sites. More recently, Meikle *et al.* (1994) have been able to immunolocalize TIMP1 within gingival connective tissue cells in specimens harvested from periodontitis patients.

In conclusion, recent knowledge regarding the function of the MMPs and TIMP in tissues has been gathered via measurements of activity in culture media or tissue extracts, immunoassays of protein or amplification of mRNA which is circumstantial.

However, the presence of these enzymes in higher level/activity in diseased than healthy sites (Ohlsson *et al.*, 1973; Heath *et al.*, 1982), their increase during experimental gingivitis (Kowashi *et al.*, 1979) and decrease after periodontal treatment (Larivèe *et al.*, 1986 and Makela *et al.*, 1991) strengthens the case for MMPs involvement in periodontal tissue breakdown. Among the MMPs both PMN and FIB-CL have the unique ability of cleaving the triple helix of type I, II and III collagens, thus initiating extracellular matrix degradation which is not shared by the other members of the family (Woessner, 1991). PMN-CL, is thought to be carried into the crevice in migrating PMNs (Birkedal-Hansen *et al.*, 1993) thus reflecting the number of emigrating PMN into the crevice

rather than the tissue destructive potential (Birkedal-Hansen, 1993). The ability of SL in activating FIB-CL and the preference of TIMP-1 to inhibit the aforementioned enzymes (Banda *et al.*, 1992) along with the fact that all these components are secreted by resident cells (MacNaul *et al.* 1990) suggest, these enzymes and enzyme inhibitor are of particular interest in GCF.

TIMP-1 is referred to as 'TIMP' throughout this manuscript.

CHAPTER 2

MATERIALS &

METHODS

2.1 Subjects

Prior to the commencement of these studies ethical approval was granted from the local ethical committee. Subjects participating in these studies were informed of the protocol and consent was obtained. All participants in the longitudinal experiment were given the option of withdrawing from the study at any time.

These individuals had no history of systemic conditions which could influence the course of periodontal disease. They were not on any medications that could affect the manifestations of periodontal disease, such as chronic antibiotic use, phenytoin, cyclosporin and calcium channel blockers.

2.1.1 Cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid

Forty patients attending the Periodontal Clinic of the Glasgow Dental Hospital participated in this study, of which 15 subjects were newly referred to the Dental Hospital whereas, the other 25 were among periodontal patients on maintenance care. Of these subjects 26 were female and 14 were male with a mean age of 43.1 ranging from 21-71 years.

2.1.2 Longitudinal study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid before and after treatment

Twenty six individuals, newly referred to the Periodontal Clinic of Glasgow Dental Hospital with advanced periodontal disease (pocket depths greater than 5.5mm in all quadrants) took part in this study. 4 subjects excluded themselves from this study due to poor compliance and one individual had to withdraw from the study because of her pregnancy. Of 21 patients who remained in this study 11 were female and 10 were male with a mean age of 43.9 ranging from 33 to 53 years.

2.2 Buffers and reagents

All chemicals were analytical grade and were obtained from BDH Chemicals Ltd., Poole, Dorset, England, unless otherwise stated.

2.2.1 Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8g NaCl, 0.2g KH_2PO_4, 0.2g KCl, 1.44g Na_2HPO_4 . 2 H_2O up to 1 litre of distilled water (pH 7.4).

2.2.2 Coating buffer (CB)

Carbonate-bicarbonate buffer prepared by dissolving 1.59g Na_2CO_3 and 2.93g $NaHCO_3$ up to 1 litre of distilled water. The pH was adjusted to 9.6 (titration with 1M HCl).

2.2.3 Incubation buffer (IB)

This was prepared by dissolving 8g NaCl, 0.2g KH_2PO_4, 0.2g KCl, 1.44g Na_2HPO_4 . 2 H_2O, 0.5g Tween 20 (SIGMA Chemical Company Ltd., Poole, Dorset), and 1g lyophilised bovine serum albumin (BSA) (SIGMA, St. Louis, USA) up to 1 litre distilled water (pH 7.4).

2.2.4 Wash buffer (PBST)

A stock solution of 10 times concentrated incubation buffer (nil BSA) was prepared. This was diluted 1/10 prior to use.

2.3 Purified standards and antibodies for enzyme linked immunosorbent assays (ELISAs)

Purified recombinant human fibroblast collagenase (FIB-CL), stromelysin (SL) and tissue inhibitor of metalloproteinases (TIMP), as well as Mac 64 and Mac 66 anti-collagenase monoclonal antibodies, Mac 19 and Mac 15 anti-TIMP monoclonal antibodies as well as Mac 78 anti-stromelysin

monoclonal antibody and Rabbit anti-stromelysin polyclonal antibody were donated by Cell Tech. Ltd. Slough, UK. Donkey anti rabbit- HRP conjugated IgG was purchased from Jackson Immuno-research Laboratories, Avondale, PA, USA.

Reagents were replaced regularly to avoid deterioration.

2.4 Clinical methods

2.4.1 Clinical indices

Two calibrated examiners (AH) and (EA) were used throughout these studies to record all clinical indices.

2.4.1.1 The Modified Gingival Index

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

0 Absence of inflammation.

1 Mild inflammation; slight change in colour, little change in texture of any portion but not the entire marginal or papillary gingival unit.

2 mild inflammation; criteria as above but involving the entire marginal or papillary gingival unit.

3 Moderate inflammation; glazing, redness, oedema, and/or hypertrophy of the marginal or papillary gingival unit.

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

This index differs from the commonly used Gingival Index (Löe & Silness, 1963) in that it eliminates the bleeding upon probing/pressure component, and provides increased sensitivity in the lower region of the scoring scale. The MGI was, therefore, preferred because it permits non-invasive evaluation of the severity of gingival inflammation, and thus facilitates GCF sampling. In both studies, unless otherwise stated, site scores refer to a single MGI recording.

2.4.1.2 The Plaque Index

The plaque index (PlI) of Silness and Löe, (1964) was used for recording plaque accumulation. The scoring system for this index is as follows:

0 No plaque in the gingival area.

1 A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may only

be recognised by running a probe across the tooth surface.

2 Moderate accumulation of soft deposits within the gingival crevice, on the gingival margin and/or adjacent tooth surface, which can be seen by the naked eye.

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

In both studies site scores refer to a single site PlI recording.

2.4.1.3 Bleeding on probing

Bleeding upon probing (BOP) was scored dichotomously using the Florida Periodontal Probe. Scoring was performed within 30s of probing.

2.4.1.4 Suppuration

Presence or absence of suppuration was recorded after applying gentle pressure on the gingival wall of the pocket at an apico-coronal direction using a ball burnisher.

2.4.2 Probing depth and attachment level measurements

In the cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid, pocket (PD) depth was recorded to the nearest mm using a manual pressure sensitive probe with a constant force of 25 g.

The Florida Probe (Florida Probe Corporation, Florida, USA) (Gibbs *et al.*, 1988) was used for probing depth and attachment level (AL) recording in the longitudinal study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid before and after treatment. This is an electronic pressure sensitive probe set at 20g. The Florida Probe records PD/AL to the nearest 0.2mm. The system consists of a pocket depth and an attachment level ('stent') handpiece, a foot switch, computer interface and portable personal computer. The measurements of PD and AL are made electronically, using the 'pocket depth' (Fig. 2.1) and 'attachment level, stent' (Fig. 2.2) handpieces respectively, and transferred automatically to the computer when the foot switch is pressed. Recordings are displayed on the computer screen which was turned away from the operator in order to allow 'blind' measurements. In addition, measurements were recorded on paper by the other examiner. The probe tip has a diameter of 0.4mm, no visible graduations along its length and it reciprocates through a sleeve. The edge of the sleeve is the reference from which measurements are recorded.

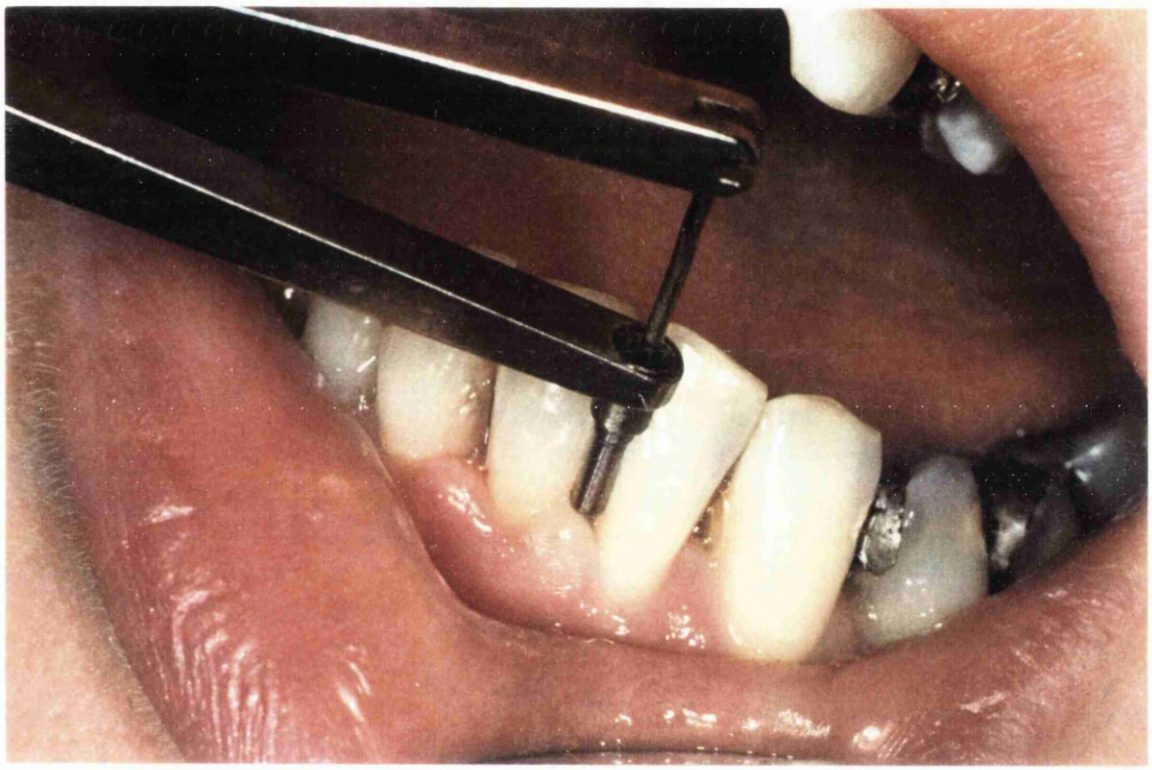

Figure 2.1 Probing depth measurement with the Florida probe.

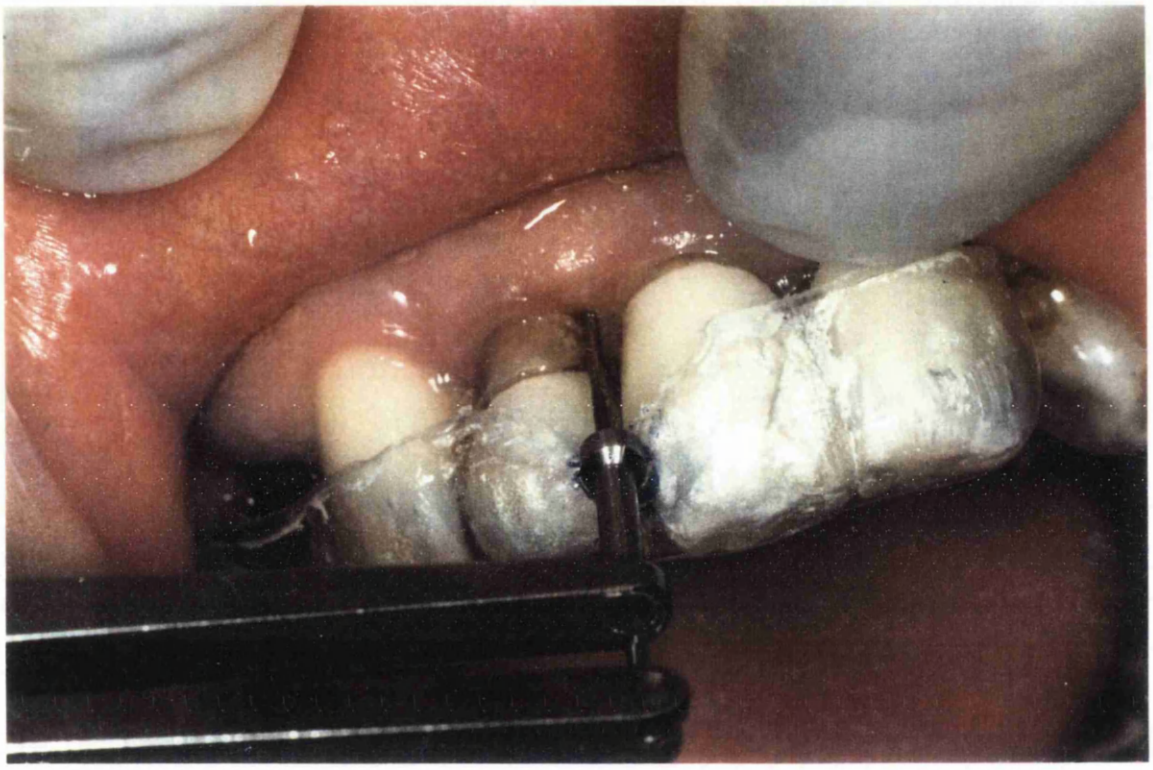

Figure 2.2 Attachment level measurement using the Florida probe and occlusal stent.

A fixed reference point is required for attachment level measurements. The Florida Probe 'stent' handpiece has a 2mm diameter disc at the edge of its sleeve which, during attachment level measurement, is seated on a custom made soft acrylic stent covering the crowns of the teeth. These stents were constructed for both upper and lower arches for each patient from a 2mm thick silicone layer using a modification of the method described by Isidor, Karring and Attström, (1984). The stents were made on individually cast models using a vacuum forming unit. The stent was then trimmed to cover approximately the coronal one third of the crowns of the teeth at the sites selected for the study. At other sites as much as possible of the stent was preserved in order to assist location and retention. Grooves approximately 1mm deep, 2mm wide and 1mm high were cut, about 1mm away from the apical edge of the stent, at the sites to be used in the study, using an inverted cone bur and a low speed handpiece. The grooves were then marked with a permanent pen for ease of identification. The disc of the probe handpiece was located in these grooves during AL measurements. Attachment level was assessed relative to the point at which the probe handpiece disc was seated on the stent (Fig. 2.2). In this way, attachment level measurements do not rely on careful location of the cementoenamel junction (CEJ). The identification of the CEJ is often complicated by its subgingival location or the presence of restorations. Buccal/lingual AL measurements were taken at the tooth's

largest curvature. Interproximal AL recordings were taken at the mid point between the contact point and the adjacent line angle. The grooves on the stent were located relatively to these points.

PD is recorded when the probe sleeve is brought into contact with the gingival margin (Fig. 2.1). Care was taken for the probe tip to be parallel to the long axis of the tooth. PD measurements were taken at the same points as AL recordings.

2.4.2.1 Change in attachment level

Attachment level change between two points in time was assessed using a modification of the tolerance method of Haffajee *et al.* (1983b). Duplicate AL measurements at each time point provide a measure of examiner error. The standard deviation of pooled duplicate measurements of a site, at the first, second and third time points, served as 'site standard deviation'. Differences between all duplicate measurements for all sites selected for the study within a patient, were used to compute the mean difference of duplicate measurements for each individual. The standard deviation of the mean difference was termed the 'patient standard deviation'. Patient standard deviations were averaged to derive a 'population standard deviation'. For the mean of the second pair of AL measurements to be considered significantly different from the mean of the

first pair, attachment level change:

a) had to exceed the 'population threshold' (which was 2 population standard deviations);

b) had to exceed the 'patient threshold' (which was 3 patient standard deviations); and finally

c) had to be greater or equal to the 'site threshold' (which was 3 site standard deviations). Significant attachment level gain or loss was assessed retrospectively on completion of the study using the above method.

2.4.3 Sample collection

2.4.3.1 Gingival crevicular fluid sampling

Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper strips (2x13mm) were used for GCF collection. Although Whatman grade 3 chromatography paper is recommended by some authors (Griffiths, Curtis & Wilton, 1988), this type of paper could not be used, as it was impossible to obtain a zero reading with the Periotron 6000 when the strips were dry. The Whatman grade 4 paper strips were cut manually using a steel ruler and scalpel. Rubber gloves were worn during their preparation in order to avoid contamination of the strips by substances from the operators hands. A line was drawn on each strip at 8mm, indicating the length of the paper strip to be inserted between the Periotron jaws and that part of the strip to be used for GCF sampling. The strips were then placed in

glass universal bottles for autoclaving and storage.

In the clinic, GCF was sampled after the PlI and MGI scores were taken but prior to any other clinical recordings which could cause irritation of the tissues and serum contamination of the sample. Such recordings included PD or AL measurements, BOP, suppuration etc. The individual crevicular site was gently air-dried in an apico-coronal direction and any visible supragingival plaque was removed. The area was carefully isolated with cotton wool rolls and saliva ejector to avoid saliva contamination of the samples. The paper strip was introduced into the crevice until mild resistance was felt, whilst care was taken to avoid mechanical injury of the tissues (Fig. 2.3). The strip was left in the crevice for 30s, and then transferred to the chairside located Periotron 6000 (Harco Electronics, Winnipeg, Manitoba, Canada) (Fig. 2.4) for volume determination. It was then stored in labelled individual sterile 1ml microcentrifuge tubes, and placed on ice until all sampling was completed. The strips were subsequently transported to the laboratory and stored frozen at -30°C until further processing.

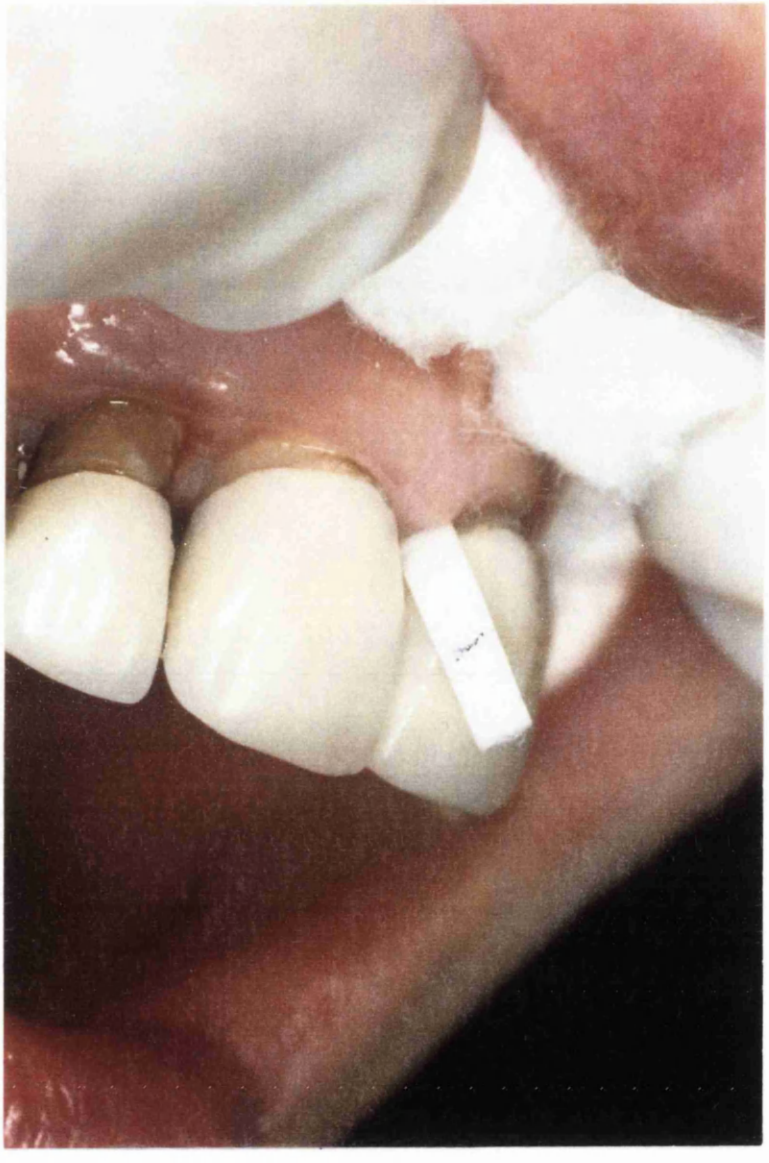

Figure 2.3 Gingival crevicular fluid sampling.

Figure 2.4 The Periotron.

2.4.4 Selection of periodontal sites

2.4.4.1 Cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid

Sites to be sampled were selected by clinical inspection and the use of pre-existing pocket charts. In this way, periodontal probing, which could cause irritation of the tissues and serum contamination of the sample, was avoided.

2.4.4.2 Longitudinal study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid before and after treatment

A conventional pocket chart, constructed on a recruiting appointment using a PC12 periodontal probe, was used for site selection in this study. Two non-adjacent sites in each quadrant, (accessible to GCF sampling and attachment level recording) exhibiting pocketing ≥ 4mm on the pocket chart were selected for this study. Interproximal sites were preferred to mid-buccal or mid-lingual sites due to their higher chance of demonstrating attachment loss (Haffajee *et al.*, 1983a). Mesial sites were preferred to distal sites as they are more accessible. On the upper posterior molars, palatal sites were preferred to buccal sites and on the lower teeth buccal/labial sites were preferred to lingual sites as these sites are less prone to

saliva contamination of the GCF sample.

2.4.5 Clinical design

2.4.5.1 Cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid

In the cross-sectional study, as the term implies, samples were obtained and clinical recordings made at one time point. Clinical indices used in this study were the MGI, PD and GCF volume. In order to establish the levels of GCF FIB-CL, SL and TIMP in relation to the clinical presentation of the sites sampled, sites were categorised into one of three clinical groups: 'healthy', 'gingivitis' and 'periodontitis'. Sites with MGI scoring of 0 or 1 and PD lower or equal to 2mm were categorised as 'healthy'. MGI between 2 and 4, and PD less than or equal to 3mm were the criteria for the 'gingivitis' sites, whereas sites with MGI between 0 to 2 and PD exceeding 4mm comprised the 'periodontitis' sites. Each subject provided the three sites (one of each).

2.4.5.2 Longitudinal study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid before and after treatment

All the clinical recordings and sample collections in this

study were carried out at three time points: i) baseline appointment; ii) reassessment appointment; and iii) follow-up appointment. Prior to the baseline appointment, at the initial recruitment appointment the consent form was signed by the patient and the clinician in charge. A medical history form was also completed by the patient and the pocket charting, to be used for site selection, was constructed in this session. An alginate impression was taken and from the resulting cast, a soft acrylic stent for attachment level measurements was constructed (section 2.4.2). Subsequently, the patient was recalled for the baseline appointment. During this appointment and in the following order:

a) PlI and MGI recordings were taken first;

b) GCF samples were then taken from the preselected sites and GCF Periotron readings recorded;

c) the first set of AL measurements at the sites sampled was subsequently made. During this procedure bleeding on probing and suppuration were also registered. The stent was then removed;

d) after changing patient position and reapplying the stent the duplicate set of AL measurements was made;

e) finally PD was assessed.

The same procedure was followed at the reassessment and the follow-up appointments.

All patients underwent hygiene phase of therapy including

oral hygiene instruction and scaling and root planing under local anaesthesia. Six weeks after last treatment session the patient was recalled for reassessment. At this visit all the procedures in the baseline appointment were repeated. After recording clinical parameters and sample collection a conventional pocket chart was constructed. This pocket chart along with the state of bleeding on probing was used to assess the overall treatment success and determine further treatment needs. At this reassessment session sites were assigned to 'no treatment', 'repeated root planing' and 'surgery' treatment groups based on the following criteria: a) no treatment: no bleeding on probing; PD<3.5mm

b) repeated root planing: bleeding on probing; PD>3.5 to 5.5mm

c) surgery: bleeding on probing; PD>5.5mm

The subjects received further treatment according to the above mentioned criteria in different sites. Six weeks after the last treatment session patients attended for the follow-up visit in which all the procedures in the reassessment appointment repeated. The follow-up examination was postponed for another 4 weeks in subjects who had received surgical treatment as visual inspection as well as gentle probing in non-study sites revealed signs of inflammation at six weeks only following the treatment session.

2.5 Experimental methods and preliminary experiments

In this section, to aid clarity, methods as well as the preliminary experiments necessary to establish these methods, and the results will be presented and discussed together.

2.5.1 Calibration of the Periotron 6000

2.5.1.1 Calibration of the Periotron 6000 for use in clinical trials

Prior to the commencement of our studies a Periotron calibration curve was constructed, using distilled water as the experimental fluid. Linear regression analysis was used to fit the standard curve. GCF volumes could then be estimated from Periotron values, using the linear function of the type I= a + b x v, where I= Periotron indication, v= volume, a= the intercept and b= the v coefficient. This type of standard curve was used when assessing GCF volumes for both cross-sectional and longitudinal studies. In order to transform the Periotron digital readings for each paper strip into volumes and also assess the accuracy of instrument, known volumes of distilled water were delivered onto Whatman grade 4 paper strips with a Hamilton microsyringe in a range of volumes (0.05-1µl). Each measurement was performed 3 times and the mean value for each volume was used in a linear regression analysis to

determine the volumes of GCF collected. In an effort to reduce daily variability and thus improve accuracy, the instrument was calibrated in the periodontal clinic each day samples were collected. The curve was split into an upper and a lower portion as described by Lamster *et al.* (1985) and two separate lines fitted by the regression method as shown in Figure 2.5. The lower portion included volumes of 0.05, 0.1 and 0.2μl whereas the upper portion included all the remaining volumes. Determination of GCF volume from given Periotron indications was performed from the corresponding part of the Periotron calibration curve using the appropriate equation. If a sample yielded a reading beyond the measuring range of the Periotron 6000, it was assigned a volume of 1.334μl which was the averaged values from 20 Periotron readings of 201 (the maximum reading) in 20 different calibration curves. However, this only occurred for 5 samples (1%) in the longitudinal study of matrix metalloproteinases in gingival crevicular fluid before and after treatment.

2.5.2 Quantification of GCF constituents

FIB-CL, SL and TIMP, in GCF were quantified using the enzyme linked immunosorbent assays (ELISAs). In both cross-sectional and longitudinal studies on matrix metalloproteinases in gingival crevicular fluid, FIB-CL, SL and TIMP were assessed in the same GCF sample.

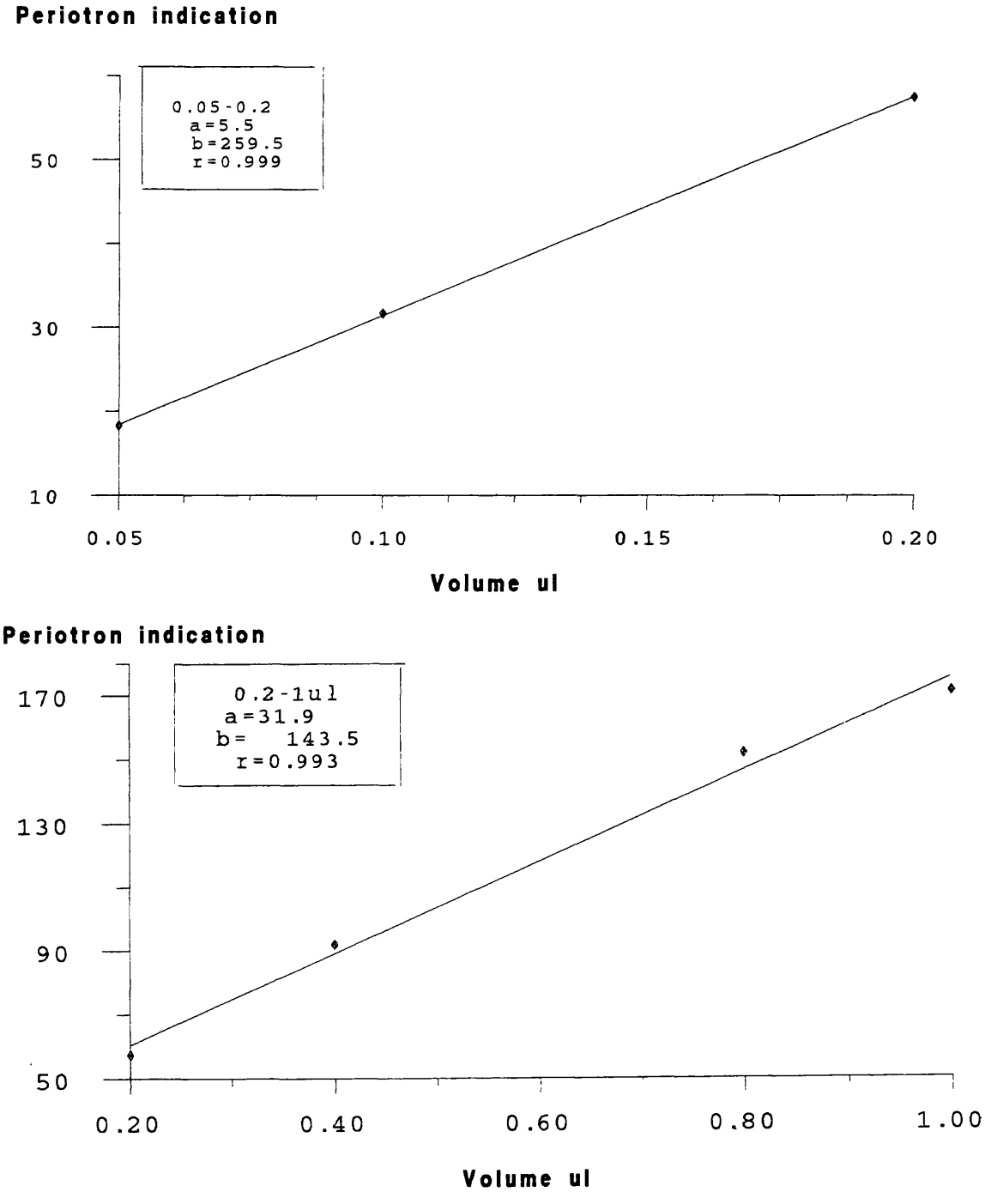

Figure 2.5 Calibration curves of the Periotron at the volume range of : (a) 0.05-0.2µl and (b) 0.2-1µl.

2.5.2.1 Sandwich ELISA methodology

The three sandwich ELISAs (FIB-CL, SL and TIMP) are based on a modification of the method of Cooksley *et al.* (1990). In summary (Fig. 2.6), the 96-well polystyrene microplate (Immulon 4 Dynatech Laboratories, Billinghurst, Sussex, U.K) was coated with the first antibody, specific to the antigen to be quantified. These antibodies were Mac 64 anti-collagenase monoclonal antibody for FIB-CL, Mac 78 anti-stromelysin monoclonal antibody for SL and Mac 19 anti-TIMP monoclonal antibody for TIMP. The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. Then the plate was incubated with a biotin-conjugated monoclonal second antibody. Finally the plate was incubated with Extr Avidin-Peroxidase (Sigma). These antibodies were biotinylated Mac 66 to reveal FIB-CL and biotinylated Mac 15 to reveal TIMP. The stromelysin assay differed in that a rabbit polyclonal antibody was used as the second antibody followed by Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG (donkey) (Jackson Immuno-research Laboratories, Avondale, USA). The anti-SL monoclonal and polyclonal and anti-FIB-CL monoclonals detected both the proenzyme and active forms of the SL and FIB-CL. Anti-TIMP monoclonals detected only free TIMP (Cooksley *et al.*, 1990). Visualisation was achieved by incubation with TMB substrate, and the reaction stopped with 2.5% NaF (sodium fluoride). The plate was read at 630 nm (dual wavelength with 490 nm as reference

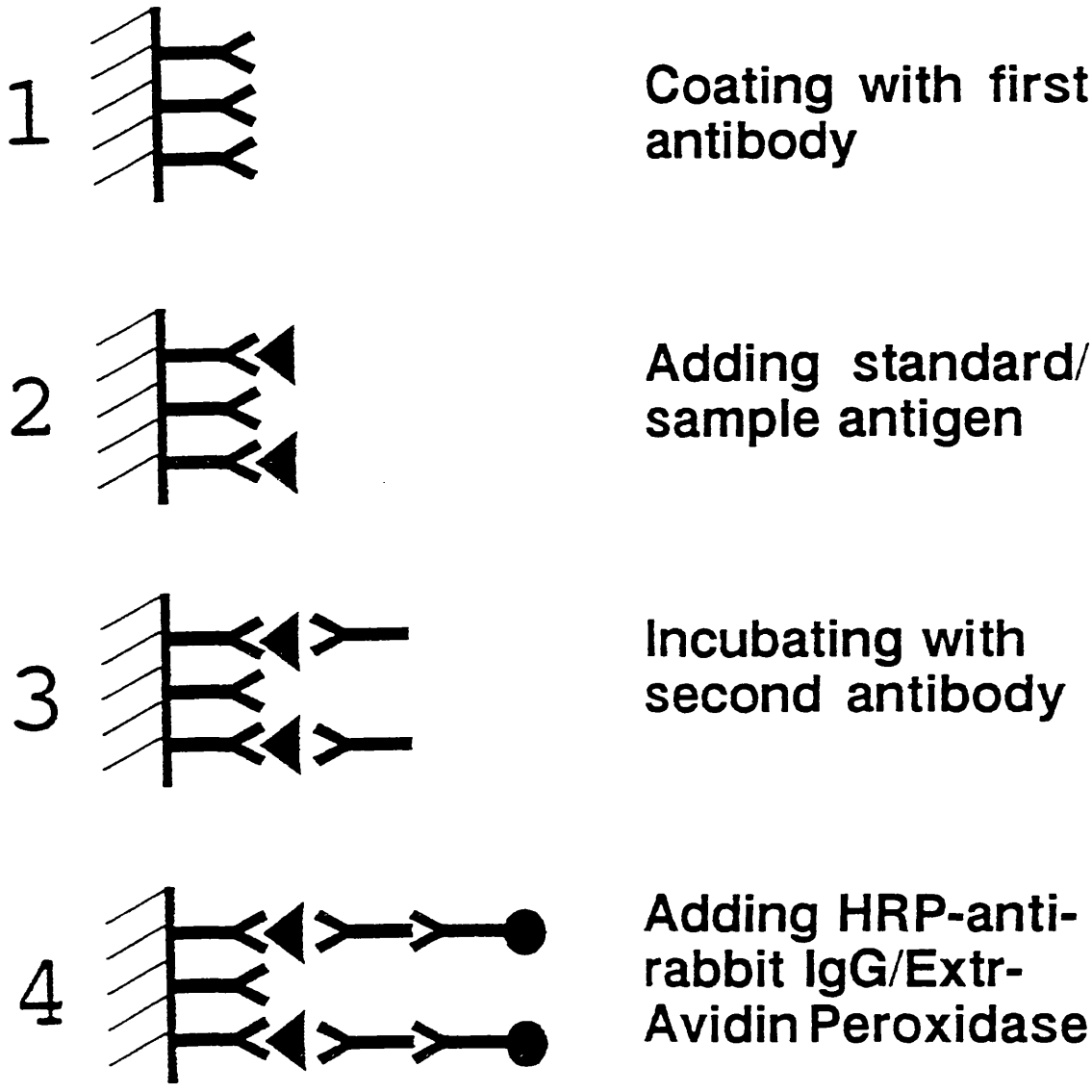

Figure 2.6 Sandwich ELISA: basic principles

wavelength), on a Dynatech MR5000 plate reader. Serial dilutions of standard antigen were run for each plate and their ODs were used in a linear regression analysis from which the slope and intercept were used to determine the samples' levels of FIB-CL, SL and TIMP.

In the sandwich assays the amount of antigen present in the sample is directly proportional to the amount of the second biotinylated antibody or antiserum (rabbit), which was quantified indirectly by the addition of the HRP conjugated anti-rabbit IgG, resulting in high ODs for high antigen concentrations. The method is described in detail in Table 2.2. The working range for each assay is given in Table 2.3. Only the central wells were used when running standards or samples (in duplicate) in an effort to avoid the edge-effect. Control wells (control 14; Table 2.1) in central wells in each plate were included which contained no sample or standard antigen in order to calculate background binding. The mean OD plus two standard deviations of these wells was taken as a cut-off point for detectability (Tijssen, 1985). The peripheral wells were used for assaying the controls (except for the control 14) which were as shown in Table 2.1. All controls were run in duplicate except for the zero-antigen (control 14; Table 2.1) which was run in quadruplicate.

Table 2.1 Control tests for sandwich ELISAs. When the addition of a reagent was omitted the equivalent volume of the respective buffer alone was added. Coating refers to the first antibody and antiserum refers to the second antibody. Standard was added at its fifth concentration in the standard curve. For volumes and concentrations of reagents refer to table 2.2.

Control	Step			
	Coating	Standard	Antiserum	HRP/Extr Avidin Peroxidase
1	-	-	-	-
2	+	-	-	-
3	-	+	-	-
4	-	-	+	-
5	-	-	-	+
6	+	+	-	-
7	+	-	+	-
8	+	-	-	+
9	-	+	+	-
10	-	+	-	+
11	-	-	+	+
12	+	+	+	-
13	+	+	-	+
14	+	-	+	+
15	-	+	+	+

Table 2.2 Steps and reagents for fibroblast collagenase (FIB-CL), stromelysin (SL) and tissue inhibitor of metalloproteinase (TIMP)sandwich ELISAs. Standard concentration range is given in Table 2.3.

SANDWICH ELISAs	
STEPS	**REAGENT**
1. Wash thrice with 200µl/well PBS for FIB-CL and CB for SL and TIMP.	
2. Coat with 50µl/well specific antiserum. Incubate overnight at 4°C.	Mac 64 anti-FIB-CL, Mac 78 anti-SL and Mac 15 anti-TIMP.
3. Wash with approximately 200µl/well PBST, 4x4+1 times and dry. Removal of excess and loosely coated antibody.	
4. Add 100µl/well IB for 1 hr at RT to block the free sites.	
5. Wash with approximately 200µl/well PBST, 4x4+1 times and dry.	
6. Add 50µl/well standard antigen or eluted samples in duplicate. Incubate for 1 hr on the shaker with speed 100, at RT.	Recombinant FIB-CL, SL and TIMP served as standard antigens.
7. Wash with approximately 200µl/well PBST, 4x4+1 times and dry. Removal of free and loosely-bound antigen.	
8. Add 50µl/well second antibody incubate for 1 hr on the shaker with speed 100, at RT.	Biotin-Mac 66 anti FIB-CL, Biotin-Mac 15 anti TIMP and Rabbit anti SL.
9. Wash with approximately 200µl/well PBST, 4x4+1 times and dry. Removal of free and loosely-bound antibody.	
10. Add 50µl/well Extr Avidin Peroxidase for FIB-CL and TIMP and HRP conjugated Anti-rabbit IgG for SL. Incubate for 1 hr on the shaker with the speed of 100, at RT.	Extr Avidin Peroxidase: 1/10000 for FIB-CL and 1/5000 in IB for TIMP. HRP: 1/20000 in IB for SL.
11. Wash with approximately 200µl/well PBST, 4x4+1 times and dry. Removal of excess Extr Avidin Peroxidase and HRP conjugated Anti-rabbit IgG.	
12. Add 50µ/well TMB. Incubate for 20 min. on the shaker with the speed of 100, at RT.	
13. Add 50µl/well NaF to stop colour development.	
14. Read at 630nm on a plate reader.	

PBST= wash buffer
CB= coating buffer
IB= incubation buffer
RT= room temperature
HRP= horse radish peroxidase
TMB= peroxidase substrate

2.5.2.2 Preliminary experiments and modification of the sandwich ELISAs

(a) Selection of microtitre plate type for the sandwich ELISAs

Based on an experiment aiming at the selection of the best plate, among five different types of Immulon (Dynatech Laboratories) microelisa plates, carried out in our laboratory, the Immulon IV plates were selected for the sandwich ELISAs in these studies.

(b) Modifying the FIB-CL, SL and TIMP sandwich ELISAs

FIB-CL, SL and TIMP sandwich ELISAs described by Cooksley *et al.* (1990) were modified in order to meet the requirements and to consider the limitations of our study. The modifications were as follows:

a) Since we had to use the eluate from the same paper strip for different ELISAs it was not possible to use three different assay buffers to elute each individual paper strip. Therefore all the paper strips were eluted in the incubation buffer (section 2.2.3).

b) Based on an experiment determining suitable volume of incubation buffer in which the paper strips were to be diluted, the samples were diluted in 500µl of incubation buffer. Since we had to use this eluate for three different ELISAs and at the same time keep some of that for

back up, it was necessary to reduce the sample volume in each well from 200µl to 50µl.

c) Considering the range of absolute amounts of FIB-CL, SL and TIMP detected in preliminary experiments, the calibration line was altered toward the right side (lower concentration).

The precision ranges of each of the four sandwich ELISAs are shown in Table 2.4. Precision range was defined as the linear part of the working range (Table 2.3). A prerequisite was that the OD of the lowest standard (mean ±SD) of the precision range differed significantly from that of the zero antigen control (mean±SD) (control 14; Table 2.1). Outwith the precision range, experimental error increased due to the typical flattening of the curve.

Figure 2.7 (a to c) shows typical standard curves (precision ranges) for the FIB-CL, SL and TIMP sandwich ELISAs. The least squares method was used to plot the best fitting line and $r^2>0.99$ were obtained for all assays. Linear functions of the type $\log_n OD = a + b \times (\log_n c)$, where OD= optical density, c= concentration, a= the intercept and b= the $\log_n c$ coefficient were thus produced, and used for the determination of sample antigen quantities. A similar standard curve was generated and a new line fitted each time an assay was run.

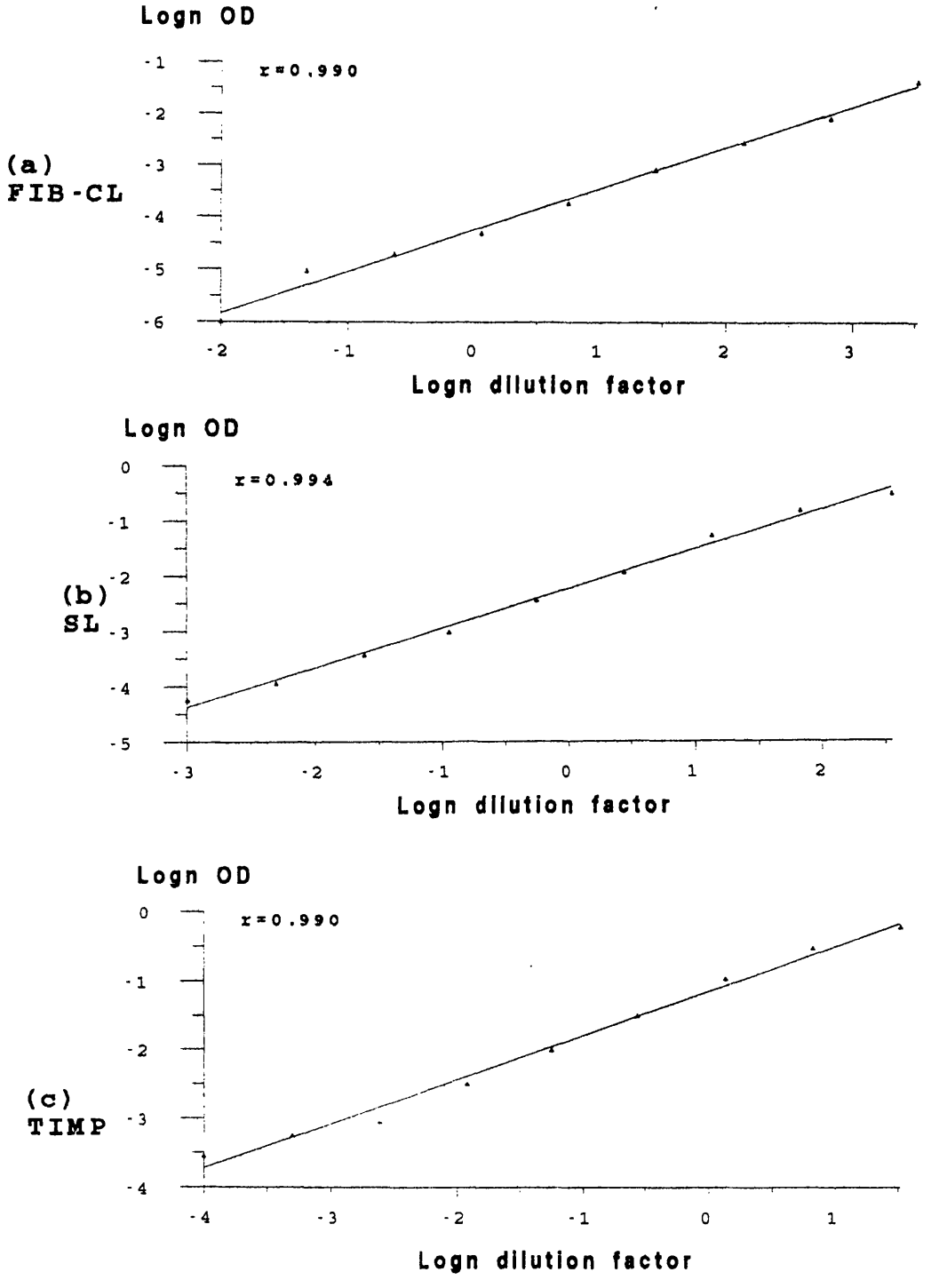

Figure 2.7 Typical calibration curves for (a) fibroblast collagenase (FIB-CL), (b) stromelysin (SL) and (c) tissue inhibitor of metalloproteinases (TIMP).

Table 2.3 Working range of the standard antigen for the fibroblast collagenase, stromelysin and tissue inhibitor of metalloproteinase (TIMP) sandwich ELISAs. This was produced by serial two fold dilutions of the standard antigen (recombinant protein) in the appropriate buffer, resulting in 10 standard solutions for the construction of a standard curve.

Assay	Working range (ng/ml in IB*)
Fibroblast collagenase	50 - 0.1
Stromelysin	12.5 - 0.025
TIMP	12.5 - 0.025

Table 2.4 Precision range of the standard antigen for the fibroblast collagenase, stromelysin and tissue inhibitor of metalloproteinase (TIMP) sandwich ELISAs. Precision range was defined the linear part of the working range (Table 3.3).

Assay	Precision range (ng/ml in IB*)
Fibroblast collagenase	25 - 0.39
Stromelysin	6.25 - 0.05
TIMP	6.25 - 0.10

IB= incubation buffer

Table 2.5 Intra and inter-plate variability in sandwich ELISAs for fibroblast collagenase (FIB-CL), stromelysin (SL) and tissue inhibitor of metalloproteinases (TIMP).

Assay	Intra-plate variation %CV*	Inter plate variation (p)*
FIB-CL	7.6	0.378
SL	4.4	0.841
TIMP	5.4	0.926

*= covariant of variation [(SDx100)/mean] is a measure of variability among repeated tests.
**= significance level (p) for repeated measures analysis of variance comparing the levels of proteins in four plates.

To investigate if serum components would interfere when assaying GCF FIB-CL, SL and TIMP and thus influence the reliability of the assay, serum at 1/500 dilution in incubation buffer was added in the FIB-CL, SL and TIMP standards and the resolution of the standard curves examined. The 1/500 dilution of serum was used for the following reasons: a) GCF samples are eluted 500μl of incubation buffer; b) assuming a maximum of approximately 1μl of GCF and a consistency similar to that of serum, the maximum concentration of serum components in the experimental eluate would be 1/500 of their original concentrations in serum. Addition of 1/500 serum in our standards did not influence the resolution of the standard curves for FIB-CL, SL and TIMP.

In an attempt to investigate the intra-plate variability, aliquots of standard proteins at different concentrations were run in one plate. The coefficient of variation (CV % = SDx100/ mean OD), which is a measure of variability among repeated tests, was calculated to measure the intra-plate variability (Table 2.5). In order to assess inter-plate variability, aliquots from the standard proteins with the same concentration were run in duplicate in four different plates. Repeated measures analysis of variance demonstrated no significant difference between the levels of GCF components in four different plates (Table 2.5).

Finally, GCF samples (FIB-CL, SL and TIMP n=4) originally

eluted in 500µl of incubation buffer were diluted a further 1/2 in incubation buffer and two serial two-fold dilutions in incubation buffer produced which were assayed for FIB-CL, SL and TIMP. This experiment confirmed that the three GCF components respond in a similar way to the highly purified standard and that the assays developed could be used for their quantitation in GCF.

The above experiment also provided some information on the amount of incubation buffer in which the paper strips were going to be eluted. Some more GCF samples were then tested and it was established that the elution of GCF samples in 500µl of incubation buffer for all three GCF components would yield concentrations in the eluate within the precision range of each ELISA.

2.5.3 Gingival crevicular fluid elution

2.5.3.1 Gingival crevicular fluid elution technique

GCF samples were eluted in 500µl of incubation buffer for 1h at room temperature using a rotary mixer. The strips were then discarded and the eluates were aliquoted and stored in microcentrifuge tubes at -30°C.

2.5.3.2 Recovery rate of fibroblast collagenase, stromelysin and tissue inhibitor of metalloproteinases from Whatman grade 4 paper strips

By the use of sandwich ELISAs it was possible to quantify all three GCF components in the same GCF sample. It therefore became imperative to determine their recovery rate when absorbed on paper strips as this would influence the determination of their relative levels in GCF.

To achieve this, the following protocol was followed for FIB-CL,SL and TIMP; 5µl of 195ng/ml stock FIB-CL and 5µl of 100ng/ml of SL and TIMP stock solution were delivered by a Hamilton microsyringe either:
a) onto Whatman grade 4 paper strips (n=7 for FIB-CL and n=9 for SL and TIMP) which were subsequently eluted in 500µl of incubation buffer for 1h at room temperature using a rotary mixer; or

b) directly (n=9) in 500µl of incubation buffer which was also rotated for 1h at room temperature.

The eluates were then stored at -30°C until further analysis. The level of each of the above GCF components in the 500µl eluate of the strips was compared against that in the 500µl eluate when direct delivery in buffer was used and the % recovery calculated (Table 2.6).

Table 2.6 Recovery rate of fibroblast collagenase (FIB-CL), stromelysin (SL) and tissue inhibitor of metalloproteinases (TIMP). 5µl of 195ng/ml recombinant FIB-CL and 5µl of 100ng/ml of recombinant SL and TIMP were delivered, either: on paper strips (n=7 for FIB-CL and n=9 for SL and TIMP) which were subsequently eluted in 500µl of IB; or b) directly (n=9) in 500µl of IB. The mean level (pg/50µl) and (SD) of eluate resulting from both methods of delivery as well as % recovery are shown.

	Level of protein (pg/50µl eluate)		Recovery
Protein	IB*	strip	rate
FIB-CL	89.3 (4.0)	82.9 (9.8)	92.3%
SL	48.7 (6.8)	42.5 (5.5)	87.3%
TIMP	51.6 (2.4)	50.6 (3.9)	98.0%

IB= incubation buffer
SD= standard deviation

Finally, an experiment was performed in order to ensure that paper strips were not contaminated by any of the proteins and inhibitor to be tested, prior to their use on patients. Sterile paper strips (n=10), were eluted in the standard procedure and the 500µl eluate tested by the sandwich ELISAs for FIB-CL, SL and TIMP. Twenty wells in each plate were run using incubation buffer. None of the strips yielded positive results at the lowest detectability limits of the assays. The results obtained from paper strips were also comparable to those of incubation buffer containing wells.

2.5.4 Fluctuation in the GCF levels of fibroblast collagenase, stromelysin and tissue inhibitor of metalloproteinases

An experiment was conducted to investigate if there is any significant difference between the level of FIB-CL, SL and TIMP in GCF samples collected from the same sites over a working week. Ten individuals participated in this study from whom eight healthy and seven gingivitis sites were sampled for five successive days. Probing pocket depth and gingival inflammation were assessed using a pressure sensitive probe with a constant force of 25 g and MGI. These clinical parameters remained unchanged over the period of study. Subjects were neither given oral hygiene instruction nor were asked to change their oral hygiene habits. Paper stirps were eluted in the standard procedure

Table 2.7 Using multivariate repeated measures analysis of variance, GCF levels (pg/30s) of SL and TIMP showed no significant difference in both healthy and gingivitis sites over a period of one week. Geometric mean and p value for MANOVA are shown. The GCF levels of these proteins were significantly lower ($p<0.001$) in healthy than in gingivitis sites.

	Healthy		Gingivitis	
Visit	**SL**	**TIMP**	**SL**	**TIMP**
Day 1	1.7	11.3	7.8	121.0
Day 2	1.6	12.0	10.5	137.4
Day 3	1.5	10.8	9.6	98.60
Day 4	1.7	14.8	8.2	112.9
Day 5	1.6	12.4	7.2	104.8
MANOVA				**p=0.798**

SL: stromelysin
TIMP: tissue inhibitor of metalloproteinases

and assayed for FIB-CL, SL and TIMP. Using MANOVA, this experiment showed no significant difference between the level of SL and TIMP over the period of the study (Table 2.7). However, FIB-CL was only detected in 6 (%8) GCF samples.

2.6 Statistical Analysis

All statistical analyses were performed on an IBM PC computer using the Minitab or SPSS/PC statistical package.

In the cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid, associations between SL or TIMP in the GCF and the clinical indices (MGI, PD and GCF volume) were determined by the non-parametric Spearman rank correlation coefficient. In the cross-sectional study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid the site was considered as the statistical unit. SL and TIMP levels in GCF were markedly skewed and required logarithmic transformation ($\log_{10}$ (x+1) x=original data value) in order to satisfy their distributional requirements prior to statistical analysis. Due to this skewed distribution, the geometric mean and 95% confidence intervals are shown rather than the standard error or standard deviation. A multivariate repeated measures analysis of variance (MANOVA) was applied in order to perform within patient comparison of healthy, gingivitis and periodontitis sites.

When a significant effect was detected by repeated measures analysis of variance, follow up analysis was performed using univariate paired t-test in order to determine the direction and location of the differences. A Bonferroni correction was applied and significance level for the t-test was set at 0.025 ($0.05/\sqrt{3}\approx0.025$, where 3=number of comparisons made) in order to compensate for multiple comparisons (Brown & Swanson Beck, 1988).

In the longitudinal study of matrix metalloproteinases and their inhibitor in gingival crevicular fluid before and after treatment, analysis of the clinical findings was performed considering both subject and site as the experimental unit. When the subject was considered as the experimental unit, GCF volumes and clinical indices as well as GCF levels of FIB-CL, SL and TIMP within the subject were averaged, and one score for each clinical index (MGI, PlI, GCF volume, AL change, BOP and suppuration) as well as one value for each biochemical parameters (FIB-CL, SL and TIMP) produced per patient. BOP and suppuration scores (0 = absence or negative and 1 = presence or positive) averages produced scores, ranging from 0 to 1, with the decimal digits being equivalent to the percentage of bleeding or suppurating sites per subject. In order to satisfy the distributional requirements of the data for the tests a $\log_{10}$ transformation was required ($\log_{10}(1+x)$, x=original data value) wherever the original data showed skewness. Multivariate repeated measures analysis of

variance (MANOVA) followed by paired t-tests were employed to investigate differences in the levels of FIB-CL, SL and TIMP as well as the differences in the GCF volumes, pocket depths (PD) and attachment levels (AL) between three examinations. Significance level for the paired t-tests was set at 0.025 ($0.05/\sqrt{3}\sim0.025$, where 3=number of comparisons made). Non-parametric repeated measures analysis of variance (Friedman test) followed by Sign tests were used to reveal differences in MGI and PlI scores between different examinations. Changes in bleeding and suppuration status (dichotomous data) between three appointments were studied using Cochran Q test followed by Chi-square tests. One way analysis of variance was employed to investigate wether FIB-CL, SL and TIMP levels are able to distinguish between sites with different pocket depths. In order to assess the effect of individual clinical parameters and their combination on the level of proteins, discriminant analysis was performed. Using Pearson correlation coefficient as well as Spearman rank correlation coefficient, the association of GCF volumes and clinical indices with the mean levels of SL and TIMP was investigated. Clinical and biochemical parameters were compared in sites which did or did not respond to treatment by the means of Mann-Whitney test (for MGI) and two sample t-test. Paired t-test and sign test were used to compare the mean levels of enzymes, the inhibitor, GCF volumes and clinical indices in sites which demonstrated attachment gain, before and after treatment.

In order to illustrate the diagnostic potential of a test based on the level of TIMP in GCF, a 2x2 table based on the data of this protein at the follow-up visit from best responding sites (BRS) and worst responding sites (WRS) was formulated. A test based on the value of TIMP was applied. Thus, the test was considered positive (successful treatment) when a site demonstrated TIMP below a designated threshold value. This threshold value represents the geometric mean of TIMP (pg/30s) in successfully treated sites at the follow-up visit. Specificity and sensitivity values as well as positive and negative predictive values were calculated using standard methods as shown in Table 2.8.

Table 2.8 Diagnostic test characteristics and definitions.

	Disease present	Disease absent	
Positive test	a True Positive	b False Positive	**Positive Predictive Value** **a/ (a+b)**
Negative test	c False negative	d True negative	**Negative Predictive Value** **d/ (c+d)**
	Sensitivity **a/ (a+c)**	**Specificity** **d/ (b+d)**	

CHAPTER 3

RESULTS

3.1 Matrix metalloproteinases and tissue inhibitor of metalloproteinases in gingival crevicular fluid from healthy, gingivitis and periodontitis sites

This cross-sectional study was carried out to examine if the level of fibroblast collagenase (FIB-CL), stromelysin (SL) and tissue inhibitor of metalloproteinases (TIMP) in gingival crevicular fluid (GCF) could differentiate between healthy, gingivitis and periodontitis sites. GCF samples were collected from one hundred and twenty sites in total, forty in each clinical group (healthy, gingivitis and periodontitis). Twenty five of the subjects were among periodontal patients on maintenance care and the other 15 patients had been newly referred to the Periodontal Clinic. In this study FIB-CL, SL and TIMP were assessed in the same GCF samples and results were expressed as absolute amounts i.e. pg/30s of sample collection. Multivariate repeated measures analysis of variance (MANOVA) and paired t-tests were employed on the $\log_{10}$ transformed data in order to determine significant differences in GCF levels of the enzymes and inhibitor among the three clinical groups. Using non-parametric Spearman rank correlation coefficients the associations between the clinical indices and the levels of SL and TIMP were assessed. In order to compensate for multiple comparisons, the significant level for the t-tests was set at 0.025 (section 2.6).

3.1.1 Clinical and biochemical findings

Table 3.1 presents the average (±SEM) clinical indices and GCF volumes of the healthy, gingivitis and periodontitis sites. The levels (95% confidence intervals) of SL and TIMP are presented in Table 3.2.

3.1.1.1 Fibroblast derived collagenase at healthy, gingivitis and periodontitis sites

FIB-CL was detectable in only 25 (20.8%) out of 120 sites, of which 7 (17.5%) belonged to the healthy group (mean=193.6 pg/30s, SD=265.9), 11 (27.5%) sites belonged to the gingivitis group (mean=286.3 pg/30s,297.3) and 7 (17.5%) sites belonged the to periodontitis group (287.2 pg/30s, SD=271.8). FIB-CL level was much higher than SL in sites where it was detected. In 20 subjects FIB-CL was not detected in any site. The association of SL and TIMP with the clinical indices was also investigated.

3.1.1.2 Stromelysin at healthy, gingivitis and periodontitis sites

SL was detected in 75 (62.5%) sites sampled of which 19 (47.5%) belonged to the healthy group and 28 (70%) both in the gingivitis and periodontitis groups. Multivariate repeated measures analysis of variance (MANOVA) demonstrated a significant effect (p=0.006) when healthy,

Table 3.1 Clinical parameters from the three categories of sites in 40 patients.

Site Status	MGI	PD	GCF
Healthy	0.17 ±0.06*	1.41 ±0.06	0.092 ±0.02
Gingivitis	3.00 ±0.07	2.37 ±0.12	0.320 ±0.05
Periodontitis	1.70 ±0.12	5.71 ±0.14	0.380 ±0.06

MGI= modified gingival index
PD= pocket depth in mm
GCF= gingival crevicular volume
*= mean & SEM

Table 3.2 Stromelysin and TIMP absolute amounts (pg/30s) in GCF from healthy, gingivitis and periodontitis sites in 40 patients. Geometric means (95% confidence intervals) are shown together with repeated measures analysis of variance (MANOVA) significance level. Follow up analysis was performed using univariate paired t-test and the results are given as footnotes.

Site status	Stromelysin*	TIMP**
Healthy	3.9 (2.4-6.10)	32.3 (19.4-53.70)
Gingivitis	8.9 (5.5-14.4)	74.0 (44.2-123.8)
Periodontitis	9.4 (5.8-15.3)	93.7 (63.2-138.9)
MANOVA p value	0.006	0.001

* healthy vs gingivitis: p=0.007, healthy vs periodontitis: p=002, gingivitis vs periodontitis: p=0.882
** healthy vs gingivitis: p=0.008, healthy vs periodontitis:p<0.001, gingivitis vs periodontitis: p=0.373

gingivitis and periodontitis sites within each patient were compared for their GCF SL levels (Table 3.2). Follow up analysis (Table 3.2) demonstrated that diseased sites (gingivitis and periodontitis) had significantly higher levels of SL than healthy sites (p= 0.007 & p=0.002 respectively). However, no significant difference could be detected between gingivitis and periodontitis sites (p=0.882).

3.1.1.3 Tissue inhibitor of metalloproteinases at healthy, gingivitis and periodontitis sites

TIMP was detected in 108 (90%) sites in total. TIMP was present at detectable level in 34 (85%) healthy, 36 (90%) gingivitis and 38 (95%) periodontitis sites. Similarly to SL, multivariate repeated measures analysis of variance demonstrated a significant effect when healthy, gingivitis and periodontitis sites were compared for their GCF TIMP levels (Table 3.2). Follow-up analysis demonstrated that gingivitis and periodontitis sites had significantly higher levels of TIMP than healthy sites (p=0.008 & p<0.001) respectively). GCF TIMP levels could not however differentiate gingivitis from periodontitis sites (p=0.373) (Table 3.2).

3.1.2 Correlation between clinical indices and GCF levels of stromelysin and TIMP

When data from healthy, gingivitis and periodontitis sites were pooled, SL and TIMP levels correlated positively and significantly with GCF volume, MGI and PD. Overall, TIMP demonstrated higher correlation coefficients than SL with clinical indices and GCF volume (Table 3.3). However, when data from different groups of sites were separately analyzed the picture changed; so that, there was neither significant nor even moderate correlation between clinical and biochemical parameters.

3.1.3 Comparison between GCF levels of stromelysin and tissue inhibitor of metalloproteinases in maintenance and newly referred patients

Healthy, gingivitis and periodontitis sites in periodontally affected patients who had newly been referred to the Periodontal Clinic showed higher levels of SL and TIMP than the similar sites in maintenance patient. However, these differences did not reach the significant level. When clinical parameters, in different categories of sites, were compared between two groups of patients except for the pocket depth in periodontitis sites no other significant difference was observed (Table 3.4). The percentages of gingivitis and periodontitis sites with detectable amount of FIB-CL were higher in newly referred

Table 3.3 Spearman rank correlation coefficient (r) between clinical indices and TIMP and SL levels in GCF from 40 patients. Probability levels are shown in parenthesis.

Parameter	Stromelysin	TIMP
Vol.	r=0.283 (p=0.002)	r=0.643 (p<0.001)
MGI	r=0.228 (p=0.012)	r=0.361 (p<0.001)
PD	r=0.256 (p=0.005)	r=0.308 (p=0.001)

Vol.= GCF volume in µl
MGI= modified gingival index
PD= pocket depth in mm

Table 3.4 Comparison between GCF levels of SL and TIMP in new and maintenance patients. Geometric means (95% confidence intervals) are shown together with two-sample t-test significance level.

	New patients (n=15)	Maintenance patients (n=25)	
Parameter	Mean (95% CI)	Mean (95% CI)	p value
SL (H)*	5.2 (2.9-7.5)	3.2 (1.5-4.9)	0.347
SL (G)**	12.5 (10.2-14.8)	7.3 (5.5-9.1)	0.282
SL (P)***	14.4 (12.3-16.5)	7.3 (5.4-9.2)	0.181
TIMP (H)	32.5 (29.0-36.0)	32.2 (30.4-34.0)	0.987
TIMP (G)	75.2 (72.5-77.9)	73.3 (71.5-75.5)	0.962
TIMP (P)	107.2 (105.1-109.3)	86.4 (84.8-88.0)	0.606

*= healthy sites
**= gingivitis sites
***= periodontitis sites
SL= stromelysin
TIMP= tissue inhibitors of metalloproteinases

patients, whereas it was not the case in healthy sites.

3.2 Longitudinal study of gingival crevicular matrix metalloproteinases and tissue inhibitor of metalloproteinases

This longitudinal study was undertaken to evaluate the changes in GCF levels of FIB-CL, SL and TIMP after periodontal treatment, their ability to predict further treatment needs, to differentiate between bleeding and non-bleeding, suppurating and non-suppurating as well as their possible association with GCF volume and clinical indices. Multivariate repeated measures analysis of variance (MANOVA) were employed on the $\log_{10}$ transformed data in order to determine significant differences in GCF levels of the proteinases and their inhibitor as well as changes in attachment levels (AL), pocket depths (PD) and GCF volumes between different appointments. When a significant effect was detected by the use of MANOVA, follow up analysis was performed using univariate paired t-tests in order to determine the direction and location of the differences. Non-parametric repeated measures analysis of variance (Friedman test) was used to determine the differences in plaque index (PlI) and gingival index (MGI) between three visits. Follow up analysis was performed by the use of Sign test to locate the differences. Attempts were made to demonstrate differences in dichotomous data (bleeding on probing and suppuration) between three examinations by

using Cochran Q test which was followed by Chi-square test in order to locate the differences. Using two-sample t-tests, the baseline levels of FIB-CL, SL and TIMP were compared between groups of bleeding and non-bleeding sites, suppurating and non-suppurating sites, BRS and WRS as well as sites which did or did not gain attachment. Using one way analysis of variance, the levels of MMPs and TIMP were compared in groups of sites with different pocket depths. In these series of analyses, because of the number of comparisons the significant level was set at 0.025. The levels of the enzyme and inhibitor in the above mentioned groups of sites were compared between different visits by the use of paired t-tests. The associations between the clinical indices and the levels of SL and TIMP were assessed using the non-parametric Spearman rank correlation coefficients. The Pearson correlation coefficient tests were performed on $\log_{10}$ transformed data to assess the association between the response to treatment and percentage of bleeding sites and those with PD>3.5mm at the reassessment and the follow-up visits. GCF samples were obtained from twenty one subjects, each provided 8 sites, with advanced periodontitis. The enzymes and inhibitor were examined in the same GCF samples and results were expressed as total amounts in pg/30s of sample collection.

3.2.1 Baseline findings

One hundred and sixty eight sites with advanced periodontal

tissue breakdown were sampled. Table 3.5 presents the mean ±SEM of clinical indices, GCF volumes and the mean levels plus 95% confidence intervals of SL and TIMP in study sites.

3.2.1.1 GCF levels of FIB-CL at baseline: comparisons between different groups of sites

FIB-CL was detectable in only 45 (26.8%) out of 168 sites. This enzyme was detected ,on average, in 2.1 out of 8 sites (26.2%) per subject. FIB-CL had much higher levels than SL in sites where it was detected. Five subjects had no detectable FIB-CL in any site examined. The enzyme was detected in only one site in 4 subjects. Two sample t-test showed no significant difference in the levels of FIB-CL between bleeding (n=131) and non-bleeding (n=37) sites (p=0.146). There was no significant difference in the mean GCF levels of enzyme when suppurating (n=36) and non-suppurating (n=132) sites were compared (two-sample t-test, p=0.721). One way analysis of variance failed to discover any significant difference between three groups of sites with different pocket depths (PD≤5mm, 5mm>PD≤6.5mm and PD>6,5mm) for their FIB-CL levels (p=0.484)(Table 3.6).

3.2.1.2 GCF levels of SL at baseline: comparisons between different groups of sites

Analysis of GCF samples showed that SL was present at

detectable levels in 82 (48.8%) sites, 3.9 out of 8 sites on average per patients. One subject did not have SL at any of the eight sites examined. Two-sample t-test demonstrated a significant effect (p=0.020) when bleeding and non-bleeding sites were compared for their GCF SL levels. There was no significant difference when suppurating and non-suppurating sites were compared (two-sample t-test, p=0.530). There was no significant difference between three groups of sites with different pocket depths (PD≤5mm, 5mm>PD≤6.5mm and PD>6.5mm) for their SL levels (one way ANOVA, p=0.824)(Table 3.6).

3.2.1.3 GCF levels of TIMP at baseline: comparisons between different groups of sites

TIMP was detected in 167 (99.4%) sites. In contrast to the SL findings, the two sample t-test did not show a significant difference between the TIMP levels in bleeding and non-bleeding sites (p=0.257), while when sites with PD≤5mm and those with PD>5mm were compared for their GCF TIMP levels a significant effect was found at p=0.024. Similarly to SL, suppurating and non-suppurating sites showed no significant difference in their TIMP levels (two-sample t-test, p=0.580) (Table 3.6). No significant difference was found when other groups of sites with different pocket depths (5mm>PD≤6.5mm and PD>6.5mm) were compared for their TIMP levels.

Table 3.5 Clinical and biochemical parameters (pg/30s) from 168 sites in 21 patients.

Parameter	Mean SEM/(95% CI)
Modified GI	3.140±0.05
Plaque Index	1.300±0.19
Pocket depth (mm)	5.580±0.10
GCF volume (µl)	0.406±0.02
Stromelysin	5.7 (3.4-8)
TIMP	229.6 (201.0-259.8)

Table 3.6 Comparison between FIB-CL, SL and TIMP levels from different group of sites using two sample t-test. Probability levels are shown against each comparison for FIB-CL, SL and TIMP. The number of sites in each groups are shown as footnotes.

Comparison	p for FIB-CL	p for SL	p for TIMP
Bleeding vs non-bleeding	0.146	0.020	0.257
Suppurating vs non-suppurating	0.721	0.530	0.580
PD≤5mm vs PD>5mm	0.793	0.834	0.024

FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases
bleeding sites n= 131
non-bleeding sites n= 37
suppurating sites n= 36
non-suppurating sites n= 132
sites with PD<5mm n= 64
sites with PD>5mm n= 104

Table 3.7 Spearman rank correlation coefficients (r) between clinical indices and SL and TIMP levels (pg/30s) in GCF from 168 sites in 21 patients at the baseline examination. Probability levels (p) are shown.

Parameter	Stromelysin	TIMP
Vol.	r=0.078 (p=0.318)	r=0.592 (p<0.001)
MGI	r=0.192 (p=0.013)	r=-0.001(p=0.989)
PlI	r=0.002 (p=0.984)	r=0.075 (p=0.334)
PD	r=0.049 (p=0.527)	r=0.193 (p=0.012)

Vol.= GCF volume in µl
MGI= modified gingival index
PlI= plaque index
PD= pocket depth in mm

In order to assess the weight of individual clinical parameters and their combination on the levels of FIB-CL, SL and TIMP discriminant analysis was performed, and this revealed the followings:

MGI had the greatest effect on FIB-CL level while BOP+suppuration had the least effect. A combination of clinical parameters including BOP+suppuration, GCF volume, MGI and PD was found to have exerted the most effect on the level of this enzyme. These clinical parameters were able to predict the presence or absence of FIB-CL in 64% of the sites.

BOP+suppuration had the greatest effect on SL level while it was the opposite for GCF volume. BOP+suppuration, PD and MGI, in combination had the most effect on the level of SL in GCF. This combination of clinical parameters was able to predict the enzyme's level in 63% of the sites.

GCF volume was found to have the greatest effect on TIMP level while BOP+suppuration had the least effect. A combination of clinical parameters including GCF volume, PD and MGI showed the greatest effect on the GCF level of TIMP. These clinical parameters were able to predict the level of TIMP in 67% of the sites.

3.2.1.4 Correlation between clinical findings and GCF levels of matrix metalloproteinases and their inhibitor at the baseline examination

The association of SL and TIMP with the clinical indices and GCF volume is shown in Table 3.7. Overall, SL and TIMP's correlation with clinical indices and GCF volume were either weak or not significant.

3.2.2 Clinical and biochemical findings at the reassessment and the follow-up visits

At least six weeks after the last treatment session of the hygiene phase therapy (HPT) all the patients were assessed at a reassessment visit. After recording clinical parameters GCF samples were collected and a conventional pocket chart was constructed. At this visit sites were assigned to 'no treatment', 'repeated root planing' and 'surgery' treatment groups based on their pocket depth and the state of bleeding on probing (section 2.4.5.2). Six weeks after repeated root planing, 10 weeks after surgical treatment and for those who did not need further treatment, almost 3 months after the reassessment examination, all the patients attended for a follow-up visit in which all the procedures in the baseline and the reassessment visits were repeated. One of the study teeth between the baseline and the reassessment visits had been extracted, thus the number of study sites decreased to 167 at reassessment and

all the comparisons between the baseline and the reassessment data were made on 167 sites. Between the reassessment and the follow-up examinations, a further study tooth was lost also, reducing the number of sites to 166. During this period one subject had a wire splint fitted which excluded another 6 teeth from attachment level measurements. Analysis was performed considering both the site and subject as the experimental unit. When the subject was considered as the unit, sites within each subjects were averaged and one score produced per subjects for each clinical and biochemical parameter. Wherever data was not normally distributed it was transformed to $\log_{10}$ in order to use parametric tests.

3.2.2.1 GCF volume and clinical indices at the reassessment and the follow-up examinations

The mean, SD and SEM for GCF volumes and clinical indices in three examinations are shown in Table 3.8. Multivariate repeated measures analysis of variance (MANOVA) as well as paired t-test was used to determine significant differences between GCF volume, attachment level (AL) and pocket depth (PD) between these three appointments (Tables 3.9 & 3.10). Non-parametric repeated measures analysis of variance (Friedman test) and Sign tests were also employed to detect changes in plaque index (PlI) and gingival index scores (MGI). The number of bleeding and suppurating sites decreased at the reassessment and the follow-up visits as

Table 3.8 Multivariate repeated measures analysis of variance (MANOVA) for clinical parameters with subjects as unit of analysis. Mean, SEM and SD are shown against each parameter for different examinations. Significance level (p value) is given.

Clinical data Average of subjects				
Parameter	**n**	**Mean**	**SEM**	**SD**
Attachment level (mm)				
Baseline	21	10.8735	0.2790	1.2784
Reassessment	21	10.1670	0.2740	1.2557
Follow-up	21	10.2732	0.2877	1.3182
Pocket depth (mm)				
Baseline	21	5.5786	0.1542	0.7065
Reassessment	21	3.9940	0.1247	0.5715
Follow-up	21	3.3134	0.1830	0.8384
GCF Volume (µl)				
Baseline	21	0.4064	0.0342	0.1568
Reassessment	21	0.3023	0.0272	0.1246
Follow-up	21	0.1833	0.0139	0.0639
Modified GI				
Baseline	21	3.1429	0.0988	0.4529
Reassessment	21	1.7662	0.1323	0.6061
Follow-up	21	1.4422	0.1169	0.5357
Plaque Index				
Baseline	21	1.2679	0.1684	0.7719
Reassessment	21	0.6922	0.1231	0.5641
Follow-up	21	0.6335	0.1048	0.484
Bleeding on probing				
Baseline	21	0.7798	0.0621	0.2848
Reassessment	21	0.3724	0.0474	0.2173
Follow-up	21	0.3189	0.0548	0.2512
Suppuration				
Baseline	21	0.2143	0.0623	0.2854
Reassessment	21	0.0298	0.0191	0.0875
Follow-up	21	0	0	0
MANOVA			**p=0.002**	

Table 3.9 Multivariate repeated measures analysis of variance (MANOVA) for clinical parameters with sites as unit of analysis. Mean, SEM and SD are shown against each parameter for different examinations. Significance level (p value) is given.

Parametric clinical data				
Parameter	**n**	**Mean**	**SEM**	**SD**
Attachment level (mm)				
Baseline	168	10.8735	0.1505	1.9504
Reassessment	166	10.1663	0.1426	1.8374
Follow-up	160	10.2244	0.1479	1.8708
Pocket depth (mm)				
Baseline	168	5.5786	0.1019	1.3201
Reassessment	167	3.9940	0.1005	1.2986
Follow-up	166	3.3229	0.1005	1.2944
GCF Volume (μl)				
Baseline	168	0.4064	0.0229	0.2964
Reassessment	167	0.3014	0.0194	0.2511
Follow-up	166	0.1835	0.0119	0.15372
MANOVA				**p<0.0001**

Table 3.10 Follow up analysis for the MANOVA of the Table 3.9 for clinical parameters. Mean differences, SD, number of pairs and significance levels (p values) for paired t-tests are given.

Parametric clinical data				
	n	**mean dif.**	**SD**	**t-test p**
Attachment level (mm)				
Baseline-Reassessment	166	0.6967	1.062	<0.001
Baseline-Follow-up	160	0.6109	1.175	<0.001
Reassessment-Follow-up	160	-0.1125	0.991	0.153
Pocket depth (mm)				
Baseline-Reassessment	167	1.5808	1.405	<0.001
Baseline-Follow-up	166	2.2229	1.527	<0.001
Reassessment-Follow-up	166	0.6398	0.239	<0.001
GCF Volume (μl)				
Baseline-Reassessment	167	0.1042	0.349	<0.001
Baseline-Follow-up	166	0.2226	0.316	<0.001
Reassessment-Follow-up	166	0.1160	0.256	<0.001

Table 3.11 Clinical indices' mean, SD, frequency distribution (f) and percentage (%) for each score in different examinations. Number of sites in each visit is shown.

Non-parametric clinical data						
	n	Mean	SD	Score		
Modified GI					f	%
Baseline	168	3.143	0.631	0	-	-
				1	1	0.60
				2	20	11.9
				3	101	60.1
				4	46	27.4
Reassessment	167	1.760	0.788	0	7	4.2
				1	55	33.0
				2	76	45.5
				3	29	17.3
				4	-	-
Follow-up	166	1.446	0.734	0	11	6.6
				1	83	50.0
				2	59	35.5
				3	13	7.90
				4	-	-
Plaque Index						
Baseline	168	1.268	0.944	0	39	23.2
				1	64	38.1
				2	46	27.4
				3	19	11.3
Reassessment	167	0.695	0.742	0	79	47.3
				1	60	35.9
				2	28	16.8
				3	-	-
Follow-up	166	0.632	0.819	0	94	56.6
				1	42	25.3
				2	27	16.3
				3	3	1.8
BOP						
Baseline	168	-	-	0	37	22.0
				1	131	78.0
Reassessment	167	-	-	0	105	62.9
				1	62	37.1
Follow-up	166	-	-	0	113	68.1
				1	53	31.9
Suppuration						
Baseline	168	-	-	0	132	78.6
				1	36	21.4
Reassessment	167	-	-	0	162	97.0
				1	5	3.00
Follow-up	166	-	-	0	166	100
				1	-	-

BOP= bleeding on probing

Table 3.12 Comparison of gingival and plaque scores between different visits using non-parametric repeated measures analysis of variance (Friedman test) followed by paired Sign tests. Dichotomous data (bleeding on probing and suppuration) are compared using Cocharn Q test followed by Chi-square tests. Significance levels (p values) are given. Base, Reass and Follow represent the baseline, the reassessment and the follow-up examinations respectively.

Non-parametric clinical data				
	Friedman p-value	Sign test p-value		
		Base-Reass	Base-Follow	Reass-Follow
MGI	<0.0001	<0.0001	<0.0001	0.0002
PlI	<0.0001	<0.0001	<0.0001	0.1824
	Cochran Q p-value	Chi-square test p-value		
		Base-Reass	Base-Follow	Reass-Follow
BOP	<0.0001	<0.0001	<0.0001	0.3211
S	<0.0001	<0.0001	<0.0001	0.1250

MGI= modified gingival index
PlI= plaque index
BOP= bleeding on probing
S= suppuration

Table 3.13 Follow up analysis of MANOVA in Table 3.8 with subject as unit of analysis. Using paired t-tests, the clinical parameters between different visits are compared. Mean differences, SD and the significance levels (p values) are given.

Parametric clinical data Average of n=21 subjects				
	n	mean dif	SD	t-test p
Attachment level (mm)				
Baseline-Reassessment	21	0.7065	0.478	<0.001
Baseline-Follow-up	21	0.6003	0.699	0.001
Reassessment-Follow-up	21	-0.1062	0.445	0.287
Pocket depth (mm)				
Baseline-Reassessment	21	1.5849	0.873	<0.001
Baseline-Follow-up	21	2.2651	1.029	<0.001
Reassessment-Follow-up	21	0.6803	0.759	0.001
GCF Volume (µl)				
Baseline-Reassessment	21	0.1041	0.157	0.006
Baseline-Follow-up	21	0.2231	0.152	<0.001
Reassessment-Follow-up	21	0.1190	0.117	<0.001
Modified GI				
Baseline-Reassessment	21	1.3767	0.453	<0.001
Baseline-Follow-up	21	1.7007	0.606	<0.001
Reassessment-Follow-up	21	0.3240	0.536	0.049
Plaque Index				
Baseline-Reassessment	21	0.5757	0.789	0.003
Baseline-Follow-up	21	0.6344	0.786	0.001
Reassessment-Follow-up	21	0.0587	0.474	0.577
Bleeding on probing				
Baseline-Reassessment	21	0.4073	0.297	<0.001
Baseline-Follow-up	21	0.4609	0.385	<0.001
Reassessment-Follow-up	21	0.0536	0.310	0.438
Suppuration				
Baseline-Reassessment	21	0.1845	0.287	0.008
Baseline-Follow-up	21	0.2143	0.285	0.003
Reassessment-Follow-up	21	0.0298	0.088	0.135

well as the percentage of sites with higher plaque and gingival scores (Table 3.11). Cochran Q and Chi-square tests were used in the cases of bleeding on probing (BOP) and suppuration (Table 3.12). When data was analyzed both on the site and subject basis, mean GCF volumes and PD measurements decreased significantly at the reassessment and the follow-up sessions from the baseline as well as at the follow-up from the reassessment visit (Tables 3.10 & 3.13). Mean attachment levels (AL), plaque index scores (PlI), bleeding on probing (BOP) and suppuration showed significant reduction at the reassessment and the follow-up appointments from the baseline on both site and subject basis whereas they failed to demonstrate any significant difference at the follow-up from the reassessment visit. However, AL measurements, at both site and subject levels, increased at the follow-up from the reassessment examination which were not significant (Tables 3.9, 3.11, 3.12 & 3.13). Mean modified gingival scores (MGI) reduced significantly at the reassessment and the follow-up visits from the baseline when it was analyzed on both site and subject basis whereas, its reduction at the follow-up from the reassessment visit was only significant when site was the unit of analysis (Tables 3.12 & 3.13).

3.2.2.2 GCF levels of fibroblast collagenase at the reassessment and the follow-up examinations

FIB-CL was detected in 29 (17.4%) sites and 20 (12%) sites

at the reassessment and the follow-up examinations respectively. The mean number of sites with detectable amounts of FIB-CL per subject dropped from 2.1 (26.2%) at the baseline to 1.4 (17.2%) and 0.95% (11.9%) sites at the reassessment and the follow-up visits respectively. The enzyme did not reach the detectable levels in any site in 10 subjects at both the reassessment and the follow-up visits. By the means of multivariate repeated measures analysis of variance (MANOVA) and paired t-test and considering the site as the unit of analysis it was revealed that GCF mean levels of FIB-CL decreased significantly at the reassessment and the follow-up examinations from the baseline ($p=0.025$ and $p<0.001$ respectively) (Tables 3.14 & 3.15). Using the same statistics and on a subject basis, the mean level of enzyme reduced significantly only at the follow-up session from the baseline ($p=0.023$)(Table, 3.17).

3.2.2.3 GCF levels of stromelysin at the reassessment and the follow-up examinations

SL reached the detectable levels in 70 (42%) and 45 (27.1%) sites at the reassessment and the follow-up visits respectively. This MMP was detected, on average, in 3.3 (41.2%) and 2.1 (26.2%) out of 8 sites per subject at the reassessment and the follow-up examinations, thus the number of sites expressing SL in each individual decreased by 7.6% and 22.5% respectively. 4 subjects at the

Table 3.14 Geometric means and 95% confidence intervals for FIB-CL, SL and TIMP (pg/30s) at different visits. Number of sites and significance level (p value) for MANOVA is given.

Protein	n	Mean	95% C.I.
Fibroblast collagenase			
Baseline	166	3.9	2.8-5.6
Reassessment	166	2.3	1.7-3.0
Follow-up	166	1.9	1.4-2.5
Stromelysin			
Baseline	166	5.6	4.2-7.4
Reassessment	166	4.0	3.1-5.2
Follow-up	166	2.4	1.9-2.9
TIMP			
Baseline	166	228.7	201.8-259.2
Reassessment	166	333.9	291.8-382.8
Follow-up	166	93.30	78.90-110.1
MANOVA			p<0.0001

Table 3.15 Comparison of GCF levels of FIB-CL, SL and TIMP on $\log_{10}$ transformed data between different visits. Follow up analysis for MANOVA of Table 3.14 for the enzymes and inhibitor. Mean differences, SD, number of pairs and significance levels (p values) for paired t-tests are given.

Protein	n	Mean Dif	SD	p
Fibroblast collagenase				
Baseline-Reassessment	167	0.2326	1.325	0.025
Baseline-Follow-up	166	0.3166	1.117	<0.001
Reassessment-Follow-up	166	0.0826	1.170	0.364
Stromelysin				
Baseline-Reassessment	167	0.5580	1.027	0.052
Baseline-Follow-up	166	0.3777	1.031	<0.001
Reassessment-Follow-up	166	0.2302	0.924	0.002
TIMP				
Baseline-Reassessment	167	-0.1656	0.489	<0.001
Baseline-Follow-up	166	0.3896	0.572	<0.001
Reassessment-Follow-up	166	0.5539	0.537	<0.001

Table 3.16 Multivariate repeated measures analysis of variance (MANOVA) for MMPs and TIMP (pg/30s) with subjects as unit of analysis. Geometric means and 95% confidence intervals are shown against each parameter for different examinations. Significance level (p value) is given.

Average of 21 subjects			
Protein	**n**	**Mean**	**95% C.I.**
Fibroblast collagenase			
Baseline	21	3.9	2.5-6.3*
Reassessment	21	2.3	1.5-3.5
Follow-up	21	1.9	1.3-2.8
Stromelysin			
Baseline	21	5.7	3.7-8.6
Reassessment	21	4.0	2.5-6.4
Follow-up	21	2.4	1.7-3.3
TIMP			
Baseline	21	230.4	185.8-285.7
Reassessment	21	336.8	264.8-428.4
Follow-up	21	93.40	72.20-120.9
MANOVA			**p<0.0001**

* The wider range of confidence intervals shown in this Table compared with those of Table 3.14 is due to the smaller sample size in the present Table.

Table 3.17 Follow up analysis of MANOVA in Table 3.16 with subject as unit of analysis. Using paired t-tests, GCF levels of MMPs and TIMP (on $\log_{10}$ transformed data) between different visits are compared. Mean differences, SD and the significance levels (p values) are given.

Average of 21 subjects				
Protein	**n**	**Mean Dif**	**SD**	**p**
Fibroblast collagenase				
Baseline-Reassessment	21	0.5278	1.303	0.078
Baseline-Follow-up	21	0.5305	0.984	0.023
Reassessment-Follow-up	21	0.0027	1.405	0.993
Stromelysin				
Baseline-Reassessment	21	0.3110	0.577	0.023
Baseline-Follow-up	21	0.5389	0.723	0.003
Reassessment-Follow-up	21	0.2279	0.684	0.143
TIMP				
Baseline-Reassessment	21	-0.1812	0.309	0.014
Baseline-Follow-up	21	0.3515	0.309	<0.001
Reassessment-Follow-up	21	0.5326	0.360	<0.001

reassessment and 7 subjects at the follow-up sessions did not have detectable SL at any site examined. Using multivariate repeated measures analysis of variance (MANOVA) and paired t-test on the site basis, the mean GCF levels of SL decreased significantly at the follow-up visit both from the baseline and the reassessment visits ($p<0.001$ and $p=0.002$ respectively)(Tables 3.14 & 3.15). However, the difference between the mean levels of SL at the baseline and the reassessment visits was just outwith the level of statistical significance ($p=0.052$)(Table 3.15). Similar statistical tests, when used on a subject basis, showed that SL mean levels reduced significantly at both the follow-up and the reassessment from the baseline examination (Tables 3.16 & 3.17). However, the levels of theses GCF components showed a large site to site variation within and between subjects.

3.2.2.4 GCF levels of tissue inhibitor of metalloproteinases at the reassessment and the follow-up examinations

TIMP was found at detectable levels in 100% of the sites (n=167) at the reassessment and in 98.2% (n=163) of the sites at the follow-up sessions. TIMP was detected in 100% and 98.2% of the 8 sites examined per individual at the reassessment and the follow-up visits respectively. Multivariate repeated measures analysis of variance and paired t-test, on both site (Tables 3.14 & 3.15) and

subject basis (Tables 3.16 & 3.17), revealed that mean GCF levels of TIMP increased significantly after hygiene phase therapy i.e. at reassessment ($p<0.001$ and $p=0.014$ respectively), whereas it showed significant decrease at the follow-up both from the baseline and the reassessment examinations with a (p) value <0.001 for all occasions.

3.2.2.5 Correlation between clinical findings and GCF levels of matrix metalloproteinases and their inhibitor at the reassessment and the follow-up examinations

With the exception of mean levels of SL and TIMP which showed positive and significant associations with the mean GCF volume all other correlations were either negative or low and failed to reach statistical significance (Table 3.18). At the follow-up visit, in addition to the positive and significant correlation between GCF volume and SL and TIMP (mean levels), mean MGI scores was associated with the mean GCF levels of SL and TIMP (Table 3.19)

3.2.3 Prediction of response to treatment at the reassessment visit according to the baseline GCF levels of matrix metalloproteinases and their inhibitor

At the reassessment visit, as part of normal clinical procedure to determine further treatment needs, all the

Table 3.18 Spearman rank correlation coefficient (r) between clinical indices and FIB-CL, SL and TIMP levels in GCF from 167 sites in 21 patients at the reassessment examination. Probability levels (p) are shown.

Parameter	Stromelysin	TIMP
Vol.	r=0.313 (p<0.001)	r=0.440 (p<0.001)
MGI	r=0.022 (p=0.782)	r=-0.036(p=0.646)
PlI	r=0.057 (p=0.461)	r=0.057 (p=0.467)
PD	r=-0.05 (p=0.523)	r=-0.029(p=0.706)

Vol.= GCF volume in µl
MGI= modified gingival index
PlI= plaque index
PD= pocket depth in mm

Table 3.19 Spearman rank correlation coefficient (r) between clinical indices and SL and TIMP levels in GCF from 166 sites in 21 patients at the follow-up examination. Probability levels (p) are shown.

Parameter	Stromelysin	TIMP
Vol.	r=0.155 (p=0.046)	r=0.696 (p<0.001)
MGI	r=0.167 (p=0.031)	r=-0.164(p=0.035)
PlI	r=0.101 (p=0.195)	r=0.058 (p=0.457)
PD	r=-0.075(p=0.336)	r=-0.058(p=0.459)

Vol.= GCF volume in µl
MGI= modified gingival index
PlI= plaque index
PD= pocket depth in mm

sites were examined for pocket depth and bleeding on probing. Treatment was considered successful i.e. a positive response, if a site did not bleed on probing and had a pocket depth of equal or less than 3.5mm. Bleeding (62 (37.1%)) and non-bleeding (105 (62.9%)) as well as sites with more (99 (59.3%)) or less (68 (40.7%)) than 3.5mm pocket, which were on the study, formed different groups and their baseline levels of MMPs and TIMP were compared. Forty three (25.7%) sites met both criteria for treatment success at the reassessment examination whereas 41 (24.6%) sites failed to do so. The baseline levels of the enzymes and inhibitor were compared in these groups of sites. The differences in MMPs and TIMP between the baseline and the reassessment visits were also assessed. Using a more stringent criteria including PD measurements, MGI scores and the state of bleeding and suppuration and in order to eliminate the smoothing effects of intermediately responding sites, the best responding sites (BRS) (n=15) and worst responding sites (WRS) (n=15) were selected (Table 3.20). The baseline levels of MMPs and TIMP, in this groups of sites, were compared to investigate their ability in predicting treatment outcome.

3.2.3.1 Prediction of response to treatment at the reassessment session according to the baseline levels of fibroblast collagenase

Using two-sample t-test, the baseline levels of FIB-CL was

compared between groups of bleeding and non-bleeding sites as well as sites with pocket depth of less or more than 3.5mm. The mean enzyme's levels at the baseline visit failed to differentiate between two groups of sites with pocket depth less or more than 3.5mm at the reassessment examination (p=0.231)(Table 3.21). Mean levels of FIB-CL at the baseline visit was not able to distinguish bleeding sites from those which did not bleed upon probing to the base of the pocket after hygiene phase therapy at the reassessment visit (p=0.082)(Table 3.22). FIB-CL mean baseline's levels, also failed to predict the response to the treatment, when both bleeding status and pocket depth were considered as criteria for treatment success (two-sample t-test, p=0.057). The baseline levels of this enzyme could not differentiate between BRS and WRS at the reassessment visit (two-sample t-test, p=0.781) (Table 3.23).

3.2.3.2 Prediction of response to treatment at the reassessment session according to the baseline levels of stromelysin

Different groups of sites, based on their bleeding status as well as their pocket depths at reassessment, were compared for their SL levels at the baseline (two-sample t-test). The mean levels of SL at the baseline was not able to differentiate between sites with PD of <3.5mm and those with >3.5mm pocket (p=0.453)(Table 3.21). There was no

Table 3.20 Comparisons of GCF volumes, gingival scores, pocket depths and GCF levels of stromelysin (SL) and TIMP between best responding sites (BRS) (n=15) and worst responding sites (WRS) (n=15) at the reassessment examination. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	BRS	WRS	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
Vol.	0.253±0.065	0.507±0.073	0.015
MGI	0.9±0.091	2.5±0.133	<0.001
PD	2.6±0.105	5.5±0.133	<0.001
SL	3.1 (1.3-7.20)	3.3 (1.5-7.2)	0.911
TIMP	379.7 (200.4-710.2)	412.3 (301.4-563.9)	0.816

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

Table 3.21 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites with less or more than 3.5mm PD after HPT, at the reassessment visit. Geometric mean, 95% confidence intervals and number of sites in each group are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels

Protein	**n**	**Mean**	**95% C.I.**
FIB-Collagenase			
PD<3.5 after HPT	68	5.1	2.8-9.3
PD>3.5 after HPT	99	3.2	2.1-5.0
t-test p-value = 0.231			
Stromelysin			
PD<3.5 after HPT	68	6.5	4.1-10.2
PD>3.5 after HPT	99	5.2	3.6-7.4
t-test p-value = 0.453			
TIMP			
PD<3.5 after HPT	68	219.9	185.5-260.7
PD>3.5 after HPT	99	235.7	197.1-281.9
t-test p-value = 0.577			

Table 3.22 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites which did or did not bleed on probing after HPT, at the reassessment visit. Geometric mean, 95% confidence intervals and number of sites in each group are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
FIB-Collagenase			
no BOP after HPT	105	3.0	2.0-4.6
BOP after HPT	62	6.0	3.1-11.3
t-test p-value = 0.082			
Stromelysin			
no BOP after HPT	105	5.1	3.6-7.2
BOP after HPT	62	6.8	4.2-11.0
t-test p-value = 0.350			
TIMP			
no BOP after HPT	105	222.8	185.8-267.2
BOP after HPT	62	240.3	207.5-278.3
t-test p-value = 0.520			

Table 3.23 Baseline levels of MMPs and TIMP (pg/30s) were compared between best responding sites (BRS) and worst responding sites (WRS) after HPT, at the reassessment visit. Geometric mean and 95% confidence intervals are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
FIB-Collagenase			
BRS after HPT	15	6.0	1.5-23.6
WRS after HPT	15	7.9	2.0-31.2
t-test p-value = 0.781			
Stromelysin			
BRS after HPT	15	8.0	3.2-20.2
WRS after HPT	15	4.8	2.0-12.0
t-test p-value = 0.440			
TIMP			
BRS after HPT	15	167.6	114.3-245.6
WRS after HPT	15	249.2	190.8-325.5
t-test p-value = 0.101			

significant difference when bleeding and non-bleeding sites, at the reassessment appointment, were compared for their SL levels at the baseline (p=0.350) (Table 3.22). The mean baseline levels of SL also could not predict the response to treatment, when the state of bleeding on probing and pocket depth were both used as criteria for treatment success (two-sample t-test, p=0.387). The baseline levels of SL failed to distinguish BRS from WRS after HPT (two-sample t-test, p=0.440)(Table 3.23).

3.2.3.3 Prediction of response to treatment at the reassessment session according to the baseline levels of tissue inhibitor of metalloproteinases

At the reassessment visit, sites with PD<3.5mm showed no significant difference over those with PD>3.5mm in their baseline GCF TIMP levels (two-sample t-test, p=0.577)(Table 3.21). Two sample t-test did not show significant difference between the baseline TIMP levels in sites which bled on probing at reassessment and those which did not (p=0.520)(Table 3.22). The mean baseline level of TIMP could not predict the response to treatment, when the state of bleeding on probing and pocket depth were both employed as criteria for treatment success (two-sample t-test, p=0.192). TIMP's baseline levels also failed to discriminate between BRS and WRS at the reassessment visit (two-sample t-test, p=0.101)(Table 3.23).

3.2.3.4 Comparison of clinical and biochemical parameters between the sites which did or did not respond to treatment at the reassessment examination

In order to investigate the effect of hygiene phase of therapy on the levels of enzymes and inhibitor in sites which did or did not respond to treatment, as was assessed by a combination of criteria, the levels of proteins as well as clinical parameters, at the reassessment visit, were compared in those sites. When different groups of sites i.e. sites with PD<3.5mm versus those with PD>3.5mm and bleeding versus non-bleeding sites were compared for their MGI (Mann-Whitney test), GCF volume as well as the mean levels of FIB-CL, SL and TIMP (two-sample t-test), no significant difference was observed.

3.2.3.5 Comparisons between different groups of sites for their GCF levels of matrix metalloproteinases and their inhibitor at the reassessment examination

The experimental sites were grouped according to their pocket depths and state of bleeding on probing at the reassessment visit. One way analysis of variance showed no significant difference between GCF levels of MMPs and TIMP in groups of sites with PD<3.5mm which did or did not bleed on probing as well as those with PD>3.5mm and different

bleeding status (Table 3.24).

3.2.3.6 Comparisons between best and worst responding sites for their GCF levels of matrix metalloproteinases and their inhibitor at the reassessment examination

Since the mean levels of FIB-CL, SL and TIMP failed to distinguish between different groups of sites which did or did not respond to treatment (sections 3.2.3.4 and 3.2.3.5) when the discriminating factors for treatment success were pocket depth and the state of bleeding on probing, using a more stringent criteria, the enzymes and inhibitor between the best responding sites (BRS)(n=15) and worst responding sites (WRS)(n=15) were compared. The BRS were those with no BOP, PD<3mm and MGI≤1 whereas the WRS bled on probing with PD>4mm and MGI≥2 at the reassessment examination. None of the sites in both groups showed suppuration on probing. Two-sample t-tests demonstrated lower levels of FIB-CL, SL and TIMP in BRS than in WRS. However these differences did not reach the statistical significance (Table 3.20).

3.2.3.7 Comparisons between before and after hygiene phase therapy of GCF volume, clinical indices, matrix metalloproteinases and their inhibitor in sites which responded to the treatment at the reassessment examination

The mean GCF levels of FIB-CL, SL and TIMP as well as GCF volume and gingival scores in sites (n=47) which responded to treatment, at the reassessment examination, were compared to their baseline levels. The mean levels of FIB-CL and SL decreased at reassessment, but the differences did not reach significance (paired t-test, $p=0.672$ and $p=0.379$ respectively), whereas TIMP's level increased significantly ($p=0.002$). GCF volume showed some reduction which was not significant, whereas PD significantly decreased (paired t-test, $p=0.082$ and $p<0.001$ respectively). The mean gingival scores reduced significantly at the reassessment examination (Sign test, $p<0.001$)(Table 3.25).

3.2.4 Prediction of response to treatment at the follow-up visit according to the baseline GCF levels of matrix metalloproteinases and their inhibitor

All subjects who either did or did not require further treatment after hygiene phase therapy were re-examined at the follow-up session. Among other clinical parameters,

Table 3.24 Comparison between GCF levels of MMPs and TIMP in different sites which were grouped according to their PD and state of bleeding on probing at the reassessment visit. Number of sites in each group and significance level (p value) for one way analysis of variance are given.

	FIB-CL	SL	TIMP
Group of sites	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)
PD<3.5mm, no BOP n=47	2.8 (1.5-5.2)	4.7 (2.9-7.7)	339.5 (251.9-457.5)
PD<3.5mm, BOP n=21	4.0 (1.4-11.2)	4.9 (2.2-10.7)	280.5 (185.3-424.4)
PD>3.5mm, no BOP n=58	2.2 (1.4-3.6)	3.6 (2.3-5.7)	310.3 (252.2-381.8)
PD>3.5mm, BOP n=41	1.4 (1.0-1.9)	3.3 (1.9-5.7)	405.2 (310.2-529.3)
p value	0.123	0.684	0.369

FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

Table 3.25 Comparisons of GCF volumes, gingival scores and GCF levels of MMPs and TIMP between the baseline and the reassessment visits in 47 sites with PD<3.5mm which did not bleed on probing at the reassessment examination. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Reassessment	
Parameter	Mean ±SEM (95% CI)	Mean ±SEM (95% CI)	p value
Vol.	0.346±0.037	0.287±0.032	0.082
MGI	3.1±0.093	1.7±0.106	<0.001
PD	4.7±0.132	2.8±0.063	<0.001
FIB-CL	3.5 (1.8-6.9)	2.8 (1.5-5.2)	0.672
SL	6.5 (3.8-11.1)	4.7 (2.9-7.7)	0.379
TIMP	219.0 (176.4-271.9)	339.5 (251.9-457.5)	0.002

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitors of metalloproteinases

pocket depths as well as the status of bleeding upon probing to the base of the pocket/sulcus were recorded. The response to the treatment was considered positive if a site did not bleed on probing and had a pocket depth of <3.5mm. At this visit 107 (64.5%) of the study sites had a pocket depth of <3.5mm, while PD in the rest of the study sites (59, 35%) was >3.5mm. Out of 166 study sites available, 113 (68%) sites did not bleed on probing whereas other 53 (32%) bled upon probing. Eighty one (48.8%) sites neither bled upon probing nor had a PD>3.5mm at the follow-up examination and 27 (16.3%) sites failed to meet these criteria. The baseline levels of the enzymes and inhibitor were compared in these groups of sites. The differences in MMPs and TIMP between the baseline and the follow-up visits were also assessed. However 58 (36.1%) sites failed to meet both criteria together. The baseline levels of MMPs and TIMP, in best responding sites (BRS) (n=17) and worst responding sites (WRS) (n=17) were also compared in order to investigate their ability in predicting treatment outcome. Clinical and biochemical parameters in BRS and WRS at the follow-up examination are shown in Table 3.26.

3.2.4.1 Prediction of response to treatment at the follow-up session according to the baseline levels of fibroblast collagenase

The mean GCF levels of FIB-CL at the baseline failed to distinguish sites with PD<3.5mm from those with pocket

depth of more than 3.5mm (two-sample t-test, p=0.971)(Table 3.27). Two-sample t-test showed no significant difference between bleeding and non-bleeding sites, at the reassessment visit, when they were compared for their baseline mean levels of enzyme (p=0.416)(Table 3.28). The mean baseline levels of FIB-CL could not predict the response to the treatment, when the state of bleeding on probing and pocket depth were both employed as criteria for treatment success (two-sample t-test, p=0.192). Using the same statistics the baseline levels of this enzyme failed to differentiate between BRS and WRS (p=0.493)(Table 3.29).

3.2.4.2 Prediction of response to treatment at the follow-up session according to the baseline levels of stromelysin

The mean levels of GCF SL at the baseline was able to predict the response to treatment at the follow-up visit. Two-sample t-test demonstrated a highly significant difference (p=0.001) between sites with PD<3.5mm and those with PD>3.5mm at the follow-up visit, when they were compared for their baseline mean GCF levels of SL (Table 3.27). The sites which did or did not bleed at the follow-up examination could also be differentiated based on their mean GCF levels of SL at the baseline visit (two-sample t-test, p=0.009) (Table 3.28). The mean baseline levels of SL was able to differentiate sites which responded to the treatment, when the state of bleeding on probing and pocket

depth were both used as criteria for treatment success (two-sample t-test, p=0.001), although its baseline level was higher in sites which responded to the treatment. No significant difference was observed, when BRS and WRS were compared for their baseline levels of SL (two-sample t-test, p=0.055) (Table 3.29).

3.2.4.3 Prediction of response to treatment at the follow-up session according to the baseline levels of tissue inhibitor of metalloproteinases

There was no significant difference between sites with PD<3.5mm and sites with PD>3.5mm at the follow-up visit, when they were compared for their mean baseline levels of TIMP (two-sample t-test, 0.077)(Table 3.27). Using the same statistical method, it was shown that the mean baseline levels of TIMP could not distinguish bleeding from non-bleeding sites at the follow-up session (p=0.192)(Table 3.28). The mean levels of TIMP at the baseline, also failed to predict the response to the treatment, when both bleeding status and pocket depth were considered as criteria for treatment success (two-sample t-test, p=0.575). TIMP's mean baseline levels was not able to differentiate between BRS and WRS (two-sample t-test, p=860)(Table 3.29).

Table 3.26 Comparisons of GCF volumes, gingival scores and GCF levels of MMPs and TIMP between best responding sites (BRS)(n=17) and worst responding sites (WRS)(n=17) at the follow-up examination. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	BRS	WRS	
Parameter	Mean ±SEM (95% CI)	Mean ±SEM (95% CI)	p value
Vol.	0.150±0.039	0.250±0.045	0.100
MGI	0.9±0.081	2.5±0.125	<0.001
PD	2.1±0.096	5.8±0.311	<0.001
FIB-CL	2.6 (0.9-7.8)	1.9 (0.8-4.5)	0.656
SL	1.8 (0.9-3.2)	4.4 (1.9-9.8)	0.082
TIMP	51.9 (25.2-107.0)	73.3 (40.1-135.3)	0.465

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitors of metalloproteinases

Table 3.27 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites with less or more than 3.5mm PD at the follow-up visit. Geometric mean, 95% confidence intervals and number of sites in each group are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	n	Mean	95% C.I.
FIB-Collagenase			
PD<3.5 at follow-up	107	3.9	2.5-6.1
PD>3.5 at follow-up	59	4.0	2.2-7.2
t-test p-value = 0.971			
Stromelysin			
PD<3.5 at follow-up	107	7.8	5.5-11.2
PD>3.5 at follow-up	59	3.1	2.0-4.70
t-test p-value = 0.001			
TIMP			
PD<3.5 at follow-up	107	250.4	217.5-288.4
PD>3.5 at follow-up	59	194.1	151.6-248.5
t-test p-value = 0.077			

Table 3.28 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites which did or did not bleed on probing, at the follow-up visit. Geometric mean, 95% confidence intervals and number of sites in each group are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
Fibroblast collagenase			
no BOP at follow-up	113	3.5	2.3-5.4
BOP at follow-up	53	4.9	2.5-9.4
t-test p-value = 0.416			
Stromelysin			
no BOP at follow-up	113	7.2	5.1-10.2
BOP at follow-up	53	3.3	2.1-5.2
t-test p-value = 0.009			
TIMP			
no BOP at follow-up	113	217.2	184.1-256.1
BOP at follow-up	53	255.6	212.4-307.5
t-test p-value = 0.192			

Table 3.29 Baseline levels of MMPs and TIMP (pg/30s) were compared between best responding sites (BRS) and worst responding sites (WRS) at the follow-up examination. Geometric mean and 95% confidence intervals are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
FIB-Collagenase			
BRS at follow-up	17	3.7	1.1-12.3
WRS at follow-up	17	6.6	2.1-20.6
t-test p-value = 0.493			
Stromelysin			
BRS at follow-up	17	7.4	2.8-19.2
WRS at follow-up	17	2.2	1.1-4.50
t-test p-value = 0.055			
TIMP			
BRS at follow-up	17	200.8	142.8-282.3
WRS at follow-up	17	193.2	148.2-251.2
t-test p-value = 0.860			

3.2.4.4 Comparison of clinical and biochemical parameters between the sites which did or did not respond to treatment at the follow-up examination

The effect of periodontal therapy on the levels of enzymes and inhibitor in sites which did or did not respond to treatment, as was assessed by a combination of criteria, was evaluated by comparing the levels of proteins as well as clinical parameters at the follow-up visit. Different groups of sites i.e. sites with PD<3.5mm versus those with PD>3.5mm and bleeding versus non-bleeding sites for their MGI (Mann-Whitney test), GCF volume as well as the mean levels of FIB-CL, SL and TIMP (two-sample t-test), at the follow-up visit. With the exception of the mean MGI scores which was significantly lower in sites with PD<3.5mm over those with PD>3.5mm as well as non-bleeding sites compared to bleeding sites at the follow-up examination, none of the comparisons reached the statistical significance.

3.2.4.5 Comparisons between different groups of sites for their GCF levels of matrix metalloproteinases and their inhibitor at the follow-up examination

Different groups of sites were compared for their GCF levels of MMPs and TIMP at the follow-up visit. Using one way analysis of variance it was demonstrated that there was

no significant difference between GCF levels of MMPs and TIMP in sites which did or did not bleed on probing and had a pocket depth of <3.5mm. The same results were obtained for sites with PD>3.5mm which showed different bleeding status (Table 3.30).

3.2.4.6 Comparisons between best and worst responding sites for their GCF levels of matrix metalloproteinases and their inhibitor at the follow-up examination

As described in section 3.2.3.6, the mean levels of FIB-CL, SL and TIMP could not distinguish between different groups of sites which did or did not respond to treatment (sections 3.2.4.4 and 3.2.4.5). The discriminating factors for treatment success used here were pocket depth and the state of bleeding on probing. Using more stringent criteria, the enzymes and inhibitor between the best responding sites (BRS)(n=17) and worst responding sites (WRS)(n=17) were compared. The criteria for the selection of the BRS and WRS have been described in section 3.2.3.6. Two-sample t-tests demonstrated lower levels of SL and TIMP in BRS than in WRS, although these differences were not statistically significant. However, the mean GCF level of FIB-CL was higher in BRS (Table 3.26). These results along with those described in sections 3.2.3.4, 3.2.3.5, 3.2.3.6, 3.2.4.4 and 3.2.4.5 suggest that it is not possible to differentiate successfully treated sites from sites which

Table 3.30 Comparison between GCF levels of MMPs and TIMP (pg/30s) in different sites which were grouped according to their PD and state of bleeding on probing at the follow-up visit. Number of sites in each group and significance level (p value) for one way analysis of variance are given.

	FIB-CL	SL	TIMP
Group of sites	Mean (95% CI)	Mean (95% CI)	Mean (95% CI)
PD<3.5mm, no BOP n=81	1.9 (1.3-2.8)	2.0 (1.5-2.7)	97.1 (76.7-122.8)
PD<3.5mm, BOP n=26	2.3 (1.0-5.0)	2.2 (1.3-3.8)	79.2 (53.9-116.5)
PD>3.5mm, no BOP n=32	1.7 (0.8-3.0)	3.1 (1.7-5.4)	116.6 (74.3-183.2)
PD>3.5mm, BOP n=27	1.8 (0.9-3.5)	3.0 (1.6-5.8)	74.2 (48.3-114.1)
p value	0.923	0.393	0.361

FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

failed to respond to treatment using a diagnostic test based on the levels of MMPs and TIMP in GCF. This is illustrated by assessing its sensitivity, specificity and predictive values. Since, FIB-CL and SL were not detected in 29 sites (85.3%) and 23 (67.7%) sites respectively, a test based on the value of TIMP was used. Thus, the test was considered positive (successful treatment) when a site demonstrated TIMP ≤ 51.9pg/30s. This threshold value represents the geometric mean of TIMP in successfully treated sites (Table 3.26). The sensitivity of the test was low (24%), whereas its specificity reached 76%. Both positive and negative predictive values were 50%, indicating that in 50% of the cases a positive test would have misclassified a site with treatment failure as a successfully treated site and vice versa (Table 3.31).

3.2.4.7 Comparisons between the baseline and the follow up levels of GCF volume, clinical indices, matrix metalloproteinases and their inhibitor in sites which responded to the treatment at the follow-up examination

At the follow-up examination 81 sites showed no signs of bleeding on probing while their PD was <3.5mm. The mean levels of FIB-CL, SL and TIMP in these sites, at the follow-up visit, were compared to their baseline levels. The enzymes and inhibitor mean levels showed a highly significant reduction at the follow-up examination (paired

t-tests, p=0.023 for FIB-CL and p<0.001 for SL and TIMP). The number of sites expressing detectable amounts of FIB-CL reduced from 20 (12%) sites at the baseline to 10 (6%) sites at the follow-up visit. Significant reductions were observed in GCF volume and pocket depth (paired t-test, p<0.001) as well as in gingival scores (Sign test, p<0.001) at the follow-up examination (Table 3.32).

3.2.4.8 Correlation between the response to treatment and the baseline levels of metalloproteinases and their inhibitor on a subject basis

In order to investigate if the average baseline levels of FIB-CL, SL, and TIMP could be associated with the percentage of sites which bled on probing or had a PD>3.5mm at the reassessment and the follow-up examinations, with subject as a unit of analysis, the Pearson correlation coefficient tests were performed on $\log_{10}$ transformed data. The average levels of the enzymes and the inhibitor were obtained by averaging the subjects' mean levels. The only significant association was found between the percentage of sites with a pocket depth of more than 3.5mm at the follow-up visit and the average baseline levels of SL (r=-0.4872, p=0.025)(Table 3.33). The significance of this finding is further reduced by Bonferroni correction which renders it insignificant. SL showed negative and insignificant correlation with sites having PD>3.5mm at the reassessment as well as with bleeding sites at both the reassessment and

the follow-up examinations. However, there was no significant correlation, either positive or negative, between the FIB-CL and TIMP's baseline levels with bleeding sites as well as sites with PD>3.5mm at both post therapy visits (Table 3.33).

3.2.5 Changes in attachment levels after hygiene phase therapy and at the follow-up examination

At each of the three examination visits, two attachment level measurements were taken from the study sites. This experimental design provided a measure of examiner error during the study as well as the appropriate measures for the use of the tolerance method for detecting attachment level changes (section 2.4.2.1).

After hygiene phase therapy, at the reassessment examination 24 (14.4%) sites, in 12 subjects, demonstrated significant attachment gain whereas, attachment loss was not observed in any site. According to the follow-up attachment level measurements, 25 sites (15%), in 11 subjects, had gained attachment from the baseline levels. However, at this visit 5 (3%) sites within 3 patients showed significant attachment loss from the baseline.

Table 3.31 Characteristics of a diagnostic test based on the levels (pg/30s) of tissue inhibitor of metalloproteinases (TIMP) at the follow-up examination. Sensitivity, specificity and positive and negative predictive values are shown.

	Successful treatment	Unsuccessful treatment	
Positive test			
TIMP≤51.9pg/30s	4	4	Positive Predictive Value 50%
Negative test			
TIMP>51.9pg/30s	13	13	Negative Predictive Value 50%
	Sensitivity 24%	Specificity 76%	

Table 3.32 Comparisons of GCF volumes, gingival scores and GCF levels of MMPs and TIMP between the baseline and the follow-up visits in 81 sites with PD<3.5mm which did not bleed on probing at follow-up examination. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Follow-up	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
Vol.	0.395±0.033	0.173±0.016	<0.001
MGI	3.1±0.074	1.2±0.069	<0.001
PD	5.3±0.139	2.6±0.065	<0.001
FIB-CL	3.6 (2.2-6.0)	1.9 (1.3-2.8)	0.023
SL	8.8 (5.8-13.2)	2.0 (1.5-2.7)	<0.001
TIMP	236.6 (202.0-277.1)	97.1 (76.7-122.8)	<0.001

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

Table 3.33 Pearson correlation coefficients on log10 transformed data between the percentage of sites in each subject that bled on probing or had PD>3.5mm at the reassessment (after HPT) and the follow-up visits and averaged baseline levels of MMPs and TIMP in 21 subjects. Correlation coefficients (r) and significance levels (p values) are given.

Response to treatment according to baseline MMP and inhibitor levels correlations n=21 subjects

Mean Protein	%sites with BOP after HPT	%sites with BOP at follow-up	%sites with PD>3.5 after HPT	%sites with PD>3.5 at follow-up
FIB-CL	r=0.126 p=0.586	r=0.155 p=0.504	r=0.013 p=0.955	r=0.135 p=0.558
SL	r=-0.279 p=0.221	r=-0.153 p=0.508	r=-0.233 p=0.310	r=-0.487 p=0.025
TIMP	r=-0.050 p=0.830	r=-0.144 p=0.534	r=0.189 p=0.412	r=-0.403 p=0.070

FIB-CL= fibroblast derived collagenase
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

3.2.5.1 Prediction of response to the treatment from the changes in the attachment levels at the reassessment and the follow-up examinations according to the baseline levels of matrix metalloproteinases and their inhibitor

Using a more accurate approach, the response to the treatment in relation to the baseline levels of the enzymes and the inhibitor was assessed. In order to investigate if the baseline levels of FIB-CL, SL and TIMP could differentiate between sites which showed attachment gain and those which remained unchanged at the reassessment visit, each site demonstrating attachment gain (n=24) was matched with an unchanged site for its GCF volume and clinical indices at the baseline. Two-sample t-test showed no significant difference between the mean baseline levels of the enzymes and the inhibitor when two groups of sites were compared (Table 3.34). The same procedure was performed for the sites which exhibited attachment gain at the follow-up examination (n=25). No significant difference was found in the mean baseline levels of FIB-CL and SL between the sites which gained attachment and their matched controls (two-sample t-test)(Table 3.35).

3.2.5.2 Comparisons between before and after hygiene phase therapy of GCF volume, clinical indices, matrix metalloproteinases and their inhibitor in sites which demonstrated attachment gain at the reassessment examination

Attempts were made to investigate if the mean GCF levels of FIB-CL, SL and TIMP in sites which responded to HPT by attachment gain differ from their baseline levels. Using paired t-tests, the mean levels of the SL and the inhibitor in sites which gained attachment at the reassessment examination were compared with their levels at the baseline. The mean level of SL decreased significantly ($p=0.029$) after HPT, whereas TIMP's level increased, although it did not reach the statistical significance ($p=0.307$). The number of sites with detectable levels of FIB-CL reduced to half at the reassessment visit (4 sites, (16.7%)). The change in the GCF volume was not significant (paired t-test, $p=0.427$). However, there was a decrease in GCF volume at this visit. The mean pocket depth (paired t-test, $p<0.001$) as well as the mean gingival scores (Sign test, $p<0.001$) showed a significant reduction at the reassessment examination (Table 3.36).

Table 3.34 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites which showed attachment gain and their baseline matched controls which did not show attachment gain after HPT, at the reassessment visit. Geometric mean and 95% confidence intervals are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
FIB-Collagenase			
AL gain after HPT	24	5.5	2.0-15.4
AL unchanged after HPT	24	2.8	1.2-6.40
t-test p-value = 0.296			
Stromelysin			
AL gain after HPT	24	9.9	4.6-21.2
AL unchanged after HPT	24	7.1	3.3-15.3
t-test p-value = 0.536			
TIMP			
AL gain after HPT	24	231.7	170.2-315.5
AL unchanged after HPT	24	290.8	217.6-388.7
t-test p-value = 0.289			

Table 3.35 Baseline levels of MMPs and TIMP (pg/30s) were compared between sites which showed attachment gain and their baseline matched controls which did not show attachment gain at the follow-up visit. Geometric mean and 95% confidence intervals are shown. Significance level (p value) for each comparison is given.

Prediction of response to treatment according to baseline MMPs and TIMP levels			
Protein	**n**	**Mean**	**95% C.I.**
Fibroblast collagenase			
AL gain at follow-up	25	5.0	1.9-13.1
AL unchanged at follow-up	25	4.3	1.9-10.4
t-test p-value = 0.826			
Stromelysin			
AL gain at follow-up	25	9.4	4.4-20.2
AL unchanged at follow-up	25	6.5	3.3-12.8
t-test p-value = 0.446			
TIMP			
AL gain at follow-up	25	251.4	192.5-328.4
AL unchanged at follow-up	25	229.9	182.6-289.5
t-test p-value = 0.614			

Table 3.36 Comparisons of GCF volumes, gingival scores, PD and GCF levels of SL and TIMP between the baseline and the reassessment visits in 24 sites which demonstrated attachment gain at the reassessment examination (after HPT). Means ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for the proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Reassessment	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
Vol.	0.397±0.057	0.341±0.058	0.463
MGI	3.5±0.104	1.7±0.138	<0.001
PD	6.5±0.293	3.7±0.234	<0.001
SL	9.9 (4.6-21.2)	3.1 (1.9-5.6)	0.029
TIMP	231.7 (170.2-315.5)	291.3 (217.0-391.2)	0.307

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
SL= stromelysin
TIMP= tissue inhibitor of metalloproteinases

Table 3.37 Comparisons of GCF volumes, gingival scores and GCF levels of SL and TIMP between the baseline and the follow-up visits in 25 sites which demonstrated attachment gain at the follow-up examination. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Follow-up	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
Vol.	0.387±0.051	0.220±0.040	0.002
MGI	3.4±0.114	1.4±0.151	<0.001
PD	6.4±0.289	3.0±0.147	<0.001
SL	9.4 (4.4-20.2)	2.0 (1.5-2.7)	0.003
TIMP	251.4 (192.5-328.4)	115.9 (71.5-188.0)	0.005

Vol.= gingival crevicular fluid volume in µl
MGI= Modified Gingival Index
PD= pocket depth in mm
SL= stromelysin
TIMP= tissue inhibitors of metalloproteinases

3.2.5.3 Comparisons between the baseline and the follow-up levels of GCF volume, clinical indices, matrix metalloproteinases and their inhibitor in sites which showed attachment gain at the follow-up examination

Twenty five sites demonstrated significant attachment gain at the follow-up visit. The mean levels of SL and TIMP at the follow-up visit were compared to their baseline levels. Both the enzyme and the inhibitor mean levels showed a highly significant reduction at the follow-up examination (paired t-tests, $p=0.003$ for SL and $p=0.005$ for TIMP). The number of sites expressing detectable amounts of FIB-CL reduced from 8 (25%) sites at the baseline to 1 (4%) site at the follow-up visit. Significant reductions were observed in GCF volume and pocket depth (paired t-test, $p=0.002$ and $p<0.001$ respectively) as well as gingival scores (Sign test, $p<0.001$) at the follow-up examination (Table 3.37).

3.2.5.4 Correlation between clinical findings and GCF levels of matrix metalloproteinases and their inhibitor in sites which gained attachment at the reassessment and the follow-up examinations

Since the reduction in the mean levels of the enzymes and inhibitor was concomitant with the a reduction in GCF volume, MGI and PD in sites with attachment gain at the

reassessment and the follow-up examinations, in order to illustrate any possible relation between clinical and biochemical parameters, the association of SL and TIMP with clinical parameters was investigated. Using Spearman rank correlation coefficients, it was found that only TIMP had a strong positive correlation with GCF volume at both reassessment ($r=0.609$, $p=0.002$) and the follow-up ($r=660$, $p<0.001$) visits, whereas SL was correlated with GCF volume only at the reassessment examination ($r=0.493$, $p=0.014$). Pocket depths and gingival scores showed no significant association with the mean levels of SL and TIMP at both visits. However, because very small number of sites expressed detectable amounts of FIB-CL, no attempt was made to correlate its GCF level with the clinical parameters.

3.2.6 Prediction of treatment needs according to the baseline levels of metalloproteinases and their inhibitor

Response to the treatment after HPT was evaluated at the reassessment examination. Sites were assigned to three groups for their further treatment needs according to their pocket depths at this visit. One way analysis of variance, was used to investigate if the levels of the enzymes and inhibitor at the baseline visit could predict further treatment required after HPT. However, the mean baseline levels of MMPs and TIMP failed to distinguish between different treatment modalities needed after HPT. These

findings suggest that the baseline levels of the FIB-CL, SL and TIMP in GCF can not be used as predictors of further treatment needs and treatment response and these proteins are probably best suited for monitoring or disclosing the current tissue host response status.

CHAPTER 4

DISCUSSION

4.1 Methodological considerations

4.1.1 Choice of threshold for further treatment needs

Generally accepted PD thresholds upon which further treatment needs are decided in routine clinical practice for sites which bleed on probing are: PD>3mm for re-root planing and PD>5mm for surgical intervention based on the findings that complete removal of subgingival deposits is difficult in PD ranging from 3 to 5mm whereas it is extremely difficult in sites with PD>5mm (Waerhaug 1973; Rabbani *et al.*, 1981). In the longitudinal study conventional and Florida probing measurements, taken at the same visit, were compared at the reassessment and the follow-up examinations. Florida probe readings were higher than conventional probing. Therefore in analyzing the reassessment and the follow-up data the above mentioned thresholds were changed to 3.5 and 5.5mm in order to compensate for the difference observed. When the readings of the two methods of probing measurements were compared at the baseline visit conventional probing measurements were found to be higher than Florida probe readings. However, Florida probe measurements were made at least one week after conventional probing at which visit patients had received oral hygiene instructions which may have influenced the gingival tissue tonus thus, resulting in lower pocket readings. The follow-up examination was

postponed for 4 weeks in subjects who had received surgical treatment as visual inspection as well as gentle probing in non-study sites showed signs of inflammation, six weeks following the treatment session.

4.1.2 Detection of attachment level changes

The selection of method for detection of attachment changes is crucial. Haffajee *et al.* (1983b) compared three different methods namely linear regression analysis, running median and tolerance method. The authors concluded that the "tolerance method" is superior to the two other methods for its ability in detecting changes over a short period of time as well as considering patient and population variances in addition to site variance. Aeppli and Pihlstrom (1989) reached the same conclusion. Fleiss *et al.* (1991) have also reported that variances for replicate measurements differs from patient to patient and even from site to site. In our longitudinal study, because of its relatively short duration and the necessity for detecting attachment level changes between individuals, the "tolerance method" was employed. It must also be kept in mind that attachment gain after periodontal therapy may not necessarily reflect the true connective tissue attachment level as the quality and tonus of gingival tissue improve following therapy which in turn offers a greater resistance to passage of the probe between the tooth and the gingiva (Magnusson & Listgarten, 1980; Fowler *et al.*, 1982).

However, no analysis was performed on sites which showed attachment loss after treatment since it might has been caused by subgingival instrumentation and remodelling of attachment levels during the healing (Badersten, Nilvèus & Egelberg, 1987; Vanooteghem *et al.*, 1990) and can not be, necessarily, taken as a sign of disease progression.

4.1.3 Quantification of gingival crevicular fluid matrix metalloproteinases and their inhibitor

The enzyme linked immunosorbent assay (ELISA) is being used increasingly in clinical laboratories for the assessment of constituents of biological fluids. ELISAs have taken the place of other methods such as radioimmunoassay, as they do not have the problem of radiation danger, as well as immunofluorescence techniques. The ELISA methodology also provides precision and low detectability limits, although visual assessment is possible, results can be automatically obtained using spectrophotometer. Performing preliminary experiments, described in chapter 2, the ELISAs used in these studies were validated and proven suitable for the quantification of MMPs and TIMP in GCF.

The ELISA methodology has the following inherent problems which may introduce erroneous results or make their interpretation difficult:

a) High background

The background of an assay can be determined by evaluating the OD readings in the control wells which contain no sample or standard. High background can readily obscure the results of positive samples, thus making the interpretation difficult. Increasing the number and efficiency of the wash steps as well as using a non-specific protein i.e. bovine serum albumin (BSA) to bind unreacted sites on the solid phase may solve the problem (Carpenter, 1992). However, in these experiments there was not such a problem as the OD for control wells were much lower than those obtained for both samples and standards ranging from 0.004 to 0.01.

b) Edge effect

It has been noticed that outside wells of a plate may absorb more protein than the inside wells thus, resulting in an assay bias (Tijssen, 1985; Carpenter, 1992). In order to avoid this phenomenon (edge effect) only the inside wells were used for running samples and controls (Tijssen, 1985).

c) Hook effect

The hook effect is an unexpected fall in the amount of a substance at the high end of the dose-response curve, resulting in a gross underestimation of the substance (Carpenter, 1992). However, in these experiments the possibility of hook effect was negated as assaying serial

dilutions of GCF eluates (chapter 2) did not demonstrate such an effect .

4.1.4 Reporting of gingival crevicular fluid constituents

The traditional method of expressing result from biological fluid analysis is in terms of concentrations (activity or amount of constituent per unit volume). When reporting data with this method, it is assumed that each sample contains an equal volume of fluid which is representative of the total fluid volume. As it has been pointed out by Lamster *et al.* (1986), expressing the data as a concentration might not be appropriate for GCF components. GCF differs from most other biological fluids in that it is produced in micro-amounts (generally <1μl) in the crevice and is constantly cleared towards the oral cavity. The amounts of GCF present in different sulci are extremely variable and usually, most or all of the fluid present in the crevice is removed by sampling. Thus, collecting standardized amounts of GCF may increase the gingival capillary permeability which results in the influx of the serum to the crevice and consequent dilution of the GCF components. When sampling time is standardized, expressing results as absolute amounts with a standardised collection time seems more appropriate for GCF biochemical components (Lamster *et al.*, 1986, 1988; Smith & Geegan, 1991). Reporting GCF constituents as absolute amounts has other

advantages as the calculation of GCF volumes is not required for its determination. As it was mentioned above GCF volumes are usually below 1μl which makes their precise measurement difficult. In the assessment of GCF volumes, the Periotron is considered accurate, however small errors in the estimation of the latter would result in large alterations in the concentrations of GCF components (Lamster *et al.*, 1988). When GCF volumes are extremely low, losses from evaporation could easily occur both during sampling and transferring the sample to the Periotron.

4.2 The choice of gingival crevicular fluid components to be examined

These studies were carried out to investigate the level of FIB-CL, SL and TIMP in GCF samples from healthy, gingivitis and periodontitis sites in the same individuals (cross-sectional study) as well as comparing their levels before and after periodontal therapy and their ability to predict treatment outcome (longitudinal study). The reasons for choosing these MMPs and TIMP are: a) these fibroblast derived enzymes and inhibitor have never been assessed before in GCF; b) most of the previous studies in the literature have focused on PMN derived enzymes in GCF (Ohlsson *et al.*, 1973; Sorsa *et al.*, 1990; Overall *et al.*, 1991); c) TIMP appears to preferentially inhibit FIB-CL and SL (Banda *et al.*, 1992); d) the ability of SL in activating FIB-CL (Unemori *et al.*, 1991); and finally e) the fact that

all these components are secreted by resident cells (MacNaul *et al.*, 1990).

A number of studies have shown that FIB-CL, SL and TIMP are locally produced by resident cells in gingival tissues (Woolley & Davies, 1981; Heath *et al.*, 1982; MacNaul *et al.*, 1990; Nomura *et al.*, 1993). However, the extent of local production of proteins can be assessed using the relative coefficient of excretion (RCE). In order to calculate RCE, the GCF:serum ratios of both protein and albumin are calculated. The ratio of these two ratios (protein ratio:albumin ratio) gives the RCE which if is >1 indicates local production of protein being more than its serum contribution and vice versa (Giannopoulou *et al.*, 1990). This experiment was not necessary to be carried out in the present studies because of the local production of these proteins and therefore, their negligible levels in serum in otherwise healthy subjects.

Some investigators have shown that repeated sampling resulted in a reduction in total amounts of GCF components (Lamster *et al.*, 1989; Persson & Page, 1990). Therefore, in order to increase the chance of a higher detection rate for these proteins stagnant samples were assayed. However, the discovery later of the difficulty of detecting FIB-CL and to a lesser extent SL even in stagnant GCF samples suggests that the use of successive samples may result in even lower detectability for these enzymes.

4.3 Cross-sectional study

4.3.1 Study design

In order to permit within subjects comparisons, samples were taken from different sites within the same individual and data were analyzed by the use of repeated measures analysis of variance (MANOVA) in which variables are not considered independent. By employing this experimental design, the host effect was also allowed for. Attempt was made to sample periodontitis sites with low gingival scores in order to be different from gingivitis sites as far as the degree of gingival inflammation of the tissues adjacent to the pocket was concerned. However, considering difficulties in finding different sites (healthy, gingivitis and periodontitis) in the same individual only three sites from each patient comprised the study sites.

4.3.2 Clinical findings

The mean GCF levels of SL was able to differentiate between healthy and diseased (gingivitis and periodontitis) sites which could render this GCF component a possible marker of inflammatory periodontal disease. Birkedal-Hansen *et al.* (1993) have also suggested that SL may act as a marker of stromal cell involvement in the process of tissue degradation. Villela *et al.* (1987), found PD to be good indicator of protease activity. However, in the present

study there was no significant difference between the GCF levels of SL in gingivitis and periodontitis sites in spite of the presence of much shallower pockets in the former. There are several explanations in the literature for this observation. a) Considering that our antibodies detected total levels of proteinases (active plus latent), this observation could be explained by the finding of Kryshtalskyj, Sodek and Ferrier (1986) who reported that periodontitis sites had higher active collagenase compared to latent enzyme whereas, in gingivitis sites latent enzyme was higher than the active form of collagenase and the observation of Hayakawa *et al.* (1994) who reported that most of the collagenase in whole saliva of healthy subjects was in latent form while, enzyme present in whole saliva from periodontitis patients was active. This is further supported by a recent report (McCulloch, 1994) indicating that active collagenase was able to distinguish between gingivitis and periodontitis sites. b) In this cross-sectional investigation probing depths were the differentiating factor between gingivitis and periodontitis sites. Probing depth measurements taken at one point in time are indicative of past history of periodontal disease and do not necessarily reflect active periodontal destruction, indicating that all the periodontitis sites may not have been active at the time of sampling. Thus, the SL's levels in these sites may not necessarily be indicative of ongoing periodontal destruction. This is supported by the finding of Lee *et al.* (1991) who found

higher collagenase activity in active sites than in inactive sites with comparable pocket depths. c) In addition, inflammation present superficially in gingivitis sites and at the depth of the pocket (Armitage *et al.*, 1977; Caton *et al.*, 1981) may in periodontitis sites obscure the picture.

The mean GCF levels of TIMP could distinguish between healthy and diseased (gingivitis and periodontitis) sites. This GCF component could also be considered a possible marker of inflammatory periodontal disease. The fact that TIMP was higher in diseased sites than healthy sites is in agreement with findings of Nomura *et al.* (1993) who found the same results for TIMP mRNA in the periodontal tissues. This may be due to the fact that bacterial stimuli initially induce MMP expression by host cells causing an increase in tissue self destruction. Thereafter, the host cells recognize the ongoing tissue destruction and attempt to defend the host by producing tissue inhibitor of metalloproteinases (TIMP). However, in contrast to the aforementioned study and the present study, Larivèe and co-workers (1986) concluded that the activity of inhibitor is higher in healthy sites than in diseased sites. This discrepancy could be attributed to different GCF sampling methodology. They collected GCF by micro-capillary tubes following a 3 minute GCF sampling by means of paper strips for determining GCF flow. Both methods of GCF collection are thought to irritate the gingival tissues (Cimasoni,

1983) and could increase the vascular permeability in the area, resulting in a dilution of the locally derived components. Moreover, considering the method they used for GCF collection, only excluding blood contaminated samples does not rule out the possibility of analyzing other mainly serum derived inhibitor e.g. α2-M. Although, it has been shown that α2-M is higher in diseased sites than in healthy ones (Adonogianaki, Mooney & Kinane, 1992). In our study a less invasive method of GCF collection (paper strips for 30s) was used. In addition to sampling methodology discrepancies, the present study reports the amount of enzymes rather than their activity as did the study of Larivèe *et al.* (1986). However, the mean GCF levels of TIMP was not able to differentiate between gingivitis and periodontitis sites. The same results have been obtained by Adonogianaki *et al.* (1992) who investigated the levels of α2-M and α1-antitrypsin (α1-AT) in healthy, gingivitis and periodontitis sites. Considering the above argument indicating TIMP's production being provoked by destructive effects of MMPs along with the observation that SL's level did not differ significantly between gingivitis and periodontitis sites, the inability of TIMP in differentiating gingivitis from periodontitis is understandable.

Generally, it could also be suggested that the alteration in the levels of these GCF constituents may be initiated during the development of gingivitis and relate to this

inflammation rather than periodontal destruction. This view could also be supported by: the findings of Cao and Smith (1989) who measured the levels of myeloperoxidase; Adonogianaki *et al.* (1992) who analyzed the GCF levels of α2-M, α1-AT and transferrin (TF) and; Ebersole *et al.* (1993) who compared PGE_2, IL-1ß and IL-2 in GCF from healthy, gingivitis and periodontitis sites.

The distribution of SL and TIMP in different diagnostic categories of sites visualised using mean values does not reflect the case in every individual. Various individuals respond differently and present with different tissue destructive potential in response to bacterial stimuli.

The observation that healthy, gingivitis and periodontitis sites in newly referred patients showed higher, although not statistically significant, levels of SL and TIMP than similar sites in maintenance patients, is consistent with the finding of Ebersole *et al.* (1993). These workers reported that the levels of PGE_2 and IL-ß were lower in healthy sites from healthy subjects than healthy sites within refractory periodontitis patients, and gingivitis sites from healthy subjects had lower levels of PGE_2 than those in adult periodontitis and refractory periodontitis patients. These observations may indicate the presence of an intrinsic contribution from the individuals with different disease status into the local host response and therefore suggests that in studying GCF biochemical

components subjects should be used as a basis for analysis.

In conclusion, this cross-sectional study showed that although the mean GCF levels of SL and TIMP were able to differentiate between healthy and diseased sites they failed to distinguish gingivitis from periodontitis sites.

4.4 Longitudinal study

4.4.1 Study design

The clinical design of this experiment allowed us to evaluate the effect of treatment on the GCF levels of MMPs and TIMP as well as the ability of these GCF constituents in predicting the response to treatment. By the measurement of both pocket depth and attachment level, it became possible to assess the above mentioned goals using different approaches. In order to prevent the possible leakage of GCF from neighbouring teeth, sampling of adjacent sites was avoided. Adult periodontitis patients were included in the study in an attempt to eliminate any possible effects of different disease category on the levels of enzymes as well as outcome of treatment. However, the inclusion of some sites in the study in each individual, while the entire dentition had to receive treatment imposed some problems when further treatment was delivered. As a result of these problems some of the study sites were overtreated due to their inclusion in a sextant

with its majority of non-study sites having deep pockets after hygiene phase therapy. By the use of MANOVA for analysis of the data, the host response was allowed for.

4.4.2 Baseline findings

The observation that FIB-CL was only detected in 26.8% of the sites is consistent with our cross-sectional study. SL was detectable in 48% of the sites which is lower than our previous finding. In spite of the two other GCF components, not surprisingly, TIMP was detected in almost all the sites (99.4%). This finding could be explained by the fact that there are other MMPs in the inflamed gingiva which collectively increase tissue destruction and this stimulates TIMP production (Nomura *et al.* 1993). Bleeding on probing and suppuration are traditionally considered to be signs of disease activity as well as indicative of further treatment need (Haffajee *et al.*, 1983a and Badersten *et al.*, 1990). Therefore, we compared the GCF levels of SL and TIMP in these different groups of sites. SL was able to differentiate between bleeding and non-bleeding sites. This enzyme failed to discriminate suppurating from non-suppurating sites although its level was higher in the latter. Mean GCF levels of TIMP could not differentiate bleeding from non-bleeding and suppurating from non-suppurating sites. However, the levels of TIMP, although not statistically significant, were higher in bleeding and suppurating sites. Analyses

were performed for three different pocket depth groups and it was noted that none of the enzymes and inhibitor could discriminate between different groups of sites. The observation that SL was significantly higher in bleeding sites and both the enzyme and inhibitor were higher in bleeding and suppurating sites may indicate the presence of a higher degree of inflammation as well as more destructive activity in these groups of sites. However, although the latter is almost impossible to prove by the use of available clinical indices in a cross-sectional manner the validity of the former seems reasonable. The fact that PD may reflect prior disease experience and not necessarily current destruction (Haffajee *et al.*, 1983a) might be the reason behind the inability of MMPs and TIMP in discriminating different pocket depth groups.

4.4.3 Changes in GCF volume and clinical indices as a result of treatment

As expected, after the hygiene phase of therapy at the reassessment visit, as well as at the follow-up visit, GCF volume, AL and PD measurements, BOP, suppuration as well as gingival and plaque scores reduced significantly with both site and subject as the unit of analysis. These findings are indicative of an overall treatment success. However, at both occasions and particularly after HPT, there were some sites which did not respond to treatment. Mean GCF volume and PD measurement reduced significantly at the

follow-up from the reassessment, indicating further improvement in gingival tissue condition. The observation that the mean AL, although not significantly, increased at the follow-up from the reassessment visit may reflect the effects of over treatment in some sites, which was inevitably performed in sextants which underwent surgical therapy due to the majority of the sextants' sites having persistent pockets.

4.4.4 Changes in GCF levels of matrix metalloproteinases and their inhibitor as a result of treatment

The changes in the mean GCF levels of MMPs and TIMP, as a result of treatment, were investigated in the total study sites as well as in different groups of sites. In the whole sample, SL levels reduced after treatment at both after treatment examinations although its reduction was not significant at the reassessment visit. After hygiene phase therapy, the mean GCF levels of TIMP increased whereas, it showed a sharp reduction at the follow-up visit. Both changes were statistically significant. These trends were observed, either directionally or significantly, when before and after treatment levels of SL and TIMP were compared in different groups of sites. The observation that the mean levels of SL, in the best responding sites (BRS), in sites which gained attachment and in sites with some signs of improvement i.e. no BOP and PD reduction,

decreased after treatment at both following examinations was not unexpected as a result of treatment success. These are in agreement with the findings of Larivèe *et al.* (1986), Gangbar *et al.* (1990), Suomalainen (1992) and Hayakawa *et al.* (1994). TIMP's level increased after HPT in these groups of sites which is in accordance with the findings of Larivèe *et al.* (1986) and Hayakawa *et al.* (1994) who reported an increase in inhibitor's level after treatment. This increase, although not significant, may reflect TIMP's involvement in the healing process. At the follow-up visit the mean level of TIMP reduced significantly from the baseline in all different groups of sites. To further elucidate this finding, the case is discussed in detail in group of sites which gained attachment after therapy. The decrease in TIMP's level at the follow-up visit following its increase after HPT could be explained by the observations that the inflammatory process has subsided and gingival tissues are being healed particularly in the light of the fact that more than one third of the sites which showed attachment gain at the follow-up visit had not received further treatment after HPT. Further evidence for this speculation is the observation that SL level showed a 3 times reduction at the reassessment visit while it decreased 4.5 times at the follow-up examination.

4.4.5 Comparison of matrix metalloproteinases and their inhibitor in sites which did or did not respond to treatment

The finding that the levels of FIB-CL, SL and TIMP in sites which failed to respond to treatment showed the same pattern of change as those which responded to treatment was unexpected, as they were still presenting with signs of disease. The following explanation could be provided to explain this observation: a) although these sites had no significant attachment gain, their clinical conditions had improved significantly after treatment; b) since our antibodies detected total enzyme (latent and active), the possibility can not be ruled out that the active enzyme has been lower in sites which gained attachment or had shallow pockets than those that showed no attachment gain or had deep pockets, as was the case in the studies carried out by Larivèe *et al.* (1986) and Suomalainen (1992); c) treatment resulted in an alteration in the composition as well as reduction in the bacterial deposits and removal of the inflamed gingival tissues in both groups of the sites; d) the overall improved clinical condition, in an individual, may exert an indirect influence on the level of MMPs and TIMP in sites which did not respond to treatment, which is in agreement with the findings of Ebersole *et al.* (1993). These workers reported that the levels of PGE_2 and IL-ß were lower in healthy sites from healthy subjects than healthy sites within refractory periodontitis patients and

gingivitis sites from healthy subjects had lower levels of PGE_2 than those in adult periodontitis and refractory periodontitis patients. Gustafsson *et al.* (1994) have also reported that elastase activity was higher in gingivitis sites from patients with periodontitis than clinically comparable gingivitis sites from gingivitis affected individuals. These results are consistent with our findings in the cross-sectional study in which the GCF levels of FIB-CL, SL and TIMP were lower in healthy, gingivitis and periodontitis sites in subjects on maintenance care (with only few diseased sites) than patients with advanced periodontal disease.

Since the best responding sites (BRS) and worst responding sites (WRS) represent two extremes of response to treatment their clinical and biochemical parameters are discussed separately.

Comparisons of the clinical and biochemical parameters between BRS and WRS at the reassessment examination revealed that although the mean GCF volume was 2 fold higher in WRS than in BRS, both groups of sites had almost the same levels of SL and TIMP in their GCF and FIB-CL was even lower in WRS. These proportional discrepancies between GCF volume, SL and TIMP was also observed in the follow-up visit with SL showing the most prominent difference (2.4 fold) between the two groups of sites. These differences between GCF volume and GCF levels of

proteins may imply the local production of FIB-CL, SL and TIMP. One interesting finding here is that TIMP's level in the BRS, at the reassessment visit, whose average PD is 2.6mm was 5 fold higher than in the WRS at the follow-up visit with an average PD of 5.8mm. The higher level of TIMP, even in the BRS, after HPT may reflect its involvement in the healing process. The lower level of TIMP, even in the WRS, at the follow-up visit from reassessment could be explained by the observations that inflammatory process has subsided and gingival tissues are healing. The levels of TIMP in both BRS and WRS at both post therapy visits showed the same pattern, despite the fact that their clinical conditions were extremely different. One explanation for this finding could be the observation that clinical conditions in general had improved significantly at the follow-up visit from reassessment, which may exert an indirect influence, particularly at the subject level, on the level of TIMP in sites which did not respond to treatment. This is supported by the findings of Ebersole *et al.* (1993), Gustafsson *et al.* (1994) and by the results of our cross-sectional study in which the GCF levels of FIB-CL, SL and TIMP were lower in all categories of sites in subjects on maintenance care compared to those with advanced periodontal disease.

These observations may indicate the presence of an individual subject based effect on the local host response

which may imply that subject should be used as the unit of analysis, when biochemical components of GCF are being investigated.

The inability of FIB-CL and SL to differentiate BRS (healthy) from WRS (periodontitis sites) was not unexpected as FIB-CL and SL were not detected in 80% and 63.3% of sites, at the reassessment visit, respectively. The percentage of sites in which the enzymes did not reach the detectable levels at the follow-up examination were 85.3% for FIB-CL and 67.6% for SL. In addition to this, the following explanation could be provided. Even though the proteinases had been detected in all sites the finding of Larivèe *et al.* (1986) that after treatment only active enzyme decreased whereas total enzyme remained unchanged and the observation of Uitto and co-workers (1990) that collagenase is mostly in latent form in health while in periodontitis it is found in active form along with the fact that our antibodies detected total enzymes would probably prevent them distinguishing successfully treated sites from those which did not respond to treatment. The inability of TIMP to differentiate BRS from WRS is demonstrated by the relatively low positive and negative predictive values of a test based on TIMP's level at the follow-up visit. The sensitivity of the test was very low (24%) demonstrating a very high rate of false negatives. However the relatively high specificity of the test may be due to the low number of positive tests. Moreover, these

observations are in contrast with our finding from the cross-sectional study in which the levels of the enzymes and inhibitor were significantly lower in healthy than in periodontitis sites. As mentioned above Larivèe *et al.* (1986) reported that the levels of total collagenase and inhibitor remained unchanged after treatment whereas they dropped dramatically at a recall visit 6 months after therapy. Therefore it could be speculated that a significant difference in the levels of MMPs and TIMP between BRS and WRS would have been found if these sites had been sampled much longer after therapy.

4.4.6 Prediction of the outcome of treatment

4.4.6.1 Prediction of treatment response: Using the pocket depth and the state of bleeding on probing as criteria

The response to treatment in a routine clinical practice is assessed by pocket depth and the state of bleeding on probing, thus sites with PD<3.5mm and no BOP were considered as responding sites. Prediction of the treatment outcome, using baseline subjective criteria, has been investigated by Vanooteghem *et al.* (1990) who reported a maximum 30% diagnostic accuracy for subjective clinical parameters. Therefore at both post therapy visits the baseline levels of the enzymes and inhibitor were compared in sites which met either one of the two or both criteria.

GCF level of SL at the baseline was able to distinguish bleeding from non-bleeding sites as well as sites with PD of less or more than 3.5mm. Quite surprisingly, on both occasions the baseline levels of SL was higher in responding sites which is very difficult to explain, although this observation may, in part, be due to the fact that our antibodies detected total enzyme.

However, failure in predicting treatment outcome by using PD and state of BOP as criteria for treatment success led us to use the gain in attachment level as an indicator of response to treatment.

4.4.6.2 Prediction of treatment response: Using the attachment level changes as criterion

Longitudinal monitoring of attachment levels is the best available tool for evaluating treatment outcome at present. Since clinical parameters demonstrated rather poor ability to predict disease progression (Haffajee *et al.*, 1983a; Badersten *et al.* 1985; Jenkins *et al.*, 1988; Badersten, Nilvèus & Egelberg, 1990; Kaldahl *et al.*, 1990), attempts have been made to investigate the association between attachment loss and biochemical components of GCF in order to predict future disease activity (Offenbacher, Odle & Van Dyke, 1986; Lamster *et al.*, 1988; Persson, De Rouen & Page, 1990). No study investigated the ability of GCF constituents to predict the response to treatment as it is

evaluated by changes in attachment levels. Pre-treatment levels of FIB-CL, SL and TIMP failed to distinguish between sites that gained attachment and those which did not, although efforts were made to eliminate the effect of variations in the baseline GCF volumes and clinical parameters between two groups of sites on their biochemical properties, sites which showed attachment gain were carefully matched for their pre-treatment GCF volumes and clinical parameters. However, within subject matching which is preferable was not possible due to limited number of sites in each individual. This approach, although very accurate, might be criticised in that it did not take into account the state of gingival inflammation and pocket depth in groups of sites which did or did not gain attachment after therapy.

4.4.6.3 Prediction of treatment response: Using a combination of clinical parameters as criteria

In order to fulfil these requirements a more stringent criteria, according which two groups of best responding sites (BRS) and worst responding sites (WRS) were selected at the both post-therapy visits, was adopted. To avoid the smoothing of the data by including intermediately responding sites e.g. sites with shallow pockets but BOP or high gingival score, sites with PD≤3mm, no BOP and MGI scores of 0-1 comprised the BRS whereas WRS consisted of those with PD>4mm, BOP and gingival scores of ≥2. However,

none of the sites in either groups and in both occasions demonstrated suppuration after probing. Although preferable, it was not possible to select BRS from those which gained attachment due to limited number of the latter. In spite of the highly significant differences in pocket depths and gingival scores as well as completely reverse status of BOP, between BRS and WRS, their pre-treatment levels of MMPs and TIMP failed to predict the success and failure of treatment. However because of the limited number of the sites with above mentioned criteria in both groups their baseline matching for clinical parameters was not possible. Considering the facts that our antibodies detected total enzyme (latent and active) and that probing depth measurements taken at one point in time are indicative of past history of periodontal disease and do not necessarily reflect active periodontal destruction thus, it could be speculated that different sites in the present study, depending on whether they are active or inactive, had different proportions of active and latent enzymes which in turn may determine the destructive potential of the inflammatory exudate, the GCF. Lee *et al.* (1991) reported that the frequency of active sites with active collagenase was higher than that of inactive sites with comparable pocket depths.

4.4.7 General findings

In this section common observations between the cross-sectional and longitudinal studies are discussed.

4.4.7.1 Low detectability of fibroblast collagenase

In the cross-sectional study FIB-CL was found in only 18.9% of all the sites and only in 20.8% of periodontitis sites and in the longitudinal study we were only able to detect FIB-CL in 26.8%, 17.4% and 12% of the sites at the baseline, the reassessment and the follow-up visits respectively. These results are consistent with the finding of Woolley and Davies (1981). The possibility that the ELISA for FIB-CL was ineffective was negated as positive samples of FIB-CL were obtained from ovarian cyst carcinoma fluids and GCF samples of patients with LJP from Dr T. Sorsa, University of Helsinki, Finland. It is also of note the level of this enzyme, whenever was detected, was 1.5 fold higher than the control wells which contained no sample. However the number of sites expressing FIB-CL reduced to half after HPT and it was eight times smaller at the follow-up examination in sites with attachment gain. The rapid binding of α2-M to FIB-CL, reported by Cawston *et al.* (1987) could also explain the low detectability of this enzyme in GCF in the light of the fact that α2-M is present in both healthy and diseased sites (Adonogianaki *et al.*, 1992).

4.4.7.2 Correlation between clinical parameters and gingival crevicular fluid biochemical components

In the cross-sectional study there was a moderate positive and significant correlation between GCF levels of SL and TIMP and clinical parameters when data from healthy, gingivitis and periodontitis sites were pooled. When data from different groups of sites were separately analyzed the picture changed; so that, there was neither significant nor even moderate correlation between clinical and biochemical parameters. In the longitudinal study correlation coefficients between clinical and biochemical parameters were either very weak or not significant at the baseline. The large site to site variation in the levels of SL and TIMP observed in this study may, in part, be responsible for the lack of correlation between these proteins and clinical parameters. TIMP showed significant positive correlation with GCF volume at both after treatment sessions; the important finding here is that GCF volume and TIMP's level changes, while correlated, were in opposite direction at the reassessment visit which is difficult to explain although may imply the local production of TIMP. SL showed the same direction of change with GCF volume at both post therapy sessions while they were only associated at the follow-up visit. The mean levels of the enzymes and inhibitor showed the same direction of change with the GCF volume, MGI and PD in sites which gained attachment at the

reassessment and the follow-up examination. Therefore, in order to illustrate any possible relation between clinical and biochemical parameters, the association of SL and TIMP with clinical parameters was investigated. Using Spearman rank correlation coefficients, it was found that only TIMP had a strong positive correlation with GCF volume at both the reassessment and the follow-up visits, whereas SL was correlated with GCF volume only at the reassessment examination. Pocket depths and gingival scores showed no significant association with the mean levels of SL and TIMP at both visits. There was no significant correlation, either positive or negative, between the FIB-CL, SL and TIMP's baseline levels with bleeding sites as well as sites with PD>3.5mm at both the reassessment and the follow-up visits. As very small number of sites expressed detectable amounts of FIB-CL at post therapy visits, no attempt was made to correlate its GCF level with the clinical parameters.

There is no consensus in the literature regarding correlation between clinical parameters and proteinases and their inhibitor in gingival tissue/fluid. Kryshtalskyj *et al.* (1986) reported strong correlation between collagenase activity and GCF flow. Villela *et al.* (1987) have also reported positive but rather weak correlation between PD and GI and collagenase activity while Gangbar et al (1990) and Teng, Sodek and McColloch, (1992) failed to find any correlation between collagenase

activity and clinical parameters. Gustafsson *et al.* (1992) reported that elastase activity in GCF showed a moderate correlation with PD and attachment loss, whereas the enzyme level did not show any correlation with these clinical parameters. Lactoferrin (LF) absolute amounts in GCF and gingival crevicular washings showed rather strong correlation with MGI, PD and GCF volume (Adonogianaki *et al.*, 1993). Different methods of GCF sampling and laboratory techniques as well as variations in reporting the results may influence the presence and extent of correlation between clinical and biochemical parameters, thus correlations of biochemical parameters and clinical indices reported in previous papers should be viewed with caution. Moreover, the observation that using exactly the same clinical and laboratory techniques in cross-sectional and longitudinal studies and even analyzing data from cross-sectional study in different ways yielded different correlation coefficients suggests that not only these requirements are necessary but also the presence of identical clinical conditions between two or more groups of study sites is required for any reproducible relationship between clinical and biochemical parameters to be obtained.

4.5 Further research

The cross-sectional and longitudinal studies have demonstrated that mean GCF levels of SL and TIMP were able to distinguish between healthy and diseased sites and have

also provided information on the changes of GCF MMPs and TIMP as a result of periodontal therapy. However, these GCF components were not able to discriminate between gingivitis and periodontitis sites and could not predict the outcome of treatment. They also failed to differentiate successfully treated sites from those which failed to respond to treatment. In order to overcome these shortcomings some changes in the design of the longitudinal study are proposed in the following section.

4.5.1 How these studies can be designed in the future

A recent paper (McCulloch, 1994) reported that active collagenase was able to distinguish between gingivitis and periodontitis sites. FIB-CL detection rate was very low in these studies. This enzyme also shows similarities to SL (Birkedal-Hansen *et al.*, 1993). Therefore, it would be of interest to investigate if active SL is able to distinguish between gingivitis and periodontitis and predict the outcome of treatment. In addition, since under conditions of the present studies, SL was not detected in all sites the GCF samples could be eluted in smaller volumes of assay buffer, resulting in higher detection rate for this enzyme.

The results of these studies have also shown that TIMP was the best candidate for investigation as it was detectable in almost 100% of the sites. On the other hand, although the difference in TIMP levels was not significant between

best responding sites (BRS) and worst responding sites (WRS) at the follow-up visit, its level in BRS was lower than in WRS. Therefore, using means and standard deviations of TIMP levels from these groups of sites at this visit, it was worked out that by increasing the number of patients to 38 (89% increase) a significant difference at 0.05 level in the amount of TIMP between responding and non-responding sites could be obtained. Since, TIMP levels showed a fluctuation over the period of the longitudinal study, in order to examine these changes more closely during the healing process the study sites should be sampled after treatment at monthly intervals for a period of 5 months. Discriminant analysis showed that PD and MGI had the greatest effect on the GCF levels of the SL and TIMP. Therefor, in selecting the study sites priority should be given to deeper sites with higher MGI scores.

4.5.2 Other lines of research

In situ hybridization could be used to locate the source of these GCF constituents as both healed and unhealed sites expressed, almost, the same levels of MMPs and TIMP. Active form of fibroblast derived gelatinase (72 k) may also be able to distinguish between periodontitis and gingivitis sites. The *in vivo* activation of MMPs is likely to be caused by plasmin. Plasminogen activator can produce plasmin from its precursor, plasminogen (Woessner, 1991). Therefore, the levels of plasminogen activator in GCF may

be able to distinguish between gingivitis and periodontitis sites.

In general, as discussed in section 4.3.2, the alteration in the levels of these GCF constituents may reflect the development of gingival inflammation rather than periodontal destruction. Therefore it seems justified to investigate the levels of GCF components which are resulted from bone destruction in order to distinguish gingivitis from periodontitis sites.

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LIST OF PUBLICATIONS

The following papers include materials presented as part of this thesis:

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Gingival crevicular stromelysin, collagenase and tissue inhibitor of metalloproteinases levels in healthy and diseased sites

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Abstract

The ability of stromelysin (SL), fibroblast type collagenase (FIB-CL) and tissue inhibitor of metalloproteinase (TIMP) to differentiate between healthy, gingivitis and periodontitis sites was investigated. SL and FIB-CL are members of a family of enzymes which are capable of degrading most of the extracellular matrix macromolecules. Extracellular control of these enzymes is performed by TIMP. 40 patients each provided 3 GCF samples from healthy, gingivitis and periodontitis sites. GCF samples were collected by means of sterile paper strips. GCF samples were eluted into 500 μl of Assay Buffer and assays for SL, FIB-CL and TIMP were performed by a sandwich ELISA. The mean amounts of SL and TIMP in diseased sites (gingivitis and periodontitis) were significantly higher than the mean amount of these GCF components in healthy sites (MANOVA p values were: 0.006 for SL and 0.001 for TIMP). GCF SL and TIMP differentiated healthy from diseased sites. Both SL and TIMP showed moderate correlation with clinical indices. FIB-CL was detectable in only 20.8% of all sites and did not correlate with disease status.

Introduction

Connective tissue degradation in periodontal disease is believed to result from the action of proteolytic enzymes. Among these, matrix metalloproteinases (MMP) are capable of degrading a variety of extracellular matrix components. MMP activity is inhibited by tissue inhibitors of metalloproteinases (TIMP) (Woessner, 1991), which are produced by host cells. The tissue degradation is further thought to be induced by an imbalance between MMPs and TIMP (Nomura *et al.* 1993). Analysis of these enzymes, thought to mediate degradation of collagen is a rational approach to diagnosis based on the importance of collagen as the major structural component of the periodontium (Lee *et al.* 1991). MMPs also seems to have an important role in the regulation of matrix turnover (Murphy *et al.* 1989). Among different members of this family of enzymes, polymorphonuclear leucocyte derived collagenase (PMN-CL), which is thought to be carried into the crevice in migrating PMNs (Birkedal-Hansen *et al.* 1993), has received more attention in the periodontal field than others (Ohlsson *et al.* 1973; Sorsa *et al.* 1990; Overall *et al.* 1991). However PMN proteases including PMN collagenase in the GCF of diseased sites may simply reflect the number of emigrating PMN into the crevice rather than the tissue destructive potential (Birkedal-Hansen, 1993). Adonogianaki *et al.* (1993) have reported a strong positive correlation between crevicular PMN numbers and the GCF level of one of its proteases, lactoferrin.

Bacterial components stimulate macrophages and lymphocytes to produce inflammatory mediators which are further thought to affect fibroblasts to

produce MMPs (Meikle *et al.* 1989; Overall *et al.* 1991; Birkedal-Hansen 1993). SL and FIB-CL are the major MMPs of fibroblast origin (Chin *et al.* 1985). It has been observed that stromelysin can activate fibroblast type collagenase (Unemori *et al.* 1991). Most of the cells capable of secreting SL and FIB-CL also produce TIMP (MacNaul *et al.* 1990). Cawston and co-workers (1987) suggested that TIMP is locally produced and its main role is defending connective tissues in the very local area around the cell from which metalloproteinases are secreted. No studies have been so far conducted on the fibroblast derived MMPs and inhibitors in GCF. In the present study we measured GCF levels of FIB-CL, SL and TIMP from healthy, gingivitis and periodontitis sites, in an attempt to elucidate whether the level of fibroblast derived enzymes and enzyme inhibitor can differentiate these disease states.

Materials and methods

Clinical Criteria

Forty patients, (26 F; 14 M; mean age 43.1 (range 21-71)) attending the Periodontal Clinic of Glasgow Dental Hospital participated in this study. These individuals had no history of systemic disease and had not received antibiotics for the past three months. The modified gingival index (MGI) (Lobene *et al.* 1986) was used to assess gingival inflammation and a pressure sensitive probe with a constant force of 25g was used to assess probing pocket depth. Sites with gingival scoring of 0 or 1 and pocket depth (PD) lower or equal to

2mm were categorized as "healthy". MGI between 2 to 4 and PD≤3mm were the criteria for "gingivitis" sites, and sites with MGI of between 0 to 2 and PD>4mm comprised the "periodontitis" sites (Table 1.). Although difficulties were experienced in finding the three types of sites (healthy, gingivitis and periodontitis) within the same patient, there were considerable advantages with this protocol as the subject variability could be excluded by 'pairing' the three sites to be compared in a repeated measures statistical analyses. The following criteria for site selection were adopted: mesial sites were preferred to distal sites as they are more accessible; upper molar palatal sites were preferred to buccal sites; and on the lower teeth buccal/labial sites were preferred to lingual sites, as these sites were less prone to saliva contamination of the GCF sample.

Gingival Crevicular Fluid (GCF) Sampling and Processing

GCF samples were collected using Whatman grade 4 paper strips (2x13 mm) (Griffiths *et al.* 1988). The site being sampled was gently air-dried and any supragingival plaque was removed. The area was carefully isolated to prevent samples from being contaminated by saliva. The paper strip was inserted into the crevice until mild resistance was felt, and left for 30 seconds. Care was exercised in order to avoid mechanical injury of the tissues. After GCF collection, the paper strip was transferred to the chairside-located Periotron 6000 (Harco Electronics, Winnipeg, Canada) for the quantification of the fluid volume. The jaws of the Periotron were wiped with pure methanol between readings. The strips were then placed in individual sterile micro centrifuge

tubes and stored at -30°C until further processing. Subsequently, the strips were eluted into 500 μl of incubation buffer at room temperature using a rotary mixer. The strips were then discarded and the eluate aliquoted and stored at -30°C. These aliquots were subsequently analyzed for the quantification of FIB-CL, SL and TIMP.

Calibration of the Periotron 6000

In order to transform the Periotron digital readings for each paper strip into volumes and also assess the accuracy of instrument, known volumes of distilled water were delivered to Whatman grade 4 paper strips with a Hamilton microsyringe in a range of volumes (0.05-1μl). Each measurement was performed 3 times and the mean value for each volume was used in a linear regression analysis from which the slope and intercept were used to determine the volumes of GCF collected.

GCF Analysis

FIB-CL, SL and TIMP quantification was performed on the eluates of GCF and all constituents were assayed by means of sandwich ELISAs, based on a modification of the method of Cooksley *et al.* (1990). The 96-well polystyrene microplate (Immulon 4 Dynatech Laboratories, Billinghurst, Sussex, U.K) was coated with the first antibody, specific to the antigen to be quantified. These antibodies were Mac 64 anti-collagenase monoclonal antibody for FIB-CL, Mac 78 anti-stromelysin monoclonal antibody for SL and Mac 19 anti-TIMP monoclonal antibody for TIMP. The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. Then the plate

was incubated with a Biotin-conjugated monoclonal second antibody. Finally the plate was incubated with Extravidin-Peroxidase (Sigma). These antibodies were biotinylated Mac 66 to reveal FIB-CL and biotinylated Mac 15 to reveal TIMP. The stromelysin assay differed in that a rabbit polyclonal antibody was used as the second antibody followed by Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG (donkey) (Jackson). The anti-SL monoclonal and polyclonal and anti-FIB-CL monoclonals detected both the proenzyme and active forms of the fibroblast SL and FIB-CL. Anti-TIMP monoclonals detected only free TIMP (Cooksley *et al.* 1990). All the antibodies were donated by Cell Tech. Ltd. Slough, UK. Visualisation was achieved by incubation with TMB substrate, and the reaction stopped with 2.5% NaF (sodium fluoride). The plate was read at 630 nm, on a Dynatech MR5000 plate reader. Serial dilutions of standard antigen were run for each plate and their ODs were used in a linear regression analysis from which the slope and intercept were used to determine the samples' levels of FIB-CL, SL and TIMP. FIB-CL and stromelysin positive samples were available from ovarian cyst carcinoma fluid and rheumatoid arthritis sera (gift from Dr. T. Sorsa) and these confirmed that our ELISA techniques were able to detect these proteins. Results were expressed as pg/30s sample. Control wells in each plate were included which contained no sample or standard antigen in order to calculate background binding. The mean OD plus two standard deviations of these wells was taken as a cut-off point for detectability (Tijssen, 1985).

Statistical Analysis

GCF levels of SL and TIMP were markedly skewed and required logarithmic transformation (log10 (x+1) x=original data value). A repeated measures analysis of variance was applied in order to perform within patient comparisons of healthy, gingivitis and periodontitis sites. When a significant effect was detected by repeated measures analysis of variance, follow up analysis was performed using univariate paired t-tests in order to determine the direction and location of the differences. A Bonferroni correction was applied and significance level for the t-tests was set at $0.05/\sqrt{3}$ (3=number of comparisons made). In order to identify associations between the clinical indices and the levels of SL and TIMP the non-parametric Spearman rank correlation coefficients were assessed. Data were analyzed using SPSS statistical package on an IBM microcomputer.

Results

One hundred and twenty sites were sampled in total, forty in each clinical group. Table 1 presents the average clinical indices and GCF volumes of the healthy, gingivitis and periodontitis sites.

The levels (95% confidence intervals) of SL and TIMP are presented in Table 2. SL was detected in 75 (62.5%) sites sampled of which 19 (47.5%) belonged to the healthy group and 28 (70%) both in the gingivitis and periodontitis groups. Multivariate repeated measures analysis of variance

(MANOVA) demonstrated a significant effect (p=0.006) when healthy, gingivitis and periodontitis sites within each patient were compared for their GCF SL levels (Table 2). Follow up analysis (Table 2) demonstrated that diseased sites (gingivitis and periodontitis) had significantly higher levels of SL than healthy sites (p= 0.007 & p=0.002 respectively). However, no significant difference could be detected between gingivitis and periodontitis sites (p=0.882). TIMP was detected in 108 (90%) sites in total. TIMP was present at detectable level in 34 (85%) healthy, 36 (90%) gingivitis and 38 (95%) periodontitis sites. Similarly to SL, multivariate repeated measures analysis of variance demonstrated a significant effect when healthy, gingivitis and periodontitis sites were compared for their GCF TIMP levels (Table 2). Follow-up analysis demonstrated that gingivitis and periodontitis sites had significantly higher levels of TIMP than healthy sites (p=0.008 & p<0.001) respectively). GCF TIMP levels could not however differentiate gingivitis from periodontitis sites (p=0.373) (Table 2). FIB-CL was detectable in only 25 (20.8%) out of 120 sites, of which 7 (17.5%) belonged to the healthy group (mean=193.6 pg/30s, SD=265.9), 11 (27.5%) sites belonged to the gingivitis group (mean=286.3 pg/30s,297.3) and 7 (17.5%) sites belonged the to periodontitis group (287.2 pg/30s, SD=271.8). FIB-CL had much higher levels than SL in sites where it was detected. In 20 subjects FIB-CL was not detected in any site.

The association of SL and TIMP with the clinical indices is shown in Table 3. SL and TIMP levels correlated positively and significantly with GCF volume, MGI and PD. Overall, TIMP demonstrated higher correlation

coefficients than SL with clinical indices and GCF volume (Table 3).

Discussion

In the present study we investigated the level of FIB-CL, SL and TIMP in GCF samples from healthy, gingivitis and periodontitis sites in the same individuals. These fibroblast derived enzymes and inhibitor have never been assessed before in GCF. Most of the previous studies in the literature have focused on PMN derived enzymes in GCF (Ohlsson *et al.* 1973; Sorsa *et al.* 1990; Overall *et al.* 1991). By taking samples from different sites within the same individual and using repeated measures MANOVA in order to permit within subjects comparisons, the host effect was allowed for. The GCF levels of FIB-CL, SL and TIMP are reported as the absolute amount in pg/30s. The rationale for expressing GCF biochemical components as absolute amounts with a standardised collection time has been discussed in detail by several investigators (Lamster *et al.* 1985; Lamster *et al.* 1986; Smith & Geegan, 1991). Our previous reports have reiterated these conclusions, finding that the expression of GCF biochemical components by concentration yields results which are always in the same direction as the absolute amount analyses but have more variability.

The observation that both SL and TIMP were able to differentiate between healthy and diseased sites could render these two GCF components possible markers of inflammatory periodontal disease. Birkedal-Hansen *et al.* (1993) have also suggested that SL may act as a marker of stromal cell

involvement in the process of tissue degradation. Villela *et al.* (1987), found PD to be good indicator of protease activity. However, in the present study there was no significant difference between GCF SL and TIMP in gingivitis and periodontitis sites in spite of the presence of much shallower pockets in the former. In this cross-sectional investigation probing depths were the differentiating factor between gingivitis and periodontitis sites. Probing depth measurements taken at one point in time are indicative of past history of periodontal disease and do not necessarily reflect active periodontal destruction. In addition, inflammation present superficially in gingivitis sites and at the depth of the pocket (Armitage *et al.* 1977; Caton *et al.* 1981) in periodontitis sites may obscure the picture. Further longitudinal studies are required in order to elucidate whether SL and TIMP levels can be used as indicators of active periodontal destruction. The fact that TIMP was higher in diseased sites than healthy sites is in agreement with findings of Nomura *et al.* (1993) who found the same results for TIMP mRNA in the periodontal tissues. This may be due to the fact that bacterial stimuli initially induce MMP expression by host cells causing an increase in tissue self destruction. Thereafter, the host cells recognize the ongoing tissue destruction and attempt to defend the host by producing tissue inhibitors of metalloproteinases (TIMP). However, in contrast to the aforementioned study and the present study, Larivèe and co-workers (1986) concluded that the activity of inhibitors is higher in healthy sites than in diseased sites. This discrepancy could be attributed to different GCF sampling methodology. They collected GCF by microcapillary tubes following a 3 minute GCF sampling by means of paper strips for

determining GCF flow. Both methods of GCF collection are thought to irritate the gingival tissues (Cimasoni, 1983) and could increase the vascular permeability in the area, resulting in a dilution of the GCF locally derived components. In this study we used a less invasive method of GCF collection (paper strips for 30 s). In addition to sampling methodology discrepancies the present study reports the amount of enzymes rather than their activity as the study of Larivèe *et al.* (1986). The distribution of mean SL and TIMP values in different disease states varied between individuals. Thus, individuals may respond differently and present with different tissue destructive potential in response to bacterial stimuli. The observation that FIB-CL was found in only 18.9% of all the sites and only in 20.8% of periodontitis sites is in agreement with the findings of Woolley & Davies (1981). Unemori *et al.* (1991) in an *in vitro* study demonstrated that stromelysin is able to enhance the collagenase activity, although the *in vivo* validity of this finding has been questioned by Woessner (1991). In this study it was found that out of 25 sites with detectable collagenase, stromelysin did not reach the detectable level in 9 sites and of the 75 sites with detectable amounts of stromelysin, 61 sites did not express FIB-CL.

In conclusion, this cross-sectional study has shown that although GCF SL and TIMP can differentiate healthy and diseased sites these tissue-derived components were unable to distinguish gingivitis and periodontitis sites.

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Table 1. Clinical parameters from the three categories of sites in 40 patients.

Site Status	MGI	PD	GCF
Healthy	0.17 ±0.06*	1.41 ±0.06	0.092 ±0.02
Gingivitis	3.00 ±0.07	2.37 ±0.12	0.320 ±0.05
Periodontitis	1.70 ±0.12	5.71 ±0.14	0.380 ±0.06

MGI= modified gingival index
PD = pocket depth
GCF= gingival crevicular volume
* = mean & SEM

Table 2. Stromelysin and TIMP absolute amounts (pg/30s) in GCF from healthy, gingivitis and periodontitis sites in 40 patients. Geometric mean (95% confidence intervals) are shown together with repeated measures analysis of variance (MANOVA) significance level. Follow up analysis was performed using univariate paired t-test and the results are given as footnotes.

Site status	Stromelysin*	TIMP**
Healthy	3.9 (2.4-6.10)	32.3 (19.4-53.70)
Gingivitis	8.9 (5.5-14.4)	74.0 (44.2-123.8)
Periodontitis	9.4 (5.8-15.3)	93.7 (63.2-138.9)
MANOVA p value	0.006	0.001

* healthy vs gingivitis: p=0.007, healthy vs periodontitis: p=002, gingivitis vs periodontitis: p=0.882
** healthy vs gingivitis: p=0.008, healthy vs periodontitis:p<0.001, gingivitis vs periodontitis: p=0.373

Table 3. Spearman rank correlation coefficient (r) between clinical indices and TIMP and SL levels in GCF from 40 patients. Probability levels are shown in parenthesis.

Parameter	Stromelysin	TIMP
Vol.	r=0.283 (p=0.002)	r=0.643 (p=0.000)
MGI	r=0.228 (p=0.012)	r=0.361 (p=0.000)
PD	r=0.256 (p=0.005)	r=0.308 (p=0.001)

Vol.= GCF volume
MGI= modified gingival index
PD= pocket depth

Effects of treatment on gingival crevicular collagenase, stromelysin and tissue inhibitor of metalloproteinases and their ability to predict response to treatment

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Abstract

Fibroblast collagenase (FIB-CL) and stromelysin (SL) are members of a family of enzymes which are capable of degrading most of the extracellular matrix macromolecules. Extracellular control of these enzymes is performed by tissue inhibitor of metalloproteinases (TIMP). During healing, inflammation and normal tissue turnover, levels of MMPs and TIMP will change. The effect of treatment on the levels of FIB-CL, SL and TIMP as well as their ability, at baseline, to predict the outcome of therapy was investigated. 21 patients each provided 8 gingival crevicular fluid (GCF) samples from sites with probing depths equal or greater than 4mm. Clinical recordings and GCF sampling were performed at three time points. Assays for SL, FIB-CL and TIMP were performed by a sandwich ELISA. Attachment level changes were detected by the "tolerance method". The ability of the GCF constituents to predict the response to treatment was assessed by comparing pre-treatment levels between sites which did or did not show attachment gain after therapy. Although no GCF constituents could reliably predict the response to treatment, SL reduced significantly ($p=0.029$) after the hygiene phase of therapy. In addition both SL and TIMP's level showed a highly significant reduction at follow-up visit ($p=0.003$ and $p=0.005$ respectively). Thus, SL and TIMP levels are reduced by treatment but these GCF proteins do not appear to have an ability to predict treatment outcome from baseline.

INTRODUCTION

Proteinases have long been implicated in the pathogenesis of periodontal disease (Ohlsson, Olsson & Tynelius-Bratthall, 1973; Uitto *et al.* 1978; Heath *et al.* 1982 and Uitto, 1987). The increase proteinases activity/level during experimental gingivitis (Kowashi, Jaccard & Cimasoni, 1979) and their decrease after periodontal treatment (Larivèe, Sodek & Ferrier, 1986 and Makela *et al.* 1991) further suggest their involvement in periodontal tissue breakdown. Of the different proteinases thought to be involved in periodontal connective tissue breakdown the matrix metalloproteinases (MMPs) have received much attention and are implicated in other chronic inflammatory conditions such as Rheumatoid arthritis (Chin, Murphy & Werb, 1985; MacNaul *et al.* 1990 and Okada *et al.* 1992). MMPs are capable of degrading a variety of extracellular matrix components and this activity is required during tissue remodelling (Murphy, Koklitis & Carne, 1989). Tissue inhibitors of metalloproteinases (TIMP) inhibit these proteinases (Woessner, 1991), and tissue degradation may result from an imbalance between MMPs and TIMP (Nomura, Takahashi & Hara, 1993). Analysis of these enzymes, thought to mediate degradation of collagen, is a rational approach to diagnosis based on the importance of collagen as the major structural component of the periodontium (Lee *et al.*, 1991) and the knowledge that it is quickly degraded in chronic periodontal inflammation.

The relationship between periodontal treatment and collagenase in GCF

has been investigated by evaluating the changes in collagenase activity before and after treatment in a group of patients with localized juvenile periodontitis (Larivèe *et al.*, 1986). The enzyme activity for active collagenase showed a significant reduction 5 weeks after periodontal surgery, whereas the total enzyme activity remained high after surgical treatment (5 weeks) and dropped significantly, at the recall visit, 6 months after the last surgery. A further study by Villela *et al.* (1987) indicated that collagenolytic activity in GCF increases with the severity of disease in the order: periodontitis > gingivitis > healthy. Collagenase and gelatinase activity has also been assessed in mouthrinse samples from patients with AP and LJP as well as healthy subjects before and after periodontal therapy by Gangbar *et al.* (1990), who reported that both collagenase and gelatinase activity reduced following treatment but was only significant for active collagenase after scaling and antibiotic therapy in LJP patients. This suggests that there are differences in host response, reflected through MMP levels, which may have a bearing on prognosis and susceptibility. However, Birkedal-Hansen et al. (1989) reported no correlation between collagenase activity and progression of periodontal disease as was evidenced by alveolar bone loss.

Most of the studies have been conducted on polymorphonuclear leucocyte derived MMPs which are thought to be carried into the crevice in migrating PMNs (Birkedal-Hansen, 1993). It has been shown that one of the PMN proteins, lactoferrin, can serve as a marker of PMNs emigrating into the crevice (Adonogianaki *et al.*, 1993; Gustafsson *et al.*, 1994). Resident gingival

cells, including fibroblasts, are other sources of MMPs and TIMP (MacNaul *et al.* 1990). Cawston, McLaughlin and Hazleman, (1987) suggested that TIMP is locally produced and its main role is in defending connective tissue constituents in the very local area around the cell from which metalloproteinases are secreted. TIMP appears to preferentially inhibit FIB-CL and SL (Banda *et al.* 1992). No studies have examined fibroblast derived MMPs and TIMP in GCF as predictors of response to treatment. In this longitudinal study we have investigated the level of GCF FIB-CL, SL and TIMP in sites with advanced periodontal breakdown before and after treatment in an attempt to determine: a) the effect of therapy on the enzymes and inhibitor's levels; b) the ability of pre-treatment levels of MMPs and TIMP to predict the outcome of treatment; c) the association between clinical and biochemical parameters.

MATERIALS AND METHODS

Twenty six individuals, newly referred to the Periodontal Clinic of Glasgow Dental Hospital with advanced periodontal disease (pocket depths greater than 5.5mm in all quadrants) took part in this study. 4 subjects dropped out from the study and one individual had to withdraw from the study because of her pregnancy. Of 21 patients who remained in this study 11 were female and 10 were male with a mean age of 43.9 years ranging from 33 to 53 years. These individuals had no history of systemic conditions which could influence the course of periodontal disease. They were not on any medications that could

affect the manifestations of periodontal disease, such as chronic antibiotic use, phenytoin, cyclosporin and calcium channel blockers. A conventional pocket chart, made at a recruiting appointment using a PC12 periodontal probe, was used for site selection in this study. Two non-adjacent sites in each quadrant, (accessible to GCF sampling and attachment level recording) exhibiting probing depth ≥4mm on the pocket chart were selected for this study. Mesial sites were preferred to distal sites as they are more accessible. On the maxillary posterior molars, palatal sites were preferred to buccal sites and on the mandibular teeth buccal/labial sites were preferred to lingual sites as these sites are less prone to saliva contamination of the GCF sample. All the clinical recordings and sample collections in this study were carried out at three time points. i) baseline appointment; ii) reassessment appointment; and iii) follow up appointment. Prior to the baseline appointment, at the initial recruitment appointment a medical history form was completed by the patient and the pocket charting, to be used for site selection, was constructed.

An alginate impression was taken and, from the resulting cast, a soft acrylic stent for attachment level measurements was constructed. Subsequently, the patient was recalled for the baseline appointment. During this appointment and in the following order: a) PII and MGI recordings were taken first; b) GCF samples were then taken from the preselected sites and GCF Periotron readings recorded; c) the first set of AL measurements at the sites sampled was subsequently made. During this procedure bleeding on

probing and suppuration were also registered. The stent was then removed; d) after changing patient position and reapplying the stent the duplicate set of AL measurements was made; e) finally PD was assessed. The same procedure was followed at reassessment and follow up appointments.

All patients underwent hygiene phase therapy including oral hygiene instruction and scaling and root planing under local anaesthesia. Six weeks after the last treatment session (3 months±1 week after baseline examination), at the reassessment appointment, the patient was assessed and all the procedures in the baseline appointment were repeated. After recording clinical parameters and collecting samples a conventional pocket chart was constructed. This pocket chart along with the state of bleeding after conventional probing was used to assess the overall treatment success and determine further treatment needs. At this reassessment session sites were assigned to 'no treatment', 'repeated root planing' and 'surgery' treatment groups based on the following criteria: a) no treatment; no bleeding on probing; PD<3.5mm b) repeated root planing; bleeding on probing; PD>3.5 to 5.5mm c) surgery; bleeding on probing; PD>5.5mm. The subjects received further treatment according to the above mentioned criteria in different sites. The follow-up examination was performed 3 months after reassessment visit (with a tolerance of 1 week) in which all the procedures in the baseline and reassessment visits were repeated. At this visit a conventional pocket chart was constructed in order to evaluate the treatment success in non-study sites. One of the study teeth between the baseline and reassessment visits had

been extracted, thus the number of study sites decreased to 167 at reassessment session and all the comparisons between baseline and reassessment data were made on 167 sites. Between reassessment and follow-up examinations, a further study tooth was lost and one subject had a wire splint fitted which excluded another 6 teeth from attachment level measurements, thus reducing the number of sites to 160. Two calibrated examiners (EA) and (AH) were used throughout this study to record all clinical indices. The Modified Gingival Index (MGI) (Lobene *et al.* 1986) was used to assess gingival inflammation. The plaque index (PII) of Silness and Löe, (1964) was used for recording plaque accumulation. The Florida Probe (Florida Probe Corporation, Florida, USA) (Gibbs *et al.*, 1988) was used for probing depth and attachment level (AL) recording. This is an electronic pressure sensitive probe with a tip diameter of 0.4mm set at 20g. Pocket depth and attachment level were recorded to the nearest 0.2mm. Since a fixed reference point was required for attachment level measurements, individual soft acrylic stents were constructed for both upper and lower arches for each patient from a 2mm thick silicone layer using a modification of the method described by Isidor, Karring and Attström, (1984). Bleeding upon probing (BOP) was scored dichotomously using the Florida probe. Scoring was performed within 30s of probing. Presence or absence of suppuration was recorded after applying gentle pressure on the gingival wall of the pocket at an apico-coronal direction using a ball burnisher.

Gingival Crevicular Fluid (GCF) Sampling and Processing

GCF samples were collected using Whatman grade 4 paper strips (2x13 mm) (Griffiths, Curtis & Wilton, 1988). The site being sampled was gently air-dried and any supragingival plaque was removed. The area was carefully isolated to prevent samples from being contaminated by saliva. The paper strip was inserted into the crevice until mild resistance was felt or in any event not more than 1mm, and left for 30 seconds. Care was exercised in order to avoid mechanical injury of the tissues. After GCF collection, the paper strip was transferred to the chairside-located Periotron 6000 (Harco Electronics, Winnipeg, Canada) for the quantification of the fluid volume. The jaws of the Periotron were wiped with pure methanol between readings. The strips were then placed in individual sterile microcentrifuge tubes and stored at -30°C until further processing. Subsequently, the strips were eluted into 500μl of incubation buffer at room temperature using a rotary mixer. The strips were then discarded and the eluate aliquoted and stored at -30°C. These aliquots were subsequently analyzed for the quantification of FIB-CL, SL and TIMP.

Calibration of the Periotron 6000

In order to transform the Periotron digital readings for each paper strip into volumes and also assess the accuracy of the instrument, known volumes of distilled water were delivered to Whatman grade 4 paper strips with a Hamilton microsyringe in a range of volumes (0.05-1μl). Each measurement was performed 3 times and the mean value for each volume was used in a linear regression analysis from which the slope and intercept were used to determine

the volumes of GCF collected.

GCF Analysis

FIB-CL, SL and TIMP quantification was performed on the eluates of GCF and all constituents were assayed by means of sandwich ELISAs, based on a modification of the method of Cooksley *et al.* (1990). The 96-well polystyrene microplate (Immulon 4 Dynatech Laboratories, Billinghurst, Sussex, U.K) was coated with the first antibody, specific to the antigen to be quantified. These antibodies were Mac 64 anti-collagenase monoclonal antibody for FIB-CL, Mac 78 anti-stromelysin monoclonal antibody for SL and Mac 19-anti-TIMP monoclonal antibody for TIMP. The eluate of the sample was then added and any antigen present was captured by the immobilized antibody. Then the plate was incubated with a Biotin-conjugated monoclonal second antibody. Finally the plate was incubated with Extravidin-Peroxidase (Sigma). These antibodies were biotinylated Mac 66 to reveal FIB-CL and biotinylated Mac 15 to reveal TIMP. The stromelysin assay differed in that a rabbit polyclonal antibody was used as the second antibody followed by Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG (donkey) (Jackson). The anti-SL monoclonal and polyclonal and anti-FIB-CL monoclonals detected both the proenzyme and active forms of the SL and FIB-CL. Anti-TIMP monoclonals detected only free TIMP (Cooksley *et al.* 1990). All the antibodies were donated by Cell Tech. Ltd. Slough, UK. Visualisation was achieved by incubation with TMB substrate, and the reaction stopped with 2.5% NaF (sodium fluoride). The

plate was read at 630 nm, on a Dynatech MR5000 plate reader. Serial dilutions of standard antigen were run for each plate and their ODs were used in a linear regression analysis from which the slope and intercept were used to determine the samples' levels of FIB-CL, SL and TIMP. Results were expressed as pg/30s sample. Control wells in each plate were included which contained no sample or standard antigen in order to calculate background binding. The mean OD plus two standard deviations of these wells was taken as a cut-off point for detectability and any reading below that was considered undetectable (Tijssen, 1985).

Statistical Analysis

Attachment level changes between three appointments were assessed using the tolerance method described by Haffajee, Socransky and Goodson (1983b) and the differences between duplicate attachment level measurements were used to compute the site, subject and population thresholds which had to be exceeded in order to declare significant attachment level change. GCF levels of SL and TIMP were markedly skewed and required logarithmic transformation (log10, (x+1) x=original data value). Sites which demonstrated attachment gain were compared with their matched controls at baseline for their pre-treatment levels of MMPs and TIMP using two-sample t-tests. Multivariate repeated measures analysis of variance followed by paired t-tests were employed to compare baseline levels of the enzymes and inhibitor with their levels at reassessment and follow-up visits. In order to identify

associations between the clinical indices and the levels of SL and TIMP the non-parametric Spearman rank correlation coefficients were used. Data were analyzed using Minitab and SPSS statistical packages on an IBM compatible computer.

RESULTS

Multivariate repeated measure analysis of variance (MANOVA) ($p<0.0001$) and Friedman test (for non-parametric data) ($p<0.0001$) followed by pair t-test and Sign test revealed a difference in clinical parameters as a result of therapy. GCF valume, PD, MGI, BOP and suppuration scores showed a significant reduction at both post therapy visits from baseline. However, AL reduced significantly only after HPT.

MANOVA demonstrated a highly significant effect ($p<0.0001$) when all the study sites over the three visits were compared for their GCF levels of MMPs and TIMP (Table 1). Follow up analysis showed a significant reduction in the level of FIB-CL from baseline at both post therapy visits ($p=0.025$ and $p<0.001$ respectively), however, this reduction was not significant for SL ($p=0.052$) after therapy, but was significant at the follow-up visit ($p<0.001$). TIMP level increased significantly ($p<001$) after therapy and demonstrated a significant reduction at the follow-up visit ($p<0.001$). Although there was an overall improvement in clinical parameters following treatment, in order to further elucidate the effects of treatment, sites were grouped for further analysis according to their response to therapy assessed by gain in attachment

level.

a) Reassessment examination

At the reassessment examination, after hygiene phase therapy, 24 (14.4%) sites, in 12 subjects, demonstrated significant attachment gain. Clinical and biochemical parameters, (except GCF volume) were lower in sites which gained attachment (n=24) than those which did not (n=143), however these differences did not reach the significance level (Table 4). The percentage of sites with detectable amounts of FIB-CL that did and did not gain attachment at reassessment were 16.7% and 17.5% respectively. Attempts were made to investigate if the mean GCF levels of FIB-CL, SL and TIMP in sites which responded to therapy by attachment gain differed from their baseline levels. Using MANOVA ($p=0.026$) followed by paired t-tests, the mean levels of SL and TIMP in sites which gained attachment at the reassessment examination were compared with their levels at baseline. The mean level of SL decreased significantly ($p=0.029$) at reassessment. The number of sites with detectable levels of FIB-CL reduced to half at the reassessment visit (4 sites, (16.7%)). The mean pocket depth (paired t-test, $p<0.001$) as well as the mean gingival scores (Sign test, $p<0.001$) showed a significant reduction at reassessment examination (Table 2). The amounts of the proteins in sites which did not show attachment gain at reassessment (n=143) were compared with their pre-treatment levels using MANOVA ($p<0.01$) followed by paired t-tests. SL level showed a reduction at this visit, although it did not reach the significance level

(p=0.249), whereas TIMP's level increased significantly (p<0.001). GCF volume, PD and gingival scores reduced significantly at reassessment examination (p<0.001) (Table 2). The number of sites that expressed FIB-CL after therapy, decreased to 25 (17.5%) from 37 (25.9%) at baseline. In order to investigate if the baseline levels of FIB-CL, SL and TIMP could differentiate between sites which showed attachment gain and those which remained unchanged at reassessment visit, each site demonstrating attachment gain (n=24) was matched with a site which did not show attachment gain at this visit (n=24) for its GCF volume and clinical indices at baseline. Two-sample t-test showed no significant difference between the mean baseline levels of the enzymes and the inhibitor when the two groups of sites were compared (Table 3), thus the pre-treatment levels of MMPs and TIMP failed to predict the response to treatment.

b) Follow-up examination

According to the follow-up attachment level measurements, 25 sites (15%), in 11 subjects, had gained attachment from the baseline levels. Sixty percent (15) of these sites were among those which gained attachment after therapy. However, at this visit 5 (3%) sites within 3 patients showed significant attachment loss from baseline. Clinical and biochemical parameters, at the follow-up visit, except for GCF volume and the level of TIMP were lower in sites which gained attachment (n=25) than those which did not (n=130), although these differences did not reach the significance level (Table 4). The

percentage of sites expressing FIB-CL with or without attachment gain was 4% and 13.1% respectively. The mean levels of SL and TIMP at the follow-up visit were compared with their baseline levels in sites that gained attachment (MANOVA, $p=0.001$). Both the enzyme and the inhibitor mean levels showed a highly significant reduction at the follow-up examination (paired t-tests, $p=0.003$ for SL and $p=0.005$ for TIMP). The number of sites expressing detectable amounts of FIB-CL reduced from 8 (25%) sites at baseline to 1 (4%) site at follow-up. Significant reductions were observed in GCF volume and pocket depth (paired t-test, $p=0.002$ and $p<0.001$ respectively) as well as gingival scores (Sign test, $p<0.001$), in sites with attachment gain, at follow-up examination (Table 5). The GCF levels of the enzymes and inhibitor in sites which did not gain attachment ($n=130$) at follow-up visit were compared with their baseline levels using MANOVA ($p<0.001$) followed by paired t-tests. A highly significant reduction was observed in SL and TIMP levels at this examination ($p=0.002$ and $p<0.001$ respectively). GCF volume, PD and gingival score showed a significant reduction also ($p<0.001$) (Table 5). The number of sites with detectable amounts of FIB-CL, at this visit decreased to 17 (13.1%) from 31 (23.8%) sites at baseline. Sites demonstrating attachment gain ($n=25$) were matched with sites which did not gain attachment at this visit (25) for their GCF volume and clinical indices at baseline. No significant difference was found in the mean baseline levels of FIB-CL, SL and TIMP between the sites which gained attachment and their matched controls (two-sample t-test) (Table 3), indicating that pre-treatment levels of the enzyme and

inhibitor are not able to predict the outcome of treatment.

Correlation between clinical and biochemical parameters

In order to illustrate any possible relation between clinical and biochemical parameters, the association of SL and TIMP with clinical parameters was investigated. Using Spearman rank correlation coefficients, it was found that only TIMP had a strong positive correlation with GCF volume at both reassessment ($r=0.609$, $p=0.002$) and follow-up ($r=0.660$, $p<0.001$) visits, whereas SL was correlated with GCF volume only at reassessment examination ($r=0.493$, $p=0.014$). Pocket depths and gingival scores showed no significant association with the mean levels of SL and TIMP at both visits. As very small number of sites expressed detectable amounts of FIB-CL, no attempt was made to correlate its GCF level with the clinical parameters.

DISCUSSION

This paper reports the effect of periodontal treatment on GCF levels of FIB-CL, SL and TIMP in a group of patients with advanced periodontal disease as well as the ability of pre-treatment levels of these GCF components to predict the response to treatment. Longitudinal monitoring of attachment levels is the best available tool for evaluating treatment outcome at present. Selection of the method for detection of attachment level changes is crucial. Haffajee *et al.* (1983b) compared three different methods namely the linear regression analysis, the running median and the tolerance method. In the present study,

because of its relatively short duration, the "tolerance method" was employed. No analysis was performed on sites which showed attachment loss after treatment since this might have been merely caused by subgingival instrumentation and remodelling of attachment levels during healing (Badersten *et al.*, 1987; Vanooteghem *et al.*, 1990). It would be more appropriate if we could have analyzed those sites which gained attachment after surgical therapy separately. However, this was not possible due to the small number of such sites. Since collecting a standard amount of GCF is a prerequisite for expressing the results in terms of concentrations (Lamster et al., 1986) and the amount of GCF present in different sulci are extremely variable the levels of GCF biochemical components were expressed as absolute amounts with a standardised collection time as currently recommended (Lamster *et al.*, 1985; Lamster *et al.*, 1986; Smith & Geegan, 1991).

The observations that the mean levels of SL, in sites which gained attachment, decreased after treatment, at both examinations is in agreement with the findings of Larivèe *et al.*, (1986), Gangbar *et al.* (1990), and Suomalainen (1992). TIMP's level increased after therapy in these sites, which is consistent with the results of Larivèe *et al.* (1986). This increase, although not significant, may reflect TIMP's involvement in the healing process. At the follow-up visit the mean level of TIMP reduced significantly from baseline. This could be explained by the fact that the inflammatory process has subsided and the gingival tissues have healed, particularly as more than one third of the sites which showed attachment gain at follow-up did not

receive further treatment after therapy. Further evidence for this speculation is the observation that SL's levels decreased 4.5 times at the follow-up examination compared to a 3 times reduction only at the reassessment visit. The fact that SL and TIMP followed the same pattern of change after treatment in sites which did or did not gain attachment could be explained as follows: a) although these sites did not demonstrate significant attachment gain, their clinical condition improved significantly after treatment; b) since our antibodies detected total enzyme (latent and active) it is possible that the active enzyme has been lower in sites which gained attachment over those that did not, which is consistent with the studies of Larivèe *et al.* (1986) and Suomalainen (1992); c) the overall improved clinical condition in an individual, may exert an indirect influence on the levels of MMPs and TIMP in sites which did not respond to treatment. This is supported by the findings of Ebersole *et al.* (1993) who reported that the levels of PGE_2 and IL-1ß were lower in healthy sites from healthy subjects than healthy sites within refractory periodontitis patients and gingivitis sites from healthy subjects had lower levels of PGE_2 than those in adult periodontitis and refractory periodontitis patients. Gustafsson *et al.* (1994) have also reported that elastase activity was higher in gingivitis sites from patients with periodontitis than clinically comparable gingivitis sites from gingivitis affected individuals. These results are in agreement with our recent cross-sectional study in which the GCF levels of FIB-CL, SL and TIMP were lower in healthy, gingivitis and periodontitis sites in subjects on maintenance care (with only few diseased sites) than in patients

with advanced periodontal disease (Haerian *et al.* in press).

Since clinical parameters demonstrated rather poor ability to predict disease progression (Haffajee *et al.*, 1983a; Badersten *et al.* 1985; Jenkins *et al.*, 1988; Badersten *et al.*, 1990; Kaldahl *et al.*, 1990), attempts have been made to investigate the association between attachment loss and biochemical components of GCF in order to predict future disease activity (Offenbacher *et al.*, 1986; Lamster *et al.*, 1988; Persson *et al.*, 1990). Prediction of treatment outcome, using baseline subjective criteria, has been investigated by Vanooteghem *et al.* (1990) who reported a maximum 30% diagnostic accuracy for subjective clinical parameters. Pre-treatment levels of FIB-CL, SL and TIMP failed to distinguish between sites that gained attachment and those which did not. Sites that gained attachment were carefully matched to their controls according to GCF volume and clinical indices at baseline and thus the analysis focused on the parameters of interest, namely attachment gain and FIB-CL, SL and TIMP. However, within subject matching which is preferable, was not possible due to the limited number of sites in each individual. In this study, immunochemical analysis of GCF using ELISAs, detected only total enzyme (latent and active). Moreover, selection of sites at baseline was made by probing depth measurements which are indicative of past history of periodontal disease and do not necessarily reflect active periodontal destruction. Thus, it could be speculated that different sites in the present study, depending on whether they are active or inactive, had different proportions of active and latent enzymes at baseline which in turn may

determine the destructive potential of the inflammatory exudate, the GCF. Lee *et al.* (1991) reported that the frequency of active sites with active collagenase was higher than that of inactive sites with comparable pocket depths.

FIB-CL was detected in only 26.8%, 17.4% and 12% of the sites at baseline, reassessment and follow-up visits respectively which is consistent with our previous study (Haerian, *et al.* in press) and the finding of Woolley and Davies (1981). The rapid binding of α2-macroglobulin (α2-M) to FIB-CL, reported by Cawston *et al.* (1987) could also explain the low detectability of this enzyme in GCF in the light of the fact that α2-M is present in healthy, gingivitis and periodontitis sites (Adonogianaki *et al.*, 1992). It is also noteworthy that the level of this enzyme, whenever detected, was 1.5 fold higher than the control wells which contained no sample. However the number of sites expressing FIB-CL reduced to half after therapy and it was eight times smaller at follow-up examination in sites with attachment gain.

GCF levels of TIMP showed significant positive correlation with GCF volume at both post-treatment visits; the important finding here is that while GCF volume and TIMP levels were correlated, their direction of change was different after therapy. This is difficult to explain although it may imply local production of TIMP. SL showed the same direction of change with GCF volume at both post therapy sessions while their relationship was significant only at follow-up visit. There is no consensus in the literature regarding correlation between clinical parameters and proteinases and their inhibitors in gingival tissue/fluid. Kryshtalskyj *et al.*, (1986) reported strong correlation between collagenase activity and GCF flow. Villela *et al.* (1987) have also

reported positive but rather weak correlation between PD and GI and collagenase activity while Gangbar *et al.* (1990) and Teng *et al.*, (1992) failed to find any correlations between collagenase activity and clinical parameters. In our cross-sectional study on GCF MMPs and their inhibitors, there was a moderate positive and significant correlation between GCF levels of SL and TIMP and clinical parameters when data from healthy, gingivitis and periodontitis sites were pooled. When data from different groups of sites were separately analyzed the picture changed; so that, there was neither significant nor even moderate correlation between clinical and biochemical parameters. Different methods of GCF sampling and laboratory techniques as well as variations in reporting the results may influence the presence and extent of correlation between clinical and biochemical parameters, thus correlations of biochemical parameters and clinical indices reported in previous papers should be viewed with caution.

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Table 1. Geometric means and 95% confidence intervals for FIB-CL, SL and TIMP (pg/30s) at different visits. Number of sites and significance level (p value) for MANOVA is given.

Protein	n	Mean	95% C.I.
Fibroblast collagenase			
Baseline	166	3.9	2.8-5.6
Reassessment	166	2.3	1.7-3.0
Follow-up	166	1.9	1.4-2.5
Stromelysin			
Baseline	166	5.6	4.2-7.4
Reassessment	166	4.0	3.1-5.2
Follow-up	166	2.4	1.9-2.9
TIMP			
Baseline	166	228.7	201.8-259.2
Reassessment	166	333.9	291.8-382.8
Follow-up	166	93.30	78.90-110.1
MANOVA			$p<0.0001$

Table 2. Comparisons of GCF volumes, gingival scores, PD and GCF levels of SL and TIMP between baseline and reassessment visits in sites which did (AG) or did not demonstrate attachment gain (AU) at the reassessment examination (after HPT therapy). Means ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for the proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Reassessment	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
GCF Volume			
AG (n=24)	0.397±0.057	0.341±0.058	0.463
AU (n=143)	0.407±0.025	0.295±0.021	<0.001
Modified GI			
AG (n=24)	3.5±0.104	1.7±0.138	<0.001
AU (n=143)	3.1±0.052	1.8±0.067	<0.001
Pocket Depth			
AG (n=24)	6.5±0.293	3.7±0.234	<0.001
AU (n=143)	5.4±0.104	4.0±0.110	<0.001
Stromelysin			
AG (n=24)	9.9 (4.6-21.2)	3.1 (1.9-5.6)	0.029
AU (n=143)	5.2 (3.8-7.00)	4.1 (3.1-5.4)	0.249
TIMP			
AG (n=24)	231.7 (170.2-315-5)	291.3 (217.0-391.2)	0.307
AU (n=143)	228.7 (199.8-261.9)	343.6 (296.0-398.7)	<0.001

Table 3. Prediction of response to treatment according to baseline MMPs and TIMP levels. Baseline levels of MMPs and TIMP were compared between sites which showed attachment gain and their baseline matched controls which did not show attachment gain at the reassessment (n=24) and the follow-up (n=25) visits. Geometric mean and 95% confidence intervals and p values are shown.

	Attachment gain	Attachment unchanged	
	Mean (95% CI)	Mean (95% CI)	p value
Visit	**Fibroblast-CL (pg/30s)**		
Reassessment	5.5 (2.0-15.4)	2.8 (1.2-6.40)	0.296
Follow-up	5.0 (1.9-13.1)	4.3 (1.9-10.4)	0.826
	Stromelysin (pg/30s)		
Reassessment	9.9 (4.6-21.2)	7.1 (3.3-15.3)	0.536
Follow-up	9.4 (4.4-20.2)	6.5 (3.3-12.8)	0.446
	TIMP (pg/30s)		
Reassessment	231.7 (170.2-315.5)	290.8 (217.6-388.7)	0.289
Follow-up	251.4 (192.5-328.4)	229.9 (182.6-289.5)	0.614

Table 4. Comparisons of GCF volumes, gingival scores, PD and GCF levels of SL and TIMP between sites that gained attachment and those which remained unchanged at the reassessment and the follow-up visits. Mean ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for the proteins (pg/30s) are shown. Significance level (p value) for each two sample t-test and Mann- Whitney test (for gingival scores) is given.

	Attachment gain	Attachment unchanged	
	Mean ±SEM (95% CI)	Mean ±SEM (95% CI)	p value
Visit	**GCF valume (μl)**		
Reassessment	0.341±0.058	0.295±0.021	0.399
Follow-up	0.220±0.040	0.174±0.013	0.179
	Modified GI		
Reassessment	1.7±0.138	1.8±0.067	0.955
Follow-up	1.4±0.151	1.4±0.062	0.805
	Pocket Depth (mm)		
Reassessment	3.7±0.234	4.0±0.110	0.274
Follow-up	3.0±0.147	3.3±0.115	0.122
	Stromelysin (pg/30s)		
Reassessment	3.1	4.1	
	(1.9-5.6)	(3.1-5.4)	0.472
Follow-up	2.0	2.5	
	(1.5-2.7)	(2.0-3.2)	0.275
	TIMP (pg/30s)		
Reassessment	291.3	343.6	
	(217.0-391.2)	(297.4-398.7)	0.307
Follow-up	115.9	89.3	
	(71.5-188.0)	(73.9-107.9)	0.288

Table 5. Comparisons of GCF volumes, gingival scores, PD and GCF levels of SL and TIMP between baseline and follow-up visits in sites which did (AG) or did not demonstrate attachment gain (AU) at the follow-up examination. Means ±SEM for clinical parameters and the geometric mean and 95% confidence intervals for the proteins (pg/30s) are shown. Significance level (p value) for each paired t-test and Sign test (for gingival scores) is given.

	Baseline	Follow-up	
Parameter	**Mean ±SEM (95% CI)**	**Mean ±SEM (95% CI)**	**p value**
GCF Volume			
AG (n=25)	0.387±0.051	0.220±0.040	0.002
AU (n=130)	0.408±0.026	0.174±0.013	<0.001
Modified GI			
AG (n=25)	3.4±0.114	1.4±0.151	<0.001
AU (n=130)	3.1±0.099	1.4±0.062	<0.001
Pocket Depth			
AG (n=25)	6.4±0.289	3.0±0.147	<0.001
AU (n=130)	5.4±0.099	3.3±0.115	<0.001
Stromelysin			
AG (n=25)	9.4	2.0	
	(4.4-20.2)	(1.5-2.7)	0.003
AU (n=130)	4.7	4.1	
	(3.4-6.40)	(2.0-3.2)	0.002
TIMP			
AG (n=25)	251.4	115.9	
	(192.5-328.4)	(71.5-188.0)	0.005
AU (n=130)	228.0	343.6	
	(197.3-263.4)	(73.9-107.9)	<0.001