An investigation of matrix metalloproteinases in gingival crevicular fluid in the guided tissue regeneration procedure

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ABBREVIATIONS

1Y:	one-year
3M:	three months
6M:	six months
***Aa*:**	*Actinobacillus actinomycetemcomitans*
AB:	antibiotic
AL:	attachment level
AP:	adult periodontitis
ATBC:	acetylbutyl citrate
b FGF:	basic fibroblast growth factor
B:	baseline
***Bf*:**	*Bacteroides forsythus*
BOP:	bleeding on probing
CAL:	clinical attachment level
CB:	coating buffer
CEJ:	cemento-enamel junction
CEJ-BD:	cemento-enamel junction to bottom of infrabony defect
CFU:	colony forming unit
CL:	collagenases
Crest-BD:	crest to bottom of infrabony defect
CT:	connective tissue
DTPA:	diethylenetriaminepentaacetic acid
ECM:	extracellular matrix
EGF:	epidermal growth factor
ELISA:	enzyme-linked immunoabsorbent assay
ePTFE:	expanded polytetrafluoroethylene
FGF:	fibroblast growth factor
FIA:	fluorescence immunoassay
FIB-CL:	fibroblast collagenase

GCF:	gingival crevicular fluid
GL:	gelatinases
GL-A:	MMP-2 (72K GL)
GL-B:	MMP-9 (92K GL)
GTR:	guided tissue regeneration
HPT:	hygiene phase therapy
HRP:	horse radish peroxidase
HSRE:	human surface respiratory cells
IB:	incubation buffer
IFN:	interferons
IL:	interleukin
JE:	junctional epithelium
LJE:	long junctional epithelium
LJP:	localized juvenile periodontitis
MMP:	matrix metalloproteinase
MMP-1:	fibroblast collagenase
MMP-10:	stromelysin-2
MMP-11:	stromelysin-3
MMP-13:	collagenase-3
MMP-2:	gelatinase-A
MMP-3:	stromelysin-1
MMP-8:	neutrophil collagenase
MMP-9:	gelatinase-B
MT-MMP:	membrane bound metalloproteinase (MMP-14)
NAB:	non-antibiotic
ng/30s:	nanogram per 30 second
ng/ml:	nanogram per millilitre
NGF:	nerve growth factor
OD:	optical density
OHI:	oral hygiene instruction

PBS: phosphate buffered saline

PBST: wash buffer

PD: probing depth

PDGF: platelet-derived growth factor

PDL: periodontal ligament

***Pg*:** *Porphyromonas gingivalis*

***Pi*:** *Prevotella intermedia*

PII: plaque index

PMN: polymorphnuclear leukocytes

PTH: parathyroid hormone

RAL: relative attachment levels

RPP: rapidly progressive periodontitis

RT: room temperature

SEM: scanning electron microscopy

SL: stromelysin

TGF: transforming growth factor

TIMP: tissue inhibitor of metalloproteinases

TMB: tetramethylbenzidine

TNF: tumour necrosis factor

W1: week one

W4: week four

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DECLARATION

This thesis is the original work of the author. The authour's contributions to this research project includes:

1) taking GCF and microbiological samples at baseline, 1W, 4W, 3M (GCF only), 6M and 1Y visit

2) 6 points PD and RAL measurements using Florida probe and Florida disc probe at baseline, 6M and 1Y visit on the test teeth

3) recording BOP sites of the test teeth after the PD measurements

4) assisting the surgical procedures

5) analyzing GCF samples for the MMP-3 levels using ELISA methodology.

SUMMARY

Matrix metalloproteinases (MMPs) have been implicated in the aetiopathogenesis of periodontitis. MMPs are degradative host proteinases that are not only involved in the destruction process but also in embryologic development, tissue remodeling and wound healing processes. Accumulating evidence has gathered over the years for their involvement in periodontal destruction. Higher level of MMPs has been found in periodontitis sites compared to healthy sites or individuals. The 'active state' of the MMP enzyme has been associated with active periodontal lesions and reductions of MMP levels have been reported following periodontal treatment.

Repair and wound healing are most likely to be influenced by the matrix-modifying enzymes, the MMPs. MMP levels have been investigated during wound healing and stromelysin (SL) were found to be overexpressed and overactivated during the wound repair process. SL-1 and -2 have also been found in a variety of chronic wound lesions and also in acute skin wounds, suggesting their roles in tissue repair. MMP-9 levels measured 48 hours after injury can predict the amount of collagen deposited later as higher levels have been linked to retarded healing.

Resident connective tissue cells produce MMP-3 and MMP-8 are produced by polymorphnuclear leukocytes (PMN) in addition to fibroblasts of gingivae, mucosae and periodontal ligament. Both PMN and fibroblasts are increased during the wound healing process. PMN predominates immediately after the injury and up to three days after,

while an increased number of fibroblasts are found in the granulation tissue after the third day onwards.

GTR is now an acceptable technique for the treatment of infrabony defects. Compromised clinical results have been associated with microbial contamination during the membrane insertion. Microbial contamination has also been demonstrated in association with a non-resorbable barrier membrane owing to the lack of tissue integration leading to pocket formation. With resorbable membranes, this factor has been eliminated and the only concern is membrane contamination during insertion. Preliminary microbiological results have demonstrated that Guidor® barrier membranes do not seem to develop facultative anaerobic bacteria and black pigmented gram-negative anaerobic rods in high numbers during the first 6 weeks post-operatively. High dose amoxycillin, a broad-spectrum penicillin active against aerobe and facultative anaerobic microflora given 1 hour prior to the guided tissue regeneration (GTR) procedure may reduce the possible contamination of the material and the wound during membrane insertion.

The purpose of the study was to investigate the clinical outcome, the microbiological colonization and the levels of matrix metalloproteinases during the wound healing following insertion of a resorbable membrane (GUIDOR®) either alone or combined with one presurgical systemic application of amoxycillin. It was also investigated whether the fluctuation of GCF levels of MMP-3 and –8 during the wound healing could be linked to the clinical outcome and microbiological colonization. Gingival crevicular fluid (GCF)

samples were collected by means of sterile paper strips and the volume was assessed using the Periotron 6000. Fibroblast derived MMP-3 levels in GCF were assayed using modification of the sandwich ELISAs described by Cooksley *et al.* (1990). Antibodies detected both the active and the latent MMP-3. MMP-8 levels were determined by a time-resolved fluorescence immunoassay (FIA) as described by Hanemaaijer *et al.*, (1997) for the detection of active MMP-8. The results were expressed as absolute amounts, that is, ng/30s of sample collection.

GCF samples were collected from test, surgical control and healthy control sites from each patient. The test sites are the sites treated with membranes and the surgical control sites were the adjacent teeth involved in the surgical flap without the membrane insertion. The MMP-3 levels were only altered at week one for the test sites, while for the surgical control and healthy control sites the levels remained undetectable at all visit. Inspection of individual data revealed that 45% of the surgical sites had detectable increase in MMP-3 at week one.

The active MMP-8 levels for the test sites were significantly higher compared to the healthy control sites. For the test there was a significant increase in the MMP-8 levels at week one. There were also increased levels for the surgical sites, but the differences were not significant from the baseline values. Based on evidence presented in the literature about cellular aspects during healing, it can be presumed that most of the MMP-8 levels present after week 1 were derived mostly from fibroblasts.

To assess the microbiological effects of placing bioresorbable membranes in deep infrabony pockets, microbiological samples were obtained from test and healthy control sites. Both sites had deep pockets with BOP but the test sites were treated by the GTR procedure. The results demonstrated that the presence of the bioresorbable membranes did not influence the number of microorganisms detected in both sites. In other words, these membranes do not appear to act as a niche for the colonization of microorganism.

To assess the clinical outcome of the GTR procedures using Guidor®, clinical measurements of pocket depth, clinical attachment level, relative attachment level, and clinical indices of plaque index and bleeding on probing were recorded at baseline and at 6 and 12 month post-surgery. At one year post-surgery, a mean of 3.2mm pocket reduction and 2.6 mm of attachment level gain were achieved. In this study there was a significant negative correlation between the CAL gain and the plaque index at 12 .months, demonstrating the fact that maintenance of good oral hygiene over the long-term is of paramount importance.

The present study found no differences between the antibiotic and non-antibiotic groups, in all clinical and biochemical aspects except that there was a greater percentage of microorganism's resistance to amoxicillin encountered in the antibiotic group. In conclusion, the clinical, microbiological and MMP results following the GTR procedure were not influenced by the administration of a high dose of pre-operative amoxicillin.

CHAPTER 1

INTRODUCTION

1.1 PERIODONTAL DISEASE

The term 'periodontal diseases' is a non-specific term that refers to any disease or disease process that affects the periodontium. The periodontium is a functional system of different tissues supporting the teeth, comprising two soft tissues and two hard tissues namely the gingivae, the periodontal ligament, the cementum and the alveolar bone. The term 'periodontal disease', as commonly used, refers to the inflammatory periodontal diseases, which are broadly grouped into two distinct entities, depending upon the regional involvement of the inflammatory response: gingivitis and periodontitis. Both gingivitis and periodontitis are bacterial plaque associated diseases. Gingivitis refers to reversible inflammatory changes which are confined to the gingivae, and are clinically manifested by a change in colour (redness), texture and appearance (swelling) of the gingivae, together with an increased tendency to bleeding on gentle probing and gingival sensitivity (Löe, Theilade & Jensen, 1965). Periodontitis occurs when advanced destruction of both the hard and soft connective tissues becomes evident as a result of bacterial downgrowth leading to pocket formation and irreversible bone loss. Periodontitis being an inflammatory, bacterially induced disease has several features including visual signs of inflammation i.e. bleeding from the gingivae on probing, pocket formation, recession, tooth mobility and migration as well as recognizable radiographic evidence of bone loss.

1.2 THE PERIODONTIUM

1.2.1 Gingivae

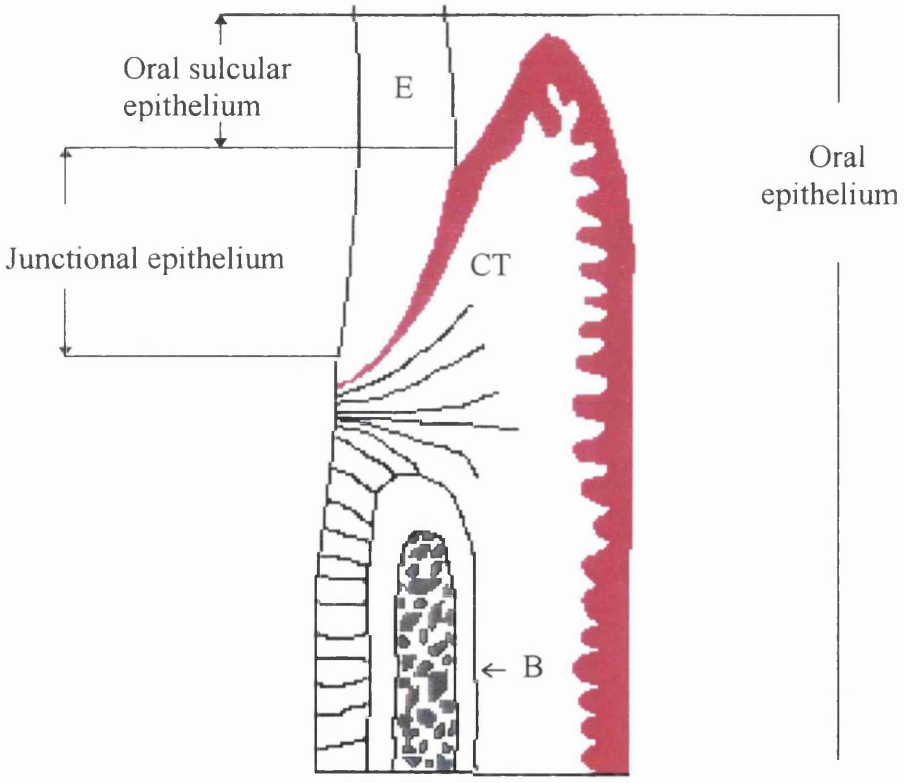
The gingivae is the most peripheral component of the periodontium that begins at the mucogingival junction and terminates as the free marginal gingivae surrounding the cervix of each tooth.

Healthy gingivae is described as "salmon or coral pink," firm in consistency and not movable upon the underlying structures. The interdental papillae are firm, do not bleed on gentle probing and occupy the entire space available below the contact area of neighbouring teeth. The surface of the gingivae is keratinised and may also exhibit stippling.

The gingivae comprises the free gingivae and the attached gingivae. The healthy free gingivae has a scalloped outline and terminates in the free gingival margin. The free gingival margin is often rounded in such a way that a small invagination or sulcus is formed between the tooth and the gingivae. When a probe is inserted into this invagination and advanced apically towards the cemento-enamel junction (CEJ) a gingival pocket is opened. The epithelium covering the free gingivae is divided into oral epithelium, oral sulcular epithelium and junctional epithelium (JE) as illustrated in Figure 1.1. The oral epithelium faces the oral cavity, the oral sulcular epithelium faces the tooth without being in contact with the tooth surface and the JE participates in the

Figure 1.1 Schematic drawing of the epithelium covering the free gingivae. The oral epithelium faces the oral cavity, the oral sulcular epithelium faces the tooth without in contact with the tooth surface and the junctional epithelium provides the contact between the gingivae and the tooth.

E: enamel; CT: connective tissue; B: alveolar bone



contact between the gingival soft tissues and the calcified structure of the tooth. JE manifests a uniform interface with the underlying connective tissue (CT) without rete ridges and is roughly 15 to 18 cells thick at the sulcus bottom and tapering to 4 to 5 cells at the most apical termination. The JE is physically attached to the tooth via hemidesmosomes. A characteristic morphological feature of the oral epithelium and the oral sulcular epithelium is the presence of invagination of epithelial ridges into the CT, so called rete pegs. These structures are lacking in normal JE but may be present in inflamed JE. The JE is permeable and serves as a pathway for diffusion of metabolic products and plaque bacteria. The size of the cells and the intercellular space in the JE are larger and wider than in the oral epithelium. The gingival epithelium forms the first line of defense against bacterial assault.

One of the earliest reported changes to occur in the periodontal tissues during development of plaque-induced periodontal inflammation is widening of the intercellular spaces between the epithelial cells of the sulcular and JE. While it is recognized that exudation occurs across the sulcular and JE both from the CT to the gingival sulcus and from the gingival sulcus to the CT, the mechanisms of intercellular widening are not understood (Brill & Krasse, 1959; Brill & Björn, 1959; Cimasoni, 1983).

The attached gingivae which extends in apical direction towards the mucogingival junction, is firmly attached to the underlying alveolar bone and cementum by connective tissue fibres and is therefore immobile. In the apical direction the gingivae is continuous with the non-attached, darker red alveolar mucosa. There is no mucogingival junction in

the palate since the alveolar process and the hard palate are both covered by attached keratinised gingivae.

1.2.2 Periodontal Ligament

The periodontal ligament is the soft, richly vascular fibrous connective tissue that surrounds the roots of the teeth and joins the root cementum with the alveolar bone. It is derived from the dental follicle. The periodontal ligament space has the shape of an hour-glass being narrowest at the mid-root level. The width of the periodontal ligament is approximately 0.25 mm $\pm$ 50%. The width of the periodontal space also varies according to the functional state of the periodontal tissues. The space is reduced in non-functional and unerupted teeth and is increased in teeth subjected to the heavy occlusal stress. With age the periodontal space narrows slightly. The presence of the periodontal ligament makes it possible to distribute and absorb forces elicited during function, into the alveolar process via the alveolar bone proper. The periodontal ligament is also related to tooth mobility, which to a large extend is determined by the width, height and quality of the ligament. Its cells form, maintain and repair alveolar bone and cementum. The periodontal ligament has a high rate of turnover compared to other soft connective tissue. The daily regeneration rate of periodontal ligament cell in normally functioning rodent is between 0.5-2% (McCulloch & Melcher, 1983).

The connective tissue fibres are mainly collagenous. The portions of the principal fibres that are embedded in the root cementum and in the alveolar bone are called Sharpey's fibres. The principal collagen species in Sharpeys's and other collagen fibres of the periodontal ligament is type I. Type III collagen appears to coat type I collagen of Sharpey's fibres.

The principal collagen fibres show different orientations in different regions of the periodontal ligament. The periodontal ligament also contains small amounts of elastin, which are present in connective tissue in association with blood vessels and in zones along cementum and bone (Lukinmaa, Mackie & Thesleff, 1991). Elastin is a very flexible and distensible protein which provides tissues the ability to stretch, bend and twist and is present in most vertebrate tissues to varying degrees (Mecham & Heuser, 1991). Oxytalan fibres are present in all connective tissue structures of the periodontium and they run mainly parallel to the long axis of the tooth in the periodontal ligament.

1.2.3 Cementum

The cementum is a specialized calcified tissue covering the root surfaces. It has many features in common with bone tissue; however the cementum contains no blood or lymph vessels, has no innervation, does not undergo physiological resorption and remodeling, but is characterized by continuing deposition throughout life. Cementum attaches the periodontal ligament Sharpey's fibres to the root and contributes to the process of repair

after damage to the root surface. Cementum is thicker apically than coronally and contains viable cells (cementocytes) in the apical 1/3 of the root. Two different types of cementum are recognized, primary or acellular cementum and secondary or cellular cementum. The primary cementum is formed in conjunction with root formation and tooth eruption and has no cells, thus denoted acellular cementum. Secondary or cellular cementum is laid down on top of the primary cementum throughout the functional period of the tooth. Both acellular and cellular cementum is produced by cementoblasts.

Changes that occur in cementum adjacent to tissue affected by periodontal inflammation includes obvious accrual of a variety of bacterial products either on the cementum surface or possibly within the cementum matrix (Hughes & Smales, 1986; Hughes, Auger & Smales, 1988), and changes in the inorganic composition (Selvig & Zander, 1962; Stepnick, Nakata & Zipkin, 1975), all of which may contribute to the reported structural changes noted in exposed cementum. The collagens of periodontally affected cementum appear to be denatured and have relatively high affinity for mineral adsorption (Furseth, 1971).

1.2.4 Alveolar Bone

The alveolar bone is part of the alveolar processes of the maxilla and mandible. It is a mineralized connective tissue. Its main function is to distribute and resorb forces generated by mastication and other tooth contacts. The alveolar process consists of bone,

which is developed both by cells from dental follicle (alveolar bone proper), and cells which are independent of tooth development. The process can be divided anatomically into two: 1) the alveolar bone proper (lamina dura, the cribiform plate)- a thin layer lining each socket, and exhibiting numerous perforations through which blood vessels, lymph channels and nerves pass; 2) the supporting alveolar bone which is made up of cortical bone and cancellous bone.

The alveolar process develops in conjunction with the development and eruption of teeth and is gradually resorbed if the teeth are lost. The alveolar bone is constantly renewed in response to functional demands. A number of cell types can be distinguished in bone. The bone-forming cells are termed osteoblasts and are found on the surface. They become trapped in their own secretion (osteoid) and subsequently become incorporated into the matrix as osteocytes. Large multinucleated cells are responsible for absorbing bone and are called osteoclasts.

1.3 EXTRACELLULAR MATRIX (ECM) OF THE CONNECTIVE TISSUE OF THE PERIODONTIUM

Most connective tissues can be divided into fibrous and non-fibrous elements. The fibrous elements include proteins such as collagen and elastin while the non-fibrous components include a variety of glycoproteins (laminin, fibronectin, proteoglycans) as well as minerals, lipids, water and tissue-bound growth factors (Bartold, 1995).

The extracellular matrix (ECM) of the periodontium or the intercellular substance is composed of a diverse number of macromolecules. Its function is to regulate cellular function by mediating the adhesion of cells and the binding of soluble growth factors, and it can also regulate the gene expression of cells. The composition of ECM can be altered by inherited disorder (Ehlers-Danlos syndrome), metabolic disorder (diabetes mellitus), inflammatory disorder (periodontitis) and drug-induced changes (phenytoin) (Mariotti, 1993).

The ECM consists of glycoproteins and proteoglycans that are secreted by cells and assembled locally into an organized network. The components of the ECM vary between tissues but generally include several types of collagen and proteoglycans (versican, decorin, biglycan, syndecan). The other components include noncollagen proteins (elastin, fibronectin, laminin, osteocalcin, osteopontin, bone sialoprotein, osteonectin, and tenascin). The proteoglycan consists of one or more sulfated polysaccharide, called a glycosaminoglycan, covalently linked to a protein core. The glycosaminoglycans are represented by several species, including chondroitin-4-sulphate, chondroitin-6-sulphate, heparan sulfate, heparin, hyaluronic acid and keratin sulfate. There are six different collagen types detected in the ECM of the periodontium. Collagen contains one or more triple-helical domains of polypeptide chains.

In the gingivae, collagen accounts for approximately 3/5 of the total protein (Page, 1972), and the majority of the collagen is type I. Gingival type I collagen is biochemically different from type I collagen found in other parts of the body e.g. skin. Other collagen types found are type III, IV, V, and VI. Type III is localized to the subepithelial CT, type V is distributed throughout the CT in a fibrillar pattern while type IV and VI are localized to basement membrane.

Changes in the gingival protein composition develop when a transition from dentate to edentulous occur and also as a result of inflammatory processes. During inflammation there is a small reduction in type I collagen (4%), 50% reduction in type III collagen and over 700% increase in type V collagen (Mariotti, 1993). Collagen type I and type III were almost entirely lost in areas of inflammation of the pocket walls in rapidly progressive periodontitis (RPP) and adult periodontitis (AP) patients (Hillmann, Dogan & Geurtsen, 1998). Furthermore, a collagen not detected in healthy gingivae appears, trimer type I collagen (Narayanan, Page & Meyers, 1980). Both the noncollagenous protein and proteoglycan content also change during inflammation.

Collagen is the principal protein found in periodontal ligament (Becker *et al.*, 1991; Eastoe & Melcher, 1971; Fullmer, 1967). The biochemical nature of collagen identified consists of type I and accounts for approximately 80% of periodontal ligament collagen (Becker *et al.*, 1991; Butler *et al.*, 1975), III (Becker *et al.*, 1991; Rahemtulla, 1992), IV (Andujar *et al.*, 1985; Romanos *et al.*, 1991), V (Becker *et al.*, 1991; Romanos *et al.*, 1991), VI (Becker *et al.*, 1991; Romanos *et al.*, 1991), XII (Dublet *et al.*, 1988). Type III

collagen is the second most common collagen found (Butler *et al.*, 1975) and, similar to type I collagen, is distributed throughout the periodontal ligament (Wang *et al.*, 1980) which form banded fibrils. Noncollagenous protein make up 10% of total protein found in periodontal ligament (Eastoe & Melcher, 1971; Guis, Slootweg & Tonino, 1973).

The periodontal ligament is one of the most metabolically active tissues in the body (Sodek & Ferrier, 1988; Sodek, 1977; Sodek *et al.*, 1977). Collagen metabolism represents most of the protein activity in the periodontal ligament (Sodek *et al.*, 1977). Sodek (1977) has demonstrated that the biological half-life of mature collagen was 20% and 17% less than found in gingivae and alveolar bone, respectively. The biological significance of the rapid turnover in the periodontal ligament is not understood, but it may have something to do with the adaptive function of this tissue due to forces of occlusion and support. An important feature of connective tissues in general and the periodontal ligament in particular, is the process of constant renewal of the extracellular matrix components (Everts & Beertsen, 1988; Sodek & Overall, 1988). Of all connective tissues studied so far it appears that periodontal ligament has the fastest turnover of collagenous proteins.

In cementum, 90% of the organic matrix is made up of type I collagen and 5% is type III collagen. Noncollagenous proteins account for the remaining components of the organic matrix.

In bone, collagen is almost solely produced by osteoblasts, is comprised predominantly of type I collagen, with small amounts of type III and type V collagen (Hauschka & Wians, 1986). Noncollagenous protein represents approximately 8% of the organic matrix.

Distribution of collagens types within the periodontal tissue

Tissue	Collagen
Healthy gingivae	I, III, IV, V, VI
Periodontal ligament	I, III, IV, V, VI, XII
Cementum	I, III
Alveolar bone	I, III, V
Inflamed gingivae	I, III, IV, V, VI

1.3.1 Collagen Turnover/Breakdown

Degradation of the ECM involves six distinct pathways (Stenman, 1990; Koivunen, Huhtala & Stenman, 1989; Birkedal-Hansen *et al.*, 1993; Sorsa *et al.*, 1997a). Matrix components may be dissolved by extracellular matrix metalloproteinase (MMP)-dependent (see 1.5), polymorphnuclear leukocytes (PMN) serine proteinase pathway, trypsin-mediated pathway, plasmin-dependent cleavage reactions, and that larger fragments may be disposed of by a phagocytic pathway by way of cleavage by lysosomal proteinases. Mineralized matrices appear to be degraded by an osteoclastic pathway

which relies on degradation by lysosomal proteinases in a narrow pericellular compartment.

The breakdown of collagenous proteins occurs via two different pathways: an intracellular and an extracellular route (Everts *et al.*, 1989; Everts & Beertsen, 1992; Murphy & Reynolds, 1993; Birkedal-Hansen, 1993a; Birkedal-Hansen *et al.*, 1993). This is shown in Figure 1.2

1.3.1.1 Intracellular Route

Under non-pathological conditions phagocytosis and intracellular digestion of collagen fibrils is a process observed at a high level in dynamic soft connective tissues such as gingivae and periodontal ligament (Ten Cate & Freeman, 1974; Beersten & Everts, 1977; Beertsen, Brekelmans & Everts, 1978; Melcher & Chan, 1981; Everts *et al.*, 1996). Fibroblasts appear to be the predominant cell involved in this process (Beertsen & Everts, 1977; Shore & Berkovitz, 1979; Yamasaki, Rose & Mahan, 1981; Everts & Beertsen, 1988). It was concluded that this pathway may be responsible for all collagen breakdown during normal turnover and therefore could be considered as the primary route of collagen degradation in soft connective tissues under steady state conditions (Shore & Berkovitz, 1979; Everts & Beertsen, 1988; Sodek & Overall, 1988; Everts *et al.*, 1989). In order for phagocytosis to occur the collagen must first be partially digested extracellularly. The mechanism by which this occur is not entirely clear. Once inside the

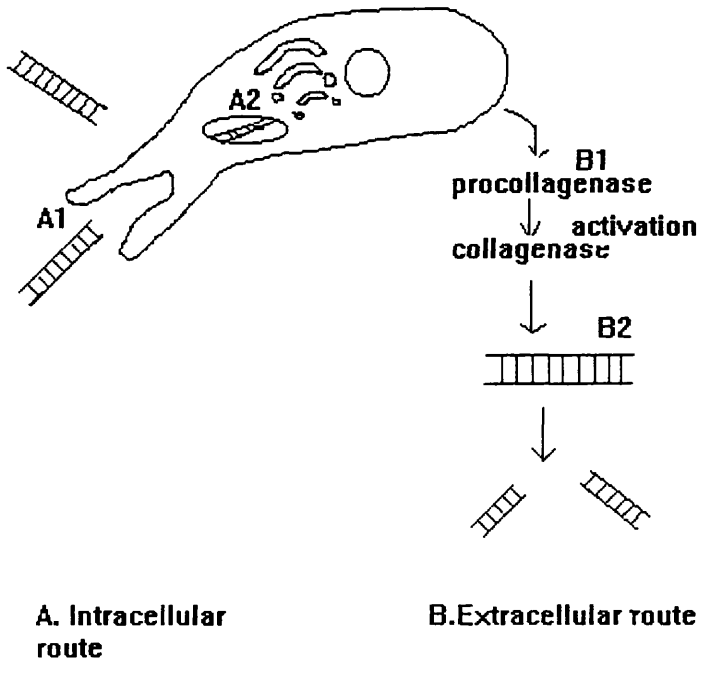


Figure 1.2 Routes of collagen breakdown

The major routes of collagen breakdown in soft tissue: [A] the intracellular pathway and [B] the extracellular pathways. The intracellular pathway primarily occur during normal turnover and remodeling. [A1] Cytoplasmic protrusion surround a collagen fibril, thus forming a phagosome. [A2] The lysosomal enzymes degrade the fibril intracellularly. [B] Following production, procollagenase is released in the extracellular environment where it may be activated. [B2] Activated collagenase degrades collagenous proteins or it may be inhibited by tissue inhibitor of metalloproteinases (TIMP).

cell the phagocytosed collagen collects in small vacuoles which appear to coalesce with lysosomes.

1.3.1.2 Extracellular Route

In pathological conditions, such as during periodontal disease, the balance between synthesis and degradation is disturbed. During early gingivitis many of the collagen fibrils in the gingivae are broken down, and replaced by inflammatory cells, changing a firm, pink gingivae into a swollen, loose and reddish tissue which has lost its integrity. When this condition becomes chronic, progression of the lesion into periodontitis may eventually occur. During the latter process collagen fibrils of the periodontal ligament that are attached to the cementum are broken down, usually together with the supporting alveolar bone.

Under such pathological conditions a different pathway of collagen degradation is likely to occur: a metalloproteinase-mediated extracellular digestion (Sodek & Overall, 1992; Birkedal-Hansen, 1993a). Interstitial collagenase (MMP-1) is the best known representative of this enzyme. It cleaves Type I and III collagen thus producing characteristic N-terminal ¾ and C-terminal ¼ fragments. MMP-2 (gelatinase), MMP-9 (type IV collagenase), MMP-3, MMP-10 and MMP-11 (stromelysin) have also been suggested to have a significant role in the initial destruction of periodontal extracellular matrix macromolecules (Birkedal-Hansen, 1993a). Neutrophil collagenase (MMP-8) is also involved in the periodontal destruction process and is the main collagenase in

gingival crevicular fluid (GCF), saliva and inflamed gingivae in adult periodontitis patients (Sorsa *et al.*, 1988; Ingman *et al.*, 1993). MMP-8 when produced by PMN is stored as mature enzymes in the subcellular granules. Among collagenases, MMP-8 most effectively hydrolyses the native type I, and II collagens, whereas MMP-1 prefers type III collagen. MMP-8 is a considerably more efficient enzymes than MMP-1 with respect to almost all substrates except type III collagens (Hasty *et al.*, 1987). The dental plaque has been shown to have the potential to serve as a reservoir and site of activation of PMN derived MMP in the periodontal inflammation (Sorsa *et al.*, 1995). Subsequent to the initial depolymerization caused by these enzymes more complete degradation of the proteins involves the action of other less specific host and bacterial enzymes

Other mechanisms of extracellular matrix disruption are associated with microbial colonization which include:

(i) release of degradative enzymes by bacterial cells

(ii) release of reactive oxygen species, or

(iii) alteration in matrix synthesis by fibroblasts, keratinocytes, endothelial cells and osteoblasts induced by a variety of cytokines and inflammatory mediators.

1.4 GINGIVAL CREVICULAR FLUID (GCF)

1.4.1 Introduction

The GCF is a pre-inflammatory serum transudate or an inflammatory exudate which exudes through the sulcular and junctional epithelium. The constituents of GCF are derived from serum, the periodontal tissues through which the fluid passes, bacteria present in the tissue and crevice, and host cells in the crevice. These constituents have been broadly classified by Cimasoni (1983) into cellular elements, electrolytes, organic compounds (including carbohydrates, proteins and lipids), bacterial products, metabolic products (including prostaglandins), and enzymes and enzymes inhibitors.

During inflammation the flow of crevicular fluid increases into the pocket. The production of GCF is primarily related to an increase in permeability of the vessels underlying the junctional and sulcular epithelia (Brill, 1959). In the case of healthy gingivae, these vessels will show signs of increased permeability, characteristics of an acute inflammatory reaction, only when stimulated, either by mechanical or chemical means. In a chronically inflamed but resting gingivae the permeability of the endothelial cells of these vessels is not normally increased, unless provoked by a stimulus in the sulcus. Even in the absence of inflammation, interstitial fluid could pass into the sulcus.

The GCF contains most of the components of serum which are present within the circulation, but in periodontitis it is selectively enriched in certain components as it

passes through the resident inflammatory cell infiltrate, such as immunoglobulin which is secreted locally by the plasma cell population. Furthermore, the GCF has high levels of metabolic products of neutrophils.

1.4.2 Passage of GCF

The epithelial covering of the gingival sulcus represents a relatively weak barrier to the penetration of foreign materials from the sulcus into the connective tissue. It is conceivable that plaque components, even of relatively high molecular weight, could pass within the gingival CT, when allowed to accumulate in the sulcus (Cimasoni, Ishikawa & Jaccard, 1977).

The state of health or inflammation of the marginal region could play a major role in the permeability of junctional and sulcular epithelia. Among the earliest ultrastructural changes during inflammation of the gingivae is the enlargement of intercellular spaces of the JE (Thilander, 1964; Freedman, Listgarten & Taichman, 1968) and the thinning and partial destruction of the basal membrane (Takarada *et al.*, 1974; Cergneux *et al.*, 1982). The basal membrane is to some extent a limiting barrier to products diffusing either from the sulcus into the connective tissue or from the connective tissue into the sulcus. The morphological alterations are even more dramatic during pocket formation, when JE gradually disappears and is replaced by pocket epithelium (Page & Schroeder, 1976).

1.4.3 Mechanism of Fluid Production

Alfano (1974) hypothesized that a very early or preinflammatory flow of gingival fluid may be osmotically mediated. In the absence of inflammation, macromolecules originated from plaque will diffuse intercellularly and reach the basement membrane where they create an osmotic gradient and attract interstitial fluid toward the sulcus. This is not an inflammatory exudate. When more macromolecules reach the basal membrane, an inflammatory reaction will start.

Gingival fluid production is modulated by the passage of fluid from capillaries into the tissues and by the removal of this interstitial fluid by the lymphatics of the gingivae. When the production of fluid from the capillaries is greater than the lymphatic uptake, fluid will accumulated as oedema or leave the area as gingival fluid.

The net flow of crevicular fluid depends on;

1) capillary filtration

2) lymphatic uptake

3) filtration coefficient of junctional and sulcular epithelium.

Due to the presence of bacterial by-products, increased production of gingival fluid occurs when the oncotic pressure of the sulcular compartment exceeds that of interstitial fluid. When inflammation is present, the oncotic pressure will be identical in both the sulcular and tissue compartments, due to the identical protein concentration in gingival fluid and serum (Bang & Cimasoni, 1971). The equality of both oncotic pressures would

thus cancel out their importance in fluid production. In inflamed gingivae, therefore the capillary pressure is more important than osmotic gradients.

GCF is derived from plasma (Pashley, 1976) and its rate of production depends on the regulation of the gingival microcirculation. It is agreed that gingival fluid flow increases several days prior to detectable clinical inflammation and persists some time after clinical inflammation has resolved (Löe & Holm-Pedersen, 1965). The GCF collected from inflamed gingivae contains the same concentration of total protein as serum (Bang & Cimasoni, 1971), but in clinical healthy gingivae the proteins are lower and similar to that of extracellular fluid.

1.4.4 Methods of Collection of Gingival Crevicular Fluid

Sampling of GCF exhibits several features, and, the diagnostic potential of GCF has been widely recognized (Page, 1992; Johnson, 1991; Lamster, 1992). For practical purposes, GCF is an inflammatory exudate from the gingival microcirculation that crosses inflamed periodontal tissues and en route collects molecules of potential interest from the local inflammatory reaction (Cimasoni, 1983). In terms of application to diagnostic tests, sampling of crevicular fluid provides advantages that are analogous to the drawing of blood by the physician (McCulloch, 1994).

Several techniques have been utilized for the collection of GCF. The choice of the technique depends on the aim of the investigation and the amount of the fluid required. The various methods include the use of paper strips/paper points, capillary tubes or micropipettes and gingival washings. Absorbing paper strips have been widely used when measuring the flow of GCF and also for quantitative analysis of fluid components. A second method based on the use of capillary tubes (Larivèe, Sodek & Ferrier, 1986) or micropipettes (Eisenhauer *et al.*, 1983) allows the collection of large volume of GCF (10-40 μl) along the upper anterior teeth.

The accurate and reproducible sampling of fluid is important. A number of studies demonstrated that the choice of collection device (Brill, 1962; Sueda, Bang & Cimasoni, 1969; Takamori, 1963; Oppenheim, 1970; Egelberg & Attstrom; 1973), the various materials for sampling (Nakashima, Demeurisse & Cimasoni, 1994), the length of collection time, the number of sample repetition (Binder, Goodson & Socransky, 1987; Persson & Page, 1990), the presence of supragingival plaque (Stoller, Karras & Johnson, 1990) and the calculation of data as absolute measures or as flow rates (Lamster, Hartley & Vogel, 1985), contribute in a large measure to overall variability. The potential depletion of the sample by a prolonged collection time (Persson & Page, 1990), the potential contamination by serum components (Lamster, Hartley & Vogel, 1985), the loss of the sample from the collection device (Rossomando, Kennedy & Hadjimichael, 1990), and the differences between sites (Smith & Gaegan, 1991) are also factors which affect variability.

Crevicular Fluid Sampling

Advantages	Uncertainties
Ease of access	Type of collection device
Atraumatic	Length of collection time
Rapid equilibration with whole pool	Number of repetitions
Can vary with time and site	Absolute or relatives value
Repeated sampling possible	Contamination by serum components
	Loss of sample over time

1.5 MATRIX METALLOPROTEINASES

1.5.1 Introduction

Matrix metalloproteinases (MMP) are members of a large subfamily of zinc- and calcium-dependent protelytic enzymes, proteinases which are responsible for remodeling and degradation of ECM components (Woessner 1991). The components degrade interstitial and basement membrane collagens, fibronectin, elastin, laminin, and proteoglycan core protein. These potent enzymes are made in a proenzyme form, and activation occurs after leaving the cell synthesizing them, the connective tissue cells. These proteinases are involved in a number of physiologic events, such as embryologic development, tissue remodeling, wound healing, salivary gland morphogenesis and tooth eruption, in addition to various pathologic process such as periodontal disease, arthritis, cancer, atherosclerosis, pulmonary emphysema and osteoporosis (Ryan, Ramamurthy & Golub, 1996).

It is now recognized that an imbalance between activated MMP and their endogenous inhibitors (excess of MMP over the inhibitors) can have profound effects on the composition and organization of the extracellular matrix during periodontitis. At the tissue level, this affects tissue morphology and function, while at the cellular level, changes in the composition of the extracellular matrix influence cell adhesion, migration, differentiation and activity (Overall, 1991).

MMP comprises at least twenty members, each of which is the product of different gene. Members of this family generally possess quite broad specificity.

There is a high degree of similarity between the amino acid sequences of metalloproteinases in each group and between groups, and 6 domain have been defined as shown in Figure 1.3 (Reynolds & Meikle, 1997). The MMP structural features include:

- a propeptide domain that contain the 'cysteine' switch (Van Wart *et al.*, 1990; Springman *et al.*, 1990),
- the catalytic zinc-binding domain,
- a C-terminal (hemopexin-like) domain (Birkedal *et al.*, 1993; Docherty *et al.*, 1990). (absent from the smallest MMP, pump-1)
- a hinge region
- a gelatin-binding domain (for gelatinases only)
- a trans-membrane (for membrane bound group)

The chief characteristics of the matrix metalloproteinases are:

- The catalytic mechanism depends on zinc at the active center
- The proteinases are secreted in zymogen form.
- The zymogens can be activated by proteinases or by organomercurials.
- Activation is accompanied or followed by a loss of M_r of about 10,000.

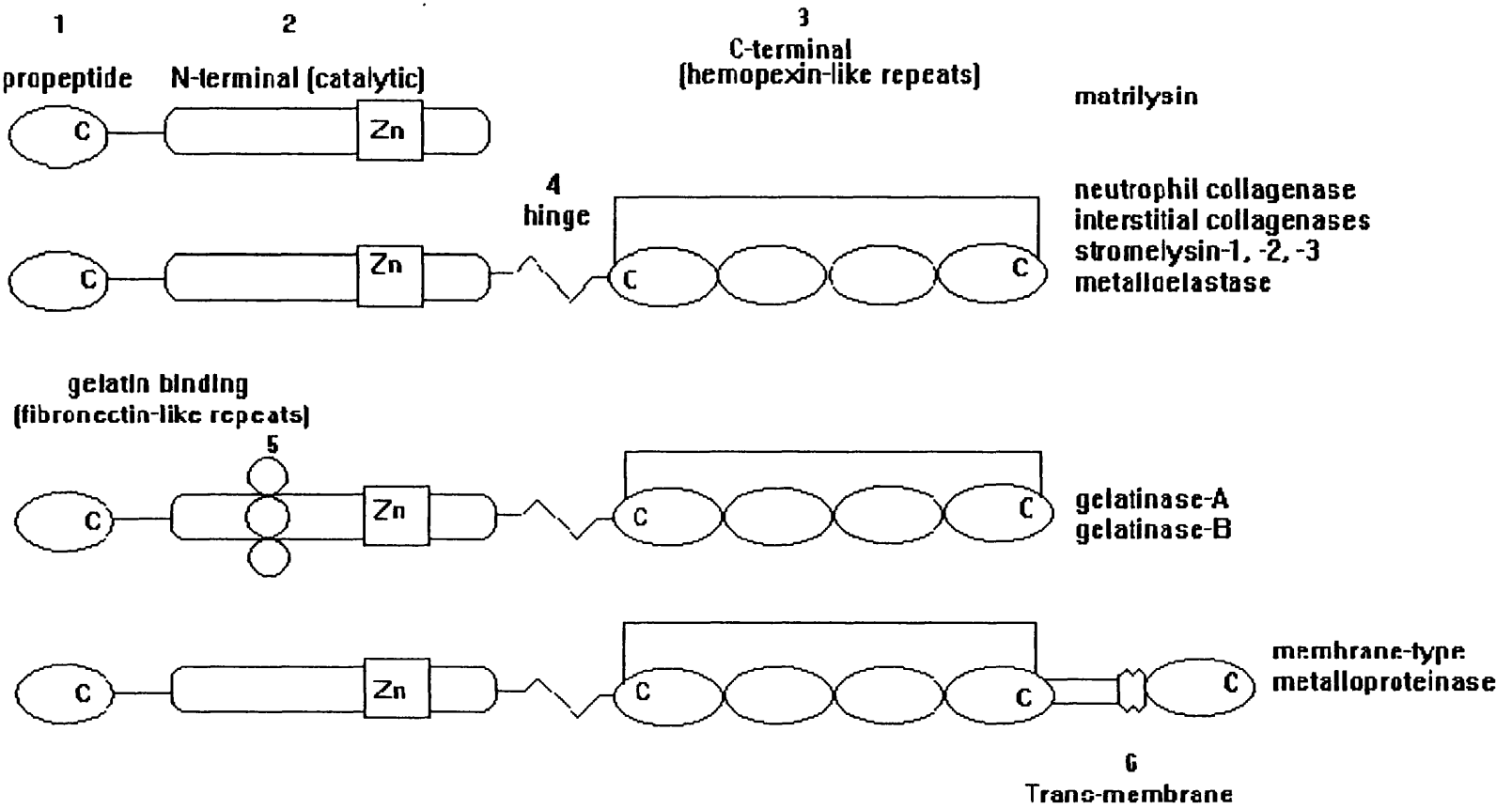


Figure 1.3 Domains of matrix metalloproteinase

The sequence of prometalloproteinases can be illustrated as six major domains. Domain 1: a propeptide region containing the conserved cysteine, which confers latency. Domain 2: a catalytic core domain at the N-terminus, which is the minimum sequence necessary for enzymatic activity, and contains Zn++ binding site. Domain 3: a C-terminus region that contain hemo-pexin like repeats, which are involved in substrate binding, and a disulfide bridge. Domain 4: a proline-rich hinge region. Domain 5: a gelatin binding domain in the gelatinases that is composed of fibronectin-like repeats. Domain 6: a putative membrane-spanning domain that locates a membrane type metalloproteinase at the cell surface, and a charged tail. Also all the MMP have a signal peptide that is first cleaved to yield the latent forms.

- The cDNA sequences all show homology to that of collagenase.
- The enzymes cleave one or more components of the extracellular matrix.
- Activity is inhibited by tissue inhibitor of metalloproteinases (TIMP).

(Woessner, 1991).

MMP family member can be simply divided into five subgroups, based on substrate characteristics:

Collagenases (MMP-1 and MMP-8)

Gelatinases (MMP-2 and MMP-9)

stromelysin (MMP-3, MMP-10 and MMP-11)

membrane-type MMPs (MT-MMPs)

and others.

Table 1.1 shows the family members of the MMPs.

1.5.2 MMP Classification

1.5.2.1 Collagenases (CL)

This is the first member of the metalloproteinase family discovered. It was discovered by Gross and Lapiere in 1962. Only the 'fibroblast' collagenase (FIB-CL/MMP-1) and the 'neutrophil' collagenase (MMP-8) can cleave native undenatured interstitial collagen (type I, II, III, VII, VIII,X) at neutral pH. MMP-1 is a neutral MMP capable of cleaving

Table 1.1 Matrix Metalloproteinase Family

Enzyme	MMP	Mr	Extracellular matrix Substrates
Collagenases			
Fibroblast-collagenase	MMP-1	57,000/52,000	Collagen I, II, III, VII, VIII, X, Gelatin
PMN-Collagenase	MMP-8	75,000	Same as FIB-CL
Collagenase-3	MMP-13	65,000	Proteoglycan core protein
Gelatinases			
Mr 72K (Gelatinase A)	MMP-2	72,000	Gelatin, Collagen IV,V, VII, X, Elastin, Fibronectin
Mr 92K (Gelatinase B)	MMP-9	92,000	Gelatin, Collagen IV, V, XI, Elastin
Stromelysins			
Stromelysin-1	MMP-3	60,000/55,000	PG Core Protein, Fibronectin, Laminin, Collagen IV,V, IX, X, Elastin
Stromelysin-2	MMP-10	60,000/55,000	Same as SL-1
Stromelysin-3	MMP-11	51,000	Elastin, fibronectin, gelatins, procollagen I, II, III
Membrane-type MMPs			
MT MMP-1	MMP-14	63,000	Gelatin, elastin, fibronectin, laminin, vitronectin, PG core protein, proMMP-2, MMP-13, collagen, I, II, III.
MT MMP-2	MMP-15	72,000	not determined
MT MMP-3	MMP-16	64,000	proMMP-2
MT MMP-4	MMP-17	70,000	not determined
Others			
Matrilysin (PUMP-1)	MMP-7	28,000	Fibronectin, Laminin, Collagen IV, Gelatin, proCL, PG Core Protein, urokinase
Macrophage metalloelastase	MMP-12	54,000	elastin, fibronectin, gelatin, laminin, vitronectin, PG core protein, collagen IV.
	MMP-18	57,000	not determined
	MMP-19	57,000	not determined
enamelysin	MMP-20	52,000	amelogenin

(modified from Nagase *et al.*, 1992)

the collagen triple helix at a single locus to yield characteristic one-quarter - three-quarter products (Gross & Nagai, 1965). The initial cleavage of native collagen by tissue collagenase (MMP-1) is essential to the degradation of collagen extracellularly. However, the activity of several related MMP, including MMP-2 and MMP-3, are also required to complete the collagenolytic process (Sodek & Overall, 1988).

MMP-1 is expressed after appropriate stimulation by a variety of cell types:

fibroblasts (Goldberg *et al.*, 1986; Stricklin *et al.*, 1977),

keratinocytes (Lin *et al.*, 1987; Petersen *et al.*, 1987),

endothelial cells (Herron *et al.*, 1986; Moscatelli, Jaffe & Rifkin, 1980),

macrophages (Campbell *et al.*, 1987; Welgus *et al.*, 1985),

osteoblasts (Otsuka *et al.*, 1984; Quinn *et al.*, 1990)

and chondrocytes (Lefebre, Peeters-Joris & Vaes, 1990).

MMP-1 is synthesized on demand in response to growth factor/cytokine activation of gene transcription. The fully functional protein emerges from the cell after 6- to 12-h lag period required for activation of the transcriptional translational machinery. MMP-1 degrade preferentially type I and III collagen over type II collagen. MMP-1 levels were more prominent in GCF of localized juvenile periodontitis (LJP) than in adult periodontitis patients (Suomalainen *et al.*, 1991; Ingman *et al.*, 1996).

MMP-8 is expressed mostly by PMN. It is synthesized and packaged in the specific (secondary) granules of PMN before the cells emigrate from the bloodstream and is

rapidly released when the cells are triggered. MMP-8 transcripts and protein can also be found in gingival and periodontal ligament fibroblasts (Salo *et al.*, 1995, Sorsa *et al.*, 1996) and also in chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996), suggesting their possible ability to also produce MMP-8. MMP-8 are also expressed by other non-PMN cells which includes mucosal fibroblast, rheumatoid synovial fibroblast, cultured squamous cell carcinoma of the tongue, and human umbilical vein endothelial cells (Cole & Kuettner, 1995). Among collagenases, MMP-8 most effectively hydrolyses the native type I, and II collagens, whereas MMP-1 prefers type III collagen. MMP-8 is a considerably more efficient enzymes than MMP-1 with respect to almost all substrates except type III collagens (Hasty *et al.*, 1987). PMN-derived MMP (MMP-8 & MMP-9) predominantly in autoactive form was detected in both supra- and subgingival dental plaque extracts of adult periodontitis patients. The activity of PMN derived MMP-8 is influence by PMN degranulation and pro-MMP-8 activation (Hasty *et al.*, 1987; Weiss, 1989; Mainardi, Pourmotabbed & Hasty, 1991) while non-PMN MMP-8, like other MMPs is also most likely regulated by inflammatory mediators such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (Hanemaaijer *et al.*, 1993; Chubinskaya *et al.*, 1996; Cole *et al.*, 1996; Schopov *et al.*, 1997; Hanemaaijer *et al.*, 1997).

1.5.2.2. Gelatinases (GL)/ Type IV Collagenases

These two gelatinases cleave types IV, V, VII, X, XI collagens, elastin, fibronectin, and perhaps other matrix substrates as well.

Mr 72K gelatinase (GL-A or MMP-2)/type IV collagenases is expressed by most cell types:

fibroblast (Seltzer *et al.*, 1981),

keratinocytes (Salo *et al.*, 1991),

endothelial cell (Kalebic *et al.*, 1983)

macrophages (Garbisa *et al.*, 1986),

osteoblast (Overall, Wiebkin & Thonard, 1987)

and chondrocytes (Lefebvre *et al.*, 1991)

but characteristically not by PMN.

The gelatinase, MMP-2 is perhaps the most widely distributed of all MMP and is present in a circulating form in plasma (Birkedal-Hansen, 1993).

Mr 92K GL (GL-B or MMP-9) is produced by PMN but also by a number of other cell types including:

keratinocytes (Salo *et al.*, 1991; Wilhelm *et al.*, 1989)

macrophages (Mainardi *et al.*, 1984)

and occasionally fibroblasts.

1.5.2.3 Stromelysin (SL) Group

Stromelysin have virtually identical substrate specificities and cleave a wide range of ECM proteins. SL-1 (MMP-3) and SL-2 (MMP-10) cleave ECM proteins: proteoglycan core protein, type IV, V, IX, X collagen, fibronectin, elastin and laminin (reviewed in Murphy, Nagase & Brinckerhoff, 1988; Nicolson, Murphy& Breathnach, 1989). Induced stromal cells, macrophages, and endothelial cells express the SL-1 gene, where as keratinocyte express the homologous SL-2 gene. SL-3 transcripts are expressed by stromal cells of human mammary carcinomas and by embryonic fibroblasts. Stromelysin-1 (MMP-3) not only degrades connective tissue component but is also involved in the proteolytic activation of MMP-1, MMP-8 and MMP-9 (Ogata, Enghild & Nagase, 1992). In a study by Tonetti, Pini-Prato & Cortellini (1993) MMP-3 transcripts were not detectable in healthy sites while they could be specifically detected in 38 % of the periodontitis sites. The presence of MMP transcripts in some but not all periodontitis sites was not unexpected in light of the current models of disease progression. Kubota *et al.* (1996) also shown that mRNA levels of MMP-3 were significantly higher in periodontitis tissue samples when compared to healthy samples.

1.5.3 Regulation of MMP Activity

The activity of MMP against ECM substrate is regulated at four "gates":

1) by transcriptional regulation of MMP genes;

2) by precursor activation;

3) by differences in substrate specificity; and

4) by MMP inhibitors.

1.5.3.1 Transcriptional Regulation

Endogenous growth factors and cytokines regulate the transcription of most MMP genes. Inflammatory and mesenchymal cells secrete them and generally they stimulate enzyme production. Stimulation or repression of growth factors and cytokine-responsive MMP genes results in up to a 50-fold change in mRNA and proteins levels.

Transcription of the CL and MMP-3 genes is induced by:

interleukin-1β (IL-1β)

Tumour necrosis factor-α (TNF-α)

platelet-derived growth factor (PDGF)

Transforming growth factor-α (TGF-α)

epidermal growth factor (EGF)

basic fibroblast growth factor (b FGF)

nerve growth factor (NGF)

and with few exceptions abrogated by TGF-β and interferons-γ (IFN-γ).

Other endogenous pathway may also regulate transcription of MMP genes. MMP-1 is induced/stimulated in osteoblasts by parathyroid hormone (PTH) and 1, 25 di (OH) D3 and MMP-1 and MMP-3 are down-regulated by glucocorticoids and by retinoids.

Regulation of MMP expression

Type of regulation	
Induction	IL-1α, IL-1β, TNF-α, TGF-α, EGF, PDGF, bFGF, NGF, TGF-β, PGE2, 1,25-di(OH)D3
Repression	IFN-γ, IL-4, TGF-β, glucocorticoids, retinoic acid

1.5.3.2 Activation of Precursors

MMPs are not stored in cells but are secreted as inactive or latent proenzymes or zymogens. In vivo, it is likely that the procollagenases are activated by proteolytic cleavage catalyzed by other proteinases such as plasmin. Also, the activity of stromelysin is probably required for complete activation of collagenases (Harris & Vater, 1982; Goldberg *et al.*, 1990).

All the MMPs have an N-terminal propeptide that determines the latency of the proenzyme form. Here, the cysteine interacts with the Zn (II) of the adjoining catalytic domain, displacing the water molecules required for catalytic activity.

In the activation process, the secreted zymogen first loses its signal peptide. Activation of most MMPs involves sequential exogenous/endogenous cleavages of the propeptide which destabilize the cysteine - Zn (II) interaction, modify the enzyme conformation, and permit further exogenous or autocatalytic processing to the final active form.

Latency is overcome by physical (chaotropic agents and detergents), chemical (oxidants and organomercurials), or enzymatic (trypsin, plasmin, and other proteinases) processes that allow the separation of the cysteine residue from the zinc in the active domain of the enzyme.

Only procollagenase and prostromelysin are activated by plasmin. Plasmin is a serine proteinase derived from plasminogen. Plasmin itself is not a proteinase in matrix degradation.

MMP-2 has no apparent plasmin- susceptible propeptide cleavage site. A number of independent studies have described a cell membrane mediated activation process for progelatinase A, the membrane bound metalloproteinase (MT-MMP, MMP-14) gives a second mechanism of activation. MT-1 metalloproteinases can also process procollagenase-3 (MMP-13) to active enzyme. The membrane "activator", which is specific for gelatinase A can be induced in a number of cell types, such as :

fibroblasts,

osteoblasts,

chondrocytes,

endothelial cells and

tumour cells

by effectors such as concanavalin A, cytochalasin D, phorbol esters,

transforming growth factor β1 or by exposure to collagen.

There is also evidence that exogenous proteases, such as those from periodontally pathogenic bacteria, can act as activators of procollagenase (Sorsa, Uitto & Suomalainen, 1992).

1.5.3.3 Substrate Specificity

The different types of substrate have been shown to be one of the factors involved in regulation of MMP activity. Accordingly, regulation of cell function is affected by changes in the extracellular matrix composition. At the tissue level, this affects tissue morphology and function, while at the cellular level, changes in the composition of the extracellular matrix, influence cell adhesion, migration, differentiation, and activity. The enzymes have some overlapping effects on the substrate, however there are some differences with respect to their function. Virtually all of the enzymes cleavage gelatin and fibronectin at some rate, and most cleave type IV and V collagens at sufficiently high

temperature. The characteristics ability of MMP-1 and MMP-8 to dissolve interstitial collagen fibrils is not shared by other members of the family.

1.5.3.4 Inhibition

Endogenous or natural inhibitor such as tissue inhibitors of MMPs (TIMPs) and α-macroglobulins bind in a high affinity, noncovalent fashion to inhibit members of the MMP family. TIMPs bind to the active sites of MMPs. TIMP is widely distributed in tissues, fluids (in serum, amniotic fluid (Welgus, 1983; Murphy, Cawston, Reynolds, 1981) and saliva (Drouin, Overall & Sodek, 1988). TIMPs are expressed by many cell types including fibroblasts, keratinocytes, monocytes/macrophages, endothelial cells, and osteoblast (Settler-Stevenson, Krutzch & Liotta, 1989; Welgus, 1983). TIMPs probably control MMP activity pericellularly, whereas α_2-macroglobulin functions as a regulators of MMPs in body fluids (Ryan, Ramamurthy & Golub, 1996). During inflammation, however α-macroglobulins, may escape the vasculature and function in the ECM.

α_2-macroglobulin is a major physiologic plasma proteinase inhibitor that is restricted in its site of activity owing to its large size. α-macroglobulin inactivates susceptible proteinase by entrapment following cleavage of a peptide bond in the bait region, by a unique venus-fly- trap mechanism (Sottrup-Jensen 1989; Sottrup-Jensen *et al.*, 1989). α_2-macroglobulin levels in the GCF of inflamed sites range from 0.2 to 2.4 mg/ml, level far above those for TIMPs (Birkedal-Hansen, 1993).

The inhibitory activity of TIMP resides in the first three loops of structures defined by six disulfide bridges (Docherty *et al.*, 1992; O'Shea *et al.*, 1992; Williamson *et al.*, 1990). It is believed that the three-dimensional structure of tissue inhibitors of TIMP uniquely determines their high affinity for MMP since disruption of disulfide bonds result in loss of activity.

TIMPs form classical irreversible non-covalent bimolecular complexes with the active forms of MMPs and, in some instances, with latent MMP precursors MMP-9 (Goldberg *et al.*, 1989; Howard, Bullen & Banda, 1991a; 1991b) in a 1:1 stoichiometry. The complex can be dissociated under acid conditions and the inhibitor is readily destroyed by reduction and alkylation (Nagase, Barrett & Woessner, 1992). TIMPs appear to regulate matrix degradation both by proteinase elimination and by blockage or autolytic MMP activation. Four members of the TIMP family, TIMP-1, -2, -3 and -4, have been identified (Aiba *et al.*, 1996).

TIMP-1 is a Mr 28K glycoprotein and is highly associated with the zymogen form of the MMP-9 and active MMP-1. TIMP-2 is unglycosylated protein, associated with MMP-2 expressed by fibroblasts and endothelial cells and perhaps by other cells as well. TIMP-2 forms a complex with the zymogen form of MMP-2. Also, in-vitro studies had shown that TIMP-2 has a higher inhibitory effect on gelatinases (MMP-2 and -9) and neutrophil collagenase (MMP-8) than TIMP-1 (Howard, Bullen & Banda, 1991a; Klein *et al.*, 1993). TIMP-2 is 2- to 10-fold more effective than TIMP-1 against the two gelatinases

whereas TIMP-1 appear more effectively inhibit CL. (Howard, Bullen & Banda, 1991b). The two TIMP genes are differently regulated. TIMP-1 expression is stimulated by growth factors (EGF, TNF-α, IL-1, TGF-β), phorbol esters, retinoids, and glucocorticoids - whereas TIMP-2 expression is down regulated by TGF-β and fails to respond to phorbol esters. In contrast to the solubility of TIMP-1 and -2, TIMP-3 is a 21K protein tightly bound to ECM (Leco *et al.*, 1994). TIMP-4, with a molecular mass of 22K, has been cloned from human (Greene *et al.,* 1996) and mouse (Leco *et al.*, 1997) cDNA libraries.

1.5.4 Role of MMP in Human Periodontal Diseases

Loss of attachment, as occurs in periodontal disease, is associated with extensive breakdown of collagen fibres in the periodontal tissues. Since the major structural protein of the periodontium is collagen, the assessment of MMP levels during inflammation will reflect the association of MMP in collagen destruction in periodontitis. The involvement of matrix metalloproteinases in pathological tissue destruction is well documented. The evidence for the role of MMPs in periodontal destruction has accumulated over the years. In earlier studies, collagenases has been identified in explanted gingival tissues and their culture fluids (Fullmer & Gibson, 1966; Bennick & Hunt, 1967; Fullmer, Taylor & Guthrie, 1972; Robertson & Fullmer, 1973; Birkendal-Hansen *et al.*, 1974; Wooley *et al.*, 1978) and in homogenates of gingival biopsies (Uitto, Turto & Saxen, 1978). Collagenases activity has also been demonstrated in GCF, and

this activity increased with the severity of inflammation (Golub, Stakiw & Singer, 1974; Smith, Rule & Rosen, 1974; Golub *et al.*, 1976; Kowashi, Jaccard & Cimasoni, 1979). Further evidence that MMP are involved in tissue destruction in human periodontal disease were summarized by Birkedal-Hansen (1993), which includes 1) cells isolated from normal and inflamed gingivae are capable of expressing a wide complement of MMP in culture, 2) several MMPs can be detected in cells of human gingivae in vivo, 3) MMP-8 and MMP-9 are readily detected in gingival crevicular fluid from gingivitis and periodontitis patients.

Each of the major cell types of human periodontal tissues is capable of expressing a unique complement of MMP when properly stimulated as been shown in Table 1. 2. The cells include PMN-leukocyte, fibroblast, keratinocyte, macrophage and endothelial cells.

Collagenases in tissues may exist in different forms, i.e. in active form, in inactive form that is an enzyme inhibitor complex, or in latent form (proenzyme). Total collagenase activity has been shown to be high in GCF of inflamed dogs gingivae (Kryshtalskyj, Sodek & Ferrier, 1986), in GCF of LJP and chronic AP patients when compared to gingivitis and control patients (Villela *et al.*, 1987a; b), in GCF of untreated LJP patients and also that the activity decreased after treatment (Larivée, 1986). Collagenase activity increased with disease severity in GCF, i.e. CAP & LJP > gingivitis > normal (Villela, 1987a; b). Ingman *et al.* (1993) demonstrated that saliva samples did not reflect periodontal tissue destruction clearly as they found only low amount of total collagenase activity in saliva of untreated LJP patient and the level was comparable to normal

Table 1.2 Expression of Matrix Metalloproteinases by Five Major Cell Types of Human Periodontal Tissues.

Cell Type	PMN-Leukocyte	Fibroblast	Keratinocyte	Macrophage	Endothelial cell
Enzymes expressed	MMP-8, MMP-9	MMP-1, MMP-3, MMP-2, MMP-7, MMP-11	MMP-1, MMP-2, MMP-9, MMP-10	MMP-1, MMP-3, MMP-2, MMP-9	MMP-1, MMP-3, MMP-2, MMP-9
Transcriptional activation	?	IL-1, TNF-α, EGF, TGF-α, PDGF, TPA	TNF-α, EGF, TGF-α, TGF-β, TPA	TPA	TPA
mobilization of enzymes	Granule release	transcriptional activation			
Response time	Seconds	6 to12 hours			
Response duration	Minutes	Days			

patients. Ingman *et al.* (1996) also demonstrated that in saliva no difference in collagenolytic activity could be seen in AP, LJP and control patients.

Latent collagenase levels were found to be higher in GCF of gingivitis sites in humans and dogs compared to periodontitis and progressive sites (Lee *et al.*, 1995; Kryshtalskyj, Sodek & Ferrier, 1986; Kryshtalskyj & Sodek, 1987), and also in normal sites (Kryshtalsky *et al.*, 1986). It was also detected in the explant of clinically normal tissue (Heath *et al.*, 1982). Higher latent collagenase levels seem to reflect either normal healthy state or gingivitis state.

Little or no active collagenase activity was demonstrated in GCF of healthy and gingivitis sites in dogs (Kryshtalsky *et al.*, 1987), but higher levels were found in GCF of LJP compared to healthy patients (Larivée, 1986). Higher levels were also detected in GCF of progressive periodontitis sites compared to stable and gingivitis sites (Lee *et al.*, 1995). In whole saliva, high active collagenases activity were demonstrated in untreated AP (Ingman *et al.*, 1993; Gangbar *et al.*, 1990) and LJP but as treatment progressed more collagenase is present in the latent form (Gangbar *et al.*, 1990). Higher levels of active collagenase levels seem to reflect diseased and active state of periodontal disease.

Collagenase inhibitors were detected in GCF of normal healthy sites (Larivée *et al.*, 1986) and low activities were found in inflamed and degenerating sites in GCF of dogs (Kryshtalsky *et al.*, 1986). Kryshtalsky *et al.* (1987) also found the highest amount of inhibitor in GCF of control normal sites in dogs. The amount of inhibitor increased in

GCF of LJP patients after treatment was carried out, though it was absent at baseline (Larivée *et al.*, 1986). From these studies it was shown that higher levels of inhibitor indicates healthy periodontal state. In contradiction to the above findings, free TIMP was found to be at significantly higher levels in GCF of gingivitis and periodontitis sites compared to the control sites (Haerian *et al.*, 1995), and also the levels were reduced by treatment in advanced periodontitis patients (Haerian *et al.*, 1996).

In the earlier studies, since the cellular source MMP was not determined, the MMP studied were just simply designated the term collagenase. Collagenase that is found in the case of periodontitis is derived from the host and not from the periopathogenic bacteria (Kryshtalsky & Sodek, 1987). Gangbar *et al.* (1990) has established that the source of procollagenase in patients with periodontal disease and inflammation are PMN cells, which is abundant in acute inflammation. Their results are in agreement with those of Sorsa *et al.* (1988).

Collagenase immunolocalization in human gingival biopsies from untreated periodontitis patients suggested the enzyme was frequently associated with inflammatory cells (Wooley & Davies, 1981). Pinchback, Gibbins & Hunter (1996) demonstrated by immunohistochemistry, that MMP-1 and TIMP-1 in advanced destructive tissue were associated with the vasculature of the connective tissue.

Once the MMP cellular source and characteristics were determined, more work was done on specific MMPs. It is established that MMP-1 and MMP-8 share the unique ability to

cleave types I, II and III collagen and serve as initiators of extracellular matrix destruction in periodontal disease. These fibrillar collagens form about 60% of the content of gingival connective tissue in healthy periodontium. Thus, synthesis and activation of MMP-1 and MMP-8 are important steps in the pathologic ECM destruction associated with inflammatory periodontal disease (Birkedal-Hansen, 1993; Lindhe, 1985). The outcome of the later studies revealed that in GCF, MMP-1 levels were much higher in LJP compared to AP and healthy subjects, while MMP-8 is the main collagenase in gingival crevicular fluid, saliva, and inflamed gingivae in adult periodontitis. (Sorsa *et al.*, 1988; Ingman *et al.*, 1993). MMP-3 (proenzyme and active form) levels in GCF of gingivitis and periodontitis sites were significantly higher than in control sites (Haerian *et al.*, 1995). Evidence also shows that MMP-1 and MMP-3 can be synthesized (and presumably activated) during adult periodontitis. It is likely that MMP-1 and MMP-3 may play a role in more chronic inflammatory processes, whereas MMP-8 contributes more to acute injury (Ingman *et al.*, 1994), conflicting with earlier findings. There is no consensus in the literatures on the role of MMP-1 and MMP-8 in periodontitis.

When the levels of the transcripts of the MMP were investigated, Nomura, Takahashi & Hara (1993) found out that MMP-1 and TIMP-1 mRNA were higher in periodontitis-affected gingivae compared to healthy gingivae. Aiba *et al.* (1996) also demonstrated the significantly elevated levels of MMP-1 mRNA in inflamed gingivae compared to normal gingivae in periodontitis patients, while there was no difference in the two groups for MMP-8 and TIMP transcript. These results suggest that MMP-1 rather MMP-8 may play

an important role in the initiation of collagen degradation in periodontal disease. However, the possibility remains that MMP-8 plays an important role in periodontal destruction. Kubota, Nomura & Takaahashi, (1996) had shown that the level of MMP-1, -3, -8 and TIMP-1 mRNA were significantly higher in the periodontitis group than in the healthy controls, and mRNA of MMP-3 > MMP-1 > MMP-8. But Tonetti, Pini-Prato & Cortellini (1993) could only demonstrate the presence of MMP-3 mRNA in 38% of the periodontitis patients, while MMP-8 mRNA was found in 46% of the periodontitis patients. TIMP-1 and -2 mRNA were both found to be elevated in the periodontitis patients compared to the minimal gingivitis group (Kubota *et al.*, 1997).

Below is a summarized tabulation of work done on MMP to support the association of MMP and destructive periodontal disease.

Enzymes	**Author and year**	**Experiments & Findings**
collagenases	Fullmer *et al.*, 1969.	From human gingival tissues, pure epithelial cells, and variably inflamed gingival connective tissue were capable of producing collagenases on culture. Collagenases was also detected in culture fluids of both pure epithelial and fibroblast-like cells derived from primary explants of gingivae.
collagenase-	Robertson *et al.*, 1973	Culture fluids of gingival biopsies of healthy and mildly

inhibitor		inflamed tissue were mixed with partially purified collagenases to show the effect of serum inhibitor on determination of the collagenolytic activity. There was a decrease in collagenolytic activity in inflamed tissue in untreated culture fluids. This showed that in-vitro determination of collagenolytic activity may be influenced by specific inhibitors.
collagenases	Woolley & Davies (1981)	Immunolocalization techniques on diseased gingival biopsies on patients who had treatments and in untreated patients. Tissues had 4-6 mm pocketing at time of biopsy. In the treated group, no significant immunoreactive enzyme was detected compared to half of the untreated group. The enzyme in the untreated group was distributed throughout the connective tissue and especially concentrated at the interface between epithelium and connective tissue and often associated with the presence of inflammatory cells.
collagenases	Uitto, Applegren & Robinson, 1981	Gingival specimens from treated periodontal patients were used. The proteinases were found to exist in the extracts mostly in latent form and the enzyme activities were significantly higher in inflamed samples than in non-inflamed samples.
Collagenases,	Heath *et al.*, 1982	Early studies showing production of MMPs and TIMP

gelatinases and TIMP		by gingival tissue. Gingival normal and inflamed tissues were obtained from patients during periodontal surgery. The explants in culture produced collagenases and gelatinases in both active and latent forms. The enzymes in clinically normal tissue were detected in the latent form. Human gingival, fibroblasts in monolayer culture produced no metalloproteinases, but did produce TIMP.
collagenases	Yanagimura, Hara & Nohara, 1983	Normal and inflamed tissues of dogs were excised. The collagenases activities in the neutral salt extract were significantly higher in inflamed gingivae after 1 week of plaque formation than in healthy gingivae. Gelatinase activity did not vary significantly with the induction of inflammation.
Collagenase	Golub *et al.*, 1984	PMN rat collagenases when treated with minocycline, doxycycline and tetracycline (16μg/ml) inhibited the activity of collagenase in-vitro by 90%.
collagenases	Golub *et al.*, 1985	GCF and subgingival plaque from periodontitis patients were used to evaluate the effect of 2-week regime of low dose (40-80mg per day) minocycline. Low-dose minocycline therapy reduced GCF flow (40 to 72 % reduction) as well as collagenolytic activity (26-93% reduction) even just after one week. The effects on the clinical parameters were less consistent and the crevicular microflora showed minimal changes except in

		spirochetes reduction. Therefore, minocycline therapy reduced collagenolytic activity by interacting with and inhibiting the host-derived collagenase rather than by antimicrobial action.
Collagenase and collagenase inhibitor	Larivée *et al.*, 1986	GCF samples of LJP and healthy control patients were used to analyze the latent, active and collagenase inhibitor levels at baseline and after treatment. At baseline collagenase activity (total) was significantly higher in diseased patients and this activity decrease substantially after clinical treatment. Latent collagenases remain high in diseased sites and in inflamed sites. LJP has higher active collagenase activity. Collagenase inhibitor was only detected in sites free of active collagenases and believed to be free of acute inflammation. It was absent at baseline in LJP, but increased after treatment was carried out.
Collagenase and collagenase inhibitor	Kryshtalsky, Sodek & Ferrier, 1986	GCF from healthy, gingivitis, and periodontitis sites were obtained from beagle dogs. Higher collagenolytic and low inhibitory activities were found in inflamed an degenerating sites. Periodontitis sites had higher active enzyme, compared to latent activities, whereas latent collagenase was predominant in control and gingivitis sites. Higher activity of collagenolytic enzyme, combined with absence of latent enzyme and collagenase inhibitor, was only found in periodontitis sites.

Collagenase	Villela *et al.*, 1987a; Villela *et al.*, 1987b.	GCF collagenase activity in healthy, gingivitis, CAP and LJP patients. Higher collagenase activity in GCF of CAP and LJP when compared to gingivitis, and in gingivitis higher than in control. Thus, collagenase activity increases with disease severity. Little or no collagenase activity is related to clinically healthy sites. Collagenase activity correlated well with pocket depth in the CAP and LJP group. GCF volume increased with disease severity in the order healthy < gingivitis < periodontitis, and stated that GCF volume was dependent on pocket depth. There is no correlation of collagenase activity and fluid volume in gingivitis and CAP. Both collagenase activity and GCF volume were correlated to PD. The collegenolytic enzyme was shown to be genuine vertebrate collagenase derived from unidentified host cells.
Collagenase and collagenase inhibitor	Kryshtalskyj & Sodek, 1987	GCF from healthy, gingivitis and periodontitis sites from beagle dogs. In the baseline, control and gingivitis samples has little or no active collagenolytic activity. The gingivitis samples contained the greatest amount of latent collagenase. The highest amount of inhibitor was found in control samples. Analysis of plaque, crevicular cell debri, saliva and serum indicated that the collagenolytic activity was derived from connective tissue and/or inflammatory

		cells, whereas collagenase inhibitor could also be derived from saliva and serum.
collagenase	Hakkarainen, Uitto & Ainamo, 1988	Collagenase activity were found to be high in periodontitis affected teeth either mobile or immobile. The collagenase activity decreased after scaling and root planing and further decreased after occlusal therapy in the mobile teeth.
PMN collagenases and gelatinase	Gangbar *et al.*, 1990.	Whole pooled saliva from AP, LJP and control patients were used. Active collagenase level were found to be significantly higher in AP and LJP compared to controls, and MMP activity not found in edentulous patients. Collagenase inhibitor were generally low in all groups. Following clinical treatment (tetracycline treatment and scaling) active collagenase and gelatinase were reduced.
Collagenase (MMP-8)	Sorsa, Suomalainen & Uitto, 1990.	The characteristics of interstitial collagenases found in inflamed gingivae, GCF and saliva are consistent with those of human neutrophils interstitial collagenases rather than the fibroblast-type interstitial collagenase. These collagenase degraded preferentially type I and II collagen as compared with type III (3) collagen and their molecular weight was about 70 K. The neutrophil collagenase was rather ineffectively activated by plasma kallikrein and plasmin, compared to trypsin,

		chymotrypsin and neutrophil cathepsin G.
collagenase (MMP-1)	Suomalainen *et al.*, 1991	GCF of untreated juvenile periodontitis patients was found to contain only vertebrae collagenase and degraded type I and III (3) collagen faster than type II collagen into ¾ and ¼ length fragments. This is indicative of collagenases produced by fibroblasts, epithelial cells and macrophages
mRNA TIMP-1, TIMP-2 and collagenase	Nomura, Takahashi & Hara, 1993	Gingival samples of healthy and periodontitis patients were used in reverse transcription-polymerase chain reaction to look for levels of mRNA of fibroblasts collagenases (MMP-1), TIMP-1 and TIMP-2. TIMP-1 mRNA level in periodontitis- affected gingivae was higher than in healthy gingivae, so was the level of mRNA of fibroblasts collagenases (MMP-1). There was no significant different between the level of mRNA TIMP-2 in the two groups.
mRNA MMP-3	Tonetti, Pini-Prato & Cortellini, 1993	MMP-3 (stromelysin) transcripts were not detectable in the soft tissues biopsies in healthy sites and only detected in 38% of the periodontitis sites. MMP-8 mRNA was detected in 17% in healthy patients and 46% in periodontitis patient.

Salivary collagenases	Ingman *et al.*, 1993	Whole stimulated saliva were obtained from untreated LJP and AP patients as well as healthy controls. Low amount of interstitial collagenase activity was found in the saliva of LJP, which was comparable to healthy controls. Tissue destruction in LJP cases was not clearly reflected in salivary samples. In AP high active collagenase levels was detected. There was no evidence of bacterial proteases.
MMP-8	Lee *et al.*, 1995.	GCF samples from progressive, stable periodontitis and gingivitis sites were obtained. Progressive periodontitis GCF had higher (5X) active collagenase activity compared to stable and gingivitis patients. Latent collagenase (2X) was higher in gingivitis group compared to progressive group. Only in sites with progressive destruction was there significant increase of active collagenase with time. At the time of detection of destruction there was 40% increase of pooled active collagenase activity. This study demonstrated that there is a direct evidence for the role of active collagenase in the destruction of periodontal connective tissue.
MMP-3, MMP-1, TIMP-1	Haerian *et al.*, 1995	(fibroblasts derived MMP and TIMP) The mean amounts of SL (proenzymes and active form) and TIMP (free) in diseased sites (gingivitis and periodontitis) were significantly higher than the mean

		amount of these GCF components in healthy sites in the same individual (permitting within subjects comparison).
MMP-1, TIMP-1, TIMP-2	Pinchback, Gibbins & Hunter, 1996.	Tissues biopsies from advanced destructive periodontitis (PD 8-13 mm) and minimally inflamed sites (PD 3 mm) were used in immunohistochemistry to locate the distribution of MMP-1. In advanced destructive tissue, MMP-1 was found pronouncedly in the vasculature of intact connective tissue, particularly in the proximity to oral epithelium. There were also scattered in the small vessels immediately adjacent to pocket epithelium. In minimally inflamed specimen only weak staining associated with a few blood vessels. TIMP-1 in advanced lesion were found particularly centered on the vasculature corresponding to MMP-1 region but more diffuse. TIMP-2 were confined to the remaining islands of connective tissues in advanced lesion. Both TIMP-1 and TIMP-2 were not found in inflamed tissues.
mRNA MMP-1, MMP-8, TIMP-1	Aiba *et al.*, 1996	Biopsies of normal and inflamed gingivae were obtained from periodontal patients during surgery. Using RT-PCR, it was shown there was an increase in MMP-1 mRNA in the inflamed gingivae compared to the control. MMP-8 and TIMP-1 gene expression did not change. When comparing the 3 mRNA in inflamed gingivae,

		TIMP-1 was the highest and MMP-8 was the lowest mRNA. MMP-1 mRNA in inflamed gingivae is 5-fold higher than that found in control gingivae. This finding of higher mRNA MMP-1 level in adult periodontitis in agreement with Nomura *et al.* (1993), while MMP-1 was found to be higher in JP (Sorsa *et al.*, 1988; Suomalainen *et al.*, 1991).
MMP-1, MMP-3, TIMP.	Haerian *et al.*, 1996.	This longitudinal study was looking at the level of FIB-CL, SL and TIMP in sites with advanced periodontal breakdown before and after treatment. It was shown that SL and TIMP levels are reduced by treatment. SL level reduction was significant 3 months after therapy (RP), but not 6 weeks after therapy. Pre-treatment levels of FIB-CL, SL and TIMP failed to predict the response to treatment or the outcome of treatment.
MMP-1, MMP-3, MMP-8, MMP-9, TIMP-1	Ingman *et al.*, 1996.	GCF and whole stimulated saliva were obtained from AP, LJP, and control subject before any periodontal treatment. MMP-1 levels were found to be higher in GCF of LJP patients in comparison to AP and healthy controls. Significantly elevated levels of TIMP-1 were also detected in LJP GCF compared to AP and control. Higher levels of MMP-8 were detected in saliva of AP patients compared to LJP and the control salivary samples. Both MMP-1 and TIMP-1 were detected in all studied saliva samples, but no significant differences

		were detected between the groups. MMP-9 levels were also significantly higher in AP compared to LJP and control GCF. MMP-8 is the main collagenase in saliva, GCF and gingival tissue in AP patients, whereas in LJP GCF it seems to be MMP-1.
MRNA MMP-1, -3, - 8 TIMP-1 and -2.	Kubota *et al.*, 1996	The levels of MMP-1, -3, -8, and TIMP-1 mRNA relative to β-actin were significantly higher in the diseased group than in the healthy controls. However, the difference in TIMP-2 was not significant between the two groups. It was shown that MMP-3 mRNA > MMP-1 mRNA > MMP-8 mRNA in periodontally affected tissues, while it was shown that in GCF MMP-8 > MMP-1 in adult periodontitis (Ingman *et al.*, 1993; Sorsa *et al.*, 1988; 1990; Suomalainen *et al.*, 1991), which contradict each other.
MMP-3 & TIMP	Pourtaghi *et al.*, 1996.	52 adult periodontitis patients previously been treated were divided into 4 groups of treatment, scaling and RP (S+RP) alone and S+RP plus 3 different locally delivered antibiotic. Stromelysin level significantly decreased in tetracycline fiber and monocycline treated group, whereas it remained unchanged in the other two groups. TIMP levels increased significantly in all three adjunctive antimicrobial treatment.

mRNA TIMP-1 & -2.	Kubota *et al.*, 1997.	Using in situ hybridization and immunohistochemistry, it was shown that the numbers of both TIMP-1 and -2 mRNA expressing cells are higher in the periodontitis than in the minimal gingivitis group. Both TIMP-1 and TIMP-2 mRNA-expressing cells were frequently observed in connective tissue where inflammatory cell infiltration was prominent was broadly observed in the gingival connective tissue while TIMP-2 mRNA was predominantly expressed in the connective tissue adjacent to the pocket epithelium.

Although there is no direct evidence for a causal relationship between MMP and periodontal tissue destruction, the involvement of collagenase in collagen breakdown during chronic inflammatory periodontal disease is highly suggestive and based on a vast amount of studies. MMP-8 is detected at high levels in GCF and saliva during gingivitis or periodontitis whereas it is undetectable in healthy individuals (Sorsa, Uitto & Suomalainen, 1992). In extracts or homogenates of diseased periodontal tissues MMP-1 is abundantly present in contrast to healthy specimens and, as with MMP-8, a positive correlation was found between its presence and the severity of inflammation (Overall, Wiebkin & Thonard, 1987; Sorsa *et al.*, 1988; Robinson *et al.*, 1992). In addition, MMP-1 has been immunolocalized in inflamed but not in healthy periodontal tissue (Wooley & Davies 1981). These data may suggest that, following production during inflammation,

most MMP-1 remains in the gingival tissue, whereas the majority of released MMP-8 finds its way to the pocket.

1.5.5 MMP and TIMP during Repair

Wound repair involves many processes including cell migration, provisional matrix deposition, and remodeling. All of these processes are likely to be affected by matrix-modifying enzymes. Members of the MMP family are physiological mediators of the ECM. It is important to monitor the early stages of postoperative wound repair in order to identify those problems associated with impaired healing. Many of the crucial cellular responses of early wound healing, such as inflammatory infiltration, angiogenesis and re-epithelization, are made possible through the action of MMP.

Stromelysins had been shown to be produced during wound healing by human surface respiratory cells (HSRE), thus involved in cell migration and extracellular matrix remodeling. Stromelysins were overexpressed and overactivated during the wound repair process, with the maximal production observed at wound closure (Buisson *et al.*, 1996).

Partially activated stromelysin were observed in the repair tissue of injured cornea, synthesized by stromal fibroblasts which decreased in a gradient radiating from the repair tissue (Girard *et al.*, 1993). Synthesis of collagenase was detected in repair tissue as long as nine months after injury. Stromelysin are also synthesized by resident corneal cells in

thermally injured corneas of rabbit, but correlated more closely to deposition of repair tissue, while MMP-9 synthesis was correlated to basement membrane dissolution which led to failure to re-epithelialize (Fini *et al.*, 1996). The expression of MMP-2 has been detected both in undamaged and damaged cornea, but this expression increased in the damaged cornea and much of it appears in the active form. These changes persisted for at least 7 months, suggesting its involvement in the prolonged process of collagen remodeling in the stromal repair tissue (Fini *et al.*, 1992).

Stromelysin-1 and -2 are found to be produced by basal keratinocytes in a variety of chronic wounds (Saarialho-Kere *et al.*, 1994), and also in acute and chronic skin wound (Vaalaamo *et al.*, 1996) and thus suggest these two MMP serve distinct roles in tissue repair. Bullard *et al.* (1997) data suggested that increased MMP-2 & -3 found in fetal skin contribute to scarless healing, as when TGF-beta were added to fetal skin, it induced scarring by decreasing the interstitial MMP production.

MMP-9 level in the early inflammatory phase can predict the amount of collagen deposited later in the wound healing process (Agren *et al.*, 1998; Tarlton *et al.*, 1997). The levels of MMP-2 and MMP-9 in wound fluid were increased post-surgery compared to control serum, although MMP-9 decreased in the wounds 24-48 hr after injury (Agren *et al.*, 1998). Tarlton *et al.* (1997) also found an increase in both MMP-2 and -9 in acute wounds, but still greater levels are found in chronic wounds. Yager *et al.* (1996) demonstrated the elevation to be more than 10-fold and 25-fold respectively. This finding indicates that uncontrolled proteolysis is a characteristic of retarded healing.

Wounds which were healing were found to expressed maximal levels of MMP-9 at 24 hr, followed by a significant decline by 48 h. Persistent elevation of MMP-9 expression was associated with infected and chronic wounds. Therefore, measurement of MMP-9 in postoperative wound fluids within 48 hour of injury provides an early indicator of impaired healing. The expression of MMP-13 has been demonstrated in fibroblasts deep in the chronic ulcer bed, but it was not detected in epidermis and acute wounds (Vaalamo *et al.*, 1997). MMP-13 most likely plays a role in the remodeling of collagenous matrix in chronic wounds.

When the levels of MMP-1 and TIMP mRNA were analyzed in normal and fibrotic fibroblasts, the MMP-1 mRNA were greatly reduced to undetectable levels whereas TIMP mRNA levels increased fourfold in fibrotic fibroblasts compared to normal (Lafuma *et al.*, 1994). TIMP-1 mRNA in granulation fibroblasts was only down-regulated by 20% between day 3 and 14 after wounding (Petri *et al.*, 1997). TIMP-1 mRNA in epithelium was not detected in chronic cutaneous wound but were expressed in acute cutaneous wound in both human and pig (Vaalamo *et al.*, 1996). TIMP-1 level were found by Bullen *et al.* (1995) to be lower in chronic than in healing wounds.

The levels of MMP-8 during wound healing or repair has not been studied before.

1.6 DETECTION OF PERIODONTAL DISEASE

Periodontal disease can be detected clinically and radiographically. Clinical detection includes visual examination - changes in colour and texture of the gingivae, probing to measure the pocket depth (PD) and the clinical attachment level (CAL), bleeding on probing (BOP) and checking the tooth mobility. Radiographs can reveal horizontal or vertical bone loss.

A diagnostic procedure is used to supply information about the type of periodontal disease, where it is located and how severe it is. The findings can then be used to formulate a treatment plan. Diagnostic procedures are also useful in monitoring patients during and after treatment and detecting recurrence of disease.

The general uses of periodontal diagnostic procedures are in:

i) screening

ii) diagnosis of specific periodontal diseases

iii) treatment planing

iv) monitoring of therapy

v) identification of sites or subjects at a higher risk of progression of periodontal destruction.

1.6.1 Probing

Use of the periodontal probe has been proved to be a simple and efficient method to measure periodontal support, gauge tissue health, and gain tactile appreciation of areas that are not visually apparent. The rationale behind probing is to detect and measure attachment loss or gain (CAL), in order to determine the extent of previous or ongoing periodontal disease activity, to assess the apical extension of the inflammatory lesion (PD) and to assess therapy and treatment efficacy. Additional findings obtained during probing include descriptions of bleeding, suppuration, and tissue consistency.

The results of probing depth measurements are conditional upon the probe tip diameter and shape (Cianco *et al.,* 1992; Gabathuler & Hassel, 1971; Van Der Zee, Davies & Newmann, 1991; Atassi *et al.,* 1992), the probe angulation (Kaarim *et al.,* 1990), the force applied (Mombelli, Mukle & Frigg, 1992; Osborn *et al.,* 1990), and the location, presence or absence of inflammation in the subepithelial connective tissue (Armitage *et al.,* 1977; Keagle *et al.,* 1989; Zappa *et al.,* 1990; Anderson *et al.,* 1991; Caton, Greenstein & Polson, 1981).

Conventional, manual probes that do not control for probing force or pressure are called first generation probes. The introduction of constant-force or pressure-sensitive probes allowed for improved standardization are called second-generation probes. Third-generation probes have computer-assisted direct data capture thus reducing examiner bias.

In the present study the first-generation probe (University of North Carolina and PCP 12 manual probe) and the third-generation probes (Florida probe) were used. University of North Carolina probes have 1 mm graduation marks with black bands of 1 mm every 5 intervals while PCP 12 probes are graduated with consecutive 3 mm black bands and 3 mm unmarked bands. The maximum depth that can be measured with these probes is 15 and 12 mm respectively.

1.6.1.1 The Florida Probe

Gibbs *et al.* (1988) developed the Florida Probe system, a third generation probe, which features constant probing force, precise electronic measurement to 0.1 mm, computerized data capturing, and sterilization of all system parts entering or close to the mouth. The system consists of a probe handpiece, a digital read-out, foot switch, computer interface and computer. The probe tip reciprocates through a sleeve and is connected to a movable arm, which transfers the movement to a transducer with a digital read-out. The edge of the sleeve which is 0.6 mm in radius is the reference from which measurements are recorded. The probe tip is 0.4 mm in diameter and the probing force is usually set at 0.25 N (1.99 N/mm^2). The actual measurement of pocket depth (or relative attachment level) is made electronically and transferred to the computer when the foot switch is pressed.

Besides the pocket depth probe, the stent probe and the disk probe are also available. The former uses an acrylic stent as a reference and for the reproducible placement of the probe tip. The latter has a small metal disc attached to the sleeve and uses the occlusal surface or incisal edge of a tooth as a reference for relative attachment level measurement. In the present study the pocket depths measurements are taken with the manual probe as well as with the Florida probe. Manual probing is also used to take measurements of the attachment level from the CEJ (CAL), while the relative attachment level (RAL) taken with the Florida disc probe. Pressure-controlled probing is more time consuming than conventional probing. However, the use of a pressure-sensitive probe at least standardizes the probing pressure employed during a study. Repeated measurements have been shown to increase the measurements sensitivity and specificity with pressure-controlled probes (Mullally & Linden, 1994). Magnusson, Batich & Collins (1988) and Osborn *et al.* (1990) found that pocket depth measurements recorded when using the manual probe with visual readings were consistently deeper than those obtained using the constant pressure probes.

Based on reports in the literature, a small diameter tip (0.4 mm) and moderate probing force (25g), would likely place the probe tip about 0.25 mm coronal to the base of the junctional epithelium in healthy sites and as much as 1.25 mm beyond the coronal level of the connective tissue in diseased sites (Polson *et al.,* 1980; Robinson & Vitek, 1979; Spray *et al.,* 1978; Caton, Greenstein & Polson, 1981; Listgarten, Mao & Robinson, 1976).

Ahmed, Watts & Wilson (1996) measured the CAL of 34 teeth scheduled for extraction clinically and in the laboratory using an automated probe (Florida probe) and Vernier calipers. Vertical grooves were made on six sites of the teeth and used for guidance in measuring the AL before extraction. After the extraction, using dissecting stereo microscopy, the distance from the most coronal attachment fibres and the center of the end of each groove were measured and compared to the measurement done clinically. In all cases there was a statistically significant correlation between clinical and laboratory measurements. However, the molar sites as a group displayed a distinct difference to the non-molar sites in that the mean clinical probing AL was 0.45 mm greater than the laboratory measurement.

Another study into the accuracy of a controlled force electronic probe and a manual probe was performed in 15 patients scheduled for tooth extraction. It was found that the electronic probe significantly underestimated the clinical probing depths (-0.48 mm) whereas no significant difference was found between the manual CAL and the actual AL (Hull, Clerehugh & Ghassemi-Aval, 1995). This study demonstrated that the constant force electronic probe under-measured the probing depths as determined in the laboratory.

In another study by Reddy, Palcanis & Geurs (1997) 3 examiners recorded AL with four different probing techniques, Florida disc probe relative to the occlusal surface, modified Florida probe relative to a stent, North Carolina probe read to 1 mm relative to CEJ or

restoration base and North Carolina probe read to 0.5 mm relative to the CEJ or restoration base. 8 subjects were involved and each quadrants were randomly measured using different methods. At a subsequent visit during the week the subjects were reexamined by repeating the experimental design in the same sequence. The highest repeatability was observed with the manual probe read to 1 mm, where as the modified constant force probe and stent represented the group with the greatest variability between measurement. The site measured on the tooth also had a significant result on the repeatability outcome. The distal surfaces, either buccal or lingual, showed significantly more variability in the repeated measurements of attachment levels. In this study a reproducibility of 0.6 mm was accomplished with both manual and electronic probes. The use of modified Florida probe with a stent had no true advantage with regard to repeatability in this study. Rams and Slots (1993) reported that periodontal pockets > 5 mm have better repeatability when measured using an electronic probe than a conventional probe. Walsh and Saxby (1989) also demonstrated better agreement between two examiners when a controlled force probe was utilized. Comparable repeatability between constant force and manual probes have been reported (Quirynen *et al.*, 1993; Mullaly & Linden, 1994; Tupta-Veslicky *et al.*, 1994; Wang *et al.*, 1995). A lack of agreement between measurements taken at the same site has also been reported (Galgut & Waite, 1990; Rams & Slots, 1993; Perry *et al.*, 1994; Hull, Clerehugh & Ghassemi-Aval, 1995) with electronic probes having a tendency toward shallower probing depth measurements.

Oringer *et al.* (1997) compared the manual probe and the automated probe (Florida probe) to measure PD and CAL in 411 interproximal sites in untreated patients in a 6 months study. Variability between the 2 passes at a single site at the same visit was significantly larger in automated probe, but when inter-visit analysis was done over the 6-month period, automated probe appeared to demonstrate better repeatability than the manual probe. This study demonstrated that automated probing may have less variability of AL over a 6-month period and it may be advantageous using automated probe for monitoring periodontitis patients in a longitudinal clinical trial.

Marks *et al.* (1991) evaluated the reproducibility of 2 models of the Florida probe, the original stent model and the modified disc model, in measuring attachment levels. The disc probe uses the occlusal surface of the tooth as a static reference point in calculating changes in attachment level. In this study, examiners using both types of probes took duplicate measurements. The results demonstrate that the disc probe yields reproducible measurements similar to the stent probe and is therefore suitable for use in longitudinal clinical studies. Osborn *et al.* (1990) has demonstrated that double passes with the Florida disc probe offers significant advantages for measuring relative attachment level in longitudinal studies as the mean intra-examiner standard deviation of differences in repeated relative attachment level measurement using the disc probe was one-half smaller (0.44 to 0.57 mm) using double passes than using the conventional probe in single pass (0.78 to 0.95 mm). Using double passes with the Florida disc probe reduced intra-examiner error by approximately 40% when compared to measurement level using one pass with a conventional periodontal probe.

1.6.1.2 Assessment of Probing Depth

When the probe is inserted into the sulcus of a healthy tissue, it easily penetrates the junctional epithelium at the bottom of the sulcus, which does not offer any significant resistance to probing. Further penetration of the probe is halted by the pressure of the dense network of collagen fibres of the connective tissue (Armitage *et al.*, 1977). The probe tip penetration is coronal to the apical end of the junctional epithelium.

In mild inflammatory conditions, the probe will likely penetrate through the junctional epithelium, but will be stopped quickly by the resistance of the impenetrable arrangement of intact connective tissue fibre bundles in the gingival connective tissue. The clinical measurement will be similar to that measured in the healthy tissue. Some minor bleeding may be observed as a result of injury to blood vessels.

When a periodontal pocket develops and the tissue is inflamed the probe will easily transverse the pocket epithelium and penetrate more or less deeply into the connective tissue, as shown by Magnusson & Listgarten (1980) and Fowler *et al.* (1982). Presence or absence of inflammation of the gingival connective tissue is an important determinant in the probe tip penetration. Fowler *et al.* (1982) stated that reduced inflammatory infiltrate level would allow for greater resistance to probe penetration. The precise location of the probe tip during the probing event remains an enigma.

1.6.2 Radiographs

In general dentistry, panoramic, bitewing and periapical radiography are widely used for diagnostic purposes. In periodontics, periapical radiographs provide the most useful views for periodontal diagnosis, but for screening purposes, panoramic radiograph can also be used. The clinical implications of radiography in the diagnosis of periodontal disease are twofold; to visualize the initial status of the bone tissue and to illustrate changes in the bone tissue over time. Assessments of bone loss in intraoral radiographs are usually performed by evaluating a multitude of qualitative and quantitative features of the visualized interproximal bone, e.g. i) presence of an intact lamina dura, ii) the width of the periodontal ligament space, iii) the morphology of the bone crest (even or angular appearance), and iv) the distance between the CEJ and the most coronal level of the normal width of the periodontal ligament space. The threshold of bone loss, i.e. the CEJ-bone crest distance considered to indicate that bone loss has occurred, varies between ≥ 1 (Schei *et a.,* 1959; Lennon & Davies 1974; Hugoson & Rylander, 1982; Mann *et al.,* 1985; Bishop *et al.,* 1995) and $\geq$ 3 mm in different studies (Blankenstein *et al.,* 1978; Latchman *et al.,* 1983; Sjödin *et al.,* 1989; Albandar, Buischi & Barbosa, 1991; Albandar *et al.,* 1995; Shapira *et al.,* 1995). Most studies have used > 2 mm as the criterion for damage (Belting, Massler & Schour,1953; Hoover, Ellegaard & Attström, 1981; Gjermo *et al.,* 1984; Kronauer, Borsa & Lang, 1986; Aass *et al.,* 1988; Aass, Preus & Gjermo, 1992; Aass, Tollefsen & Gjermo, 1994; Källestål & Matsson, 1989; 1991; Sandholm *et al.,* 1989; Sjödin & Matsson 1992; 1994; Sjödin *et al.,* 1993; Bimstein *et al.,* 1994), and two studies have indicated that this is the appropriate threshold for radiographic

assessment of bone loss (Källestål & Matsson 1989; Hausmann, Allen & Clerehugh, 1991).

1.6.2.1 Infrabony Defects

Pockets are classified into suprabony or infrabony depending on the location of the bottom of the pocket to its relationship to the alveolar crest. These horizontal or vertical bone loss can be revealed by radiographs. A suprabony pocket is defined as a pathological sulcus where the base of the pocket is coronal or occlusal to the alveolar crest, while infrabony is defined as a pathological sulcus where the bottom of the pocket is apical to the alveolar crest. Infrabony defects are sometimes called angular bony defects. Goldman *et al.* (1958) proposed a classification of the infrabony pocket on a morphologic basis which is dependent on the number of osseous walls present in the defect. These are: 1) three osseous walls defect, 2) two osseous walls defect, 3) one osseous wall defect and 4) combination defect (Figure 1.4). Prichard (1967) classified infrabony defects as intrabony defects, hemisepta or craters. According to his classification, a crater is a wide mouthed cup or bowl shaped defect, with bone destruction about equal on the roots of contiguous teeth; and the side walls are formed by marginal bone of the vestibular and lingual surfaces. When a tooth is affected and the septal bone adjacent to it is destroyed without affecting the contiguous tooth, a

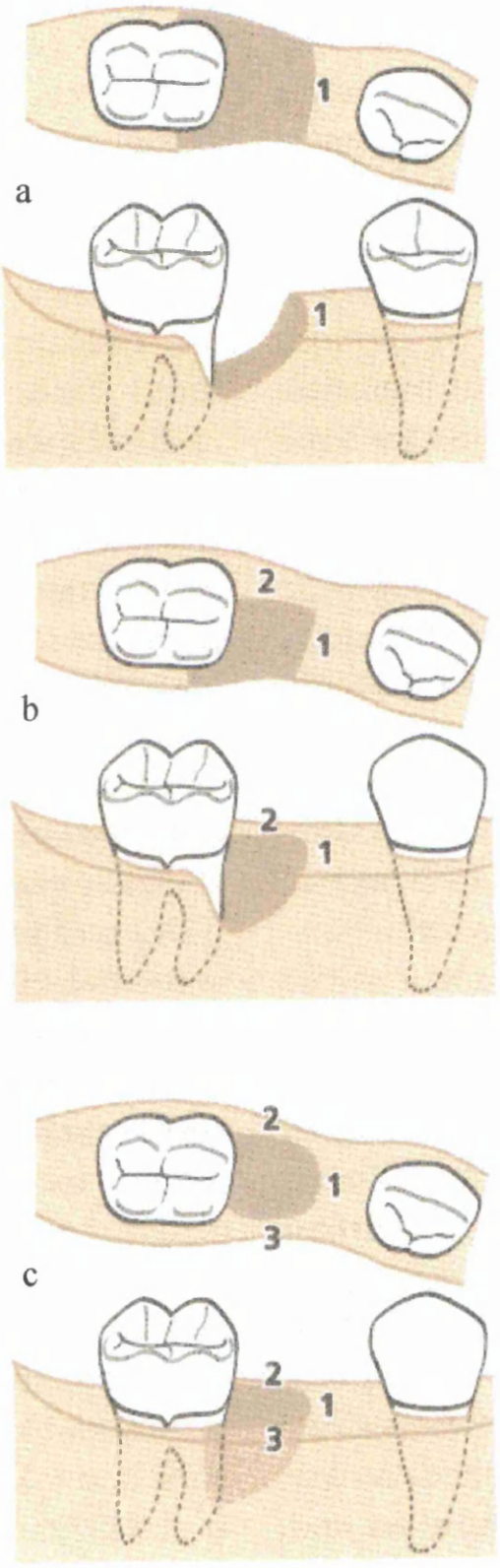


Figure 1.4 Schematic drawings illustrating the classification of infrabony defects based on the number of surrounding osseous walls,

a) 1-wall

b) 2-wall

c) 3-wall defects

hemiseptum would exist. The intrabony defect is surrounded by bony walls on three sides with the tooth root forming the fourth wall.

Subgingival plaque has been demonstrated to play a major role in the pathogenesis of the infrabony defect as observed in 48 periodontally involved extracted teeth with radiographic evidence of infrabony pocket. Subgingival plaque was found covering the root surface to a distance of 1 to 2 mm from its apical limitation (Waerhaug, 1979a). Based on autopsy results, Waehaug (1979b) also observed that the configuration of interdental septum of the infrabony defects was dependent on the level of plaque of the two neighbouring teeth. If plaque had proliferated down to different levels on the two neighbouring teeth, the crest of the interdental septum was oblique and angular defect hereby established. There was no evidence to indicate that trauma from occlusion had been involved in the pathogenesis of the infrabony defects in both studies.

Anatomic factors have also been assumed to influence the alveolar bone loss pattern, e.g. distance between neighboring teeth - open contact points with resultant food impaction, morphology and quality of the alveolar bone, concavities/fissures on the root surface, position of the tooth, the relationship of adjacent marginal ridges and cementoenamel junctions.

Radiographically detectable angular bony defects are a common finding in adults with a reported prevalence ranging from 18 to 32% (Nielsen, Glavind & Karring, 1980; Wouters *et al.,* 1989). An increasing frequency of periodontal infrabony defects with age

has been observed in both human skull material (Larato, 1970b) and in clinical and radiographic study (Nielson *et al.*, 1980). As regards the teeth and tooth surfaces most frequently involved, varying findings have been reported. When Lorato (1970b) examined 337 dry human skull for three wall defects he found that in the maxilla, the second molar was the tooth most affected, while the site most affected was the mesial. In the mandible, it was the wisdom tooth and the mesial site was the most common area affected. Based on examination during periodontal surgeries in all 4 quadrants in 30 patients, Manson & Nicholson (1974) observed more infrabony defects in the maxilla than in mandible. Interdental crater was more common in the posterior segment of the mandible, while 3-, 2-, 1-walled defects and hemiseptum were found almost exclusively in the maxilla, and mostly in the anterior segment. A clinical and radiographic study by Nielsen, Glavind & Karring (1980) on 209 dental patients, revealed that there was no significant difference between the prevalence of defects in male and females, but the defects occurred most frequently in the older age group. Only 47 individuals out of the 209 were affected with osseous defects which had a higher incidence in the distal than the mesial surfaces and most frequently in the mandible. The prevalence is lower than that reported by Larato (1970b), 36% in 17-29 age group and 72% in 60 and above, compared to 34% and 14%. Periodontal infrabony defects have also been observed in skulls of children (Larato 1970a).

It is well known that infrabony defects can present special therapeutic problems (Steffensen & Weber, 1989; Heins *et al.*, 1990) but it has not been clearly established if their elimination is mandatory for long-term periodontal stability. Based on the results of

a 10-year retrospective study of a population without any special periodontal therapy, a higher percentage of teeth were lost which had angular bony defects (32.2%) than teeth without such defects (12.7%) (Papapanou & Wennström, 1991). However, results from two retrospective studies of treated and maintained patients suggest that sites with residual angular bony defects are not at a higher risk of breaking down than sites without these defects (Pontoriero, Nyman & Lindhe, 1988; Heins *et al.,* 1989).

1.7 TREATMENT OF PERIODONTAL DISEASE AND HEALING PROCESS

The main aim of periodontal treatment is to eliminate disease. Ideally, treatment should resolve inflammation, arrest disease progression, maintain aesthetics, maximize patient comfort, regenerate lost periodontium, and create an environment that deters recurrent disease. This can be carried out by several measures, which include initial (cause-related) measures, corrective measures, and maintenance measures.

1.7.1 Initial Therapy

The main aim of initial therapy is to eliminate or control the recurrence of supra- and subgingivally located bacterial deposits from the tooth surfaces. Instructions in oral hygiene measures, scaling and root planing and in combination with removal of retention factors for plaque, and extraction of teeth with poor prognosis are included in this

category. Subsequent check-ups and reinstruction are carried out to maintain good oral hygiene. The patients are motivated and educated on proper oral hygiene techniques. To facilitate proper oral hygiene the use of toothbrushes, interdental cleaning aids such as dental floss or tape, toothpicks and interproximal brushes, are introduced to the patients.

Scaling is a procedure aimed at the removal of plaque and calculus from the tooth surface. Root planing denotes a technique of instrumentation by which the altered cementum is removed and the tooth surface is made hard and smooth. Subgingival instrumentation aims at resolving the inflammation in the gingivae and arresting the progressive destruction of the attachment apparatus by removing the microbiota of the gingival pocket.

Once this therapy is finished the patient is re-examined in order to reevaluate the outcome of the treatment and whether further treatments or guidance is needed. If the results from the reexamination, made at the termination of the initial treatment phase show that periodontal disease has been brought under proper control, the corrective treatment may be carried out.

1.7.2 Corrective Therapy

The corrective treatment includes periodontal surgery, extraction, hemisection, and also prosthodontic treatment. Periodontal surgery can be carried out in those areas of the

dentition where the inflammatory lesions had not been resolved by scaling and root planing. The planning of the type and the extent of surgical treatment should be based on the pocket depth and "bleeding on probing" measurements made at the end of the initial phase of treatment.

Surgical treatment of the periodontal lesions offers obvious advantages over a nonsurgical approach: the root surfaces can be inspected and cleaned by direct vision. By doing surgery the diseased pocket epithelium can be excised and removed and the flap can be replaced either coronally or apically. If an apically repositioned flap procedure is carried out the pockets are virtually eliminated as the soft tissue flaps are repositioned to the level of the alveolar bone. The modified Widman flap, which is also recognized as the open flap curettage technique, is usually used for the anterior segment of the dentition as it exposes less of the root surfaces while giving access for removal of pocket epithelium and granulation tissue. Other periodontal surgical procedures include, distal wedge procedure which excise a triangular wedge of tissue to remove the distal pocket of a distal molar, and gingivectomy procedures removing excess gingival tissue.

1.7.3 Maintenance Therapy

Following completion of cause-related and corrective therapy the patient must be enrolled in a recall system which aims at preventing the recurrence of disease. The

ability of the patient to maintain a proper oral hygiene standard determines the time interval between the recalls appointments.

At the recall appointments the oral hygiene standard should be assessed and scaling and polishing should be carried out if indicated. At least once a year a comprehensive examination should be performed to assess the overall dental health and particularly the periodontal health by checking the depth of pathologically deepened pockets, furcation involvement, tooth mobility and alterations of the alveolar bone level.

1.7.4 Wound Healing

Definitions

- Repair is defined as 'healing of a wound by tissue that does not fully restore the architecture or the function of the part.'
- Reattachment is defined as 'the reunion of surrounding soft tissue and a root surface with preserved periodontal ligament tissue.'
- New attachment is defined as 'the formation of new cementum with inserting collagen fibres on a root surface deprived of its periodontal ligament tissue.'
- Regeneration is defined as 'reproduction or reconstitution of a lost or injured part in such a way that the architecture and function of the lost or injured tissue are completely restored.'

Wound healing is a response to various kinds of injury toward the restoration of normal structure and function. Healing is divided into regeneration and repair. Regeneration is the complete restoration of damaged tissues by proliferation and differentiation of the original parenchymal elements. In comparison, repair (incomplete regeneration) is healing by the replacement of lost tissues by non-specialized connective tissue (granulation tissue) maturing to fibrosis and eventuating in scar tissue formation.

The cells of the body are divided into three groups according to their mitotic activity, i.e., labile cells, stable cells, and permanent cells. Labile cells (epithelial cells, fibroblasts cells, hematopoietic cells, lymphoid cells) undergo continuous replication even under normal physiological condition. Stable cells, including glandular parenchymal cells of the liver, pancreas, endocrine glands, and so forth, have a decrease or loss of mitosis in adults but reenter the cell cycle as the situation demands. Permanent cells, e.g. neurons and cardiac myocytes, lose their ability to regenerate. Tissue composed of labile and stable cells can be expected to regenerate, but permanent cells cannot be renewed if lost.

Periodontal tissues are composed of cells belonging to the labile or stable groups. These cells are expected to exhibit their high ability to proliferate when the periodontal tissues are damaged.

In periodontitis, cells from gingival epithelium, gingival connective tissue, bone cells, and cells from the periodontal ligament participate in the healing of the damaged

periodontal tissues. Melcher (1976) proposed a theory on the repair potential of periodontal tissue and postulated that the cells that repopulate the exposed root surface determine the nature of the attachment that will form. If the epithelium cells proliferate along the root surface, precisely on the surface of the granulation tissue of the gingivae, long junctional epithelium (LJE) will result. If cells from the gingival connective tissue populate the root surface, the attachment occurs in the form of connective tissue adhesion, and root resorption may occur. If bone cells migrate into contact with the tooth surface, root resorption and ankylosis occur. The ideal new connective tissue attachment develops if cells from the periodontal ligament proliferate to cover the denuded surface. The formation of the new connective tissue together with bone restoration by the cells from periodontal ligament is described as regeneration.

1.7.4.1 Healing After Root Planing

Coronal attachment of the periodontal tissues as a result of root planing and soft tissue curettage have been described (Beube, 1947; Ramfjord, 1951; Waerhaug, 1952; Schaffer & Zander, 1953; Ramfjord *et al.,* 1968). The increased resistance to probing following root planing and soft tissue curettage may be due to a more coronal connective tissue attachment, and/or to the formation of a long junctional epithelium, or increased resistance of the less inflamed tissue. A LJE was demonstrated following subgingival plaque control in humans (Waerhaug, 1978) and following periodontal flap surgery in primates (Caton & Kowalski, 1976; Yukna, 1976). Caton & Zander (1979) concluded

that the coronal attachment of gingival tissues to the root surface commonly reported following root planing and soft tissue curettage appears to result from formation of a long junctional epithelium rather than a new connective tissue attachment. This work was based on a non-human primate model.

It was stated that teeth with a LJE were not more prone than other areas to develop new pocket formation (Magnusson *et al.*, 1983; Beaumont *et al.*, 1984; Aukhill *et al.*, 1988). Aukhil and coworkers concluded that the type of attachment (connective tissue vs. LJE) present was irrelevant and that plaque control was the critical element that promoted health (Aukhill *et al.*, 1988).

1.7.4.2 Healing After Conventional Surgical Procedure

Healing of surgical wounds in skin or oral soft tissues begin with the chemoattraction of cells to the wound area and end with the formation and maturation of a new extracellular matrix. This matrix bridges the margins of the wound, supports cells and a regenerating vasculature, and restores the resistance of the tissues to functional stress. Superficially, epithelial cells migrating from the wound margins rapidly cover the maturing fibrin clot in the wound space.

In a periodontal defect, the gingival flap is seated against a vascularized wound margin, namely the gingival connective tissue and the alveolar process, and also it opposes the

avascular, calcified, and rigid root surface. Healing of such lesions has generally been characterized by maturation of the gingival connective tissue, some regeneration of alveolar bone and cementum, and most significantly, epithelialization of root surface (Caton & Zander, 1976; Listgarten & Rosenberg, 1979; Steiner, Crigger & Egelberg, 1981). The dentogingival epithelium seems to be the 'sprinter' in the race, thus traditional flap surgery routinely results in healing with a long junctional epithelium lining the debrided root surface (Caton, Nyman & Zander, 1980; Magnusson *et al.*, 1983; Beaumont *et al.*, 1984; Listgarten & Rosenberg, 1979; Yukna, 1978), with no new connective tissue reattachment. The cells which repopulate the root surface determine the nature of the attachment that will form. The epithelial downgrowth prevents the formation of a new connective tissue attachment by preventing repopulation of the root surface by cells derived from the periodontal ligament. However, the coverage of the root surface by an epithelial layer has a beneficial barrier effect; i.e., the prevention of root resorption and ankylosis, which otherwise could be induced by gingival connective tissue and bone.

The sequence of healing based on experimental incision and excision wounds, primarily in the skin, is commonly divided into three overlapping phases; 1) inflammation; 2) granulation tissue formation; and 3) matrix formation and remodeling.

Early healing events at the dento-gingival interface have been examined using dentine blocks implanted in edentulous ridges in dogs by Wikesjö *et al.* (1991). Immediately following wound closure, a granular precipitate of plasma proteins with aggregates of red

blood cells seems to adhere to the dentine surface. A few hours later, the intercellular matrix appears more organized with fibrin formation around erythrocyte aggregates and at the dentine surface. Neutrophils are present throughout the wound space at this time. As the inflammatory phase progresses, the numbers of neutrophils migrating towards the dentine are increasing. Strands of fibrin gradually become more distinct throughout the clot, binding to the connective tissue as well as to the dentine surface. Degradation of the erythrocytes begins.

Granulation tissue formation may be visualized within three days of healing. Macrophages can be seen at the dentine surface at this time and fibroblasts now occur in the wound space, which, however, is still dominated by inflammatory cells. Further degradation of fibrin clot is evidenced by loss of structure and presence of fragmented erythrocytes.

Seven days following wound closure, the wound site is dominated by a cell-rich granulation tissue with fibroblasts and young collagen fibres. At this stage, collagenous elements appear parallel to the dentine surface. Within 14 days, the newly formed collagen fibres may show arrangement indicative of physical attachment to the dentine. Cementum formation is not seen until at least three weeks after wound closure (Bjorvatn, 1986; Selvig, Bogle & Claffey, 1988). However, a functional fibre arrangement followed by cementum formation does not always occur.

Reported changes produced by conventional periodontal therapy in humans included pocket depth reduction mediated by recession of the gingival margin and gain of clinical attachment (Caton, Proye & Polson, 1982; Proye, Caton & Polson, 1982). Until the mid-1970s, the favorable changes produced by conventional periodontal therapy were often interpreted to represent a true periodontal regeneration. Indeed, longitudinal human clinical studies from several different research centers demonstrated that conventional periodontal therapy followed by good periodontal supportive therapy was effective in stabilizing periodontal status and maintaining periodontal health (Becker, Berg & Becker, 1984; Knowles *et al.,* 1979; Lindhe & Nyman, 1984; Lindhe *et al.,* 1987; Pihlstrom *et al.,* 1983; Ramfjord *et al.,* 1987).

Caton *et al.* (1980) showed histological evidence of re-formation of an epithelial lining (long junctional epithelium) facing the instrumented root surfaces with no new connective tissue attachment after surgical and nonsurgical treatments in animals. The development of a long junctional epithelium occurred in sites with suprabony pockets as well as sites with infrabony pockets. New bone formation (bone fill) in infrabony defects was a frequent findings, but a long junctional epithelium was always interposed between the newly formed bone and the root. The formation of a long junctional epithelium was reported by Bowers (1989) to occur also in humans following flap curettage of infrabony defects. Therefore, conventional periodontal therapy results primarily in repair rather than regeneration of the periodontium (Caton *et al.,* 1980; Listgarten & Rosenberg, 1979). These studies were a turning point in the standards of evaluation of therapy and approach to modifying therapy to favor regeneration.

1.7.4.3 Time for Healing and Maturation of the Periodontium to Occur

Following scaling and root planing, Proye and coworkers (1982) have reported a gain of clinical attachment after 3 weeks. Morrison, Ramfjord & Hill (1980) demonstrated that healing took at least 4 weeks. Kaldahl and colleagues (1988) demonstrated that the repair process extended for a year. It was concluded in the Proceedings of the World Workshop in Clinical Periodontics in 1989 that it appears that the greatest changes with respect to probing depth reduction and gain of clinical attachment can be recorded after 4 to 6 weeks, but gradual repair and maturation of the periodontium may occur over 9 to 12 months.

1.8 REGENERATION

1.8.1 Definition

Periodontal regeneration is defined as the restoration of the various components of the periodontium, i.e. alveolar bone, periodontal ligament, root cementum, and gingivae lost due to disease, in their appropriate locations, amounts, and relationships to each other, thereby locating the epithelial attachment at a more coronal position before treatment. For the regenerative process to take place, it is vital to prevent the epithelium from contacting the root surface. It is believed that by placing a physical barrier between the gingival connective tissue and the root surface the following may be achieved: (a) epithelial cells are impeded from apically migrating and interfering with connective tissue-root surface interactions; (b) gingival connective tissue from the flap is excluded from healing sites; and (c) progenitor cells from the periodontal ligament are favoured to repopulate the coronal root surface, thereby facilitating formation of new periodontium

1.8.2 The Regenerative Capacity of the Periodontal Tissue

In the wound healing process following surgical treatment, the denuded root surface can be repopulated by cells derived from the dentogingival epithelium, the gingival connective tissue, the alveolar bone and the periodontal ligament. As stated before, the dentogingival epithelium seems to be the "sprinter" in the race. During the early healing

phase, dentogingival epithelium migrates apically along the wound surface that faces the root, effectively preventing the formation of a new connective tissue attachment. This epithelial proliferation extends to or close to the bottom of the defect, resulting in the formation of so-called long junctional epithelium.

A critical step in periodontal regenerative therapy is to alter the periodontitis-affected root surface to make it a hospitable substrate to support and encourage migration, attachment, proliferation and proper phenotypic expression of periodontal connective tissue progenitor cells.

In order for regeneration to occur, cells having the capacity to form cementum, bone and periodontal ligament must occupy the periodontal defect and produce these specialized tissues. Current thought is that these periodontal progenitor cells reside in the periodontal ligament remaining around the tooth (Melcher, 1976; Melcher *et al.*, 1987; Nyman *et al.*, 1982). It has been shown by Karring, Nyman & Lindhe (1980) that ankylosis and root resorption occurred where bone tissue had grown into direct contact with the ligament deprived root surface. Gingival connective tissue has also been shown to lack the potential to induce the formation of new CT attachment to a root surface that has been deprived of its original periodontal ligament, and actually cause root resorption (Nyman *et al.*, 1980). Karring *et al.* (1985) has demonstrated that extensive formation of new CT in the coronal portion of the roots occurred when the periodontal ligament in the apical half was maintained undamaged. This was taken as evidence that granulation tissue originating from the periodontal ligament possess the ability to form new

attachment. Another study that supported this idea was the formation of cementum and periodontal ligament on implants placed in close contact with retained roots. Only osseointegration with no cementum or periodontal ligament formation occurred on implants with no direct contact to retained roots, thus suggesting that the progenitor cells for cementum formation reside in the periodontal ligament and not in the alveolar bone (Buser *et. al.*, 1990).

1.8.3 Root Surface Conditioning and/or Bone Grafts or Bone Substitutes to Enhance Regeneration

Removal of the cementum layer by citric acid has been shown to promote new attachment in animals (Register, 1973; Crigger *et al.*, 1978; Nilvéus, Johansson & Egelberg, 1978; Ririe, Crigger & Selvig, 1980). The citric acid exposes the collagen fibrils of the dentine matrix and a subsequent interdigitation of these fibrils occurs with collagen fibrils of the adjacent soft tissue flap (Ririe, Crigger & Selvig, 1980; Selvig *et al.*, 1981; Polson & Proye, 1982). Formation of a new connective tissue attachment, without any sign of new cementum on the root surface following treatment of root surfaces with citric acid, have been observed in experimental studies (Bogle, Claffey & Egelberg 1985; Caffessee *et al.*, 1985; Isidor *et al.*, 1985). Formation of a long junctional epithelium (Nyman *et al.*, 1981) and increased frequency of root resorption (Gottlow, Nyman & Karring, 1984b; Magnusson *et al.*, 1985b; Dreyer & van Heerden, 1986) have been reported in animals studies. Comparative studies in humans failed to

detect clinically significant differences between acid treated and non-acid treated teeth (Renvert *et al.,* 1981; 1985; Smith *et al.,* 1986; Moore, Ashley & Waterman, 1987).

Most investigators have focused upon bone regeneration as being the prerequisite for new attachment formation. If bone regeneration could be induced it is hypothesized that this will also lead to induction of cementum formation (Hiatt *et al.*, 1978). To accomplish this goal several bone grafting materials or bone substitutes have been used mostly in the treatment of infrabony defects and furcation defects.

In a number of clinical trials and animal studies, surgical procedures have been combined with the placement of bone grafts or implant materials to promote regeneration in furcation and infrabony defects. The grafting materials can be divided into 1) autogenous grafts, 2) allografts, 3) hetero- or xenografts, and 4) alloplastic materials.

The rationale behind the use of bone grafts or alloplastic materials is the assumption that they may either be osteoproliferative, osteoconductive or osteoinductive and that they may favour cementogenesis (Melcher *et al.,* 1987). Inconsistent results have been obtained following grafting procedures. Successful results with bone regeneration and gain in probing AL, in angular bony defects, has been demonstrated by Schallhorn (1977), Dragoo (1981) Nabers (1984), Hiatt *et al.* (1986), and Schrad & Tussing (1986). Other studies, however have failed to demonstrate any beneficial effect of using bone grafts compared to curettage alone (Ellegaard & Loe, 1971; Carraro, Sznayader & Alonso, 1976; Renvert *et al.,* 1985b). In a study by Ellegaard *et al.* (1974) healing of

angular bony defects in monkeys was evaluated. It was observed that the use of grafts in defects favoured regeneration, but the placement of fresh autogenous marrow resulted in ankylosis and root resorption. Moskow, Karsh & Stein (1979) reported new bone formation associated with placement of cancellous bone, but noted the presence of long junctional epithelium between the grafts and the root surface. It has been indicated that by using bone graft the effect is more physical rather than biological, acting like a filler supporting the mucogingival flaps.

In summary, root surface conditioning and bone grafting in addition to conservative treatment only resulted in limited formation of a new attachment when evaluated histologically. Most frequently healing occurs with the formation of a long junctional epithelium extending to the base of the previous defect.

1.8.4 Earlier Studies on Guided Tissue Regeneration

The principal of guided tissue regeneration was born as an extension of the window technique and first introduced by Nyman *et al.* (1982b). The window technique is a procedure where a U-shaped incision was made on the buccal mucosa to expose the root surface underneath the alveolar bone. By doing this dentogingival epithelium was prevented from interfering with the wound, and by placing a membrane in between the flap and the root surface the connective tissue was prevented from contacting the root surface. The guided tissue regeneration technique is based on the argument that cells

proliferating from the periodontal ligament bordering the periodontal defect should preferentially and selectively be guided to repopulate the denuded root surface. To achieve that purpose, a piece of physical barrier, i.e., a non-resorbable membrane or biodegradable membrane material is placed over the denuded root surface and covered by mucosal tissue. The membrane extends apically from the outer bone surface, laterally to the root defect and underneath the gingival margin, and allows cells of the adjacent periodontal ligament and of alveolar bone to repopulate the blood clot that fills the space between root and barrier, and later the denuded root surface. At the same time, the barrier prevents gingival epithelium and connective tissue from contacting that surface.

In a study by Minabe (1991), a new cementum matrix was formed along up to 70% of the denuded root surface extending from the apical or lateral border of the still intact periodontium coronally for distances varying between 1 and 5 mm. The regenerated cementum was thin, tapered coronally, overlapping the old cementum apically but did not adhere to the established root surface, neither the old cementum nor dentine (Gottlow, Karring & Nyman, 1984a; 1990; Ettetl *et al.*, 1989). In humans, the outer surface of this new cementum is covered by connective tissue, rarely showing dense aggregates of collagen fibres oriented perpendicularly to and inserting into the new cementum, in contrast to dogs and non-human primates in which dense fibres were seen. Cells of the periodontal ligament along the intact periodontium bordering the denuded root surface proliferated in a very limited zone of 200 to 300 μm during the first 7 days after surgery. There is some evidence that periodontal ligament cells actually migrate towards the denuded root surface (Aukhil & Iglhaut, 1988; Iglhaut *et al.*, 1988).

Nyman et al. (1982) and Gottlow, Karring & Nyman (1984a) presented the first studies that demonstrated the formation of a new attachment and bone as a result of the GTR treatment. The results of these studies have been confirmed in a number of studies (Aukhil, Petterson & Suggs, 1986; Aukhil, Simpson & Schaberg, 1983; Caffesse *et al.,* 1990; 1988; Caton *et al.,* 1992; Claffey *et al.,* 1989; Gottlow, Karring & Nyman, 1990; Magnusson *et al.,* 1985a). The first report of a human tooth treated according to the principle of GTR was by Nyman *et al.* (1982). The GTR procedure was performed on a single mandibular incisor scheduled for extraction, with a cemento-enamel junction to bottom of infrabony defect (CEJ-BD) distance of 11 mm and a cemento-enamel junction to bony crest (CEJ-Crest) distance of 9 mm giving 2 mm of infrabony defects, using a Millipore membrane. After 3 months of healing, a block biopsy revealed new cementum and inserting fibres had formed corresponding to 7 mm of new attachment on the exposed root surface. Case reports on 12 teeth in 10 patients were presented by Gottlow *et al.* (1986). Histological and clinical results were obtained from 5 and 7 teeth respectively, some with furcation lesion. Histological results were obtained 3 months after membrane placement and clinical results were measured 3 months after barrier removal. Histological result demonstrated new attachment. New attachment following the GTR treatment has also been shown histologically by Becker *et al.* (1987), Stahl, Fronum. & Tarnow, (1990), and Stahl & Froum (1991).

In the study by Gottlow *et al.* (1986), the clinical results following the GTR treatment of 7 teeth with furcation class II or angular bony defects revealed a mean gain of clinical

attachment of 5.6 mm, and the corresponding bone regrowth of 5.1 mm. Regeneration of bone was restricted to sites where angular bony defects were present before. Gains in probing attachment level and probing bone level following the GTR treatment have been reported in several short-term clinical studies, e.g., those by Becker *et al.* (1988), Schallhorn & McClain (1988), and Cortellini *et al.* (1990), and in long-term clinical study by Gottlow, Nyman & Karring (1992).

Controlled clinical studies with intra-individual comparison of the GTR therapy and conventional flap surgery in the treatment of class II furcation defects have been presented by Pontoriero *et al.* (1988), Lekovic *et al.* (1989), and Caffesse *et al.* (1990). All studies showed statistically significantly more gain in probing attachment level at GTR-treated sites. The study by Pontoriero *et al.* (1988) comprised 21 patients and evaluated treatment of Class II furcation involvement in mandibular molars. GTR therapy resulted in clinical closure of 19 of the 21 test defects compared to 6 out of the 21 defects in the control group (conventional surgery). In another study, Pontoriero *et al.* (1989) treated 21 patients with through and through mandibular molars furcation involvement in both sides of the jaw. Only 8 out of the 21 defects treated by GTR were completely closed, 10 were partially closed and three teeth showed remaining through-and-through furcation involvement after therapy. On the control side (non GTR treated), none of the 21 defects were completely closed, 10 showed partial closure, while 11 showed remaining through-and through furcation involvement. It was found that the healing result at the test teeth was related to the size of the entrance opening to the original Class II defects.

It can thus be concluded that regeneration of the periodontium, i.e. the formation of new cementum with inserting connective tissue fibres as well as new bone formation, can be accomplished if the treatment procedure is based on the biological principle of guided tissue regeneration.

1.8.5 Nonresorbable Barrier Material

The first commercially available barrier for GTR was Gore-Tex® manufactured from expanded polytetrafluoroethylene (ePTFE). They were initially chosen because of the microporosity, which allowed for passage of liquid and nutritional products but not for cells, and they could be sterilized. It consists of an open microstructure collar and an occlusive apron. The microstructure facilitates early clot formation and collagen fibre penetration during healing and the occlusive apron excludes the gingival epithelium and connective tissue from the root surface. The barrier is preformed into various sizes and shapes to fit periodontal defects of different morphology and location. The safety and efficacy of this barrier has been confirmed in numerous animal and clinical investigation as illustrated in Table 1.3.

The first generation of GTR devices are non-resorbable, which calls for a second surgical procedure. This is a negative factor both from a cost-benefit point of view and with respect to the additional trauma to the patient and to their newly formed, immature

Table 1.3 Clinical outcomes of GTR using ePTFE membrane barrier

Study	Animal model/number/ defect	observation period	Treatment effects
Gottlow *et al.* (1984)	Monkey/3/dehiscence/experimental periodontitis	3 months	New attachment: Expt: 77% Control 33%
Gottlow *et al.* (1986) case reports	human/10/Class II & III furcation + angular bony defects	3/6 months	New attachment 40%. Probing attachment gain 5.6 mm
Pontoriero *et al.* (1987)	Human/37/Class II and III furcations	6 months	completely closed sites class II 67%/expt 10%/con class III 25%/expt 0%con
Becker *et al.* (1987)	Human/3/ I, II wall, class III furcations	3/6 months	Probing attachment gain (PAG) I wall: 2-4mm 2 wall : 4 mm Class III: 4mm
Pontoriero *et al.* (1988)	Human/21/class II	6 months	PAG Vert: 4.1mm/expt 1.5mm/con Horz: 4.1mm/expt 1.9mm/con

Becker (1988)	Human/27/class II & III furcations; 3 wall defects	6 months	PAG Class II: 2.3mm Class III: 1.3mm 3 wall: 4.5mm
caffesse (1990) case reports	Human/9/class II furcations	6 months	CAL gain expt:1.8 mm con: 0.6mm
lekovic (1990)	Human/15/Class II furcations	6 months	CAL gain GTM only: 2.40 ± 0.48mm GTM + PHA grafts: 2.93 ± 0.64 mm.

periodontal tissues which may jeopardize new attachment formation. Such problems could be overcome if the GTR devices were bioresorbable.

Other drawbacks observed when using GTR-barriers are the frequent incidents of apical downgrowth of the dentogingival epithelium along the surface of the connective tissue flap facing the barrier, even though they had a cervical collar with large pores aimed at preventing epithelial downgrowth along the barrier by ingrowth of connective tissues into the pores. However, this collar is often exposed to the oral cavity and therefore does not fulfill its intended function, but instead harbours large amounts of oral microorganisms rather than being filled with ingrowing connective tissue. This may seriously disturb the healing process, markedly reducing or even preventing regeneration. To increase the clinical benefit of GTR treatment, it was therefore necessary to find out if a barrier could be designed so that there would be minimal gingival flap recessions and epithelial downgrowth from the gingival margin when it was placed between the root surface and the soft tissue flap.

In conclusion, among the problems associated with the use of non-resorbable barrier membranes are:

1) the need for a second surgical procedure for membrane removal. This is associated with increased morbidity for the patients, is time consuming for the surgeon, and can interfere with the maturation of the regenerated tissues during an early and delicate stage of healing (Tonetti *et al.*, 1993; Cortellini, Pini Prato & Tonetti, 1995b).

2) the possibility of membrane contamination and/or infection whenever the membrane is exposed to the oral environment.
3) the optimal timing of membrane removal has not been determined in a human population (Caton *et al.*, 1992).

In 1988 a research team was established with the purpose of developing and researching/testing a bioresorbable barrier. The objectives for the team were to develop the best possible barrier design and the best possible barrier material to enable a safe and effective single step GTR therapy (Lundgren, Mathisen & Gottlow, 1994).

Based on the knowledge gained from previous GTR research and the studies of the implant-soft tissue integration, a list of design criteria was set up to characterize a well-functioning bioresorbable barrier for periodontal regeneration:

- rapid integration with the adjacent connective tissue
- maintenance of space for selective tissue ingrowth
- maintenance of barrier function during a sufficient healing period
- effective anchorage to the tooth.

The main requirements of a material for use as a single-step GTR-barrier are:

1) bioresorbability
2) biocompatibility
3) a purpose-made resorption pattern

4) initial dimensional stability

5) suitable malleability

1.8.6 Resorbable Barriers

A variety of resorbable membrane materials have been used for GTR including collagen, polyglycolic acid, polylactic acid, or copolymers of these materials (Bluementhal, 1988; Card *et al.,* 1989; Caton, Greenstein & Zappa, 1994; Fleisher, Waal & Bloom, 1988; Kon *et al.,* 1991; Magnusson, Batich & Collins, 1988; 1990; Pitaru *et al.,* 1987; 1988; 1989;Quiñones *et al.,* 1990a; 1990b; Tanner, Solt & Vuddhakanok 1988; Warren *et al.,* 1992). As the barriers resorb they do not require a second surgical procedure for their removal, thus eliminating any trauma that may occur to the immature, newly regenerated periodontal tissues during barrier removal.

Studies on type I collagen barrier were done by Pitaru *et al.* (1987;1988) the second study of which showed healing by repair with a long junctional epithelium. Another study by Pitaru *et al.* (1989) confirmed this result. Tanner, Solt & Vuddhakanok (1988) showed that application of the microfibrillar collagen in the GTR procedures did not prevent the apical migration of epithelium and failed to facilitate periodontal regeneration.

Magnusson, Batich & Collins (1988b) evaluated the use of a polylactic acid polymer barriers in the treatment of surgically created dehiscence-like defects in 2 dogs.

Histometric analysis after two months revealed more new attachment and bone regeneration in the test group compared to the control. A second study (Magnusson *et al.,* 1990) using this barrier for 1-wall vertical and horizontal defects however failed to demonstrate a difference between experimental and control groups.

When our study was initiated, only two of these bioresorable barriers, Guidor® (John O. Butler Co., Chicago, IL) and Resolut® (W.L. Gore & Associates) have been approved by the U.S. Food and Drug Administration and commercially available for periodontal applications.

1.8.6.1 Resolut®

Resolut ® is a bioresorbable barrier composed of a synthetic copolymer of polyglycolic and polylactic acids. Histological evaluation of this bioresorbable barrier in beagle dogs found comparable results to those achieved with the non-resorbable ePTFE barrier. Evaluations performed at 1, 3, and 6 months demonstrated a minimal inflammatory reaction and the reformation of a connective tissue attachment with these barriers. New cementum with inserting collagen fibres occurred on the previously denuded root surfaces isolated by either the non-resorbable or the resorbable barriers (Caffesse *et al.,* 1994; Warren, Sanchez & Karring, 1994). Furthermore, studies in monkeys by Quiñones *et al.* (1994) and Huerzeler *et al.* (1994) proved the benefits of this barrier when

compared to open flap procedure in interproximal infrabony defects and class II furcations, respectively.

The clinical efficacy of this bioresorbable material in the treatment of deep infrabony defects in human has been evaluated and compared to the non-resorbable barrier membranes (ePTFE) or access flap procedure by Cortellini, Pini Prato & Tonetti, (1996c). Both the groups treated with bioresorbable and non-resorbable barrier materials gained significantly more CAL at 1 year than the access flap procedure group and there was no statistically significant difference in the CAL gain in both the membrane groups.

Another study was done by Caffesse *et al.* (1997) in human, evaluating the efficacy of this material and compared it to the proven non-resorbable ePTFE barrier material in the treatment of Class II furcations and infrabony lesions. Both treatments produced significant improvement from baseline in probing reduction, and gain in clinical attachment. No statistical differences were found when results were compared between the two different barrier membranes as shown earlier by Cortellini, Pini Prato & Tonetti, (1996c).

1.8.6.2 Guidor®

A resorbable barrier called Guidor® was commercially available when this study was initiated. It has been approved by the responsible authorities as a safe and effective

method of promoting periodontal tissue regeneration. The composition of Guidor® is based on bioresorbable polylactic acid, softened with a citric acid ester to accomplished malleability and facilitate clinical handling. Apart from being bioresorbable, this product has a matrix design, which also allows for integration, prevention of gingival recession and formation of pseudo-pockets around the device and subsequent infection and gingival inflammation, problems frequently observed with available non-resorbable GTR products. This material is designed to maintain a barrier function for a minimum of 6 weeks. During this period, both the design of the matrix and the stability of the polymer are maintained.

Afterwards, the matrix slowly resorbs and is gradually replaced by periodontal tissue. The process of degradation is by hydrolysis resulting in lactic acid which is absorbed by the surrounding tissues. The polymer fragments are eliminated by macrophages and the end degradation products are ultimately metabolized to water and carbon dioxide. Bioresorption is completed 6 to 12 months after placement.

Gottlow *et al.* (1992a) first evaluated the safety and efficacy of this barrier in recession-type and interproximals defects in monkeys in a 12-month study period. This study showed clinical healing with minimal or no inflammation and histological healing revealing cementum with inserting periodontal ligament fibres extending to the coronal border of the barrier after 1 month. Extensive new bone formation had occurred after 3 months, and after 6 months complete resorption of the barrier had taken place. Gottlow *et al.* (1993) also compared Gore-Tex and Guidor® membranes. It was found that more

membrane exposure occurred by using the nonresorable membrane, and it was speculated whether the difference in the result in new connective tissue regeneration (72% in resorable, 63% in nonresorable) and bone regeneration (89% and 87%) was due to this fact.

The clinical use of the bioresorbable matrix barrier (Guidor®) for the GTR therapy, was also evaluated in humans (Laurell *et al.*, 1992; Gottlow *et al.*, 1992b), where furcation class II defects and infrabony defects were followed for six months or more after the GTR treatment. The result of the therapy was evaluated by assessing PD and CAL prior to surgery and after 6 months. At furcation defects mean PD was reduced from 5.6 to 3.0 mm. The mean gain of CAL-vertical was 3.2 mm and CAL-horizontal 3.1 mm resulting in complete closure of 7 of the 12 defects. At infrabony defects mean PD was reduced from 8.9 to 3.1 mm (5.8 reduction). It was concluded that the use of bioresorbable matrix barrier in GTR resulted in pronounced gain of CAL and a very low incidence of gingival pathology, gingival recession, and device exposure.

In a case report by Laurell *et al.* (1994) Guidor® was used in 19 class II furcation and 47 infrabony (2 or 3 walls, or combination) defects in 59 patients scheduled for GTR therapy. Inflammation was found adjacent only to 3 defects and limited to the first month after membrane placement. Only 10 devices were exposed during the $1^{st}/2^{nd}$ week of healing and never more than 2 mm. The PD reduction in the furcation defects was 3.7 mm. Mean gain in vertical CAL was 3.4 and 1.4 mm for horizontal CAL gain. At the infrabony defects mean PD reduction was 5.4 mm and mean gain of CAL 4.9 mm. These

results were quite similar to the one described above, and this study also showed low incidence of gingival pathology, gingival recession and device exposure.

A study by Falk *et al.* (1997) involving 203 defects in 143 patients treated in three periodontal specialist practice evaluating the effect of GTR using Guidor® after 1 year showed comparable results in clinical attachment gain and bone fill to previous case studies and controlled clinical trials. Mean PD was reduced from 9.0 $\pm$ 1.0 mm to 3.3 $\pm$ 1.0 mm. Mean CAL gain amounted to 4.8 $\pm$ 1.5 mm corresponding to 79 $\pm$ 13% of the initial infrabony defects. Bone fill averaged 3.2 $\pm$ 1.8 mm. In this study it was also shown that the depth of infrabony defects were positively correlated to CAL gain and bone fill and also early barrier exposure and plaque in the defect area at the time of surgery negatively influenced the treatment outcome.

In a study by Weltman *et al.* (1997) periodontal regeneration of infrabony defects utilizing either Guidor® or ePTFE were assessed in 30 patients randomly divided into two groups; each patient having one infrabony defects of probing depth $\geq$ 6mm. In this study, significantly more attachment gain and shallower probing depths were found with ePTFE, if the data were analyzed using the deepest site of the defect. However, less inflammation was noticed in Guidor® sites at 2 and 4 weeks compared to the ePTFE sites. Comparisons of PD reductions, CAL and bone fill after 12 months of healing between treatments reveals no statistically significant differences when the averaged-site data were analyzed between groups.

When an in vitro study was done to examine the colonization of different bioresorbable and non-resorbable membranes by *P. gingivalis* it was established by Ricci *et al.* (1996) that Guidor® membrane do not harbour *P. gingivalis* even though they easily let the bacteria through, even after 6 hours.

Thus, Guidor® barrier membrane has been proved clinically to be effective in treatment of infrabony defects in GTR procedures and have shown low incidence of gingival pathology, gingival recession and device exposure.

1.8.7 Factors Affecting the Outcome of the GTR procedure

The factors affecting the outcome of the GTR relates to:

1) patient selection
2) morphology of defect
3) the GTR procedure and the healing period.

1.8.7.1 The patient

Plaque control

The ability to maintain high levels of plaque control has been associated with better outcomes following the GTR procedures (Tonetti *et al.,* 1995; Tonetti, Pini-Prato & Cortellini, 1996; Cortellini *et al.,* 1994). It should be emphasized that the clinical attachment gain detected in patients with less than 10% of surfaces covered with plaque were 1.85 mm greater than the ones observed in patients with at least 20% full mouth plaque score (Tonetti, Pini-Prato & Cortellini, 1996). Presence of plaque in the defect area at the time of surgery has been shown to negatively influence the GTR treatment outcome (Falk *et al.,* 1997).

Residual infection

Another important variable associated with GTR outcome is the level of residual periodontal infection in the dentition, evaluated clinically as a percentage of sites with bleeding on probing or microbiologically as persistence of periodontal pathogens after completion of initial therapy (Tonetti, Pini-Prato & Cortellini, 1993; Machtei *et al.,* 1994). Control of patient's oral hygiene and residual periodontal infection in the oral cavity has also been shown to be strongly associated with clinical outcomes of both regenerative and conventional surgical procedures (Tonetti, Pini-Prato & Cortellini, 1996). GTR procedures should be deferred until an adequate level of control of periodontitis has been achieved in the dentition.

Smoking

Smoking is a strong risk marker and probably a true risk factor for the development of periodontitis (Feldman, Bravacos & Rose, 1983; Bergström, 1989; Bergström, Eliasson & Preber, 1991). Smokers present a less favourable result following both non-surgical and surgical periodontal therapy (Preber & Bergström, 1985; 1990). Cigarette smoking has also been associated with reduced GTR outcomes: attachment level gains of 2.1 ± 1.2 mm were observed in subjects smoking more than 10 cigarettes per day, whereas nonsmokers gained 5.2 ± 1.9 mm of clinical attachment (Tonetti, Pini-Prato & Cortellini, 1995). The negative correlation of smoking to CAL gain following GTR therapy has also been demonstrated by Trombelli *et. al.* (1997), and they also showed higher incidence of membrane exposure in the smokers.

The negative impact of smoking in GTR treated sites and sites treated with subgingival debridement (root planing) were demonstrated in a long-term study by Cortellini, Pini Prato & Tonetti, (1996a). The patients were divided into a loser group and a stable group comparing GTR sites which were matched to RP sites. The loser group included patients who lost CAL ≥ 3 mm in at least 3 sites from different teeth. When CAL loss between 1 and 5 years were compared in the two groups, the loser group lost 2.4 ± 1.2 mm CAL in GTR sites and 2.2 ± 1.5 mm in RP sites, while the stable group only lost 0.3 ± 0.7 mm in GTR sites and 0.7 ± 0.9 mm in RP sites. Most of the patients in the loser group were smokers and had not complied to the recall system.

1.8.7.2 Defect Morphology

Considerable evidence indicates that defect morphology plays a major role in the healing response following GTR therapy of infrabony defects. It has been demonstrated that the deeper the baseline defect, the greater the amount of probing attachment and bone gained at 1 year (Tonetti, Pini-Prato & Cortellini, 1993; 1996; Garrett *et al.*, 1988). This relationship is in agreement with several studies on the effect of conventional surgical therapy of infrabony periodontal defects without the application of barrier membranes (Knowles *et al.*, 1979; Hill *et al.*, 1981; Pihlstrom, Oritz-Campos & McHugh, 1981; Lindhe *et al.*, 1982) and also with barrier membranes application in class II furcation defects (Machtei, *et al.*, 1994).

Early observations suggested that defect morphology in terms of number of residual bony walls could be associated with the outcomes of regenerative surgery without membrane application (Goldman & Cohen, 1958; Schallhorn, Hiatt & Boyce, 1970). An investigation by Renvert *et al.* (1985a) has failed to demonstrate a significant association between the number of residual bony walls and the clinical outcome. Other investigations has also observed good clinical results irrespective of defect configuration in terms of one-, two-, and three-wall defects (Tonetti, Pini-Prato & Cortellini, 1993).

Based on these studies and the treatment objectives, the most positive outcome from guided tissue regeneration can be achieved in deep and narrow infrabony defects.

1.8.7.3 The Guided Tissue Regeneration Procedure and the Healing Period

Different membranes, i.e. resorbable versus non-resorbable membranes, possess different abilities to create and maintain the necessary space for regeneration. The amount of available space has been associated with the extent of observed clinical improvements (Tonetti, Pini-Prato & Cortellini, 1996), as evidenced by significantly higher gains for titanium reinforced membranes with the modified papilla preservation technique versus the conventional GTR with ePTFE membranes.

A study by Sander & Karring (1995) in monkeys has demonstrated that significantly more new connective tissue attachment and bone formation could be achieved if bacterial contamination was limited by submerging the roots and the barrier material (Gore-tex® or Vicryl Mesh®) compared to non-submerged roots. A recent investigation by Falk *et al.* (1997) has also supported this fact by demonstrating a significantly greater gains in attachment level observed in sites where resorbable membrane were completely covered as compared with sites with partial exposure (5.0 $\pm$ 1.8 mm versus 4.2 $\pm$ 2.1 mm, respectively). Membrane exposure has been repeatedly reported to be a major complication with prevalence in the 70% to 80% range (Becker *et al.*, 1988; Cortellini *et al.*, 1990; Cortellini, Pini Prato & Tonetti, 1993; Selvig, Kersten & Wikesjö, 1993; Murphy 1995; DeSanctis *et al.*, 1996a; DeSanctis *et al.*, 1996b). Early barrier exposure was found to be a negative predictor of CAL gain (Falk *et al.*, 1997). Probing bone level (PBL) gain was significantly greater in patient without membrane exposure (Selvig *et al.*,

1992; Schallhorn & McClain 1994; Trombelli *et al.,* 1997) than in patient with membrane exposure, while there was no significant difference in reduction in PD or gain in CAL. The membrane exposure can be highly reduced with the use of an access flap specifically modified to preserve the interdental tissues (Cortellini, Pini Prato & Tonetti, 1995a; 1996b). It was found that higher incidence of membrane exposure occur in smokers and also, the amount of gingival recession was significantly greater (increased by a factor of 2) in smokers than in non-smokers (Trombelli *et al.,* 1997).

Once the membrane is exposed to the oral environment a bacterial biofilm is formed on the surface of the implanted barrier, thus affecting the outcome of GTR surgery. The presence of an organized bacterial biofilm, comprising a variety of bacteria including periodontal pathogens, was detected on both the outer and the inner aspects of the membrane (DeSanctis *et al.,* 1996a; DeSanctis *et al.,* 1996b; Selvig *et al.,* 1992; Grevstad & Leknes, 1993; Nowzari, Matian & Slots, 1995b; Novaes *et al.,* 1995).

The amount of newly formed tissue present at membrane removal positively predicts the expected outcome of 1-year CAL gain (Tonetti, Pini-Prato & Cortellini, 1996).

1.8.8 Long Term Results of GTR

In a long-term study, Gottlow, Nyman & Karring (1992) assessed the long term stability of clinical attachment gained by GTR procedures. Eighty sites in 39 patients which 6

months after surgery exhibited a gain of clinical attachment of $\geq$ 2mm were monitored during the additional periods of 1-5 years. Of the 80, 65 were monitored for 2 years, 40 for 3 years, 17 for 4 years and 9 for 5 years. Six sites lost CAL $\geq$ 2 mm in the first year, three in the second and third year, while in the fourth and fifth year, follow up sites retained the new gained CAL. The results of this study indicate that attachment gain initially obtained can be maintained on a long term basis.

Results by Becker & Becker (1993) of followed-up patients with infrabony defects treated by GTR procedures demonstrated clinically significant reductions in PD (6.2 mm), gains in CAL (5.5 mm), and gains in bone fill (4.4 mm) after 3 years and 5 months. The patients who were re-entered and examined 3 times had sustained PD reduction and CAL gains for an average of 4 years and 3 months. The results from this study indicate that deep, multi-walled infrabony defects can be predictably treated by GTR procedures and the results can be sustained for a long term.

In another long-term followed-up study by Wiegel *et al.* (1995) treating patients in 1985/1986, 19 sites monitored for 4 years following GTR procedures. A statistically significant clinical attachment lost of all sites of 1.27 mm was seen from 1 to 4 years examination. However, between baseline and the 4 year examination there was no statistically significant loss of clinical attachment. During the maintenance phase, 1.42mm of probing attachment were lost at the deepest sites of the teeth, but compared to baseline, 1.37mm of new attachment was maintained. Out of the 19 sites, 7 showed a continued loss of probing attachment of $\geq$ 2mm during the maintenance period. Patients

that lost new attachment during the observation period tended to lose attachment in the entire dentition. There was an increase in plaque scores from baseline to 4 years examination which could contribute to the results which were not as positive as the result reported by Gottlow, Nyman & Karring (1992).

The long term stability of attachment gained through GTR procedures was also compared to the attachment gained through root planing by Cortellini, Pini Prato & Tonetti, (1996a). In this study, 44 patients were involved, full mouth root planing was carried out before one infrabony defect in each patient was treated with GTR. After the one year, the GTR treated sites were matched to a RP site with matching interproximal CAL, similar PD and if possible the same tooth type. These sites were then followed-up for a further four years. At the one year examination, GTR sites gained 4.0 $\pm$ 2.1 mm CAL and root planing sites gained 1.1 $\pm$ 1 mm CAL. On average GTR-treated sites lost 1.2 $\pm$ 1.4 mm and RP sites lost 1.3 $\pm$ 1.3 mm between 1 and 5 years. The difference in CAL loss between GTR and control sites was not statistically significant. This study shows no significant differences in terms of CAL loss between GTR and root planing treated sites during a 4 year follow-up period, indicating that GTR treated sites were no more susceptible to long term periodontal breakdown than root planed sites. In addition, stability of CAL was not limited to the experimental and control sites, but in general observed in the whole of the dentition.

A recent study by Eickholz, Kim & Holle (1998) evaluated the effects of GTR with non-resorbable (ePTFE) and biodegradable barriers (Polyglactin 910) in a split-mouth study,

24 months after surgery. Similar PD reduction was obtained in both groups at 12 and 24 months (2.2 $\pm$ 1.73 mm in ePTFE group vs 2.97 $\pm$ 1.90 mm in resorbable group). This study demonstrated that more favourable vertical attachment gain was obtained after therapy with biodegradable barrier membranes (2.02 $\pm$ 1.83 mm) compared to ePTFE membranes (1.18 $\pm$ 1.50 mm). While the mean vertical attachment gain in the biodegradable group remained stable over the complete observation period, there was a mean loss of 0.9 mm from 12 to 24 months post-surgically in the ePTFE group. Hence attachment level gains after GTR therapy using biodegradable barriers seem to be more stable.

1.8.9 Microflora Associated with the Placement of Physical Barrier Membranes

A study by Mombelli, Lang & Nyman (1993) reported the microbiological culture results from ePTFE membranes retrieved after 6 weeks of placement and indicated a high total bacterial counts. 1/3 of all cultivable organisms was Gram-negative anaerobic rods. This included considerable proportion of black-pigmented rods and *Fusobacterium.* The presence of bacteria on retrieved ePTFE membranes has also been shown by other authors (Selvig *et al.,* 1990; Tempro & Nalbandian, 1993; Frandsen *et al.,* 1994; Nowzari & Slots 1994).

The presence of a certain microflora associated with the removed ePTFE membrane at 6 week may act as a negative or positive predictor in GTR. The presence of *Porphyromonas gingivalis* are associated with loss of attachment; *Prevotella intermedia, Peptostreptococcus micros, Campylobacter rectus* are associated with no gain in attachment (Nowzari & Slots, 1994; Nowzari, Matian & Slots, 1995b); *Actinobacillus actinomycetemcomitans* and *Bacteroides forsythus* are associated with failing GTR (Nowzari, London & Slots, 1995a). In contrast, species of viridans streptococci and *Actinomyces* are associated with sites of significant healing (Nowzari, Matian & Slots, 1995b). Nowzari *et al.* (1996) has demonstrated that the total microbial counts and the percentage of *Peptostreptococcus micros* and *Capnocytophaga* species at baseline, and of motile rods on the ePTFE membrane surface facing the gingivae at 6 weeks, were statistically significant negative predictors of clinical attachment following GTR. In their study, the patients were divided into two groups, group AB, patients with no other residual pockets except the test site, while group NAB consisted of patients with residual pockets. Group AB gained more clinical attachment level and at baseline and 6 weeks no putative periodontopathogens were noted in the pockets and on the inner surface of the membrane respectively. Group NAB exhibited high levels of tested putative periodontopathogens at baseline and at 6 weeks on both sides of the membrane. Detection of *P. gingivalis* by DNA probe technique at 3 minutes of intraoral membrane manipulation was a significant negative predictor of attachment gain. Membranes showing specific microorganisms at 3 minutes also tended to exhibit the same species after 6 weeks of insertion, suggesting long-term survivability of bacteria on barrier membranes. This study emphasizes the fact that in patients with high subgingival levels

of putative periodontopathogens, the barrier membranes are at risk of becoming contaminated with motile rods, *P. gingivalis, P. intermedia, Spirochetes, P. micros, Propionibacterium* species, or other pathogens within 3 minutes of intraoral membrane manipulation. GTR procedures should only be carried out after other deep pockets are eliminated as this would lead to optimum clinical results, as been shown in this particular study.

Results from several studies have revealed that distribution of periodontal pathogens on the outer and inner surfaces of the barrier membrane (ePTFE) determines the amount of clinical attachment gain following GTR (Nowzari *et al.,* 1995b; Yoshinari *et al.,* 1998).

SEM analysis was carried out on the tooth facing surface of polyglycolactic barrier membrane which at 5 weeks were clinically exposed and underwent a second surgery for membrane removal (De Sanctis, Zucchelli & Clauser, 1996a). The membrane was divided into three portions a) the coronal area (clinically exposed portion), b) the mid-portion (clinically unexposed) and c) the apical portion. 13 of the 20 membrane (65%) were exposed supragingivally. Gain in attachment level was statistically greater at sites with clinical unexposed membranes. The SEM analysis of the inner surface of the membrane revealed that all the randomly selected fields examined in the coronal portion were positive for bacterial colonization. In the mid-portion 41% of the fields were positive, while no bacteria field was observed in the most apical portion. Colonization of the mid-portion of the inner surface of the membrane is a critical factor, as bacteria-positive fields had less attachment gain (3.0 ± 0.5) compared to bacteria-negative fields

(3.8 ± 0.4) and this difference was statistically significant. The gain in attachment in the bacteria negative field of the mid-portion is comparable to the gain in the unexposed membrane. The predominant microorganism morphotypes were cocci and short rods. Thus this study shows the importance of complete coverage of the membrane material during healing. But, caution has to be taken with these results as the removal of the membrane could cause trauma to the new tissue and could influence the healing process. Bacterial colonization of this membrane material was observed to be of a magnitude similar to that previously observed in ePTFE (De Sanctis, Zucchelli & Clauser, 1996b).

In the Nowzari *et al.* (1995b) study, sites free of pathogens on the membrane surface towards the tooth gained the most clinical attachment, even in the presence of various pathogens on the gingivae facing membrane surface. Pathogenic bacteria on the tooth-facing surface of the barrier membrane seemed to be particularly detrimental for periodontal regeneration.

In a very recent study by Yoshinari *et al.* (1998), using light microscopy the bacterial colonization on the ePTFE material was studied by dividing the membrane into vertical (coronal, middle and apical portions) and horizontal planes (outer, middle and inner portions). The majority of the bacteria were Gram-positive and the number of bacteria statistically decreased toward the apical portion in the vertical plane. There was a tendency for the bacteria to be observed in almost the same number in the outer and inner parts in the direction perpendicular to the long axis of the ePTFE membrane. These data indicated that bacteria grow downwards into and onto the unexposed portion of the

membrane. Mononuclear cell types of T lymphocytes, B lymphocytes and macrophages were seen in ePTFE membrane and in tissue adhering to them. The total number of cells statistically decreased towards the apical portion vertically and towards the inner part horizontally. Moreover, this study showed the negative influence of bacterial colonization of ePTFE membranes on the amount of gain in the probing attachment following the GTR procedure.

In summary, ePTFE barrier membranes could be colonized as early as three minutes after intra-oral manipulation. The microflora on these membranes were shown to be gram-negative rods particularly black-pigmenting rods and Fusobacterium. The presence of periodontopathogens can act as a negative predictor in the GTR procedure. The colonization of the microflora was found to be more detrimental if occurred on the tooth-facing side. It is advisable to remove all infective sites in the patients before commencing on GTR procedure to obtain the best outcome.

1.8.10 Application of Antibiotics in Relation to the GTR Treatment

It has been demonstrated that new attachment and bone formation were favoured considerably in teeth which were crown-resected and completely covered with the mucoperiosteal flap during the membrane insertion period in order to eliminate or minimize microbial colonization of the wound (Sander & Karring, 1995).

Newman's review paper (1993) stated that infection and bacterial complications associated with regenerative therapy can either occur pre-surgically, intra-operatively, immediate postoperatively or during short- and long-term healing. Potential sources of contamination must be eliminated by thorough root preparation and instruction in oral hygiene procedures pre-surgically. By doing this, the bacterial load could be reduced, thereby reducing inflammation and improving the tissue tone.

Intra-operative procedures appear to be important factors in determining whether or not the patient will get post-surgical infection (Newman & Fleming 1992; Fleming & Newman, 1990). Adequate root preparation, suture technique and material, flap design and manipulation and the use of particular regenerative materials are included in this variable.

Systemic antibiotic therapy is often prescribed, even though there is no specific antibiotic regime recommendations for regenerative procedures (Newman & Fleming, 1990; Topazian, 1992). Timing of administration of the drug is of utmost importance if the clinician decide to prescribe an antibiotic. A preferred timing would be to have the antibiotics consumed before surgical treatment begins (Newman & Fleming, 1992; Topazian, 1992; Classen *et al.*, 1992), thus bactericidal concentrations can be achieved at the time of surgery.

It is essentials that clot stability and a clean environment is maintained in the early post-surgical management of regenerative surgical wounds (Mombelli, Lang & Nyman, 1993). Minimal intervention immediately post-treatment will assist in the maturation of the clot. During this critical time, the most common treatment is to rely on topical antimicrobial (chlorohexidine) for reducing infection and inflammation. Another post-surgical complication is exposure of membranes during the healing phase. The material exposure into the oral cavity permits communication between the oral environment and the newly-forming tissues thus possibly leading to infection and/or induction of inflammation.

It is recommended that supportive periodontal treatment is increased in its frequency and additional adjunctive antimicrobial anti-plaque become standard during the maintenance of the regenerative tissues (Zablotsky & Meffert, 1991).

Studies on GTR treatment with additional use of antibiotics is difficult to evaluate due to the differences in 1) the AB that has been used, 2) the dosage,3) the time of starting the AB therapy, 4) the duration of taking AB, 5) time lapse after membrane placement and the sampling.

Systemic antibiotic treatment has been used in conjunction with GTR procedures to prevent infection and bacterial colonization of the membrane material. Various antibiotic regimes have been used by different investigators including AB given either prior to surgery only, prior to surgery and after surgery for a number of days or for a number of

days after surgery only. Localized application of an antibiotic during placement of the membrane has also been tested

The aim of a study by Demolon *et al.* (1993) was to determine whether the presence and development of specific target periodontal pathogens differed in subjects treated with a systemic antibiotic from subjects not treated with antibiotic following the placement of ePTFE membrane. A broad spectrum antibiotic, amoxycillin (given prior and after surgery) was only selected after information about microbial and clinical characteristics were known in subjects not treated with an antibiotic. In each patient, 2 sites were used for microbiological sampling; the test sites with the ePTFE membrane and a control site. The target microflora at control sites did not differ over time or between groups of patients. Even though there was no difference in total bacterial loads for both group in both sites from baseline to week 4, there was a significantly greater level of *P. intermedia* at week one and *F. nucleatum* at week one and week two in the non-antibiotic group. Also at week 4, the total bacterial load was significantly greater at the ePTFE site than at control sites and with significantly greater presence of *B. forsythus* at test site in the non-antibiotic group. A high level of *B. forsythus* was also found on all membranes in the non-antibiotic group at time of removal, while only low levels were found in two of the membranes in the antibiotic group. The use of amoxycillin reduced or prevented most of the target pathogens, but did not prevent the total Gram-negative flora from reaching the preoperative level. The results also suggested that the bactericidal effect on *P. gingivalis* and *B. forsythus* during the 10 days extended to the end of week four.

In a study by Nowzari *et al.* (1995b) a broad spectrum systemic AB, Augmentin was prescribed 1 hour pre-op and for 8 days following the procedure. The antibiotic group showed significant fewer organisms at the time of membrane removal at 4 to 6 weeks (52.2×10^6 vs. 488.6×10^6 log 10 CFU) and also exhibited a significantly higher mean CAL gain compared to control patients without antibiotic cover (36.5% of potential gain to CEJ vs. 22.4 %). The number of organisms recovered from the most apical part of the membrane surface facing the gingivae and the tooth did not differ in the two groups. *B. forsythus*, *P gingivalis* and *P. intermedia* were the microorganisms most frequently recovered from the control group. It was concluded that Augmentin seemed to augment healing when administered in association with the GTR procedure.

In a split-mouth design study (Sander *et al.*, 1994; Frandsen *et al.*, 1994) metronidazole gel (250 mg) was applied locally on the outer surface of the ePTFE membrane during placement and microbiological and clinical parameters were compared to a control site, i.e., membrane without local antibiotic. At the entry of the study no significant difference was observed in the subgingival microflora between test and control teeth with regard to total anaerobic counts, anaerobic/aerobic ratio, percentage of pockets harboring black-pigmented Gram-negative anaerobic rods *P. Gingivalis*, *P. intermedia*, *A. actinomycetemcomitans*, or *Staphylococci*, or proportions of these bacteria in the pockets. A statistically significant reduction in the median total anaerobic count between presurgical and one week post -surgery was observed in the test group while the control pockets showed an increase. However, after one week the values were very similar in

both groups all throughout the study. This indicated that the local effect of the metronidazole gel lasted only for 1 week. At 6 weeks when the membranes were retrieved they were found to be heavily colonized with bacteria. There was no significant difference in test and control for the anaerobic/aerobic ratio, even though, the control had a consistently higher ratio. After membrane removal, the ratio decreased below presurgical ratio. At the time of removal, the membranes harboured a microflora which was as anaerobic as the pocket flora before surgery. The relative gain in attachment level was significantly higher in the test group, even though the antibiotic effect only lasted a week, which might indicate that initial healing is one of the important aspects in GTR.

Another study on the use of systemic antibiotic in conjunction with ePTFE placement was carried out by De Sanctis *et al.* (1996b) using Augmentin 1g/day for 2 weeks after membrane placement. The SEM analysis results for the exposed against the non-exposed membranes were as follows: in the exposed membrane, all the fields in the coronally exposed portion were positive for bacterial colonization, while only 66% in the unexposed. In the mid-portion, 57% for exposed, 17% in the unexposed, in the apical portion, 19% in the exposed and negative for the non-exposed. The figures in the mid- and apical-portion in the exposed membranes were slightly higher compared to the figures in the exposed polyglicolactic membranes indicating that ePTFE material harbours more bacteria compared to polyglicolactic membranes even in the presence of systemic AB. Gain in probing attachment levels were negatively correlated to microbial colonization of the mid part of the membrane. The presence of microorganisms on the membrane could either be due to contamination of the barrier membrane during surgery

or represent true microbial colonization, as even in those cases where the barrier membrane had remained unexposed, microorganisms were found in the collar portion of the membrane. But in this study, it was ascertained that the microorganisms found were due to the microbiological colonization which could either arise from the gingival pocket or from residual microbial foci in other areas. The antibiotic regime in this study may not be sufficient to eliminate and/or prevent the recolonization of the pathogens.

Systemic use of amoxycillin (1g per day for 14 days after membrane placement) has also been used in relation to application of a bioresorbable membrane. Polyglicolactic barrier membranes if clinically exposed at 5 weeks underwent a second surgery to remove the barrier membranes (De Sanctis *et al.*, 1996a). The tooth facing surface was analyzed using scanning electron microscopy (SEM) which revealed that bacterial colonization occurred in the coronal portion of the exposed membrane. This indicated that the systemic antibiotic regime and local application of chlorhexidine did not prevent bacterial colonization of the exposed membrane material. In the mid-portion 41% of the field were positive, while no bacteria were observed in the most apical portion. Colonization of the mid-portion of the inner surface of the membrane is a critical factor, as bacteria-positive fields had less attachment gain (3.0 ± 0.5) compared to the bacteria-negative fields (3.8 ± 0.4) and this difference was statistically significant. The gain in attachment level was statistically greater at sites with clinically unexposed membranes and is comparable to the gain in the bacteria negative field of the mid-portion. The predominant microorganism morphotypes were cocci and short rods. Thus this study showed the importance of complete coverage of the membrane during healing. But,

caution has to be taken with this results as the removal of the membrane could cause trauma to the new tissue and could influence the healing process. Bacterial colonization of this membrane was observed to be of a magnitude similar to that previously observed in ePTFE (De Sanctis, *et al.,* 1996b).

In summary, none of the antibiotic regimes reported above has been successful in complete prevention of the colonization of the membrane, though giving antibiotic prior to and after the membrane placement gave better overall results in respect to the clinical results and the amount of colonization that occurred (Nowzari *et al.,* 1995b). De Sanctis *et al.* (1996a; b) has demonstrated that ePTFE membrane seem to harbour more bacteria than the polyglicolactic membrane even with systemic antibiotic given after the placement of membrane.

1.8.11 MMP and the GTR Procedure

The success of GTR depends on the restoration of lost tissue and the maintenance and preservation of the new tissue following regeneration. Proteases may play an important role in the outcome of the bone regenerative process especially during the initial remodeling phase when bone stroma has been regenerated but is not be fully mature and mineralized. In this phase of regeneration, proteases may degrade the newly formed extracellular matrix and may be a major factor in determining the clinical result.

Only a few studies have investigated MMPs in relation to GTR procedures. When the MMPs produced by cultured cells derived from the ePTFE membrane were compared to the MMP produced by cultured periodontal ligament and fibroblasts gingival cells, the latter cells expressed MMP-1, MMP-2, MMP-3, and TIMP-1 (Grosso *et al.*, 1997). Cells isolates from ePTFE membrane from GTR procedures expressed MMP-1 (Wakabayashi *et al.*, 1996), MMP-2, TIMP-1 (in a less amount compared to the periodontal ligament cells and fibroblasts cells), and low molecular proteases below 52 kDa. When clinically failed GTR cases were compared to successful GTR cases, the former produced multiple MMPs including low molecular weight MMPs which were usually not typical of either human gingival fibroblasts or periodontal ligament cells, in addition to MMP-2 and MMP-1. Successful cases were shown to produced MMP-1 and MMP-2 only. Therefore in this study, the best correlation is between the expression of multiple proteinases in cells and clinical failure of the subsequent regenerative procedure.

Wakabayashi *et al.* (1996) compared protease profiles of gingival fibroblast, keratinocytes and periodontal ligament cells retrieved from ePTFE membrane with those of human gingival fibroblasts, keratinocytes and periodontal ligament cells. All cell types except keratinocytes produced 72kD protease (MMP-2). In contrast, only keratinocytes produced 92 kD (MMP-9) gelatinase. All cell lines produced TIMP-1. All cell lines retrieved from the barrier membrane and periodontal ligament cells produced MMP-1.

1.9 AIMS OF THE STUDY

It has previously been established that healing following the guided tissue regeneration procedure is compromised if bacterial contamination of the membrane material occurred either during surgery or during the postoperative phase (Gottlow *et al.*, 1986; Demolon *et al.*, 1993). Sequentially, some investigators had administered systemic antibiotics to patients during the first 2 to 4 weeks after membrane insertion to prevent postoperative wound infection (Becker *et al.*, 1988; Demolon *et al.*, 1993; Cortellini *et al.*, 1990; Schallhorn & McClain, 1988; Proestakis *et al.*, 1992; Kersten *et al.*, 1992). Despite the systemic antibiotics administration, occurrence of postoperative wound infection and abscess formation related to implanted barrier membranes have been noticed (Cortellini *et al.*, 1990; Schallhorn & McClain, 1988). The reason may be either the administered antibiotic is not directed against the microorganisms responsible for the wound infection or that the drug does not reach a concentration sufficiently high enough at the infected site. This finding has lead to investigations of the use of a local antibiotic (Elyzol dental gel 25%) applied during insertion of ePTFE (Gore Tex®) membranes to obtain a high local concentration of an adequate drug in the healing wound. Clinical results from a study carried out by Sander *et al.* (1994) demonstrated that the gain in probing attachment levels as a percentage of the initial defect depth was statistically significantly higher in sites treated with ePTFE membrane and locally applied antibiotic than in the control sites inserted with ePTFe membrane alone (92% for test defects and 50% for control defects). Microbiologically, a reduced median number of cultivable bacteria

from the presurgical examination was sustained until one week following the insertion, whereas an increase was noticed in the same period in the control group.

Microbial contamination of teflon membranes is a common finding due to the lack of tissue integration between the gingival flap covering the material and the outer surface of the membrane (Nowzari *et al.*, 1995b; Selvig *et al.*, 1992; De Sanctis1996b). This is primarily caused by apical proliferation of the epithelium on the inner aspect of the covering tissue flap thus forming a "pocket" thereby exposing the material to the oral environment. Many bacteria preferentially colonize the material on both the inner and outer surfaces (Grevstad & Leknes 1993; Selvig *et al.* 1990).

A bioresorable membrane (Guidor®) has been designed so that tissue integration is possible between the outer layer of the barrier matrix and the covering gingival connective tissue flap. It can thus be anticipated that since there is no epithelial downgrowth there is little or no postoperative microbiological colonization of the healing wound and the membrane material. It is important to prevent and reduce contamination of the barrier membrane at the time of insertion thus providing optimal conditions for periodontal regeneration to take place. In preliminary microbiological studies on resorbable barrier matrices it has been shown that facultative anaerobic bacteria and black pigmented gram-negative anaerobic rods do not seem to develop in number in the first 6 weeks postoperatively, thus metronidazole appears to be of limited use (personal communication, L.Sander, Aarhus Royal Dental College). An antibiotic selective for an aerobe and facultative anaerobic microflora may reduce the possible contamination of the

material and the wound during membrane insertion. It can be hypothesized that one presurgical application of a high dose of penicillin could influence the healing.

Amoxycillin, a broad-spectrum penicillin was used in the present study. It is a derivative of ampicillin, and is active against certain Gram-positive and Gram-negative organisms. It is better absorbed than ampicillin when given orally and absorption is not affected by the presence of food in the stomach. The dosage used is 3g given 1 hour pre-operatively.

MMPs are degradative proteolytic enzymes responsible for remodeling and degradation of ECM components. The predominant ECM component of the periodontium is collagen. During the inflammatory process of the periodontium, collagenous breakdown is taking place. MMP-3 levels had been shown to be higher at gingivitis and periodontitis sites than healthy sites (Haerian *et al.*, 1995) and active MMP-8 levels were higher in progressive periodontal sites (Lee *et al.*, 1995). In repair processes MMP-3 had been demonstrated to be produced in both acute and chronic wounds (Saarialho-Kere *et al.*, 1994; Vaalaamo et al., 1996) and had been associated with scarless healing in fetal skin (Bullard *et al.*, 1997). During the healing process the PMN and fibroblast cells increased in numbers. These cells are responsible for the production of MMP-3 and -8.

The purpose of the study was to investigate the clinical outcome, the microbiological colonization and the levels of matrix metalloproteinases (MMP-3 & -8) of the wound healing following insertion of a resorbable membrane (GUIDOR®) either alone or combined with one presurgical systemic application of amoxycillin. The clinical and

microbiological outcomes were also investigated against the levels of the MMPs to see whether the fluctuation of the MMPs level could be linked to the clinical outcome and the microbiological colonization.

CHAPTER 2

MATERIALS AND METHODS

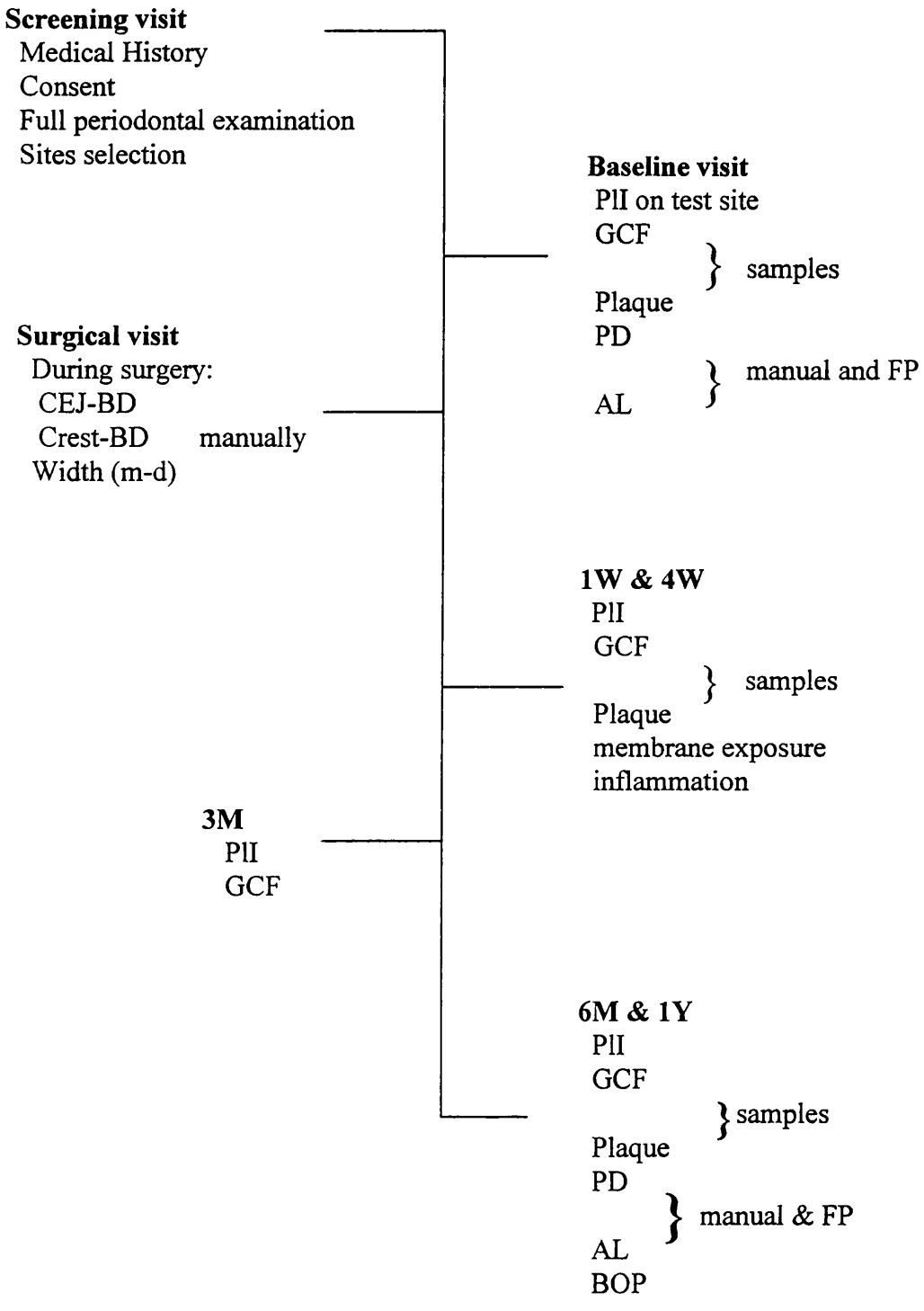
2.1 SUBJECTS

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

Patients with a history of systemic disease, likely to influence periodontal health, and patients taking medicines which affect the periodontal tissues, were excluded. Antibiotic usage 3 months prior to the baseline visit was a further exclusion criterion. Previous allergic reactions towards penicillin also lead to exclusion.

Thirty-eight patients attending the Periodontal Clinic of the Glasgow Dental Hospital agreed to participate in this study. The study population consisted of patients who had been undergoing hygiene phase therapy (HPT) including OHI and scaling and root planing, and possibly periodontal surgery but still had at least one deep infrabony pocket of $\geq$ 6mm in association with a radiographically distinct 2 or 3 wall infrabony defect with a depth of at least 4 mm measured on intraoral radiographs as the distance from the alveolar crest to the bottom of the defect. Of these subjects, 6 patients dropped-out of the study before the six months. Of the 32 left, 19 were female and 13 were male with a mean age of 39.2 ranging from 23-66 years old. 20 subjects were categorized as early-onset periodontitis patients, and 12 subjects as adult periodontitis patients. The patients were divided into treatment group AB (membrane and antibiotic), consisting of 14

Figure 2.1 Study design



Microbiological sample was obtained using sterilized size 40 paper points from the experimental site and from a contralateral or opposing site (control) with a deep pocket of $\geq$ 6 mm and BOP. These control sites were monitored all throughout the study by checking the PD, BOP and the AL every three monthly. Any sites with a loss of $\geq$ 2mm attachment or bleeding on probing would be root planed after sampling.

At baseline visit, the following measurements were taken in the following order:

1) Plaque Index (PlI) (Silness and Löe, 1964) was recorded at the six sites of the experimental tooth.
2) GCF samples were taken from the test site, surgical control site from an adjacent tooth and a healthy control site and the GCF volumes were recorded with the Periotron.
3) Microbiological samples were taken from the test site and a contralateral or opposing site with a deep pocket.
4) The first set of pocket depth (PD) measurements was performed with the electronic pressure sensitive probe (Florida probe) at six sites of the experimental tooth.
5) Absence or presence of bleeding on probing scores was recorded at the six sites of the experimental tooth.
6) The first set of relative attachment levels (RAL) measurements was performed with the electronic pressure sensitive probe (Florida probe) at six sites of the experimental tooth.
7) The second set of PD and RAL measurements (Florida probe) were carried out.

8) A set of PD and CAL measurements was carried out manually using the PCP-12 probe.

The patients were stratified according to age, tooth type, number of infrabony walls, depth of infrabony defects and smoking status. Each infrabony defect was randomly placed either in treatment group AB: application of the matrix barrier (Guidor®) combined with one presurgical systemic application of amoxycillin (3g one hour preoperatively) or treatment group NAB: application of the matrix barrier alone. There were only 3 smokers in the whole study group.

2.3 CLINICAL MEASUREMENTS

One examiner (SS), carried out all the clinical measurements using the Florida probe throughout the study, while another examiner (LS) performed all the manual measurements in order to allow non-biased measurements. The computer screen where the PD & RAL measurements using the Florida probe was displayed was turned away from the operator to allow 'blind' measurements. In addition, measurements were recorded on paper by another examiner. The plaque index (PlI), PD, BOP and AL parameters were recorded for the patients at baseline, 6-month and 1 year post-surgical. GCF samples were taken at baseline, week 1, week 4, 3 months and 12 months, while microbiology samples were taken at baseline week 1, week 4, 6 months and one year. Clinical measurements on the test tooth were again performed at 6 months and one year. The order in which the sampling and the measurements were performed has been

previously explained.

2.3.1 Plaque Index

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

0 No plaque in the gingival area.

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

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

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

2.3.2 The Gingival Crevicular Fluid Sampling Method

The GCF samples were taken after the PlI scores but before any other clinical recordings which could cause tissue irritation and serum contamination of the sample. The site to be sampled was isolated with cotton wool rolls and supragingival plaque was carefully removed. The region was dried with a gentle air stream and 30s later GCF was collected

with a Whatman grade 4 paper strips (Whatman Labsales Ltd., Maidstone, Kent) measuring 2 x13 mm (Griffith, Chatis & Wilton, 1988). The paper strip was inserted into the crevice until mild resistance was felt and left there for 30 seconds (Figure 2.2). Strips visually contaminated with saliva or blood were discarded. The sampling method is reliable and causes no significant disturbance of the gingival blood vessels (Gustafsson *et al.,* 1992). The fluid volume on the strip was measured immediately after sampling with a Periotron 6000 (Figure 2.3) (Harco Electronic, Winnipeg, Canada), and the strips were then placed in individual sterile tubes and stored at -30 degree centigrade (°C) until further processing was carried out. The jaws of the periotron were wiped with pure methanol and then dried between readings. Subsequently the strips were eluted into 500 microlitres (µl) of incubation buffer at room temperature using a rotary mixture. The strips were then discarded and the elute were aliquoted into sterile tubes and stores at -30°C. These aliquots were subsequently analyzed for the quantification of SL (MMP-3) and MMP-8.

2.3.3 Microbiological Sampling and Processing

Microbiological sampling was performed at two sites, that is from the test site (membrane treated site) and from a control site in a different quadrant with a PD $\geq$ 6 mm and BOP. These samples were taken at baseline, W1, W4, 6 months and 1 year post-surgical. The sampling sites were isolated with cotton wool rolls and supragingival plaque removed carefully. The region was then dried with a gentle stream of air. Three

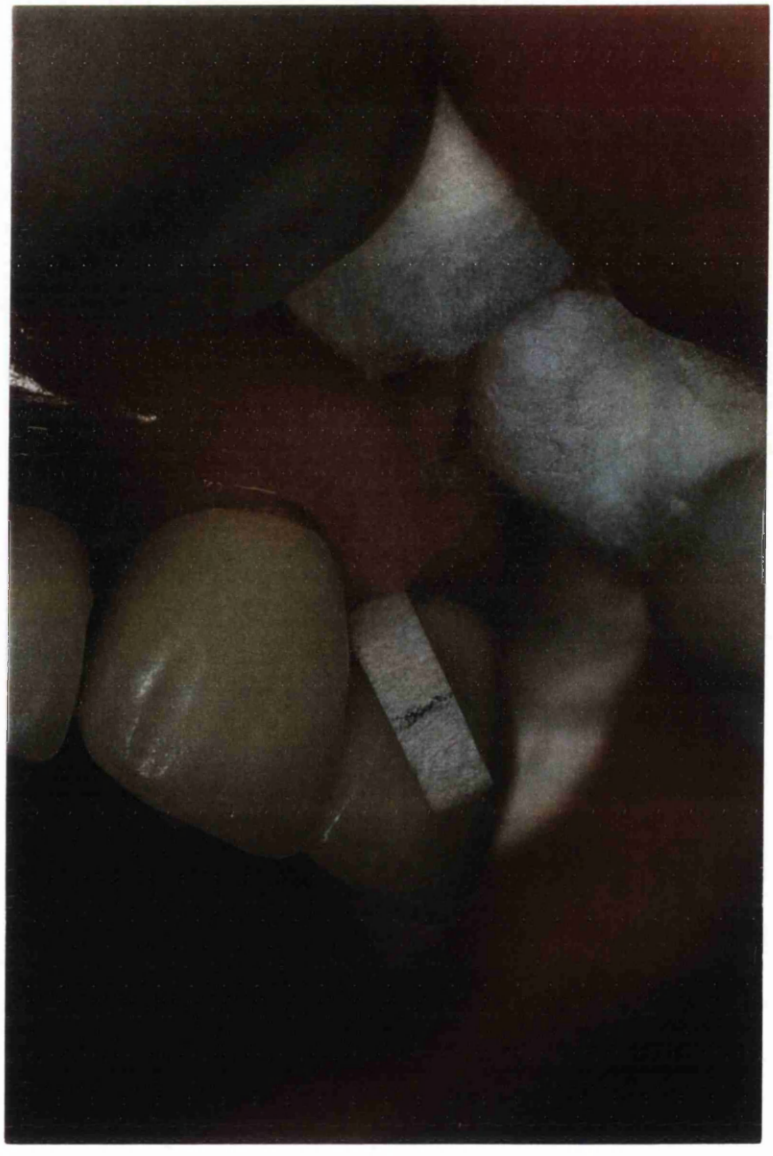


Figure 2.2 Gingival crevicular fluid sampling with a paper strip

Figure 2.3 The Periotron 6000

sterilized size 40 paper points were inserted in the crevice for 30 seconds. The paper points were immediately placed in 0.5ml of pre-reduced FAB (Lab M, Bury, England) and transported to the lab within 30 minutes of collection. In the laboratory samples were vortex mixed for 30 seconds and serial dilution made in FAB from neat to 10^{-4}. Appropriate dilutions were inoculated using a spiral plater (Don-Whitely, Shipley, UK) onto the following media;

- Columbia agar (Pro-lab diagnostics, Cheshire, UK) containing 7.5 % v/v sterile defibrinated horse blood and 1% v/v vitamin K/haemin (Life Technologies, Paisley, UK) dilutions 10^{-4} and 10^{-3} and incubated at 37°C in a CO_2 incubator (5% CO_2 and 95% air) for 48 hours for total aerobic count and *Capnocytophaga* species. Additional plates were incubated anaerobically in an atmosphere of nitrogen, hydrogen and carbon dioxide (80:10;10 by volume) for 7 days for detection of *P. gingivalis, P. intermedia, E. corrodens, Fusobacterium* species, anaerobic *streptococci* and the total anaerobic count.
- Actinobacillus agar (neat and 10^{-1} dilutions) consisting of Tryptic Soy Bacitracin Vancomycin (TSBV) agar (Gibco BRL, Life Technologies, Paisley, Scotland) and supplemented with sterile 105 horse serum (v/v) (Gibco BRL, Life Technologies, Paisley, Scotland) and incubated in a CO_2 incubator for three days for detection of *A. actinomycetemcomitans; and*
- Mitis Salivarius agar (dilutions 10^{-2} and 10^{-1}) (Difco, Surrey, UK) containing 20 units/ml bacitracin (Sigma) and incubated in CO_2 incubator for three days for total Streptococcal counts.

For the detection of amoxycillin resistant colonies a 5μg amoxyl disc was placed on a Columbia blood agar plate inoculated with neat sample and resistant colonies were picked off and identified.

Identification of predominant colonies

Following incubation each plate was processed by the predominant cultivable flora technique. Briefly, this consisted of ten fold dilutions of the specimen prepared in reduced transport medium and appropriate dilutions, as well as neat specimen, plated out in duplicate on Columbia agar plates containing 7.5% v/v sterile defibrinated horse blood and 1% vitamin K/haemin. Duplicates plates were incubated anaerobically and air plus 10% CO_2 at 37°C for 7 days. Following incubation the plates were examined and twenty colonies were selected at random from one of the anaerobe plates and identification attempted to species level. The isolates were sub-cultured for purity, the Gram stain and the atmospheric requirements determined by incubation in air, air plus 10% CO_2 and anaerobically. Identification was carried out as outlined below.

Identification of microbial isolates

Where possible, a minimum of eight black-pigmented colonies were sub-cultured from each positive sample on Columbia agar plates. Subsequently colonies were stained by Gram's method; the atmospheric requirements for growth determined and where

appropriate, identification was completed with the API 20A strips (API- Biomeriux, Basingstoke, UK).

P. gingivalis

Isolates that were strictly anaerobic Gram-negative coccobacilli which produced black-pigmented colonies, an acceptable biochemical profile on the API 20A strip and the ability to agglutinate sheep erythrocytes (Slots & Genco 1979) were identified as *P. gingivalis.*

P. intermedia

Isolates that were strictly anaerobic Gram-negative bacilli which produced black-pigmented colonies and an acceptable biochemical profile on the API 20A strip and agglutinated sheep were identified as *P. intermedia.*

E. corrodens

Isolates that were strictly anaerobic Gram-negative bacilli which produced pitting, sensitive to metronidazole and urease positive were identified as *E. corrodens.*

Fusobacterium species

Isolates that were strictly anaerobic Gram-negative bacilli and an acceptable biochemical profile on the API 20A were identified as *Fusobacterium* species.

Anaerobic *streptococci*

Isolates that were strictly anaerobic Gram-positive cocci and sensitive to metronidazole were identified as anaerobic streptococci.

Capnocytophaga species

Isolates that had a characteristic colony morphology (flat spreading), Gram negative rod and growth on $C0_2$ incubated plates were identified as Capnocytophaga species.

A. actinomycetemcomitans

After incubation for three days, the TSBV agar plates were examined and a minimum of four colonies exhibiting morphology characteristics of *A actinomycetemcomitans* were subcultured to Columbia agar plates (Gibco, BRL. Life Technologies, Paisley, Scotland) supplemented with 7.5% v/v sterile defibrinated horse blood in a CO_2 incubator. Colonies which consisted of capnophilic, catalase positive, Gram-negative coccobacilli that fermented glucose and maltose but not lactose, sucrose, salicin or arabinose, were recorded as *A. actinomycetemcomitans.*

2.3.4 Probing Depth and Attachment Levels Assessments

In this study pocket depth (PD) and relative attachment level (RAL) were recorded to the nearest 0.2 mm using the Florida probes (Florida probe Corporation, Florida, USA). The PD and AL were also measured manually using the PCP-12 probe. Another examiner (LS) did these manual measurements. Gibbs *et al.* (1988) developed the Florida Probe system, which features constant probing force, precise electronic measurement to 0.2 mm, computerized data capturing, and sterilization of all system parts entering or close to the mouth. The system consists of a probe handpiece, a digital read-out, foot switch, computer interface and computer (Figure 2.4). The probe tip reciprocates through a sleeve and is connected to a movable arm, which transfers the movement to a transducer with a digital read-out. The edge of the sleeve, which is 0.6mm in radius, is the reference from which measurements are recorded. The probe tip is 0.4mm in diameter and the

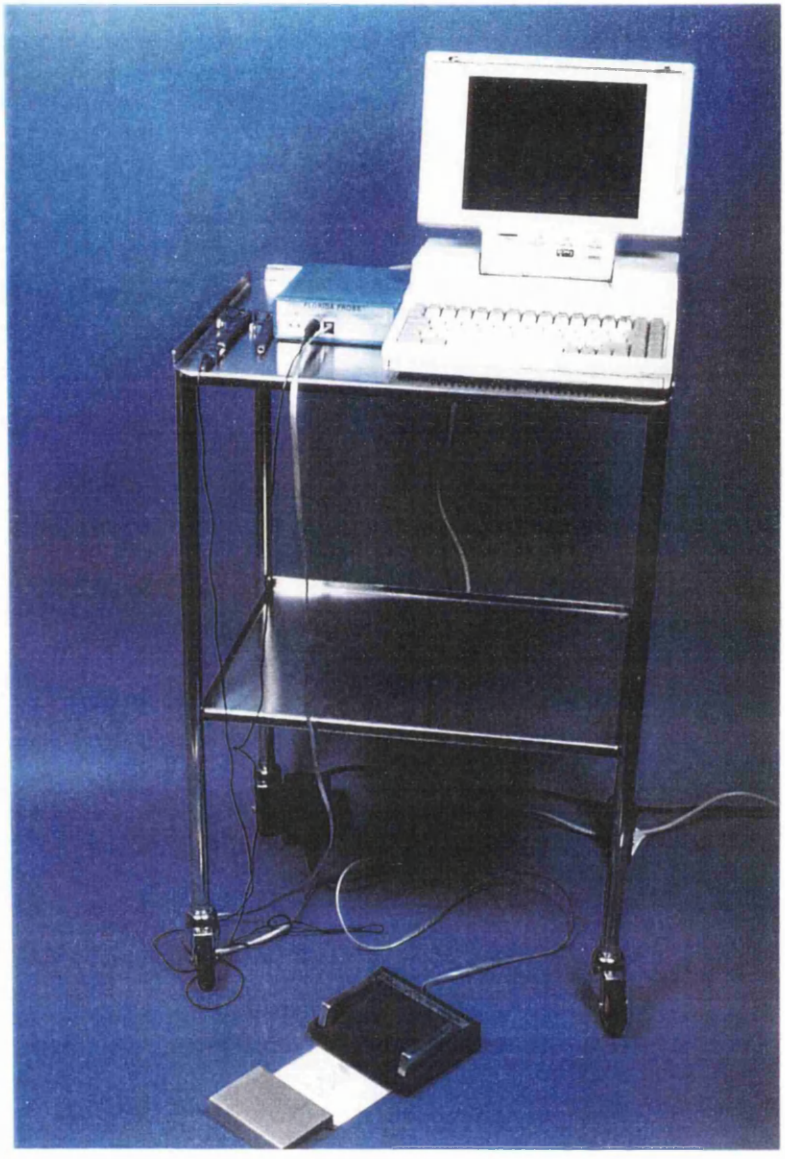


Figure 2.4 The Florida probe system

probing force is usually set at 0.25 N (1.99 N/mm^2). The actual measurement of pocket depth (or relative attachment level) is made electronically and transferred to the computer when the foot switch is pressed.

Besides the pocket depth probe, the disk probe is also available (Fig 2.5). The disc probe has a small metal disc attached to the sleeve and uses the occlusal surface or incisal edge of a tooth as a reference for relative attachment level measurement. In this study the pocket depths measurements were taken using the manual probe as well as the Florida probe. Manual probing was also used to obtain attachment levels measurement from the CEJ, while a relative attachment level was obtained with the disc Florida probe. Duplicate reading of each test sites was taken with the Florida Probe as high levels of reproducibility in the measurements could be achieved as shown by Mullally and Linden (1994).

The Florida probe records clinical pocket depth as the probe sleeve contacts the gingival margin once the probe has passed into the pocket and its downward passage has been stopped at the clinical base of the pocket with its probing force of 25g. Care was taken to ensure that the probe was held parallel to the long axis of the tooth and the probe should be placed as further towards the interproximal point as the sleeve would go.

Option-3 method (Clark *et al.*, 1993) was employed for the PD and AL measurements with the Florida probe. This method required two measurements to be taken; the difference of which should not be greater than 1mm (the threshold). If the difference was

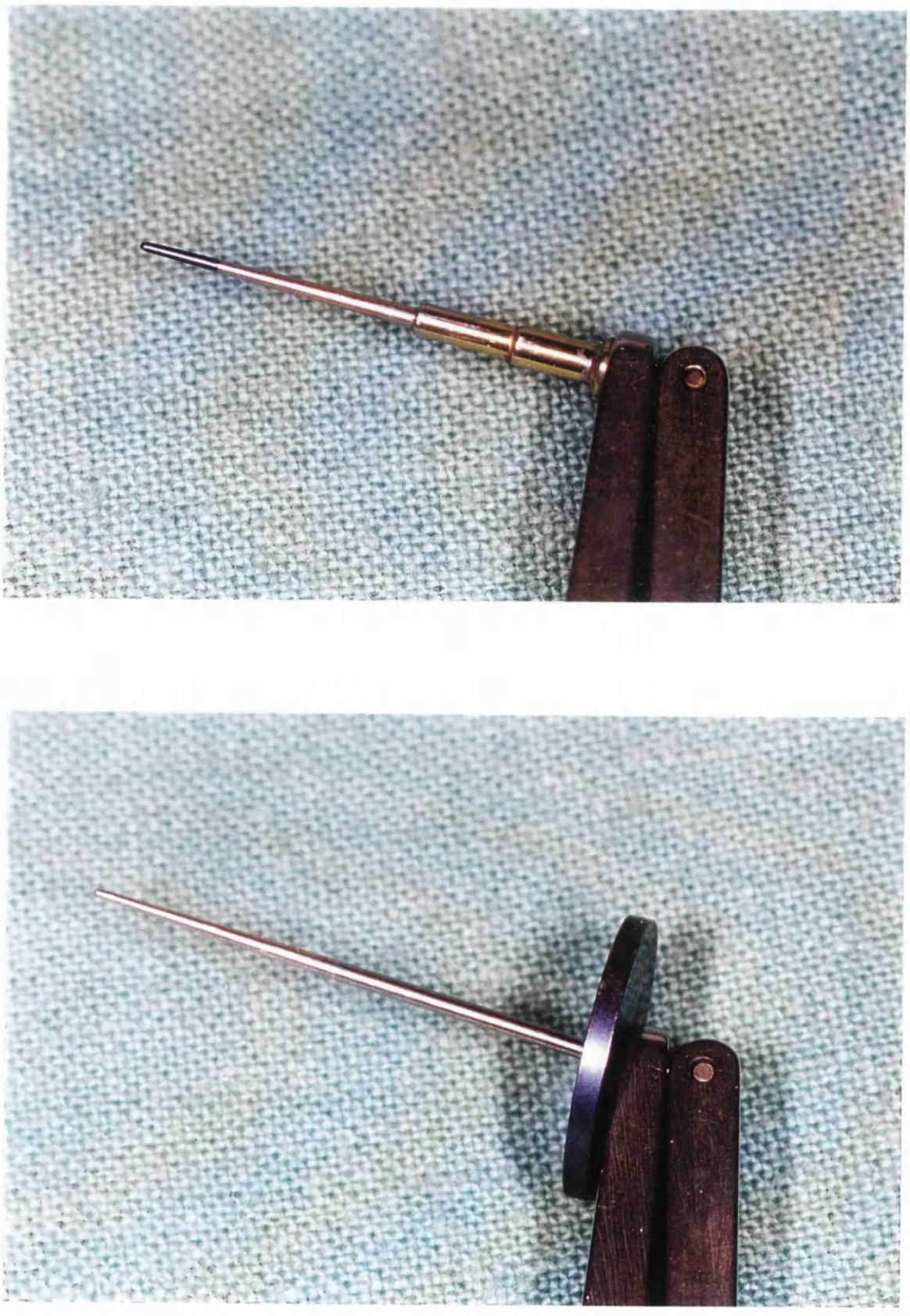


Figure 2.5 The Florida pocket probe (top) and the Florida disc probe (bottom)

more than 1 mm, recording of one or more additional measurements were taken until the difference between two measurements was less 1mm. After the first measurement of the PD, the BOP were assessed and recorded. The manual PD and AL were done last.

2.4 SURGICAL TREATMENT

A single operator (LS), performed all the surgical treatment throughout the study. Patients in treatment group AB were given 3 g of amoxycillin orally one-hour pre-operatively. Neither the patients nor the observers were blinded to the patients' grouping. After local anaesthesia (Xylocaine 2% with adrenaline 1:80000), intracrevicular incisions were made and buccal and lingual/palatal full mucoperiosteal flaps were raised and the defect exposed. A vertical releasing incision was placed when needed for optimal access to the defect and to facilitate coronal repositioning of the flap. Also, it was aimed to preserve the interproximal papillae as it is important to ensure future interproximal coverage of the barrier matrix (GUIDOR®).

The defects were carefully debrided and root surfaces scaled and planed using hand instruments. Granulation tissue and epithelium on the inner aspect of the flap were removed and the surgical area irrigated with sterile saline. The configuration of the defects were then measured and recorded. The depth of the defects from bottom of the infrabony defects to the bone crest (crest-BD) and to the CEJ (CEJ-BD) and the width of

the defects (mesio-distally) were measured with a University of North Carolina probe with 1 mm graduations.

Before handling the barrier membrane, a resorbable membrane (GUIDOR®), new pairs of gloves were put on by the operator and assistant to prevent contamination of the membrane. Guidor® matrix barrier is provided in a range of configurations, but only one configuration (molar curved) was used for this study (Figure 2.6). This configuration were trimmed to the required shape and dimension to cover the defect and the exposed root surface. Before placing the membrane on the defect, it was ascertained that the side facing the gingiva had the larger rectangular perforations for connective tissue integration and the side facing the root had the smaller circular spacers. The membrane was then attached to the tooth with the ligature, preplaced in the device. A horizontal releasing incision through the periosteum on the inner aspect of the flap was then performed to facilitate coronal displacement of the flap in order to cover the barrier material. The flaps were coronally repositioned and sutured interdentally with a modified vertical mattress suturing technique. The suture used was Gore-tex (ePTFE) suture which is non-resorbable and maintains its function for 2-4 weeks. The sutures on the infrabony site were left for 4-weeks before removal.

Figure 2.6 The Guidor® matrix barrier in molar-curved configuration

2.4.1 Post Surgical Period

The patients refrained from mechanical toothbrushing for a period of 6 weeks, postoperatively, in the area where surgery had been performed. It is of utmost importance for the success of the procedure that the area is kept free from plaque accumulation and care should be taken not to disrupt the wound by mechanical means. During this period, plaque control was accomplished by rinsing with 0.2 percent chlorhexidine solution twice daily, and the patients were seen 1-week and 4-week post-surgery. After six weeks of healing, tooth brushing and interproximal cleaning were resumed. Plaque control, including supragingival professional tooth-cleaning, was continuously monitored on an individual basis until three months after surgery. Thereafter the patients were seen every third month until the final examination 12 month after surgery.

Some gingival recession and matrix exposure could occur during the initial healing process. But since the external layer of GUIDOR® is designed to integrate with the surrounding tissues, matrix exposure does not imply a risk of continued epithelium downgrowth, pocket formation and infection. Exposed matrix material was not trimmed because of the possibility of disrupting tissue that has migrated into the matrix. It disappeared 6 to 8 weeks after surgery because of combined resorption and attrition. The patients were instructed to keep the exposed matrix clean by using chlorexidine rinse until the exposed material has disappeared.

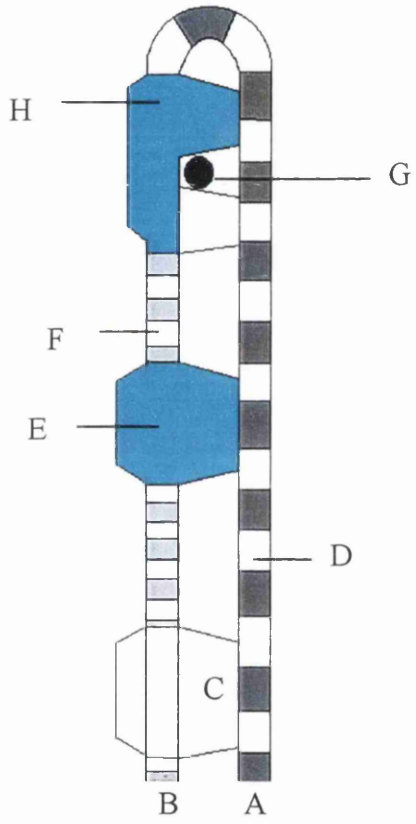
During the post-surgical visits at one week and four weeks, the occurrence of soft tissue inflammation and device exposure were recorded.

2.5 THE GUIDOR® BARRIER MEMBRANE

The Guidor® membrane is a bioresorbable matrix barrier designed to achieve a single-step GTR therapy. The Guidor® membrane is made from a bioresorbable polylactic acid (a blend of poly-D,L-lactide and poly-L-lactide) blended with a citric acid ester, acetylbutyl citrate (ATBC). The Guidor® matrix has to be stored at a temperature between 36° and 46° F (2° to 8° C). At low temperature the material is fairly stiff and almost brittle, but it softens at room temperature and can be trimmed. When placed in the oral cavity, it becomes malleable within seconds and easy to adapt to the tooth and bone surfaces. It has a double-layered matrix with two different perforated layers (Figure 2.7):

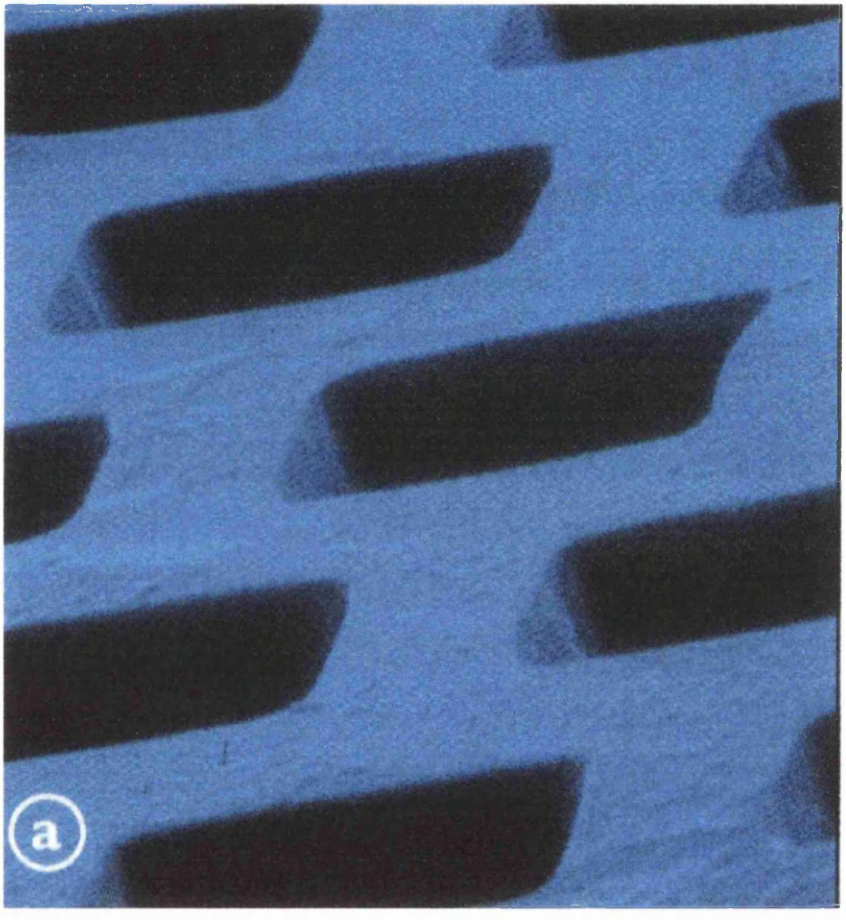
- an external layer facing the gingiva has proportionally large rectangular perforations (Figure 2.8a) to enable gingival connective tissue to penetrate quickly into the matrix during initial healing thus preventing downgrowth of gingival epithelium. This is designed to minimize gingival recession, device exposure, and pocket formation, which in turn, reduce the risk of infection and inflammation.
- an internal layer facing the tooth which has many minute circular perforations - as many as several thousand per cm^2 (Figure 2.8b). The small size of the perforations is designed to retard tissue penetration but still allow for nutrition

Figure 2.7 Illustration of double-layered matrix of GUIDOR®



A = external layer facing the gingival tissue
B = internal layer facing the tooth
C = internal spacers between the two layers, into which tissue can migrate
D = rectangular perforations on the external layer (400-500 per cm^2)
E = outer spacers on the root side of the matrix which form part of internal layer
F = smaller circular perforations on the inner layer (4000-5000 per cm^2)
G = bioresorbable ligature is placed on the coronal portion
H = a bar at the top of the internal layer which function as a seal between the matrix and the tooth

Figure 2.8(a-b). SEM illustration of Guidor® matrix barrier



a) Large rectangular perforations in the outer layer

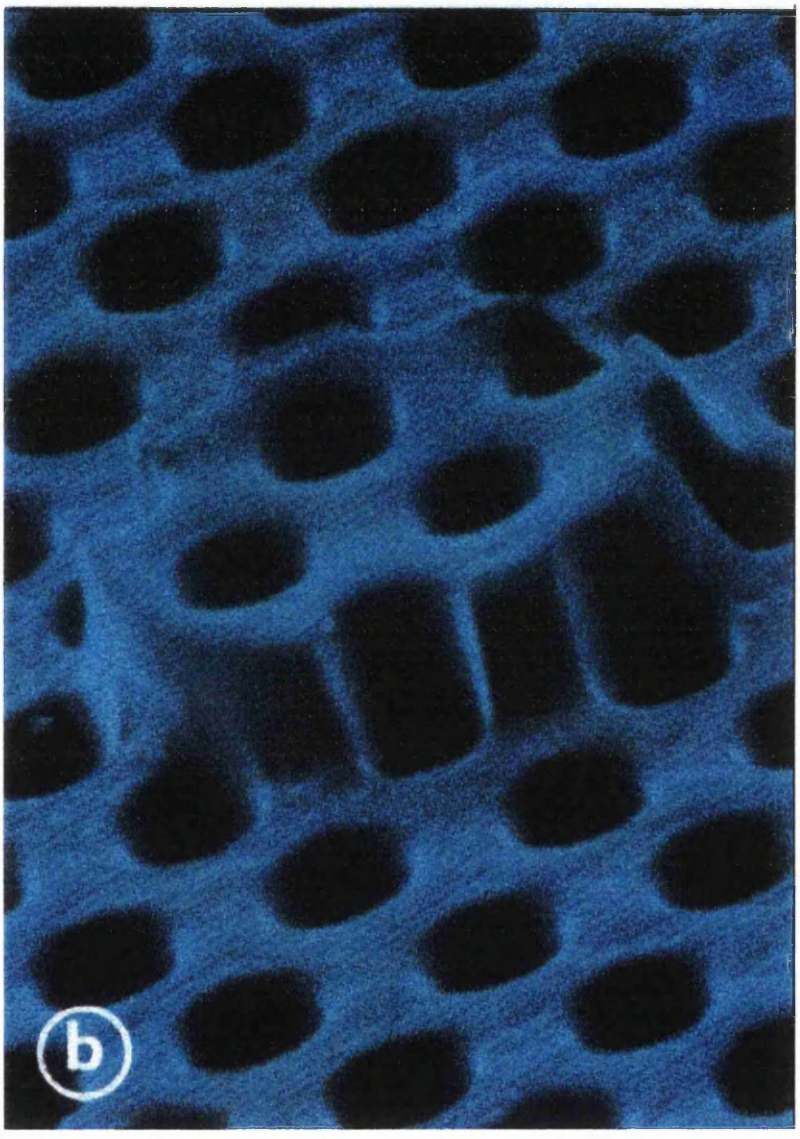


Figure 2.8b Inner layer with small perforations and spacers

Many inner spacers separate the two layers to form an interspace into which the tissue can grow. The internal layer has outerspacers projecting on the root side to ensure space for natural coronal growth of periodontal ligament in areas where the matrix run close to the root. The matrix has a coronal portion in which a ligature is preplaced.

The material is designed to maintain a barrier function for a minimum of 6 weeks. During this period, both the design of the matrix and the stability of the polymer are maintained. Afterwards, the matrix slowly resorbs and is gradually replaced by periodontal tissue. The process of degradation is hydrolysis resulting in lactic acid which is absorbed by the surroundings tissue. The polymer fragments are eliminated by macrophages and the end degradation products are ultimately metabolized to water and carbon dioxide (Figure 2.9).

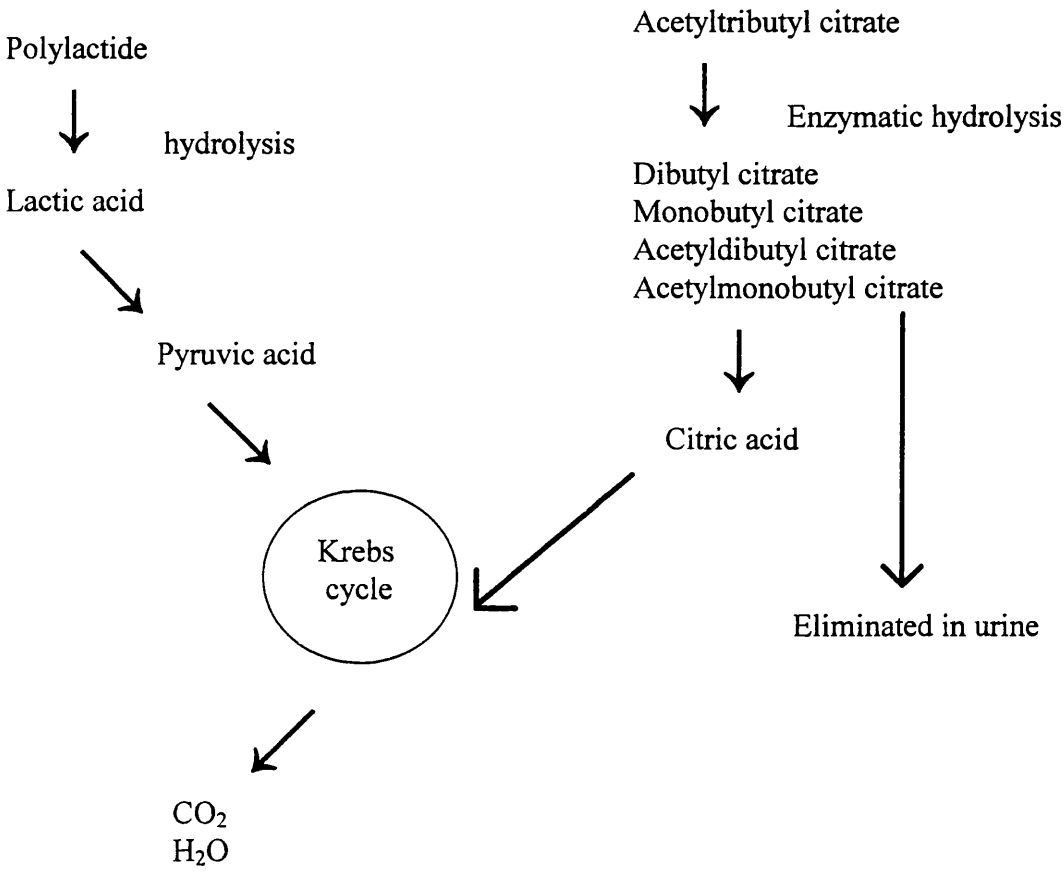
2.6 EXPERIMENTAL MATERIALS

2.6.1 Whatman Grade 4 Paper Strips

Rubber gloves were worn during the manual preparation of the paper strips in order to avoid contamination by substances from the operators hands. Whatman grade 4 (Whatman Labsales Ltd., Maidstone, Kent) paper were used for the collection of GCF, and are described by the manufacturer as a thin pure cellulose paper with an average thickness of 0.21 mm. The paper were cut manually to a standard size, 13 mm in length

Figure 2.9

The metabolism of the polylactides and ATBC



and 2 mm wide, using a steel ruler and scalpel as recommended by previous investigators (Griffith *et al.,* 1988). A line was drawn on each strip at 8 mm, indicating the length of paper strip to be inserted between the Periotron jaws and which was the part of the strip to be used for GCF sampling in the crevice. The strips were then placed in glass universal bottles for autoclaving and storage.

2.6.2 ELISA Buffers and Reagents

The buffers employed were as followings:

1) Coating buffer (CB)

Carbonate-bicarbonate buffer was prepared by dissolving 1.59g Na_2CO_3, 2.93g $NaHCO_3$ in 800 ml distilled H_2O. The pH was adjusted to 9.6 at just under 1 litre, with 1M HCl before making up to 1 litre in a volumetric flask. It was stored in a sterilised bottle at 4°C for a maximum of 1 week.

2) Incubation buffer (IB)

8g NaCl, 0.2g KH_2PO_4, 1.44g Na_2HPO_4. $2H_2O$, 0.2g Kcl, 0.5g Tween 20 (SIGMA chemical company Ltd., Poole, Dorset) was dissolved in 800 ml of distilled H_2O, and made up to 1 litre. 1g of lyophilised bovine serum albumin was added (BSA) (SIGMA, St. Louis, USA). This was layered on the surface without mixing. It was stored at 4°C for a maximum of 1 week (pH 7.4).

3) Wash buffer (PBST)

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

4) Phosphate buffered saline (PBS)

PBS was prepared by dissolving 8g NaCl, 0.2g NaCl, 0.2g KH_2PO_4, 0.2g KCl, 1.44g NaH_2PO_4 $2H_2O$ up to 1 litre of distilled water (pH 7.4).

2.6.3 Antibodies and Purified Standards for ELISAs

The antibodies which were used in the ELISA (enzyme-linked immunoabsorbent assay) included: Mac 78 anti-stromelysin monoclonal antibody and rabbit anti-stromelysin polyclonal antibody, and purified recombinant human stromelysin (SL) were donated by Cell Tech. Ltd. Slough, UK. Donkey anti rabbit-HRP conjugated IgG was purchased from Jackson Immuno-research Laboratories, Avondale, PA, USA.

2.7 EXPERIMENTAL METHODS

2.7.1 Calibration of the Periotron 6000 for Use in Clinical Trials

In order to transform the Periotron digital readings for each paper strip into volumes, and also to verify the accuracy of the instrument the following procedure was carried out. First the blank filter paper strip was placed between the jaws of the machine and the reading dial was set to zero. Known volumes of PBS plus serum (in equivalent volume) were delivered to Whatman grade 4 paper strip with a Hamilton microsyringe at a range of volumes (0.05-1 μl) and Periotron readings taken. The reciprocal jaws were cleaned with pure methanol and dried before the filter paper strips was inserted between its jaws. Each measurement was performed 3 times. 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 the GCF collected. Using linear regression analysis, the points plotted were resolved into two lines. The first line being determined by fluid volumes from 0 to 400 nl, and the second line by fluid volumes from 400 to 1000 nl (Figure 2.10). The calibration was performed in the periodontal clinic each time the samples were taken, in order to reduce variability and thus improve accuracy. Determination of actual GCF volume from Periotron indications was performed from a given Periotron calibration curve using the following equation, $Y = mx + c$, where Y = periotron readings, m = gradient of the slope, x the unknown volume of GCF in μl and c is the intercept (Figure 2.11). If a sample yielded a reading beyond the measuring range of the Periotron 6000, it was assigned a volume of 1111.77 nl which was the averaged values from 20 Periotron readings of 200 (the maximum reading) in 20 different calibration curves.

Figure 2.10 Calibration curves of the Periotron at the volume range of 0-1000nl. It can be seen that there is 2 sets of lines, meeting at 400nl. The first line is for 0-400nl and the linear equation in the form of $y = mx + c$, is $y = 3.40x + 6.41$ and the second line is $y = 7.93x - 469.18$ for 400-1000nl where y = Periotron reading, m = the slope, x = volume in nl, and c = the intercept.

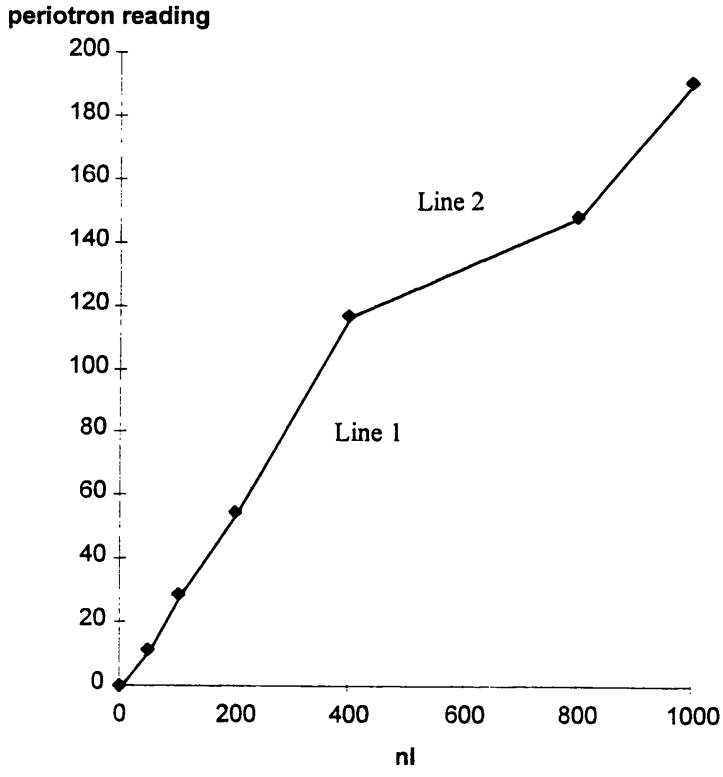
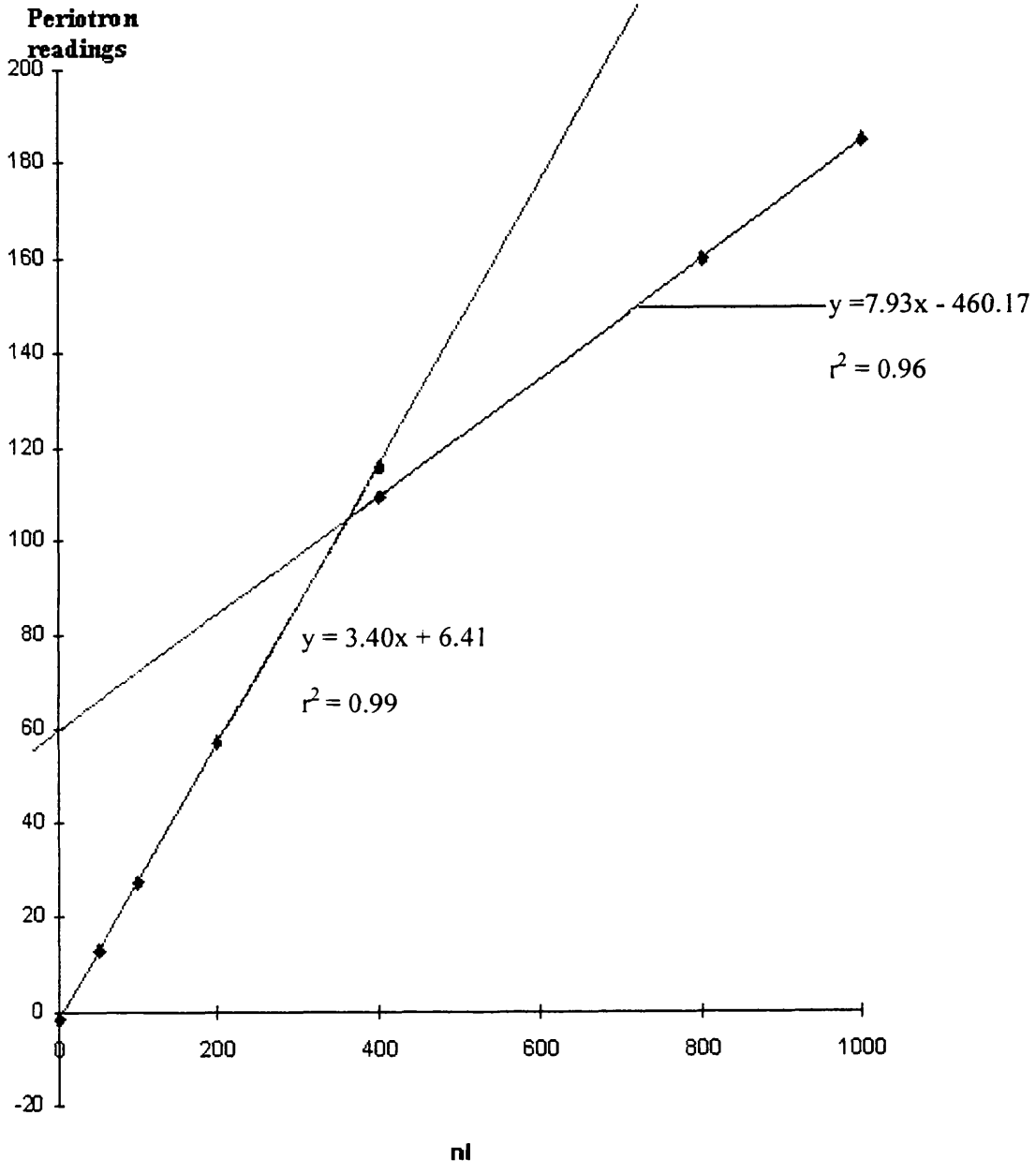


Figure 2.11 Linear regression analysis lines used to transform the Periotron digital readings for each paper strips into volumes (nl).



2.7.2 Gingival Crevicular Fluid Elution

GCF samples were eluated in 500 µl of incubation buffer for 1 hour at room temperature using a rotary mixer. The strips were then discarded and the eluates were aliquoted and stored at -30°C, until further analysis. A previous study (Haerian, 1994) has demonstrated that the elution of GCF samples in 500 µl of incubation buffer would yield concentrations in the eluate within the precision range of each ELISA.

2.7.3 Sandwich ELISA Methodology for Stromelysin

The sandwich ELISA method was a modification of the technique described previously by Cooksley *et al.* (1990). The modifications used were as follows: a) the sample volume in each well was reduced from 200 µl to 50 µl; since we diluted the samples in 500 µl of incubation buffer and required sufficient volume for two different ELISAs and at the same time some back up volume; b) all the paper strips were eluted in the incubation buffer; since we had to use the elute from the same paper strip for different ELISAs it was impossible to use two different assay buffers to elute each individual paper strip; c) the calibration line was altered toward the lower concentration, based on the range of absolute amounts of SL detected in preliminary experiments.

SL quantification was performed on the elutes of GCF, and all constituents were assayed by means of sandwich enzyme linked immunosorbent assays (ELISA), based on a

modification of the method of Cooksley *et al.* (1990). This method assays the amount of antigen present in the sample as directly proportional to the amount of the second antiserum (rabbit), which was quantified indirectly by the addition of horse radish peroxidase (HRP) conjugated anti-rabbit IgG (donkey) (Jackson Immuno-research Laboratories, Avondale, USA) resulting in high optical densities (ODs) for high antigen concentration.

The results of experiments already carried out in our labratory (Haerian, 1994) demonstrated that among five different types of Immulon microplates (Dynatech Laboratories), the Immulon IV plates showed the least variability for the sandwich ELISAs. In summary, the 96-well polystyrene microplate (Immulon 4 Dynatech Laboratories, Billingshurst, Sussex, U.K) was coated with the first antibody, specific to the antigen to be quantified. The antibody used was Mac 78 anti-stromelysin monoclonal antibody. After overnight incubation at 4°C, these were washed with wash buffer and dried, blocked with incubation buffer for 1 hour and then incubated with serial dilutions of recombinant human stromelysin of concentration 12.5-0.025 nanogram per millilitre (ng/ml) or eluted GCF samples in incubation buffer containing 0.1% protease free BSA (Calbiochem), for one hour at 25°C with constant mixing. Any antigen present was captured by immobilised antibody. The standard and a total of 20 samples were run in duplicate vertically in each plate. The plate was then washed and incubated with a rabbit-anti-human (polyclonal) stromelysin antibody in the same buffer for one hour at 25°C with constant mixing. The plates were then washed and incubated with Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG (donkey) (Jackson Immuno-

research Laboratories, Avondale, USA) in the same buffer for one hour at 25 C with constant mixing. The anti-SL monoclonal and polyclonal detected both the proenzyme and active forms of the SL. Visualization was achieved by incubation with tetramethylbenzidine (TMB) substrate for 15 minutes at 25°C with constant mixing, giving a blue colour. The plate was read at 630 nm on a Dynatech MR5000 plate reader. The method is described in detail in Table 2.1. Serial dilutions of standard antigen were run for each plate. The working range of the standard antigen for the stromelysin was 12.5-0.025 nanogram per millilitre (ng/ml) in incubation buffer (IB) and their optical densities (ODs) were used in a linear regression analysis from which the slope and intercept were used to determine the level of SL in the samples. The precision range was defined as the linear part of the working range. 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.

The central wells were used on each plate for standards or samples in order to avoid the edge-effect (Tijssen, 1985). The peripheral wells were used for assaying the controls (except for the control 14), and were run in duplicate. Zero antigen wells (Controls 14) were run in quadruplicate. Control wells (control 14, Table 2.2 and Table 2.3) in the central wells in each plate were included which contained no sample or standard antigen,

Table 2.2

IMMULON 4 ELISA PLATE FOR SL.

	1	2	3	4	5	6	7	8	9	10	11	12
		1		2		3		4		5		6
A		12.5 ng/ml	6.25	3.13	1.56	0.78	0.39	0.19	0.09	0.05	0.025	
B												
C	15											7
D												
E												8
F												
G	14			13		12		11		10		9

CONTROL WELLS

1) 2, 6, 7, 8, 12, 13, 14
2) 3, 6, 9, 10, 12, 13, 15
3) 4, 7, 9, 11, 12, 14, 15
4) 5, 8, 10, 11, 13, 14, 15

1)	Coat with anti-stromelysin Mac 78 overnight at 4°C	50µl
	Block with IB -1 hr RT	100µl
2)	Standard or eluted GCF samples - 1 hr on shaker at RT	50µl
3)	Rabbit anti-SL -1 hr on shaker at RT	50µl
4)	HRP-anti-rabbit IgG -1 hr on shaker RT	50µl

in order to calculate background binding. The mean plus two standard deviations of this background reading was taken as the cut-off point for detectability (Tijssen, 1985).

Results were expressed as nanogram per 30 second (ng/30s) sample, the absolute amount of SL activity in the samples.

2.7.4 MMP-8 Immunofluormetric Assay

MMP-8 levels in the GCF samples were determined by a time-resolved fluorescence immunoassay (FIA) as described by Hanemaaijer *et al.* (1997). The method involves the use of i) fluorescent labels with relatively long half-lives and ii) a pulsed source for exciting fluorescence with a time-resolved fluorometer for eliminating unwanted background fluorescence, which usually has a short decay period. The monoclonal MMP-8 specific antibodies 8708 and 8706 Medix Biochemica) were used as a catching and tracer antibody respectively. The tracer antibody was labeled using europium-chelate (Hemmilä *et al.*, 1984). The assay buffer contained 20mM Tris-HCl, pH 7.5, 0.5 M NaCl, 5mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/litre diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for 1 hour, followed by incubation for 1 hour tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8

Table 2.3. 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.

Control	Step			
	coating	standard	antiserum	HRP
1	-	-	-	-
2	+	-	-	-
3	-	+	-	-
4	-	-	+	-
5	-	-	-	+
6	+	+	-	-
7	+	-	+	-
8	+	-	-	+
9	-	+	+	-
10	-	+	-	+
11	-	-	+	+
12	+	+	+	-
13	+	+	-	+
14	+	-	+	+
15	-	+	+	+

2.7.5 Preliminary Experiments

The results of experiments already carried out in our laboratory (Haerian, 1994) demonstrated that:

a) Among five different types of immulon (Dynatech Laboratories) microelisa plates, the immulon IV plates were selected for the sandwich ELISAs in these studies.

b) There was no significant difference between the levels of GCF components on different plates at the same concentration and the intra-plate variability was low for levels of SL.

c) Serum components did not interfere in the assaying of GCF SL and TIMP.

d) GCF SL respond in a similar way to the highly purified standard and it was concluded that the assays developed could be used for their quantification in GCF.

e) The elution of GCF samples in 500µl of incubation buffer would yield concentrations in the eluate within the precision range of each ELISA.

f) The SL sandwich ELISAs described by Cooksley *et al.* (1990) were suitably modified.

2.8 STATISTICAL ANALYSIS

All statistical analysis was performed using the Minitab and SPSS/PC statistical packages on an IBM PC computer. Various Statistical analyses were used as appropriate.

The clinical measurements were treated as two groups, AB and NAB, and the Mann-Whitney test was performed for the clinical measurements at each visit for the two groups. The null hypothesis was rejected at $p<0.05$. The Mann-Whitney test were also carried out to compare the PD reduction and AL gain between the two groups.

For the GCF volume, the Mann-Whitney test was used to compare the three different sites for intra- and inter-sites comparison. The patients were then divided into the two groups, AB and NAB, and comparison of these two groups were analyzed using the same test.

The SL and MMP-8 levels for each site and visit were tested using the Mann-Whitney test to compare their levels between the different sites at each visit and between different visit of different sites. Further analysis using the AB and NAB grouping were carried out.

The associations between SL & MMP-8 in the GCF and the clinical indices (GCF volume and PD at baseline) were determined by the non-parametric Spearman rank

correlation coefficient. This was also used to seek any correlation between the changes in pocket depth reduction, attachment level gain and each of the variables (plaque at 6 and 12 months, SL and MMP-8 at baseline). Spearman's rank-order coefficient of correlation is symbolized by the Greek letter rho (ρ) which is similar to that of Pearson's r. The significance (p) of this test was performed to test the null hypothesis of zero rank correlation.

For the microbiology results the 95% confidence interval of the median for test and control sites was calculated using the binomial distribution ($p=0.05$). Differences in the microbiological parameters between test and control sites at each time of examination were tested by the Wilcoxon's signed rank test for matched pairs. The difference in the microbiological parameters for test and control sites over time were tested with the same test.

LINICAL RESULTS

ıseline findings

was analyzed as two separate groups, antibiotic (AB) and non-antibiotic
These data were derived from 32 patients although 38 were recruited
the 'dropped-out' patients had poor attendance and therefore were not
c time and one patient emigrated before completing the study. Clinical
PD, CAL and RAL were obtained at baseline, 6 month and 1 year using
la probes. Measurements were taken from six sites of the test tooth, i.e.
ıccal, disto-buccal, disto-lingual/palatal, lingual/palatal and mesio-
he deepest site of each test tooth was chosen for the statistical analysis.
ıurement results are shown in Table 3.1 and 3.2. The mean PDs for
ın-antibiotic groups at baseline were similar at 7.20 and 7.19 mm
en the Florida probe was used to measured the PD, the measurement
55 mm for the AB and NAB groups respectively. These were slightly
ımpared to baseline manual probing measurements. The manual probe
ıts for the AB and NAB groups were 8.07 and 8.16 mm respectively
ıurements with the Florida probe were 14.05 and 13.52 mm.

Table 3.1

a) Mean values (± SD) of test sites PD and ΔPD for the antibiotic and non-antibiotic groups using the manual and Florida probe (mm)

	antibiotic		non-antibiotic	
	Manual PD	FP PD	manual PD	FP PD
baseline	7.20 ± 1.15	6.98 ±1.32	7.17 ± 0.99	6.55 ± 1.10
6 months	4.00 ± 1.04	4.25 ±1.28	4.28 ± 1.32	4.32 ±1.21
12 month	4.07 ± 1.19	4.70 ±1.41	3.83 ± 0.72	3.90 ± 1.08
⌖ b-6m	3.20 ± 0.90	2.80 ± 1.30	3.10 ± 1.10	2.40 ± 1.3
⌖ b-12m	3.10 ± 1.00	2.10 ± 0.90	3.30 ± 1.00	2.80 ± 1.2

b) Medians and interquartile range for PD in the antibiotic group

	antibiotic			
	Manual PD	Q1-Q3	FP PD	Q1-Q3
baseline	7.00	6.00 - 8.00	6.60	6.00 - 8.00
6 months	4.00	3.00 - 4.25	4.00	3.35 - 5.10
12 month	4.00	3.00 - 4.00	4.35	3.70 - 5.42

c) Medians and interquartile range for PD in the non-antibiotic group

	non-antibiotic			
	Manual PD	Q1-Q3	FP PD	Q1-Q3
baseline	7.00	6.00 - 8.00	6.30	5.95 - 7.13
6 months	4.00	3.00 - 5.00	4.00	3.60 - 4.5
12 month	4.00	3.00 -4.00	3.90	2.83 - 5.0

Table 3.2

a) Mean values (± SD) of CAL and RAL for the antibiotic and non-antibiotic groups using the manual and Florida probe(mm)

	antibiotic		non-antibiotic	
	Manual CAL	FP RAL	manual CAL	FP RAL
baseline	8.07 ± 2.02	14.05 ± 2.9	8.16 ± 1.39	13.52 ± 3.32
6 months	5.50 ± 2.03	13.47 ± 2.7	5.44 ± 1.82	11.98 ± 3.15
12 month	5.53 ± 2.07	13.53 ± 2.8	5.73 ± 2.01	11.87 ± 3.42
Δ b-6m	2.64 ± 0.93	0.70 ± 2.5	2.61 ± 1.09	1.41 ± 1.64
Δ b-12m	2.61 ± 0.87	0.42 ± 2.4	2.54 ± 1.04	2.21 ± 1.21

b) Median and interquartile values of CAL and RAL for the antibiotic groups using the manual and Florida probe (mm)

	antibiotic			
	Manual CAL	Q1-Q3	FP RAL	Q1-Q3
baseline	8.00	7.00 - 9.00	12.60	12.20 - 16.20
6 months	5.00	4.00 - 7.30	13.40	11.10 - 14.90
12 month	5.00	4.0 - 7.00	12.80	11.30 - 15.90

c) Median values of CAL and RAL for the non-antibiotic group using the manual and Florida probe (mm)

	non-antibiotic			
	Manual CAL	Q1-Q3	FP RAL	Q1-Q3
baseline	8.00	7.00 - 9.00	13.40	10.20 - 16.80
6 months	5.00	4.00 - 6.00	12.15	9.95 - 13.80
12 month	5.00	4.00 - 6.00	11.90	9.20 - 14.00

3.1.2 Pocket Depth Reduction following the GTR Procedure for the AB and NAB Groups

Table 3.1a shows the PD reduction following GTR procedures using the GUIDOR® membrane after 6 months and one year. A reduction of 3.2 and 3.1 mm were achieved for AB and NAB group respectively at 6 months. There was no significant difference in PD reduction in the two groups (Table 3.3). After one year, the reduction in the PD is quite similar to the 6 months results, indicating that after 6 months no more pocket depth reduction could be achieved. With respect to the PD reduction as measured by Florida probe, 2.8 and 2.3 mm reduction was achieved for the AB and NAB respectively. There was a discrepancy between the PD reduction obtained by manual measurements and the PD reductions obtained by Florida probing measurements.

3.1.3 Clinical Attachment Gain and Relative Attachment Level Gain after 6 Months and One Year Following the GTR Procedure

The CAL and RAL gain at 6 months and one year following GTR using GUIDOR® is shown in Table 3.2a. At 6 months, the CAL gain was 2.64 and 2.61 mm for the AB and NAB group respectively. The CAL gain at one year was quite similar. The RAL gain in the AB group was 0.71 mm at 6 months and 0.42 mm at one year. For the NAB group the RAL gain was 1.41 and 2.21 mm respectively for 6 months and one year. When the Mann-Whitney tests were performed, there was no significant difference in CAL and RAL between AB and NAB group; the p values are shown in Table 3.3. Again,

Table 3.3

Significance values (p) of Δ PD and Δ AL for the antibiotic and non-antibiotic groups using the Mann-Whitney test

		Group		p values
		Antibiotic	non-antibiotic	
manual PD	Δ b-6m	3.00	3.00	0.92
	Δ b-12m	3.00	3.00	0.62
FP PD	Δ b-6m	2.75	2.30	0.66
	Δ b-12m	2.00	2.50	0.09
manual CAL	Δ b-6m	2.50	3.00	0.74
	Δ b-12m	3.00	3.0	0.60
FP RAL	Δ b-6m	0.65	1.35	0.42
	Δ b-12m	1.20	1.80	0.10

For statistical significance a level of $p<0.05$ is set (*). No significant difference was noted.

Table 3.4

Mean of PD reduction and AL gain for sites with membrane exposure and without membrane exposure (mm)

	without membrane exposure	with membrane exposure
PD reduction	3 00 ± 0.93	3.14 ± 0.96
AL gain	2.67 ± 0.87	2.71 ± 0.96

No significant difference was noted.

Table 3.5 Clinical parameters (PD, AL, BOP, PII) at baseline, 6 months and 12 month and also the changes in PD and AL at 6 and 12 month from baseline.

pt	PD b	PD 6	PD 12	AL b	AL 6	AL 12	PII b	PII 6	PII 12	BOP b	BOP 6	BOP 12	PD b-6	PD b-12	AL b-6	AL b-12	Gingival margin
1	6	6	5	9	10	9	0	0	1	1	0	1	0	1	-1	0	-1
2	7	3	4	8	5	5	0	0	0	1	0	0	4	3	3	3	0
3	6	4	3	7	4	4	0	0	0	1	0	0	2	3	3	3	0
4	9	5	5	9	5	5	0	0	0	1	1	0	4	4	4	4	0
5	6	3	3	7	4	4	0	0	0	1	0	0	3	3	3	3	0
6	7	3	3	7	4	4	0	1	1	1	0	1	4	4	3	3	-1
7	7	3	4	8	5	5	0	1	1	1	0	1	4	3	3	3	0
8	6	4	3	8	6	6	0	0	1	1	0	0	2	3	2	2	-1
9	7	4	4	9	6	5	0	1	0	1	1	0	3	3	3	4	1
10	9	6	4	12	10	10	0	0	1	1	0	0	3	5	2	2	-3
11	8	4	4	8	6	-	0	0	1	1	0	0	4	4	5	5	-
12	8	4	4	9	6	6	0	1	0	1	0	0	4	4	3	3	-1
13	7	3	4	7	4	5	0	0	0	1	0	0	4	3	3	2	-1
14	6	4	4	6	4	4	0	1	0	1	1	0	2	2	2	2	0
15	8	4	5	8	5	5	0	1	1	1	1	1	4	3	3	3	0
16	7	5	-	7	5	-	0	1	-	1	0	-	2	-	2	-	-
17	7	4	4	7	4	4	0	0	0	1	0	0	3	3	3	3	0
18	8	4	4	9	5	5	0	1	0	1	0	0	4	4	4	4	-
19*	6	4	4	6	4	4	0	0	1	1	0	1	2	2	2	2	0
20*	6	3	4	8	5	5	0	1	1	1	0	0	3	2	3	3	1
21*	7	4	4	7	5	4	0	0	0	1	0	1	3	3	2	3	0
22*	7	4	4	7	4	4	0	1	0	1	0	0	3	3	3	3	0
23*	9	4	4	14	10	10	0	1	0	1	0	0	5	5	4	4	-1
24*	9	6	7	10	8	9	0	0	0	1	1	0	3	2	2	1	-1
25*	9	6	6	9	8	7	0	0	0	1	0	1	3	3	1	2	-1
26*	7	5	4	9	7	7	0	0	1	1	0	1	2	3	2	2	-1
27*	6	3	4	6	3	4	0	0	0	1	0	0	3	2	3	2	0
28*	6	3	3	8	5	5	0	0	0	1	0	0	3	3	3	3	0
29*	6	3	3	6	4	4	0	0	0	1	0	0	3	3	2	2	-1
30*	7	4	3	8	6	5	0	1	0	1	1	0	3	4	2	3	-1
31*	8	3	3	8	4	4	0	0	0	1	1	0	5	5	4	4	-1
32*	8	4	5	8	4	5	0	0	0	1	0	0	4	3	4	3	0

* = AB group

discrepancies between the attachment level gain obtained by the different measurement methods could be seen. Due to this fact only manual measurements were shown in Table 3.5.

3.1.4 Pocket Depth Reduction and Attachment Level Gain for Sites with Exposed and Non-Exposed Membranes Following the GTR Procedures

Exposure of the bioresorbable barrier membrane occurred in 21 out of the 32 test sites. More membranes were exposed at week 4 compared to week 1 (15 vs 6). When the means of PD reduction and the CAL gain were analyzed as an exposed and a non-exposed group, there was no statistical significant difference between these two groups. The PD reduction and the CAL gain is shown in Table 3.4

3.1.5 Plaque Index (PlI) and BOP at Each Visit

Plaque index at the test sites for all the patients were zero at baseline. At 6 months, 12 patients had a PlI of 1, and 9 patients exhibited the same amount of plaque at 12 months. Of these, 4 patients exhibited PlI 1 at both visits. All the test sites at baseline exhibited bleeding on probing, but only 7 sites were bleeding on probing both at 6 months and 12 months. The patients clinical data is shown in Table 3.5.

3.1.6 Correlation between PD Reduction and CAL Gain with Baseline PD

When the correlation test was carried out using the Spearman rank test, the ρ values was 0.58 ($p<0.001$) for pocket depth reduction and only 0.29 ($p<0.13$) for changes in attachment level (Table 3.6).

3.1.7 Correlation between PD Reduction and CAL Gain with the Plaque Index at 12 Months for the Test Sites

When the PD reduction and the CAL gain were correlated to the plaque index at 12months, there was a significant negative correlation with the CAL gain. The ρ values is shown in Table 3.6.

3.2 GCF VOLUME RESULTS

3.2.1 A Cross-Sectional and Longitudinal Analysis of the Association between Median GCF Volume and the Type and Clinical Parameters of the Three Sampled Sites

A total of 480 GCF samples were taken from 32 periodontitis patients; three sites from each patient. The samples were taken from i) the experimental sites where the bioresorbable membranes were placed (test site), ii) the adjacent tooth which was involved in the flap but without any membrane placement (surgical control), and iii) a site without a deep pocket to act as a healthy control. 96 GCF samples were taken at each visit i.e. at baseline, week 1, week 4, three months and 1 year. The volumes of the GCF were not normally distributed, thus non-parametric tests were performed using the Mann-Whitney test.

3.2.2 Inter-Site Comparison at Each Visit

A cross-sectional study of median GCF volumes for the three different sites were carried out at different time points in the study to determine whether the GCF volume differed between healthy and diseased sites. The results of the median values of the GCF volume are shown in Table 3.7. At all time points in the study when samples were taken, the greatest GCF volumes were those of the test sites. The second highest volumes were

Table 3.7

Median values of GCF volume (nl) for the three sampled sites

	Median	Q1-Q3
Test site		
baseline	353.50	137.40-705.20
week 1	776.60	409.60-1109.80
week 4	407.60	269.30-873.80
week 12	305.90	210.60-637.70
week 52	231.00	128.90-373.90
Surgical control site		
baseline	195.30	84.70-348.40
week 1	350.10	166.30-804.30
week 4	351.40	167.20-576.20
week 12	200.40	140.80-319.50
week 52	183.40	94.90-258.20
Healthy control site		
baseline	130.60	93.20-350.90
week 1	135.70	71.10-311.00
week 4	186.80	48.10-267.60
week 12	203.80	91.50-311.00
week 52	214.00	84.70-350.10

seen in the surgical control sites at baseline, week1 and week 4 only (Figure 3.1 & Figure 3.2).

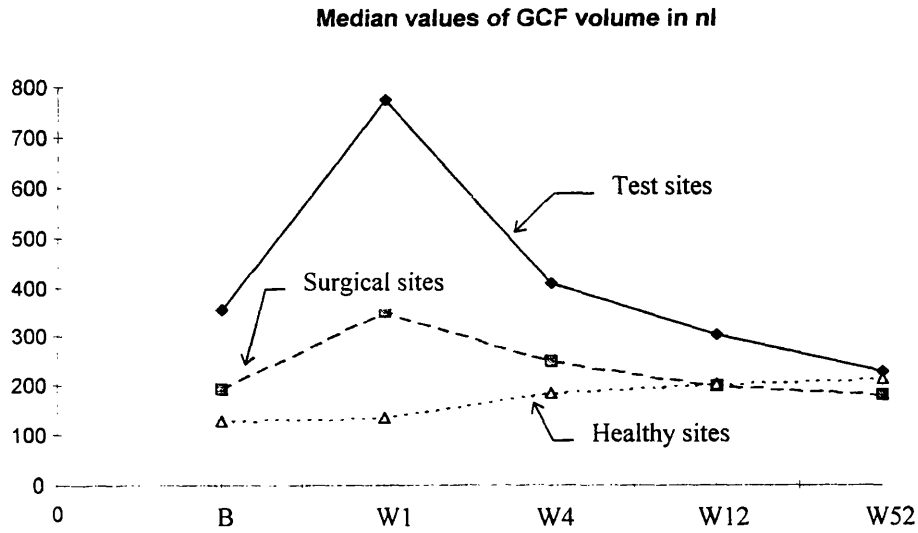
At baseline, the test sites had the highest GCF volume and were also the sites with the deepest pocket depths of the three sites. There was a statistically significant difference in volume of the test sites when compared to both the surgical control ($p<0.001$) and healthy control sites ($p< 0.001$) (Table 3.8). A week after membrane placement, the same trend of volume distribution was seen, with the test sites having the highest volume (776.70 nl) and the healthy control the lowest volume (135.70 nl). The differences in volume were statistically significant when the test sites were compared to both the surgical control and the healthy control. The same trend was seen at week four and three months, with the exception that at three months the volume for the healthy control site was just slightly higher compared to the surgical control. At one year, there was no statistically significant difference seen in the volume distribution.

3.2.3 Intra-Site Comparisons between Visits

The volume distribution and comparisons are best illustrated by the graph in Figure 3.1. For the test site, the volume increased to a maximum value at week one, and decreased to almost the baseline value at four weeks. Then the value decreased slowly to a lower value than the baseline from week four to 1 year. A week after the membrane placement at the test sites, the GCF volume at these sites increased two-fold when compared to the

Figure 3.1 Graphs showing the comparison of GCF median levels between the sites

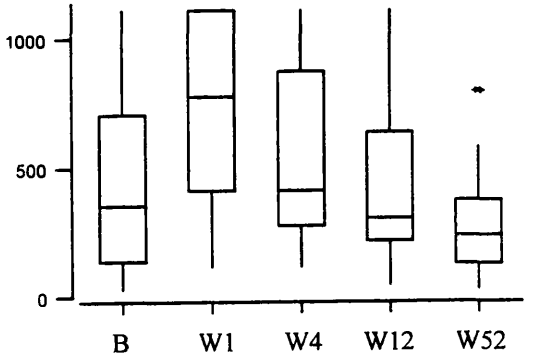
(test = membrane site, surgical = surgical control site, healthy = non-surgical control site)



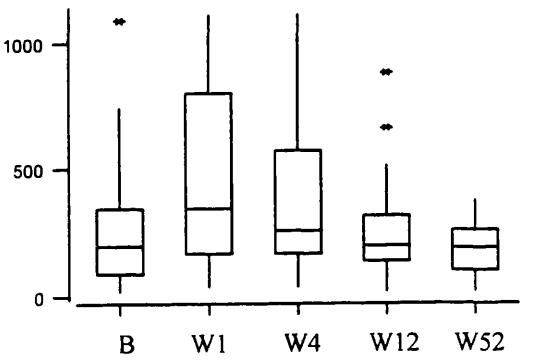
Significance difference were noted between test and surgical sites and between test and normal sites at B, W1, W4, and W12, but only at W1 and W4 between surgical and control sites (Table 3.8)

Figure 3.2 Median values of GCF volume (nl) at each visit

a) for test sites



b) for surgical control sites



c) for healthy control sites

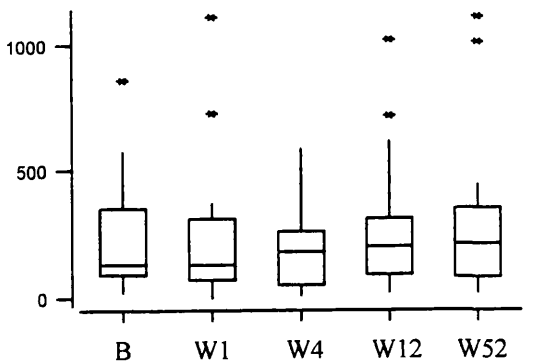


Table 3.8

p values of inter-sites comparisons of volume using the Mann-Whitney test

	Test site				
surgical control site	b	W1	W4	W12	W52
b	**0.001***				
W1		**0.001***			
W4			**0.001***		
W12				**0.001***	
W52					0.07
healthy control site					
b	**0.001***				
W1		**0.001***			
W4			**0.001***		
W12				**0.001***	
W52					0.55

	Surgical control site				
Healthy control site	b	W1	W4	W12	W52
b	0.84				
W1		**0.001***			
W4			**0.01***		
W12				0.77	
W52					0.26

* statistically significant ($p<0.05$)

baseline value. The same picture was reflected by the surgical control sites, but the values attained were much lower. For the healthy control sites, the values from baseline to one year were lower, with the lowest value found at baseline which then increased slowly from week one to one year.

For the test sites there was a statistically significant difference between baseline and week 1 ($p < 0.001$), between week 1 and week 4 ($p < 0.05$), between week 1 and week 12 ($p < 0.001$), between week 1 and week 52 ($p < 0.001$) and between week 4 and week 52 ($p < 0.001$). For the surgical control site the statistical significance differences were seen between baseline and week 1, baseline and week 4, week 1 and week 12, week 1 and week 52 and week 4 and week 52. For the healthy control, there was no statistically significant difference between any of the time points. The p values can be seen in Table 3.9.

3.2.4 GCF Volumes in AB and NAB Group

When the data were analyzed as two separate AB and NAB groups, there were no significant differences between the two groups as can be seen from Table 3.10.

Table 3.9

p values of intra sites comparisons of volume using the Mann-Whitney test

a) for test site

	Test site			
test site	b	W1	W4	W12
W1	**0.001***			
W4	0.24	**0.02***		
W12	0.88	**0.001***	0.09	
W52	**0.01***	**0.001***	**0.001***	0.09

b) for surgical control site

	Surgical control site			
Surgical control site	b	W1	W4	W12
W1	**0.001***			
W4	**0.04***	0.15		
W12	0.56	**0.001***	0.10	
W52	0.42	**0.001***	**0.01***	0.16

c) for healthy control site

	Healthy control site			
Healthy control site	b	W1	W4	W12
W1	0.50			
W4	0.45	0.79		
W12	0.75	0.42	0.30	
W52	0.69	0.28	0.19	0.74

* statistically significant ($p<0.05$)

Table 3.10

Median levels of GCF volumes (nl) and the p-values for non-antibiotic and antibiotic group.

time	site	non-antibiotic	antibiotic	p-values
baseline	test	339.90	372.20	0.49
	surgical control	273.50	132.30	0.07
	Healthy control	159.50	123.80	0.18
week one	test	1101.90	641.70	0.15
	surgical control	305.90	416.00	0.43
	Healthy control	254.80	106.80	0.07
week four	test	358.60	748.80	0.09
	surgical control	249.70	268.40	0.62
	Healthy control	220.80	161.20	0.96
week twelve	test	304.20	309.20	0.84
	surgical control	173.10	244.60	0.08
	Healthy control	191.90	227.60	0.89
week fifty-two	test	179.90	283.90	0.31
	surgical control	135.70	195.30	0.85
	Healthy control	193.60	273.50	0.64

3.2.5 Correlation between GCF Volume and PD

The PD data for the three sites at baseline were pooled and correlated to the corresponding GCF volume. The Spearman rank correlation coefficients (ρ) was 0.44 ($p<0.001$), indicating a significant positive association between these two parameters.

3.3 MMP LEVELS IN THE GCF SAMPLES - STROMELYSIN (SL)

3.3.1 Stromelysin Baseline Findings

All the available samples taken at B, W1, W4 and 3M were analyzed for MMP-3 and MMP-8 regardless of whether the patients completed the study or not. At baseline, when individual data was inspected, out of the 105 samples obtained only 13 sites had detectable SL, 6 from the test sites, 5 from the surgical control and 2 from the healthy control sites.

3.3.2 Findings at W1, W4, and 3M

The only change in the median SL levels between time points was at week one for the test sites, where the median levels increased. However, when each of the sites were analyzed at week one, 74.2% of the test sites and 45.7% of the surgical sites had detectable increased levels of stromelysin. The percentage of sites with detectable amounts of SL at each visit is presented in Table 3.11 and Figure 3.3.

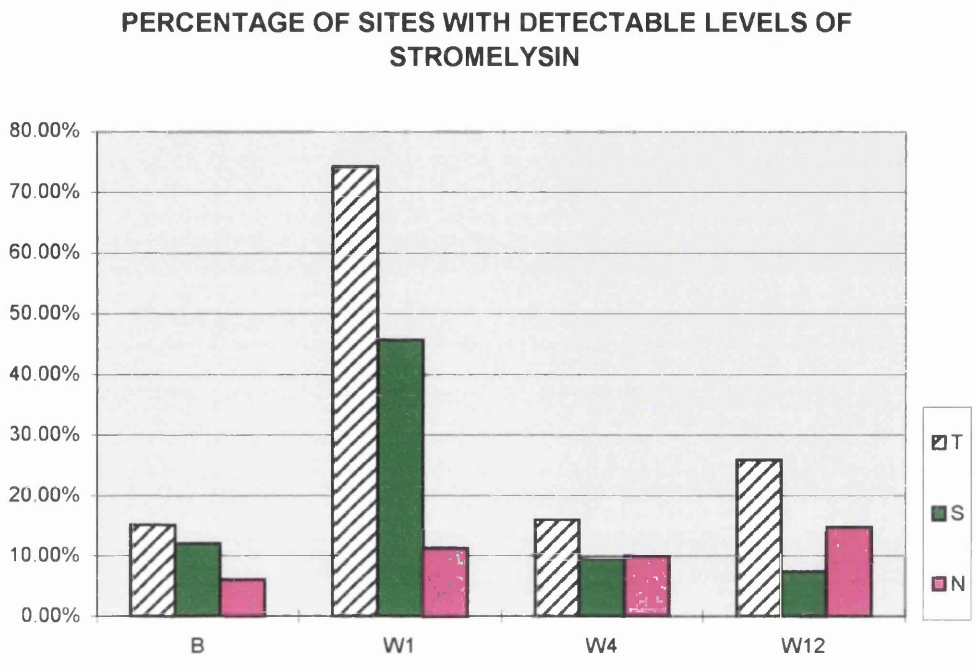


Figure 3.3 Percentage of sites with detectable levels of stromelysin. The cut-off point for detectability was taken as the means OD plus 2 SD of the control wells which contain no sample or antigen (control 14).

Table 3.11

Percentage of sites with detectable levels of stromelysin

	% of sites
Test site	
baseline	15.2
week 1	74.2
week 4	16.1
week 12	25.9
Surgical control site	
baseline	12.1
week 1	45.7
week 4	9.6
week 12	7.4
Healthy control site	
baseline	6.1
week1	11.4
week4	10.0
week12	14.8

3.3.3 Intra-Site Comparison of Stromelysin Levels in the GCF

When non-parametric Mann-Whitney tests were carried out for the intra-sites comparison, there was a statistically significant difference for the test and surgical sites at baseline and week 1 ($p< 0.001$ for test sites and $p<0.01$ for surgical control sites), week 1 and week 4 ($p< 0.001$) and at week 1 and week 12 ($p<0.001$) (Table 3.12).

3.3.4 Inter-Site Comparison of Stromelysin Level in the GCF

At week 1 statistically significant differences could be observed between test and healthy control sites ($p< 0.001$) and also between surgical control and healthy control sites ($p< 0.001$). There was also a statistically significant difference in stromelysin level between test and surgical control sites at week 4 ($p<0.01$). The p values can be seen in Table 3.13.

3.3.5 Correlation between SL and GCF Volume

When stromelysin levels from test, surgical control and healthy control sites at baseline were pooled and correlated to the GCF volume at baseline, the ρ value was equal to 0.21 ($p<0.19$). These findings do not support a correlation between SL and GCF volume.

Table 3.12

p values of intra sites comparisons of stromelysin levels using the Mann-Whitney test

a) for test site

	Test site		
test site	b	W1	W4
W1	**0.001***		
W4	0.95	**0.001***	
W12	0.46	**0.001***	0.51

b) for surgical control site

	Surgical control site		
Surgical control site	b	W1	W4
W1	**0.01***		
W4	0.84	**0.001***	
W12	0.72	**0.001***	0.87

c) for healthy control site

	Healthy control site		
Healthy control site	b	W1	W4
W1	0.70		
W4	0.52	0.77	
W12	0.34	0.54	0.73

Table 3.13

p values of inter-site comparisons of stromelysin levels using the Mann-Whitney test

	Test site			
surgical control site	b	W1	W4	W12
b	0.87			
W1		0.07		
W4			**0.01***	
W12				0.21
healthy control site				
b	0.30			
W1		**0.001***		
W4			0.66	
W12				0.47

	Surgical control site			
Healthy control site	b	W1	W4	W12
b	0.40			
W1		**0.001***		
W4			1.00	
W12				0.60

3.3.6 Antibiotic and Non-Antibiotic Group

When the data were analyzed as two separate antibiotic and non-antibiotic groups, there was no significant difference between the two groups.

3.4 MMP-8 LEVELS IN THE GCF SAMPLES

3.4.1 MMP-8 Baseline Findings

The median levels of MMP-8 at baseline for the three different sites is shown in Table 3.14. The test sites have the highest amount of MMP-8, followed by the surgical control sites and then the healthy control sites. While there is no statistically significant difference between the test and surgical control sites and between the surgical control and healthy control sites, there was a statistically significant difference between the test and healthy control sites ($p< 0.02$).

3.4.2 Findings at W1, W4, 3M and Inter-Sites Comparisons

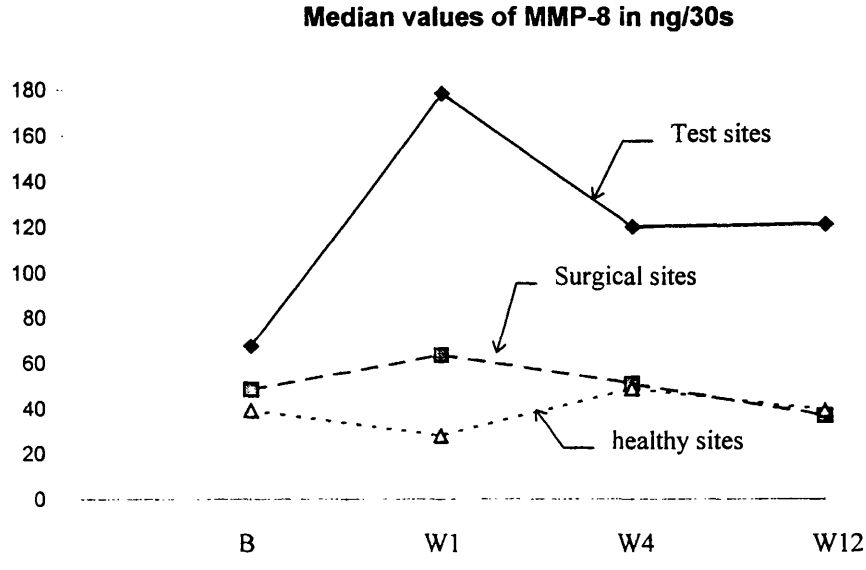
The pattern of change of median values of MMP-8 is best illustrated by a graph as shown in Figure 3.4 and Figure 3.5. At week 1, a dramatic increase in the MMP-8 level

Table 3.14

Median values (ng/30s) of MMP-8 levels in GCF for the three sampled sites

	Median	Q1-Q3
Test site		
baseline	67.60	36.20-165.20
week 1	178.30	66.60-299.90
week 4	120.00	49.70-199.40
week 12	121.30	63.70-206.4
Surgical control site		
baseline	48.30	13.90-113.00
week 1	63.50	25.60-137.00
week 4	50.90	24.70-114.80
week 12	36.90	18.70-133.70
Healthy control site		
baseline	39.1	12.60-76.50
week1	27.90	11.90-93.90
week4	48.80	7.40-92.30
week12	39.20	14.90-138.60

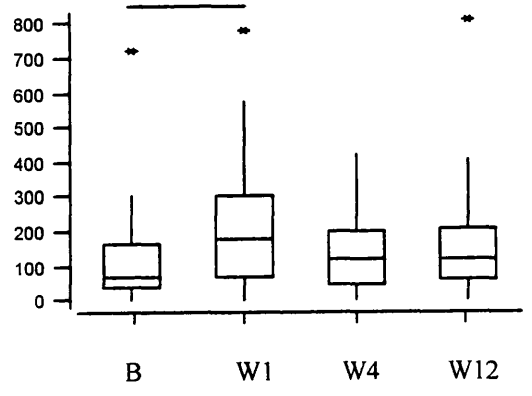
Figure 3.4 Graphs showing the comparison of MMP-8 median levels between the sites (test = membrane site, surgical = surgical control site, healthy = non-surgical control site)



Significance differences were noted between test and surgical sites at W1, W4 and W12, and at all visit between test and normal sites but only at W1 between surgical and control sites.

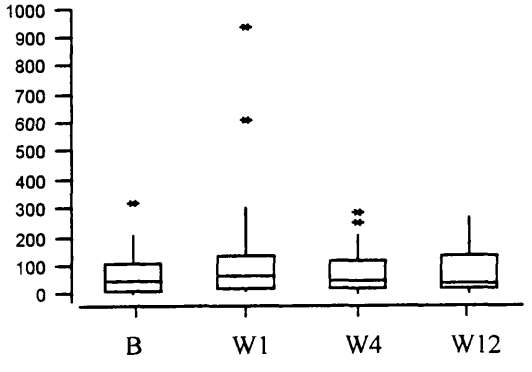
Figure 3.5 Median values of MMP-8 in ng/30s

a) for test sites

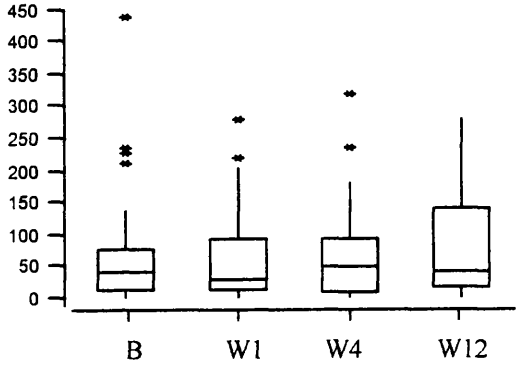


p<0.01 *

b) for surgical control sites



c) for healthy control sites



was shown by the test sites. There was a slight increase for the surgical control sites but for the healthy control sites the slight change was in a negative direction. When the Mann-Whitney test was used to compare the data, there was a statistically significant difference between the test and surgical control sites ($p<0.001$), the test and the healthy control sites ($p<0.001$) and between the surgical control and healthy control sites ($p< 0.03$), as shown in Table 3.15.

At week 4 there was a drop in the MMP-8 levels for both the test and surgical control sites, but the drop for the test site was above the baseline values. Interestingly, there was a slight increase above the baseline value in the MMP-8 values for the healthy control sites. There is a statistically significant difference for the test and surgical control sites ($p<0.001$) and also for the test and healthy control sites ($p<0.001$), but there was no significant difference for surgical control and healthy control sites.

At three months, the values for the test sites seemed to plateau from the 4W values, while there was a drop for the surgical control sites below the baseline values, the healthy control sites seemed to drop to its baseline values. There is a statistically significant difference between the test and surgical control sites ($p<0.001$) and also between the test and the healthy control sites ($p<0.01$).

Table 3.15

Significance values (p) of inter-sites comparisons of MMP-8 levels using the Mann-Whitney test

	Test site			
surgical control site	b	W1	W4	W12
b	0.09			
W1		**0.001***		
W4			**0.001***	
W12				**0.001***
healthy control site				
b	**0.02***			
W1		**0.001***		
W4			**0.001***	
W12				**0.01***

	Surgical control site			
Healthy control site	b	W1	W4	W12
b	0.42			
W1		**0.03***		
W4			0.24	
W12				0.89

* statistically significant ($p<0.05$)

3.4.3 Intra-Site Comparisons

When the MMP-8 levels were compared at different time points of the study, for the test sites only between baseline and week 1 was there a significant difference ($p<0.01$). There was no statistically significant difference for both the surgical control and the healthy control sites at any two time difference (Table 3.16).

3.4.4 Comparisons of MMP-8 Levels at Each Visit for the AB and NAB Group

When the MMP-8 data were analyzed as two separate antibiotic and non-antibiotic groups, there was no significant difference between the two groups as in the SL case. The median and significance values are tabulated in Table 3.17.

3.4.5 Correlation between MMP-8 and PD at Baseline

When the MMP-8 levels for all the three sites at baseline were pooled and correlated to the corresponding PD, the ρ value was 0.36 ($p<0.01$), showing a significant positive association between these two parameters.

Table 3.16

p values of intra sites comparisons of MMP-8 levels using the Mann-Whitney test

a) for test site

	Test site		
test site	b	W1	W4
W1	**0.01***		
W4	0.15	0.13	
W12	0.26	0.17	0.94

* Significant difference was noted for test site between baseline and week 1.

b) for surgical control site

	Surgical control site		
Surgical control site	b	W1	W4
W1	0.24		
W4	0.40	0.54	
W12	0.92	0.24	0.42

c) for healthy control site

	Healthy control site		
Healthy control site	b	W1	W4
W1	0.83		
W4	0.78	0.72	
W12	0.71	0.47	0.69

Table 3.17

Median levels of MMP-8 in the GCF and the p-values for the comparison between the non-antibiotic and the antibiotic group.

time	site	non-antibiotic	antibiotic	p-values
baseline	test	100.2	59.30	0.44
	surgical control	57.27	48.15	0.654
	Healthy control	38.10	39.10	1.00
week one	test	166.90	189.80	0.3992
	surgical control	69.60	50.00	0.29
	Healthy control	32.87	24.05	0.64
week four	test	138.30	76.60	0.08
	surgical control	61.08	50.38	0.71
	Healthy control	31.70	57.40	0.43
week twelve	test	121.30	124.20	1.00
	surgical control	53.50	29.00	0.12
	healthy control	91.40	32.60	0.24

No significant difference was noted.

3.4.6 Correlation between MMP-8 and GCF Volume

When both the MMP-8 levels and the GCF volume were pooled and correlation Spearman rank test were performed, the ρ value obtained was 0.22 ($p<0.18$).

3.4.7 Correlation between MMP-8 and Stromelysin Levels

When both the stromelysin and MMP-8 levels were pooled and Spearman rank correlation test was carried out, the ρ value was 0.47 with $p<0.001$, indicating a positive significant correlation.

3.4.8 Correlation between PD Reduction and CAL Gain with Stromelysin and MMP-8 Level at Baseline

The coefficient values for the Spearman correlation test can be seen in Table 3.18. Both the pocket depth reduction and the attachment gain were poorly correlated to the levels of stromelysin at baseline. There were significant positive associations between MMP-8 levels and the PD reduction and also the CAL gain.

Table 3.18

Spearman rank correlation coefficients (ρ) between pocket depth reduction and attachment level gain (Δ b-6m) with other variables for test sites. Probability levels (p) are shown.

Parameters	Pocket depth reduction	Attachment level gain
SL b	0.33 ($p<0.11$)	0.20 ($p<0.33$)
MMP-8 b	0.39 ($p<0.05$)*	0.71 ($p<0.01$)*

* significant positive association noted

3.5 MICROBIOLOGICAL RESULTS

Two microbiological samples were taken from each patient at baseline, week 1, week 4, 6 months and 12 months. The two sites chosen had deep pockets $\geq$ 6mm with BOP. One site acted as a test site where the membrane was placed and the other site acted as a control without any membrane placement. Three sterilized paper points of size 40 were placed at each predetermined site for 30 seconds. Samples were taken from 30 patients, 14 from the antibiotic and 16 from the non-antibiotic group. Two patients from each group dropped out before the 6 months results were obtained. None of the control sites need any treatment during the study.

3.5.1 Microbiological Results at Each Visit

At baseline, no significant difference was observed between the test and control sites with regard to total aerobic, total anaerobic, anaerobic/aerobic ratio, total *streptococcal* and *A. actinomycetemcomitans* count for both AB and NAB groups.

The median $\log_{10}$ total anaerobic counts of test and control sites for AB and NAB group are presented as Figure 3.6. The values were very similar throughout the study. The median values of anaerobic/aerobe ratios of test and control sites for AB and NAB during the investigation period are presented in Table 3.19. No statistically significant difference between test and control for both AB and NAB groups in total aerobic, total

Figure 3.6 **Mean counts of total anaerobic bacteria isolated from GTR test and control sites**

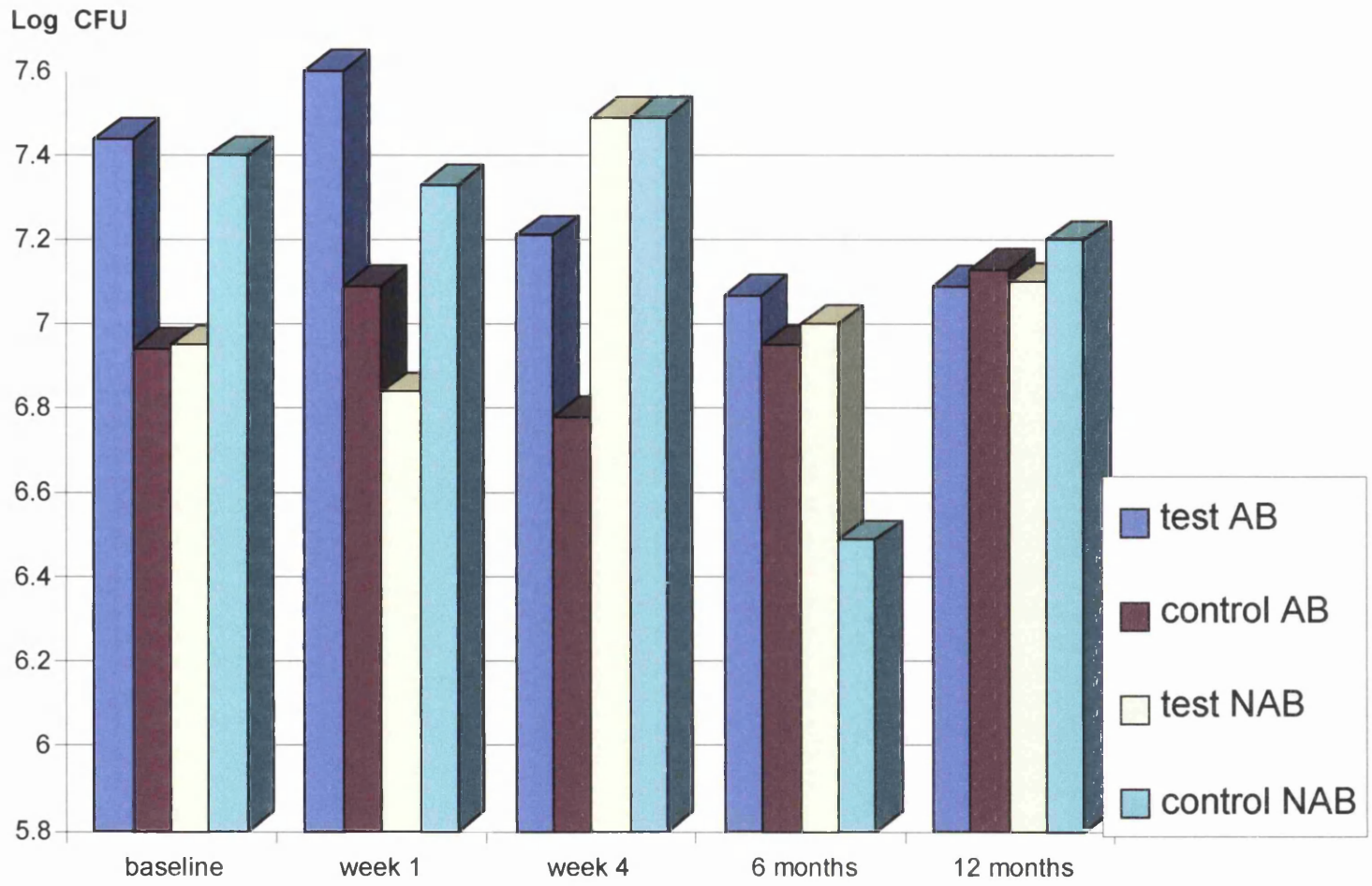


Table 3.6

Spearman rank correlation coefficients (ρ) between pocket depth reduction and clinical attachment level gain (Δ b-12m) with other variables for test sites. Probability (p) levels are shown

	Δ PD	Δ CAL
PD b	0.58 (p<0.001)*	0.29 (p<0.13)
Δ PD	-	0.61 (p<0.001)*
plaque 12M	-0.16 (p<0.43)	-0.41 (p<0.03)*

* significant association are noted

Microbiology results

Table 3.19 Bacteriological counts expressed as the mean $\log_{10}$ CFU

a)

Antibiotic group	Test sites					Control sites				
	b	W1	W4	26W	52W	b	W1	W4	26W	52W
n	13	14	14	13	12	13	14	14	13	12
Aerobic (O_2)	6.62	6.57	6.68	6.70	6.15	6.31	6.65	6.75	6.41	6.32
95%	6.59	6.51	6.4	6.73	6.00	6.36	6.81	6.86	6.28	6.19
Anaerobic (AnO_2)	7.44	7.62	7.21	7.07	7.09	6.94	7.09	6.78	6.95	7.13
95%	7.50	7.77	7.01	6.94	6.90	7.03	7.18	6.70	6.68	7.12
AnO_2:O_2	1.13	1.15	10.8	1.05	1.15	1.10	1.07	1.00	1.08	1.13
Strep	5.88	6.13	6.27	5.97	5.69	5.42	5.81	6.03	5.70	5.78
95%	5.78	6.05	6.18	5.95		5.22	5.90	5.97	5.61	5.92
Aa	6.06	4.64	4.35	5.31	3.61	5.26	4.87	4.85	4.95	3.19
95%	6.21	4.67	4.26	5.04	3.182	5.47	5.03	4.54	4.76	3.21

b)

Non-antibiotic group	Test sites					Control sites				
	b	W1	W4	26W	52W	b	W1	W4	26W	52W
number	14	16	16	11	11	14	16	16	11	11
Aerobic	5.99	6.25	6.85	6.65	5.99	6.25	6.00	6.69	6.75	6.09
95%	5.90	6.05	6.80	6.51	5.78	5.91	5.82	6.77	6.76	5.91
Anaerobic (AnO_2)	6.95	6.84	7.49	7.00	7.00	7.40	7.33	7.49	6.49	7.20
95%	6.93	6.54	7.62	6.87	7.04	7.53	7.32	7.6	6.34	7.08
$An0_2$:0_2	1.16	1.09	1.09	1.05	1.19	1.18	1.22	1.12	0.96	1.18
Strep	5.57	5.61	6.31	6.25	5.76	5.78	5.57	5.87	5.71	5.61
95%	5.4	5.5	6.4	6.5	6.0	6.04	5.55	5.83	5.83	5.76
Aa	4.4	4.66	5.17	4.65	4.9	4.73	4.56	4.92	4.75	5.50
95%	3.1	3.9	5.3	6.5	6.1	4.722	4.43	4.71	4.46	5.39

anaerobic, ratio of anaerobic and aerobic, streptococcal and *A. actinomycetemcomitans* counts was observed at any time throughout the study.

When the proportion of samples in which *P. intermedia* could be isolated was analyzed, at baseline a higher proportion of the control sites in NAB group was found to harbour *P. intermedia* compared to the rest. At week one no samples was found to harbour *P. intermedia.* At week 4, *P. intermedia* was found in 7% of the test site in the AB group and at 6 months only the control sites (8%) in AB group harbour this microorganism. At one year, only the control sites in the AB did not harbour *P. intermedia*, while the rest had a similar percentage of sites affected (Figure3.7).

When the presence of *E. corrodens* were analyzed at baseline, 31% of the test AB sites were affected, 23% in control AB, 29% in test NAB and 0% in control NAB sites. At week one, 13% of the NAB group (both test and control sites) were found to harbour this bacterial species. All the results for the different periodontal pathogens present in the sites at each visit are shown in Table 3.20 and in Figure 3.7 & 3.8.

There is no obvious pattern of distribution that can be seen with the proportion of samples affected by the periopathogens. At week one, there was no statistical significant difference between the AB and NAB group for the test site for all the periopathogens, indicating that placement of a bioresorbable membrane did not affect or influence the microbiological counts. In fact, there is no statistical significant difference in the

Table 3.20 Proportion of samples in which periodontal pathogens were detected

a)

Antibiotic group	Test sites					Control sites				
	b	W1	W4	26W	52W	b	W1	W4	26W	52W
number	13	16	16	11	11	13	16	16	11	11
P.intermedia	8%	0%	7%	0%	8%	8	0	0	8	0
B. corrodens	31	0	7	15	8	23	0	14	8	8
Fusobacterium	8	0	0	8	0	0	0	8	0	8
Ana. Strep	23	50	0	8	42	23	43	8	15	25
Aa	0	0	0	15	0	8	0	8	15	8
P. gingivalis	0	0	0	0	0	0	0	0	0	0
Capno	0	7	0	23	0	16	14	29	8	0
AB R	8	21	14	0	0	16	29	21	8	0

b)

Non-antibiotic group	Test sites					Control sites				
	b	W1	W4	26W	52W	b	W1	W4	26W	52W
number	13	16	16	11	11	13	16	16	11	11
P.intermedia	7	0	0	0	9	14	0	0	0	9
B. corrodens	29	13	0	9	18	0	13	6	0	9
Fusobacterium	7	0	0	0	9	7	0	6	0	9
Ana. Strep	21	38	38	18	18	14	31	0	0	9
Aa	7	0	0	9	9	0	13	0	18	9
P. gingivalis	0	0	0	0	0	0	0	6	0	0
Capno	0	13	6	18	0	7	13	6	9	9
AB R	14	0	6	9	0	21	6	9	9	0

Figure 3.7 Proportion of samples in which *P. intermedia* was isolated

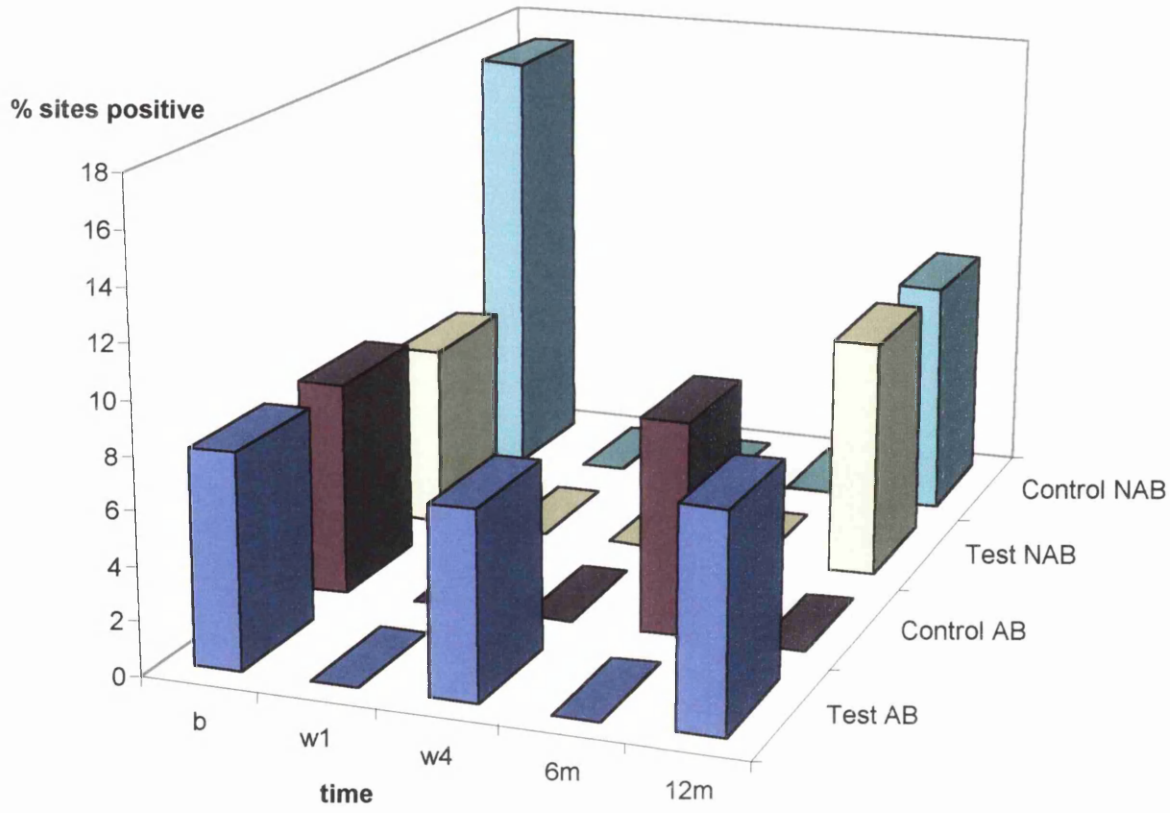
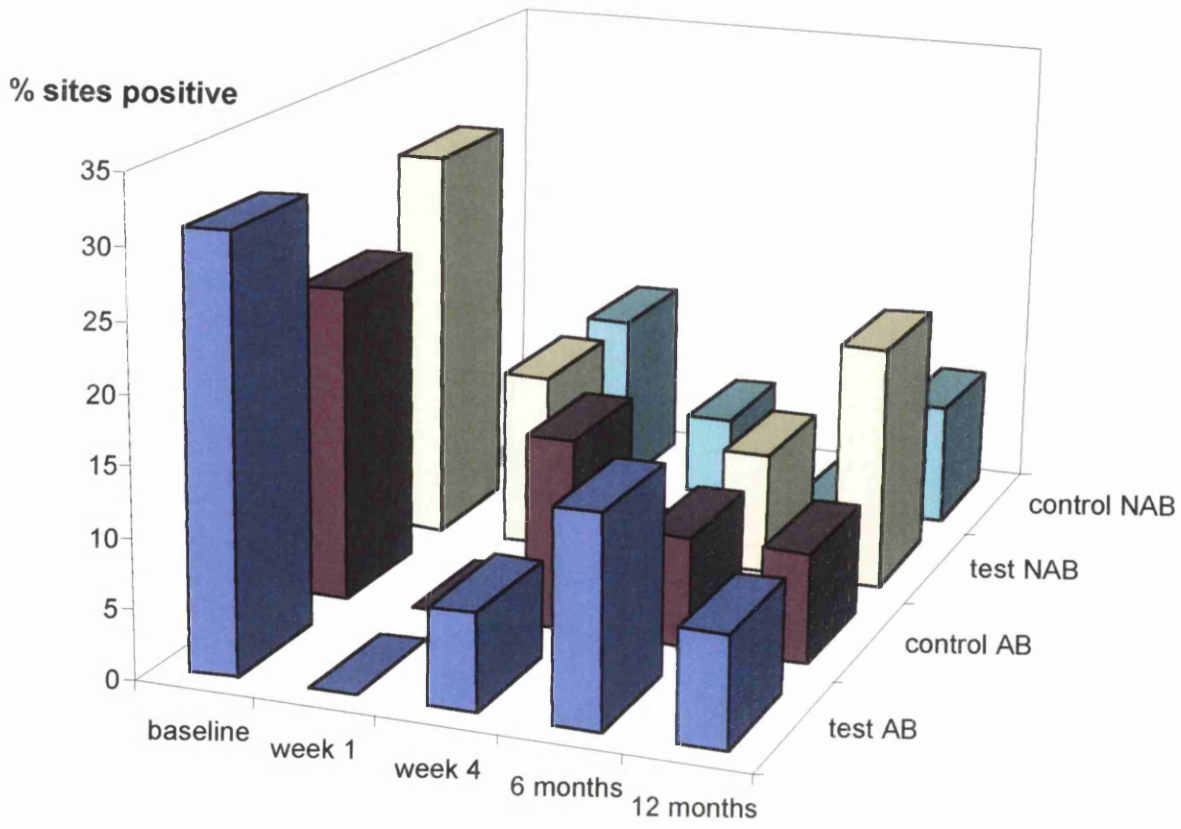


Figure 3.8 Proportions of samples in which *E. corrodens* was isolated



proportion of samples in which periopathogens were detected from either groups and test and control sites at all the time interval.

Although there was a trend of increased recovery of amoxycillin resistant isolates in the antibiotic group particularly one week after the surgery for both the test and control sites, there was no statistically significant difference observed for AB and NAB group.

CHAPTER 4

DISCUSSION

4.1 METHODOLOGICAL CONSIDERATION

4.1.1 Study design

GCF samples were taken from 3 sites from each patient to evaluate the effect of placing a bioresorbable membrane and to compare this effect with an adjacent tooth which was involved in the surgical flap design and with a healthy control site not involved during the surgery. The adjacent tooth acting as a surgical control was not chosen for their pocket depths measurements but because that tooth was going to be involved in the flap design, so unavoidably the PD varies in these subjects.

While the GCF volume and microbiological samples were analyzed up to a year, the MMPs levels were only analyzed up to 3 months from membrane placement. The rationale for this was that i) there was little activity change in SL after 1 week of membrane placement, and ii) we were mainly interested in the MMP activity during the early healing phase following the GTR procedure.

4.1.2 Clinical Measurements

To evaluate the effect of the GTR procedures using the bioresorbable barrier membranes (Guidor®), measurements of PD, CAL and RAL were obtained at baseline, 6 months and 12 months using a manual probe and an electronic probe (Florida probe). PD

There is no consensus in the literature whether manual or electronic probing measurement is more reliable, sensitive or accurate, therefore in this study both methodologies were used.

4.1.3 Antibiotic of choice

Amoxycillin, 3g given 1 hour pre-operatively was given so that a high concentration could be achieved during placement of the membrane so as to reduce the risk of biomaterial contamination of the healing area. Studies have shown that the regenerative process is compromised if bacterial contamination on the membrane material during insertion occurs. With the ePTFE barrier membrane tissue integration is not possible and formation of a pocket between the gingival flap and the matrix occurs due to the apical proliferation of the epithelium leading to bacterial colonization. As the barrier membrane used in this study was bioresorbable, tissue integration between the material and the gingivae will take place, thus limiting the bacterial contamination from the oral cavity during the healing period. As the concern is only for bacterial contamination and colonization during insertion, a pre-operative dosage of antibiotic was most appropriate in this study. Preliminary microbiological studies performed in Aarhus Dental School laboratory have shown that the microflora associated with Guidor® predominantly consist of facultative anaerobic bacteria and that black pigmented Gram-negative anaerobic rods did not seem to develop in number during the first 6 postoperative weeks

(personal communication). Application of a high systemic dose of penicillin selective for aerobe and facultative anaerobic microflora would be most suitable, thus one pre-surgical systemic application of amoxycillin was the antibiotic regime of choice.

4.1.4 Quantification of Gingival Crevicular Fluid Matrix Metalloproteinases

The enzyme linked immunosorbent assay (ELISA) is being used increasingly in clinical laboratories for the assessment of constituents of biological fluids. The ELISAs methodology provides precision and low detectability limits, although visual assessment is possible, results can be automatically obtained using spectrophotometry. It is also a simple method and a large numbers of samples can be run at a time. ELISA used in these studies were validated and proven suitable for the quantification of MMPs (Haerian, 1994).

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

a) High background

The background of an assay can be determined by evaluating the ODs readings in the control wells that contain no sample or standard (control 14). High background can easily obscure the results of positive samples, 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).

b) Edge effect

It has been noticed that perimeter wells of a plate may absorb more protein than the inner wells thus, resulting in an assay bias (Tijssen, 1985; Carpenter, 1992). In order to avoid this phenomena only the inside wells were used for running samples (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). This is particularly a problem with patients samples that contain an extremely high level of an analyte. When the standard assay dilution is used a low to moderately high result will occur. On further dilution, nonetheless, the result will be out-of range high or, if it is diluted will give extremely elevated value. However, in these experiments the possibility of hook effect was not demonstrated as assaying serial dilutions of GCF eluates did not demonstrate such an effect.

4.1.5 Reporting of GCF Constituents

GCF is an osmotically mediated transudate and the amount of fluid in any crevicular location tends to increase with inflammation and capillary permeability (Brill & Krasse,

1959; Brill and Björn, 1958; Egelberg, 1966a, b). The volume of GCF in individual crevicular locations is both very small (generally < 1 μl) and extremely variable depending on the clinical situation, however, it has a very high constituent concentration in small volumes. Unlike serum, a standard amount of GCF cannot be collected and analyzed. Considering that the amount of fluid present at individual crevicular sites is extremely variable, and variations in fluid volume depend on the state of the tissues from which they are derived, reporting the constituent concentration is not acceptable and is inadequate.

In the present study GCF data was reported as absolute amount of GCF constituents per 30s sample, and does not include the volume of GCF in its calculation. The rationale for expressing results of GCF constituents as absolute amount has been discussed extensively (Lamster *et al.*, 1986; Lamster *et al.*, 1988). Standardization in sampling is required and this is achieved by sampling all sites for the same length of time (30s) with a reproducible collection procedure.

4.2 The Choice of GCF Components to be examined

These studies were carried out to investigate 1) the effect of antibiotics on GTR outcome, 2) the MMP levels in GCF during the healing period following the placement of bioresorbable membrane in the treatment of infrabony defects. The levels of MMP-3 and -8 in the membrane treated sites were also compared to surgical and non-surgical

healthy sites during the first 3 months of the healing period. By looking at the MMP levels during the wound healing the role of the degradative phase of the regenerative process can be ascertained. The reasons for choosing MMP-3 and MMP-8 for the analysis of the early wound healing following the GTR procedure are:

a) MMP-3 has been shown to be overexpressed and overactivated during wound repair process (Buisson *et al.*, 1996) and is correlated to deposition of repair tissue (Fini *et al.*, 1996). Increased MMP-3 levels have also been shown to contribute to scarless healing (Bullard *et al.*, 1997).

b) MMP-3 is secreted by fibroblasts, endothelial, and macrophages cells (resident cells) while MMP-8 is produced by PMN cells, gingival and periodontal ligament fibroblasts (Salo *et al.*, 1995, Sorsa *et al.*, 1996) and also chondrocytes (Chubinskaya *et al.*, 1996; Cole *et al.*, 1996). Fibroblast and macrophages are the principal cellular source of matrix components constitutive of the healing granulation tissue. MMP-3 also degrades the core protein of proteoglycan in addition to its role in MMP-1, MMP-8 and MMP-9 proteolytic activation. In this study, only the fibroblast-derived SL was quantified.

c) The major fibrous constituents of the periodontal connective tissues are collagen type I and III and MMP-8 degrades both these collagen types. MMP-3 can degrades type IV collagen which is present in healthy and inflamed gingiva, and also it degrade type V collagen in inflamed gingiva.

d) To test the view that collagenase biosynthesis is essential for collagen turnover during the early repair process (Cionni *et al.*, 1986; Davidson & Galbavy, 1985; 1986) in periodontal tissue. Girard et al. (1993) has earlier proved this in cornea.

4.3 Clinical Findings

When the manual and the electronic PD measurements were compared to each other at baseline, 6 month and 12 months, the measurements were comparable, even though the Florida probe measurements were generally lower than the manual measurements. It has to be emphasized that the manual probe is recorded to the nearest mm, while the Florida probe measurements are displayed to the nearest 0.2mm. In addition, the two different methods of measurement could be measuring probing depths at different locations. The manual probe obtains measurements at a more interproximal location compared to the Florida probe as the sleeve of the automated probe restricts the placement of the probe relative to the interproximal area where the deeper probing depths are usually encountered.

For the CAL and the RAL only the changes between baseline and 6 months and between baseline and 12 months can be compared for the two different measurement methods. These two measurements are not comparable as the CAL value is measuring from the CEJ to the bottom of the pocket, while the RAL value is recording measurement from the incisal/cuspal tip to the bottom of the pocket. There was a big difference between the changes calculated using the manual probe compared to the changes calculated using the Florida disc probe. Between baseline and 6 months the changes were 2.75mm manually and 1.00mm using Florida disc probe, and 3.00mm manually and 1.5mm electronically between baseline and 12 months. Due to this fact, only the manual measurements for both PD and CAL were used for the statistical analysis. Eight anterior

teeth, 13 premolars and 11 molars were the teeth used in this study. The large variability of the differences could be explained by the fact that the Florida disc probe has quite a long dimension and this creates difficulty when inserting the probe in the molar region especially if the patient has a limited opening. Due to this difficulty the probe could be inserted not along the long axis of the tooth but slightly slanting, thus causing the variability. When the probe is placed not parallel to the long axis of the tooth the value measured would be higher than the actual value. Due to the high resolution of the automated probe, even slight alteration in the probe placement between passes would result in some variability. Also owing to the slender dimension of the probe, it is quite flexible and thus can bend easily while taking measurement of the molar region. The difficulty in measuring the molar region leading to measurement variability has been shown by Ahmed *et al.* (1996) who also noted that the distal site of either buccal or lingual aspects showed significantly more variability in repeated measurements reflecting the degree of 'awkwardness' in measuring this site.

4.3.1 CAL Gain following the GTR Procedures

CAL gain in this study was found to be 2.61 $\pm$ 0.87 for the AB group and 2.54 $\pm$ 1.04 mm for the NAB group. Higher CAL gain has been reported by several investigators using the Guidor ® membrane, Falk *et al.* (1993) achieved 4.5 $\pm$ 1.6, Laurell *et al.* (1994) achieved 4.9 $\pm$ 2.2 and Falk *et al.* (1997) achieved 4.8 $\pm$ 1.5 mm gains. The initial PD of the present study was 7.18 $\pm$ 1.04 mm which were slightly lower compared

to the above-mentioned studies which was between 8.1 to 9.0 mm. Several investigators have also showed that there was a positive correlation between the mm gain of clinical attachment and the initial PD (Tonetti *et al.*, 1993; 1996; Garrett *et al.*, 1988; Falk *et al,* 1997).

When the PD reduction and AL gain were compared between AB and NAB groups at 6 and 12 months, there was no statistically significant difference between the two groups. This indicates that the antibiotic regime using amoxycillin 1 hour pre-operatively did not influence the pocket depth reduction and the attachment level gain in the GTR procedure using Guidor®.

In the present study membrane exposure occurred in 21 out of the 32 test sites. 6 of these sites were exposed at week 1 and 15 exposed at week 4. In all cases the extent of the exposure was minor, and in most instances limited to a small portion of the interdental tissue. The exposed part of the membranes disintegrated within several weeks. Membrane exposures do not influence the PD reduction and the AL gain in the present study. This finding is in agreement with other studies (Selvig *et al.*, 1992; Schallhorn & McClain 1994; Trombelli *et al.*, 1997) which emphasized the fact that only the probing bone level and not PD reduction and AL gain were influenced by membrane exposure. However, contrasting observations have also been reported where membrane exposure was found to be a negative predictor of AL gain (De Sanctis *et al.*, 1996a; Falk *et al.*, 1997).

4.3.2 Correlation between the PD Reduction and CAL Gain with other Clinical Parameters

The baseline pocket depth seems to moderately influence the pocket depth reduction, and the pocket depth reduction was moderately correlated to the attachment level gain. When the gain in AL was analyzed with the Spearman rank correlation test, there was only a weak correlation with the baseline PD (refer Table 3.6). This finding is in contradiction to the established fact that generally the deeper the baseline defect, the greater the amount of attachment gain. This can be explained by the fact that, even though our pocket depths were considerably deep when we started, a number of patients had residual deep pockets which were root planed every 3-monthly. The presence of residual periodontal infection in the oral cavity has been shown to be strongly associated with the clinical outcomes of both regenerative and conventional surgical procedures (Tonetti *et al.*, 1996). The criteria of patient selection in our protocol were that the patients had completed initial therapy (oral hygiene instruction and root-planing) or in maintenance and had at least 2 deep pockets $\geq$ 6mm with BOP, one of them had to have an infrabony defect.

The AL gain was significantly negatively associated with the plaque index at 12 month. This is in agreement with other studies which emphasized that oral hygiene influenced the outcome of the GTR procedure (Tonetti *et al.*, 1995; 1996; Cortellini *et al.*, 1994; Falk *et al.*, 1997).

The effect of smoking on the clinical outcome was not analyzed as we had a limited number of smokers in this study group.

4.4 CHANGES IN GCF VOLUME

The median levels of GCF volumes were investigated at baseline, week 1, week 4, 3 months and 1 year following the placement of the bioresorbable membrane barrier. The values obtained at baseline for the 3 different sites are explicable by the clinical and pathological differences of the three sites. The lowest value was seen at the healthy control sites while the highest was seen at the test site with PD $\geq$ 6mm. This was expected as it has been shown that the degree of gingival inflammation is positively correlated to the GCF volume obtained (Brill, 1960; Egelberg, 1964; Löe & Holm-Pederson, 1965b). A week after the surgical procedure, both the test and the surgical control sites showed an increase in volume, though the test sites have statistically higher volume than the surgical control sites. This implies that placement of a membrane barrier creates more inflammation as the surgical control sites which were also involved in the surgery did not have this large increase in volume. This is further illustrated at week 4 when the same pattern occurred though in lower volumes. At one year, there was a slight increase in volume compared to the baseline visit for the healthy control sites. This can be explained by the fact that the plaque control at one year was not as good as when the study was initiated.

There was no statistically significant difference between the two groups for the GCF volume, thus demonstrating that AB given 1 hour pre-operatively did not influence the GCF level at any visit.

4.5 MMP-3 LEVELS

MMP-3 has been shown to be at a significantly higher level in diseased sites (gingivitis and periodontitis) compared to healthy sites (Haerian *et al.*, 1995) and the level was reduced after periodontal therapy (Haerian *et al.*, 1996). Haerian *et al.* (1994) has also shown that MMP-3 level was higher in newly referred periodontitis patients (at healthy, gingivitis and periodontitis sites) compared to maintenance patients. It was shown that during the wound healing which would invariably include inflammation and remodeling, SL level increased, thus we expect increases in MMP-3 level at the test and surgical control sites. The levels of MMP-3 would be expected to be more at the membrane site than at the surgical control site as the inflammation would be more at the membrane site, i.e. from the remodeling and from the host reaction to a foreign implant.

When the MMP-3 levels at baseline were analyzed, it was not surprising to find low levels at all the sites, even though the test sites had PD $\geq$ 5mm, these sites had received scaling and root planing treatment. Only 13 out of the 105 sites analyzed at baseline had detectable MMP-3. When the overall results were analyzed, at week 1 there was an

increase in the MMP-3 levels for the test sites only. When the individual MMP-3 data was analyzed at that same visit, 78% of the test sites and 45% of the surgical control sites exhibited detectable MMP-3 levels. Whenever SL was detected, it was at least 1.5 fold higher than the control wells which contains no samples. The surgical control sites exhibited lower levels when compared to the test sites. This implies that the increased SL level was due to both the inflammatory healing process and the presence of the resorbable membrane barrier. The membrane placement caused more increase in the fibroblast-derived SL than the surgical procedure alone. Thus both the inflammatory process and the membrane placement provided a stimulus for the fibroblasts to produce more MMP-3. Even though these cells were increased in number for both the test and surgical control sites during the healing period, only the cells at the test sites were producing more overall detectable MMP-3 levels, implying that the presence of the membrane was responsible for the increased MMP-3 levels. The SL detected by the ELISA method was in the proenzyme and the active enzyme state only, but it has to be born in mind that it is highly likely that active MMP-3 reaching the crevice would readily bind to inhibitors and causes MMP-3 in GCF not to be detected by this method.

Though an increase in the inflammatory process is expected when the bioresorption process began at week 6, unfortunately no samples were taken at this time to study MMP-3 levels.

4.5.1 Correlation between GCF Volume and MMP-3

There is a very weak correlation between these two variables. This demonstrated that an increase in the GCF volume does not necessarily increase the amount of stromelysin levels in that GCF. This makes sense as MMP-3 is locally derived from resident cells and not part of the serum transudate.

4.6 MMP-8 LEVELS

MMP-8 has been demonstrated as the main collagenase in GCF, saliva and inflamed gingivae in adult periodontitis patients (Sorsa *et al.*, 1988; Ingman, 1996), but the mRNA levels of this protease has been shown to be the same in inflamed and normal gingivae in periodontitis patients (Aiba *et al.*, 1996). mRNA of MMP-8 was also shown to be detectable in healthy patients (in 17%) as well as in periodontitis patients (46%) by Tonetti *et al.* (1993).

The levels of MMP-8 active form were predominantly quantified by the ELISA used in this study. At baseline, the highest activity was seen for the test sites which was statistically significantly different to the healthy control sites but not to the surgical control sites. Even though the test sites were active sites (BOP positive) they were not classified as progressive sites, thus the amount of active MMP-8 was not very much different to the adjacent site. Active sites are expected to have higher amount of active

MMP-8 than stable periodontitis and gingivitis sites based on observations by Lee *et al.* (1995).

Previously MMP-8 was thought to be exclusively produced by PMN, but based on recent evidence MMP-8 has also been shown to be expressed by gingivae, periodontal ligament and mucosal fibroblasts (Chubinskaya *et al.,* 1996; Cole *et al.*, 1996; Cole & Kuettner, 1995). PMN derived MMP-8 is influenced by PMN degranulation and pro-MMP-8 activation while non-MMP-8 is most likely to be regulated by inflammatory mediators. The release of the latent PMN enzymes to the surrounding tissues can be stimulated by cytokines synthesized and secreted by host cells during the inflammatory process or by the interaction of PMN cells with whole microbial cells or microbial products (Page 1991; Birkedal-Hansen, 1993b; Ding *et al.*, 1995).

One week post-surgery, the activity of MMP-8 was increased for both the test and surgical control sites. Based on a review paper by Wikesjö *et al.*, (1992) on early healing events, at week 1, the wound healing process would have gone through the early and late phase of inflammation while the granulation tissue formation process was still going on. During the early phase PMN predominates in the infiltrates and thus most probably contributes most of the MMP-8, while during the granulation tissue formation phase, fibroblasts are predominant. Based on this fact, it was postulated that the increase in the MMP-8 levels at week 1 was due to the fibroblasts. The presence of the bioresorbable membrane also seemed to be a factor determining the MMP-8 level, as the test sites with the membrane had higher levels compared to the sites without membrane.

By the fourth week, the granulation tissue formation phase is almost over (Wikesjö *et al.*, (1992), thus the levels of active MMP-8 decreased for both the test and surgical control sites. For the test sites, the MMP-8 levels did not decrease to the baseline levels, but halfway between the baseline and the week 1 values. However, the MMP-8 levels for the surgical control sites dropped to the baseline values. The difference seen could be related to the presence of the membranes at the test sites as stated earlier. The membranes in some way triggered more MMP-8 activation at these sites as evidently shown by the MMP-8 level at three months.

4.6.1 Correlation between MMP-8 Levels and Other Clinical Parameters

The baseline pocket depths for the three sites significantly influence the amount of MMP-8 present at baseline ($\rho = 0.35$, $p<0.01$). The deeper the initial pocket depth the higher the levels of MMP-8 found at that site. The levels of MMP-8 have been associated with disease activity as previously demonstrated by Lee *et al.*, (1995). The GCF volume did not influence the MMP-8 levels. There was a significant positive correlation between the stromelysin level and the MMP-8 level ($\rho = 0.49$). This can be explained by the fact that MMP-3 has the ability to activate pro MMP-8. Active MMP-8 levels has been shown to be high at actively diseased sites and in the present study was associated with early wound healing after surgery. The amount of active MMP-8 pre-operatively for the test sites was significantly and positively associated with the pocket

depth reduction. This implies that the higher the diseased activity of the sites as measured by MMP-8 level the more the pocket depth reduction to be expected.

4.7 MICROBIOLOGICAL ASPECTS OF THE STUDY

The use of the Guidor® barrier membrane did not result in an increase in adverse bacteriological parameters, such as an increased proportion of anaerobic Gram negative black pigmented bacilli at wound sites. This is shown by the similar log CFU count of the total anaerobic flora at the pre-operative period and one week after membrane placement for the test and control sites in both groups. If the barrier membrane provided a colonization niche the level of microflora for the test sites would be much higher compared to the control sites which did not have the barrier membrane.

There was no significant difference in periodontal pathogens recovered from patients receiving antibiotics compared to no antibiotics. In view of the trend to increasing proportion of amoxycillin resistant isolates from the antibiotic group we would suggest that good oral hygiene measures and wound toilet are probably of more importance for successful surgery. From the microbiological point of view, the use of systemic antibiotics is not necessary to enhance the success rate of the GTR with resorbable membranes.

4.8 GENERAL FINDINGS & ASPECTS OF FUTURE RESEARCH

The healing event in this study was uneventful, with no obvious gingival pathology caused by insertion of the barrier membranes. It is established in the present study that the use of 3g amoxycillin 1 hour-pre-operatively for the GTR procedures using Guidor® did not affect the clinical outcome, microbiological and protease level. The use of Guidor® membrane in infrabony defects resulted in PD reduction and a general gain in attachment levels as reported by other investigators (Gottlow *et al.*, 1992a; 1992b; Laurell *et al.*, 1992;1994). It has been shown that the rate of periodontal destruction is higher in sites with infrabony defects compared to sites with horizontal destruction (Papapanou & Wennström; 1991). Recent evidence (Ehnevid & Jansson, 1998) suggests that healing in proximal sites with horizontal destruction after non-surgical treatment was impaired by the presence of a vertical destruction in the adjacent proximal site. Earlier the same investigators (Ehnevid *et al.,* 1997) demonstrated that healing after non-surgical treatment was impaired in vertical defects compared to horizontal defects. So, not only is the vertical defect area at risk of further destruction, but the healing of the adjacent horizontal defects are also impaired. GTR procedure is one of the treatments of choice in treating infrabony defects the success of which has been demonstrated over the years.

The present study also demonstrated that to achieve the best possible clinical outcome following the GTR procedure, strict patient selection should be implemented. Patients should not have any deep infective pockets, as the presence of these pockets can harbour

periodontopathogens which may affect the clinical results (Tonetti *et al.,* 1993; Machtei *et al.*, 1994).

This study has provided the evidence that MMP-3 and -8 are involved in the early remodeling phase following guided tissue regeneration and surgical procedures in the periodontium. This is not surprising as the periodontium is made up primarily of type I and III collagen which can be degraded by MMP-8 while MMP-3 degrades the core of the proteoglycan and is also involved in the activation of MMP-8.

For future studies, it would be most interesting if the different forms of MMP-3 and -8 could be analyzed using Western Blotting. Western Blotting can characterize the different molecular sizes of the different state of the MMPs. This method has been used for MMP-8 and -9 (Sorsa *et al.*, 1995; Ding *et al.*, 1995; 1996; 1997) molecular forms and sizes characterization. By knowing the levels of each state at different points in the study, it may give a clearer picture of the role of MMPs in surgical wound healing and disease.

In conclusion, the present study provides evidence that collagen biosynthesis is taking place during the early phase of wound repair in the periodontium following conventional surgical treatment and the GTR procedure.

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