EVALUATION OF ORAL RINSE ELASTASE IN PATIENTS WITH PERIODONTAL DISEASES

by

NATHALIE CHRISTINE PAULETTO
D.M.D., The University of Montreal, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in
THE FACULTY OF GRADUATE STUDIES
(Deptartment of Oral Medical and Surgical Sciences)
We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
May 1997
© Nathalie Christine Pauletto, 1997
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of ORAL MEDICAL AND SURGICAL SCIENCES

The University of British Columbia
Vancouver, Canada

Date July 21, 1997
ABSTRACT

The present study is a follow-up to a previous study by Uitto et al. (1996) on salivary elastase activity in periodontally diseased patients. It was carried out in an attempt to understand why oral elastase activity is low in a small patient population with periodontitis while it is high in most patients. Oral rinse samples were collected at one or more occasions in patients with different levels of periodontal disease (n=43) and in healthy subjects (n=20). Subgroups of smokers (n=11) and patients with early onset forms of periodontal disease (EOP) (n=3) were also included in the study. Salivary elastase activity and PMN and epithelial cell counts were determined for each patient. Post operatory salivary samples for some of the patients (n=9) were also measured for elastase activity. The specificity of the salivary elastase test was 85%. Smokers and EOP patients affected the sensitivity of the salivary elastase test as a majority of these patients had low elastase activity in their samples. The study also found a correlation between the number of PMNs and elastase activity in patients with periodontitis compared with healthy subjects. These cells were twice as numerous in periodontitis patients compared to healthy subjects. Since a majority of periodontitis patients showed true positive results this test may have some value as a home diagnostic test prior to any dental appointment in subjects who are at risk for periodontal disease. Future studies are needed however to determine if this test should be limited to subjects in a certain age group category and/or in subjects who are otherwise in good general health and those who do not smoke.
TABLE OF CONTENTS

Abstract
Table of Content
List of Tables
List of Figures
Acknowledgments

INTRODUCTION

CHAPTER I

1. Review of the literature

1.1. Periodontal diseases

1.2. Pathogenesis of periodontal diseases

1.2.1. Bacterial flora
1.2.2. Host defense mechanisms in periodontal diseases

1.3. Polymorphonuclear leukocytes and periodontal health

1.3.1. PMNs and general health
1.3.2. PMNs and periodontal health

1.4. Elastase activity in relation to various health disorders

1.4.1. Elastase and its inhibitors
1.4.2. Elastase and periodontal health

1.5. Smoking and periodontal disease

1.5.1. Smoking and general health
1.5.2. Smoking and periodontal health
CHAPTER 2
Aims of the study

CHAPTER 3
Materials and Methods
3.1. Subjects
3.2. Collection of salivary samples and preparation of smears
3.3. Experiments with pure elastase
3.3.1. Pancreatic elastase
3.3.2. Leukocyte elastase
3.4. Oral rinse leukocytes and epithelial cells
3.5 Statistical analysis

CHAPTER 4
4. Results
4.1. Description of oral rinse sample collection for all subjects
4.2. Effects of the time of collection of oral rinse samples on the elastase activity
4.2.1 Effects of smoking on the elastase activity
4.3. Correlation of the numbers of epithelial and PMN cells to the elastase activity
4.3.1. Correlation between cells numbers (PMN and epithelial cells)
4.3.2. Correlation between disease severity and number of cells (PMN and epithelial) in oral rinse samples of periodontitis patients 50

4.3.3 Effects of periodontal disease on PMN and epithelial cell counts (Photo of smears) 51

4.4. Effects of periodontal therapy on the elastase activity 51

4.5. Effects of smoking on epithelial and PMN cell counts 51

4.6. Effects of addition of leukocyte elastase and pancreatic elastase to oral rinse samples of selected subjects 52

4.7. Sensitivity and specificity of the oral rinse test for elastase activity 53

CHAPTER 5

Discussion 54

CHAPTER 6

Conclusion 61

BIBLIOGRAPHY 63

Appendix 1 Tables 1-7 87

Appendix 2 Figures 1-12 94
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Patient population of the study</td>
<td>87</td>
</tr>
<tr>
<td>Table 2</td>
<td>Patients with low elastase activity</td>
<td>88</td>
</tr>
<tr>
<td>Table 3</td>
<td>Elastase activity in oral rinse samples of patients who reported smoking in their health history</td>
<td>89</td>
</tr>
<tr>
<td>Table 4</td>
<td>History of all patients who showed low elastase activity at two or more time periods</td>
<td>90</td>
</tr>
<tr>
<td>Table 5</td>
<td>Leukocyte and epithelial cell counts in all subjects</td>
<td>91</td>
</tr>
<tr>
<td>Table 6</td>
<td>Analysis of patients in Fig. 10 and Fig. 11 who showed low elastase activity despite additions of leukocyte and pancreatic elastase</td>
<td>92</td>
</tr>
<tr>
<td>Table 7</td>
<td>Specificity and sensitivity of the oral rinse test</td>
<td>93</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Pages</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Figure 1</td>
<td>Elastase activity in selected oral rinse samples of periodontitis patients and controls</td>
<td>94</td>
</tr>
<tr>
<td>Figure 2a</td>
<td>Elastase activity of two different oral rinse samples for control subjects</td>
<td>95</td>
</tr>
<tr>
<td>Figure 2b</td>
<td>Elastase activity of two or more samples for diseases patients</td>
<td>96</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Elastase activity of oral rinse samples of three patients at three times of the same day</td>
<td>97</td>
</tr>
<tr>
<td>Figure 4a</td>
<td>Number of epithelial cells/ml in oral rinse samples of patients with periodontitis in relation to elastase activity in their samples</td>
<td>98</td>
</tr>
<tr>
<td>Figure 4b</td>
<td>Number of PMN cells/ml in oral rinse samples of patients with periodontitis in relation to elastase activity in their samples</td>
<td>99</td>
</tr>
<tr>
<td>Figure 5a</td>
<td>Number of epithelial cells/ml in oral rinse samples of healthy subjects in relation to elastase activity in their samples</td>
<td>100</td>
</tr>
<tr>
<td>Figure 5b</td>
<td>Number of PMN cells/ml in oral rinses of healthy subjects in relation to elastase activity in their samples</td>
<td>101</td>
</tr>
<tr>
<td>Figure 6a</td>
<td>Correlation between the number of PMN cells to epithelial cells in oral rinse samples of patients with periodontitis</td>
<td>102</td>
</tr>
<tr>
<td>Figure 6b</td>
<td>Correlation between the number of PMN cells to epithelial cells in oral rinse samples of healthy subjects</td>
<td>103</td>
</tr>
</tbody>
</table>
Figure 7a  Number of epithelial cells/ml in the oral rinse samples of patients with periodontitis in relation to the % of pocket depths $\geq$ 5mm 104

Figure 7b  Number of PMN cells/ml in the oral rinse samples of patients with periodontitis in relation to the % of pocket depths $\geq$ 5mm 105

Figure 8  PMN and epithelial cells in smears of periodontitis patients and healthy subjects 106

Figure 9  Pre and post treatment oral elastase activity for eleven patients 107

Figure 10  Effect of oral rinse samples of periodontitis patients on pancreatic elastase 108

Figure 11  Effect of oral rinse samples of periodontitis patients on leukocyte elastase 109

Figure 12  Effect of oral rinse samples of healthy subjects on leukocyte elastase activity 110
ACKNOWLEDGMENTS

Research studies of this kind can only be accomplished through the generous assistance of many colleagues. It is my pleasure to acknowledge them here.

I wish to begin by thanking Dr. Hannu Larjava for all his editorial assistance. In particular his guidance and patience during the past two and a half years it took to complete my thesis was greatly appreciated.

Many thanks to Dr. Douglas Waterfield and Dr. Veli-Jukka Uitto for their insights and help with the experiments. Thanks also to Dr. Edward Putnins, who as the external examiner helped me consider certain aspects of my thesis in a different light and bring the final touches to it.

Special thanks to Mrs. Ginny Cathcart who made herself so readily available during the initial phase of the research and who organized the selection of the subjects at the Vancouver Community College. It was a pleasure to work with her. I also would like to express my appreciation to the graduating hygiene students of the class of 1995, and the following Graduate Periodontic students: Dr. Todd Jones, Dr. Colin Wiebe and Dr. Laura Noce for helping in the collection of the oral rinse samples. In addition I wish to acknowledge Dr. Leo Tjäderhane, Miss Sara Hamidi, Mr. Christian Sperantia for their technical support and Mr. Bruce McGaughey for the visual aids enabling the presentation of my thesis.

Finally, may heartfelt thanks to my family and friends for their support and understanding throughout these past three years: particularly my parents, Elie and Monique, my sister Catherine, my brother John and my friend Dr. Anita Orendi. Their constant encouragement made it possible for me to carry forth when times were particularly difficult.
INTRODUCTION

Initially periodontal disease progression was described as being linear but recent studies have proposed a burst theory for its progression (Socransky et al., 1984; Lindhe et al., 1983; Goodson et al., 1982). The new theory as opposed to the traditional continuous progressive disease model, explains the progression of periodontal disease as being episodic with bursts of disease activity followed by indefinite period of quiescence. In this concept, only a few teeth and even tooth surfaces are involved at one time, and it is also believed that certain gingival sites could be free of destructive periodontal disease for a lifetime.

Despite all the recent developments being achieved in the periodontal field, the periodontal probe along with other clinical signs such as bleeding on probing, suppuration and fremitus, still remains the only tools available to assess periodontal disease progression (Wilson, 1996; Philstrom et al., 1986). Authors have come to agree that a loss of clinical attachment of 2 mm was necessary in order to achieve 100% reproducibility of all measurements and that only at that point does intervention by the therapist become necessary (Wang et al., 1995; Janssen et al., 1987; Badersten et al., 1984; Glavind & Loe, 1967). In other words a 2 to 3mm change in clinical attachment loss has to occur for one to consider that progression of disease has occurred (Best et al., 1990; Aeppli et al., 1985; Lang et al., 1986; Lang et al., 1990). Because of this crude scale used to measure attachment loss, the reliability of the burst theory has been questioned (Jeffcoat, 1991). In the past ten years, automated devices used to measure attachment loss have been developed and seem to show less errors in measurements (Gibbs et al., 1988; Osborn et al., 1990; Hull et al., 19950
Because of the relatively poor reproducibility of the measurements taken with the periodontal probe, the need for a rapid and reliable test to determine the periodontal disease progression has been emphasized. There are presently no reliable and simple chemical indicator of periodontal disease which are available (Uitto et al., 1996). In the past studies have used sulcular fluid as a source of indicator material for periodontal status (Golub & Kleinberg 1976; Villela et al., 1987; Cox et al., 1990; Palcanis et al., 1992; Armitage et al., 1994).

Proteolytic enzymes responsible for tissue destruction may originate from several cellular sources including polymorphonuclear neutrophils (PMNs), macrophages, activated connective tissue fibroblasts and epithelial cells and pathogenic oral bacteria (Makela et al., 1994). Saliva contains a number of enzymes; esterases, transferases, glycosidases and proteases (Chancey, 1961; Nakamura & Slots, 1983). For practical purposes it would be interesting to find a salivary enzyme that would reflect the periodontal status of a patient. Salivary elastase for example has proven to be a promising new aid in this evaluation of periodontal health (Uitto et al., 1996; Nieminen et al., 1993). Neutrophil elastase is an important player in the degradation of extracellular matrix during periodontal inflammation (Bieth, 1978; Cergneux et al., 1982; Janoff, 1985). Oral elastase measurements may actually reflect the actual disease process. It appears that detection of salivary elastase activity of adult patients with periodontitis would serve as an adjunct to the periodontal examination and follow up of periodontal healing after therapy. The procedure of collecting saliva and performing the enzyme assay are simple and quick to perform (Nieminen et al., 1993).

Because most oral leukocytes migrate to the oral cavity along with sulcular fluids (Wright, 1964; Schiott & Loe, 1970) the measurements of salivary elastase could reflect oral PMN counts (Uitto et al., 1996). Orogranulocytic migratory rate has been shown to be a reliable measurement of oral health (Skougaard et al., 1969) and the migratory
rate of leukocytes to the oral cavity may be increased as much as 150 times in periodontitis (Klinkhamer, 1968).

In addition, several microbial and cellular enzymes are partly responsible and relevant to epithelial cell detachment. They include among others; β-glucuronidase, collagenase and hyaluronidase as well as lysosomal enzymes and microbial enzymes (Neiders, 1972). Activated but not resting PMNs, can cause detachment of human epithelial cells (Altman et al., 1992, Sugiyama et al., 1992). Sugiyama showed that a culture of PMNs with Actinobacillus actinomycetemcomitans (Aa) and human gingival keratinocytes led to non lytic detachment of the keratinocytes, thus PMN are capable of affecting cell adhesion when interacting with certain bacteria. A positive correlation between general protease activity and the number of epithelial cells in the oral cavity was also demonstrated (Watanabe et al., 1981; Smith et al., 1994). When one considers the above findings, assessment of the PMN and epithelial cell counts and their relation to the salivary elastase activity in patients with periodontal diseases becomes of particular interest.

Recently a study by Uitto et al. (1996), has shown a direct correlation between elastase activity and periodontal disease severity. However, a small number of patients with severe periodontal disease did not show this positive correlation. Numerous factors together may be responsible for such negative results. For example, certain medical conditions such as diabetes mellitus, localized juvenile periodontitis, neutropenia, agranulocytosis are associated with defective PMN functions (Van Dyke & Hoop, 1990; Cutler et al., 1991; Miyasaki et al., 1991). Low levels of elastase activity may also be related to a quiet or an active stage of disease or to minimal inflammatory reaction seen in some types of periodontitis such as localized juvenile periodontitis (Uitto et al., 1996).

Finally, smoking habits and the presence of higher levels of elastase inhibitors in the saliva of these patients have been suggested (Giannopolou et al., 1994).
The purpose of this paper was to attempt to clarify whether oral elastase activity correlates with the PMN counts in the oral cavity and to find answers to questions such as why do some patients with advanced disease show low elastase activity. The hypothesis was that these patients have defective PMNs and therefore would be at higher risk for periodontal destruction.
CHAPTER 1

1. Review of the literature

1.1 Periodontal diseases

Because of the high inherent error found with manual probes, an increase of 2.0-3.0 mm in probing depth is necessary before a diagnosis of progressing attachment loss can be made with any reliability. Because traditional tests for periodontal disease such as visual inspection, pocket probing and radiographs, all detect anatomic changes that occur late in this model, a new test for periodontitis would be advantageous to detect disease activity before significant further destruction of bone and connective tissue occurs (Jeffcoat, 1994). Periodontal diagnostic tests have been reviewed in recent articles (Louie & Larjava, 1994; Page, 1992; Lamster & Novak, 1992).

The type and severity of periodontal disease may reflect, more the competence of the host defense than the virulence of commensal oral microorganisms. It reflects the appropriateness of the host response to bacterial challenge (Clarke & Carey, 1985). As a result more emphasis has been placed lately on the development of a test which would evaluate the activity of particular enzymes as they relate to periodontal disease activity and progression and therefore reflect the hosts response to the pathogens. In general it has been difficult to establish a convincing correlation between enzyme levels and disease activity, probably due to the high site-to-site, person-to-person, and day-to-day variation (Birkedahl-Hansen, 1993; Page, 1992).

Most of the earlier studies involved the measurement of enzymes and immunoglobulins (alkaline phosphatase, prostaglandin-E2, asparate aminotransferase, collagenases, β-glucuronidase (βG), elastase, cathepsin G and IgG4 antibody subclass) (Page, 1992) in the gingival crevicular fluid (GCF) and have been associated with disease activity as measured by attachment loss of 2 mm or more.
High levels of PGE$_2$ in the crevicular fluid represent a significant risk factor for human periodontal attachment loss (Van Dyke et al., 1990). Offenbacher et al. (1993) emphasizes the importance of host response rather than bacterial etiology for periodontal diseases and discuss PGE$_2$ as being a predominant factor for the tissue destruction observed. The crevicular fluid levels of PGE$_2$ are found to be elevated in disease active sites (Offenbacher et al., 1986). The amount of PGE$_2$ released from lipopolysaccharide-stimulated monocytes may be under genetic regulation. This variation in responsiveness is found to be linked to human leukocyte antigen-D loci (HLA-D) (Genco & Loe, 1993). The cell of the monocyte lineage predominantly produce large amounts of interleukin 1$\alpha$ and interleukin 1$\beta$ (Tatakis, 1993) and stimulate PGE$_2$ and thromboxane synthesis. As the end result, prostaglandin E release causes connective tissue destruction as well as bone resorption (Offenbacher et al., 1993). In addition, an increase in PGE production by human periodontal ligament fibroblasts have been reported in response to interleukin-1 (Offenbacher et al., 1993). Neutral proteases that participate in periodontal destruction are produced by different cell types present in the gingiva (Soumalainen et al., 1989). In gingival fluid samples from patients with advanced periodontal disease, neutral protease activity was found to be significantly elevated (Bowers & Zahradnik, 1989). GCF collagenase activity increases with the severity of periodontal disease (Overall et al., 1987). The active form of the enzyme has been related to diseased sites, whereas its latent form and enzyme inhibitors are associated with healthy sites (Larivee et al., 1986). 

$\beta$-glucuronidase and asparate aminotransferase have been studied extensively (Chambers et al., 1991; Persson & Page, 1992; Lamster et al., 1994; Lamster et al., 1995). This last study by Lamster et al. found persistently elevated levels of $\beta$G in the GCF samples of patients with clinical attachment loss.
Tests which involve the measurement of enzymes in oral fluid samples of periodontitis patients include; collagenases (Uitto et al., 1990), gelatinase (Gangbar et al., 1990; Makela et al., 1994), peptidases and glycosidases (Nieminem et al., 1993). This latter study showed that the enzyme activity of trypsin-like protease, elastase-like protease, general protease, \( \alpha \)-glucosidase and \( \beta \)-galactosidase were significantly higher in the whole saliva of adults with advanced periodontitis than in healthy patients. A decrease in the protease activity was shown in response to different phases of treatment, this activity then approached that of control values. Similar results were found with metalloproteinases MMP-2 and MMP-9 both type IV collagenases. A significant correlation between elastase and gelatinase activities was also demonstrated in that same study, however some adult periodontitis patients demonstrated high elastase and low gelatinase levels (Makela et al. 1996). This may be explained by the results of another study which concluded that PMN elastase activity may be a possible MMP activator (Okada & Nakanishi, 1989). Ongoing studies with GCF elastase and in oral rinse are also being carried out (Nieminem et al., 1993; Uitto et al., 1996).

A site specific test permits the evaluation of areas in the mouth which may be of concern, and would permit the identification of disease-active versus disease-inactive periodontal sites. For example following initial examination and initial therapy, specific teeth with deeper pocket depths which may still be showing some disease activity could be monitored more closely with the test and used in conjunction with the clinical tools presently available. Since disease progression is presently considered infrequent and episodic and most progression occurs in a small proportion of highly susceptible individuals, a more general test such as an oral rinse test would permit the screening of patients prior to their initial visit at a periodontist office. This test would therefore be used as a "home test" to determine if there is a need for a periodontal consultation even prior to any visit with a specialist.
The patient's dentist may play a role in the diagnosis by making appropriate recommendations. For a group of patients who require closer attention (rapidly progressive periodontitis patients) it could be used in the intervals between recall appointments to determine the frequency of these appointments. Ultimately a perfect test would be one which would show 100% specificity and sensitivity. As we are still far from developing such a test, the clinician is left with the periodontal arsenal at hand and will need to decide whether ruling out disease (specificity) is more or less important than detecting disease (sensitivity) (Jeffcoat, 1994).
1.2. Pathogenesis

1.2.1. Bacterial flora

In the 1950s and the 1960s the concept that untreated gingivitis progressed to periodontitis emerged (Marshall-Day et al., 1955). Ranney later suggested that gingivitis does not invariably progress to periodontitis and that certain measures of host response (antibody and PGE₂ in GCF) are possible predictors of clinical outcome (Ranney, 1986). Most gingivitis lesions are transient or persistent but not progressive. A small proportion of gingivitis sites will in some patients, progress to periodontitis.

Periodontal diseases are infections caused by organisms whose products penetrate into the gingival connective tissue and in some forms of the disease, the bacteria appear to penetrate or invade into the gingival connective tissue. *Actinobacillus actinomycetemcomitans* (Aa), a facultative anaerobe non-motile coccobacillus mostly found in localized juvenile periodontitis cases (LJP) is believed to invade into the periodontal tissue in periodontal lesions (Zambon, 1985). *Black pigmented bacteroides* (BPB) are also often associated with severe forms of periodontal diseases. They are rod shaped anaerobes gram-negative and non-motile. *Porphyromonas gingivalis* (Pg) and *Prevotella intermedia* (Pi) are the most common (Van Winkelhoff et al., 1988). *Spirochetes* although present in the adult forms of periodontal diseases are detected in higher numbers in the tissues of the crevicular pockets in cases of acute necrotizing ulcerative gingivitis. (Loesche & Laughon, 1981).

Other bacteria which are commonly associated with periodontal disease activity are *Fusobacterium nucleatum*, *Eikenella corrodens*, *Wolinella recta*, anaerobic Vibros and others such as *B. forsythus* (Tanner et al., 1984; Dzink et al., 1985).
Therefore bacteria are the primary causative agents of gingivitis and the various forms of periodontitis. The host response to periodontal pathogens plays a major role in preventing their spreading to other areas of the body. The net effect of the host response to the organisms causing periodontal infections is localized tissue destruction in the periodontium and protection from extensive local and systemic infection with these pathogens.
1.2.2. Host defense mechanisms in periodontal disease

Periodontal inflammatory responses are primarily the results of an immunologic process. The mechanisms of tissue destruction can be classified as direct and result from the action of bacterial components that damage tissue directly and indirect which results from the destructive host responses triggered by the infecting organisms (Genco, 1990). A multitude of gram negative bacteria such as *A. actinomycetemcomitans, Porphyromonas gingivalis, Treponema denticola, and Prevotella intermedia* colonize the periodontal pocket. These bacteria contain potent virulence factors that cause disruption in the integrity of the periodontium and lead to attachment loss (Van Dyke & Vaikuntam, 1994). The pathogenesis of inflammatory periodontal disease still remains consistent with the model of Page and Schroeder (1976). Gingivitis typically begins with the initial lesion which appears 2 to 4 days after plaque accumulation in a previously healthy gingiva and it is localized to the gingival sulcus and includes the junctional epithelium and most of the coronal part of the connective tissue. Vasculitis, loss of perivascular collagen, increased migration of leukocytes into the junctional epithelium and sulcus, alteration of the coronal part of the junctional epithelium and increased crevicular fluid are all observed. Four to seven days after plaque accumulation the early lesion follows with a dense lymphoid infiltrate subjacent to the junctional epithelium. Collagen loss increases and may reach 60% to 70% in the inflamed tissue. The established lesion reflects the chronic clinical adult gingivitis which occurs 2 to 3 weeks after the beginning of plaque accumulation. Plasma cells with a predominance of IgG and IgA but rarely IgM are seen extravascularly. Acute inflammation persists and connective tissue loss increases without appreciable bone loss. Lateral and apical extension of the junctional epithelium is observed.
The early pocket formation may or may not occur and the established lesion may remain stable for extended periods of time. When gingivitis progresses to periodontitis an advanced lesion is observed. It is characterized by apical proliferation and ulceration of the pocket epithelium. Loss of bone with attachment loss resulting in the formation of a true pocket is seen. Plasma cells still predominate with formation of copious exudate and even purulent discharge (Page & Schroeder, 1976).

Neutrophils (PMNs) are the predominant leukocytes found in the oral cavity. It has been shown that half of the leukocytes infiltrating the junctional epithelium and 90% of those in the gingival crevicular fluid are PMNs (Van Dyke & Vaikuntam, 1994). The nucleus of the PMN is multilobular which contributes to the elasticity of the cell, allows its migration through small openings as those between junctions of endothelial cells. The normal response of PMNs to microbial invasion is migration to the site of infection (Van Dyke & Hoop, 1990). PMN predominate in the junctional epithelium and the gingival crevice whereas lymphocytes and monocytes/macrophage predominate within the subjacent connective tissue (Page & Schroeder, 1976). The concentration of PMNs in the periodontal tissues exceeds the concentration of PMNs in the blood. In mild gingivitis, $2.5 \times 10^7$ PMNs/cm$^3$ infiltrate the gingival connective tissue and $1.7 \times 10^8$ PMNs/cm$^3$ are found in the junctional epithelium (Kraal, 1979).

PMN response to microbial invasion is summarized as follows. The PMN's adhere to the vascular endothelium via specific molecules which are present on the surface of the PMN and endothelial cell. The PMN travels to the site of microbial injury in response to specific chemical agents. Surface adhesion molecules (a group of glycoproteins with $\alpha$ and $\beta$ subunits; CD11$\alpha$/CD18, CD11$\beta$/CD18, CD11c/CD18) on the PMN interact with adherence molecules on the surface of endothelial cells; intercellular adhesion molecule-1 and 2 (ICAM-1 and ICAM-2) and endothelial leukocyte adhesion molecule.
ICAM-1 in particular is induced by tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (INF-γ) (Genco, 1992) and permits transendothelial migration of cells. CD18 epitote mediates adhesion and migration of PMNs while the CD11β/CD18 complex is a major receptor on PMN surface for CR3 (Lundegren-Akerlund et al., 1993). In other words, when an infecting organism enters the body it triggers macrophages to produce cytokines (IL-1, IL-6, INF-γ and TNF) which then initiates the expression of leukocyte adhesion molecule ELAM-1 and increase expression of intercellular adhesion molecules ICAM-1 and ICAM-2. These endothelial adhesion molecules interact with PMN receptors (ICAM-1 binds to LFA-1 which is on PMN) which will then adhere and transverse the endothelium. The PMN then traverses the extracellular matrix in response to chemotaxins such as IL-8, the subfragment of complement 5 (C5a) and leukotriene B4 derived from the host. The PMN ultimately kills the infecting agent by phagocytosis and the release of oxidative and nonoxidative bactericidal mechanisms.

Later opsonisation occurs, the invading organism is coated with molecules such as IgG and C3b (opsonins) which are plasma proteins. The role of antibodies in this system is linked to the IgG class. Its role involves the opsonisation of the microorganism and its preparation for phagocytosis by the PMN and monocyte. The Fc fragment of the IgG binds to the Fc receptors on these cells and the Fab fragment bind to the antigens on the bacteria. This complex then becomes phagocytosed by PMNs and macrophages (Genco, 1992). The phagocytosed particle fuses with the PMN granules and a discharge of the granule contents into phagosome follows and intracellular killing results. At times extracellular killing occurs but this is not as desirable because it results in the destruction of the host tissue as well as the inactivation of the organisms (Van Dyke & Vaikuntam, 1994).
Periodontal tissues are therefore protected against microbial attack primarily by the PMN which releases lysosomal enzymes into the crevice and by phagocytosis, destroying the invading organisms (Socransky et al., 1991). Essentially protective in nature the PMN can also cause destruction of local tissue by releasing potent lysosomal enzymes and oxygen radicals which are potentially damaging to the periodontal ligament. These products released by the host during periodontal inflammation include enzymes such as metalloproteinases, elastase and also cytokines, prostaglandins, (Genco, 1992), oxygen radicals, superoxide anion, hydrogen peroxide, hydroxyl radical, and the potentially cytotoxic hypochlorous acid (Altman et al., 1992; Shapira et al., 1991; Van Dyke & Vaikuntam, 1994) which may damage the periodontal ligament especially when released extracellularly. The interaction of bacterial antigens with inflammatory cells results in the production of cytokines. Some of the major cytokines which are known to regulate the PMN functions include; IL-1, IL-8, TNFα secreted by monocytes and many other cell types. IL-1 is a potent inflammatory mediator and it affects several cell. It promotes the chemotaxis of phagocytes and enhances demargination and adhesion of PMN (Schleimer & Rutledge, 1986; Sullivan et al., 1987). This cytokine permits the synthesis of prostaglandins and metalloproteinases such as collegians which are responsible for both controlling tissue invasion by the microorganisms and the host’s tissue destruction. PMNs also respond to IL-8 by migration and release of their granular enzymes (Bickel, 1993). TNFα is another cytokine which is secreted by monocytes and macrophages in response to lipopolysacharides (De Titto et al., 1986; Klebanoff et al., 1986). Among other roles it is responsible for the priming of PMN for enhanced degranulation and production of oxygen products (De Titto et al., 1986; Klebanoff et al., 1986). Finally colony-stimulating factors are a group of cytokines that control production, proliferation and differentiation of leukocytes (Donahue et al., 1986).
Once the PMNs have left the circulation they encounter a complex array of molecules, the extracellular matrix proteins (ECM) which include fibronectin, laminin and serum amyloid P component which upregulate phagocytosis (Brown, 1986).

The PMN's cytoplasmic granules are; primary (azurophil) granules, secondary (specific) granules and tertiary (secretory) granules. The following table gives a description of these granules and their contents.
Table 1: The neutrophil granules and their contents.

<table>
<thead>
<tr>
<th>Granules</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary (azurophil) granules</strong></td>
<td></td>
</tr>
<tr>
<td>-Cellular myeloperoxidase</td>
<td>Anti-microbial</td>
</tr>
<tr>
<td>-Lysozyme</td>
<td></td>
</tr>
<tr>
<td>-Low molecular weight cationic proteins</td>
<td>Histamine release</td>
</tr>
<tr>
<td>-Acid hydrolases</td>
<td>Enhance phagocytosis</td>
</tr>
<tr>
<td>-β-Glucuronidase</td>
<td></td>
</tr>
<tr>
<td>-Acid phosphatase</td>
<td>Degradation of the microbial and extracellular matrix components</td>
</tr>
<tr>
<td>-Neutral protease</td>
<td></td>
</tr>
<tr>
<td>-Elastase</td>
<td></td>
</tr>
<tr>
<td>-Cathepsin B/D/G</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary (specific) granules</strong></td>
<td></td>
</tr>
<tr>
<td>-Lysozyme</td>
<td>Hydrolysis of bacterial cell wall peptidoglycan</td>
</tr>
<tr>
<td>-Alkaline phosphatase</td>
<td></td>
</tr>
<tr>
<td>-Collagenase</td>
<td></td>
</tr>
<tr>
<td>-Vitamin B12 binding protein</td>
<td></td>
</tr>
<tr>
<td>-Lactoferrin</td>
<td></td>
</tr>
<tr>
<td><strong>Tertiary (secretory) granules</strong></td>
<td></td>
</tr>
<tr>
<td>Gelatinase (MMP-9)</td>
<td></td>
</tr>
</tbody>
</table>

The PMN/antibody/complement axis is therefore critical for protection against periodontal bacteria. Increase susceptibility to periodontal disease is seen in patient who present with abnormalities in this system.
1.3. Protective role of polymorphonuclear leukocytes

1.3.1. PMNs and general health

The inflammation process involves a number of cells which include PMNs, macrophages, monocytes that secrete proteases and basophils and mast cells that release histamine, as major components (Sklar et al., 1984). PMNs are major mediators of host defense and injury, they are short lived cells which constitute about 70\% of the peripheral blood leukocytes and are produced in large numbers in the bone marrow (Genco, 1992). Their main function is to help eliminate pathogens from the body (Gadek, 1992). Recruitment of PMNs occurs as part of the normal host defense response with the goal of eliminating infectious organisms and assisting in the repair process. Activated leukocytes are a major source of cytokines and other inflammatory mediators that may directly injure tissues (Clark et al., 1995).

In the lung, PMNs play a vital role in protection from microorganisms. PMNs produce a number of short lived reactive oxygen species, including hydrogen peroxide, hydroxyl radicals, and superoxide anions. The major proteases produced by the PMN are: elastase, gelatinase and collagenase. PMNs cause injury by means of these oxidative and proteolytic processes (Leavell et al., 1996). Because PMNs are not capable of regulating their own functions, α1-antitrypsin (α1-AT) and α2–macroglobulin are some of the protease inhibitors responsible for regulating the degree of tissue damage caused indirectly by the releases of enzymes and oxidative agents from leukocytes.
An appropriate equilibrium between proteases and anti-proteases is essential for maintaining the health and integrity of the tissues. Factors that alter this balance can result in severe damage to the host (Gadek, 1992). Studies suggest that leukocytes may also contribute to significant organ injury. A number of important cardiovascular diseases in which leukocyte and endothelial activation play a destructive role have been identified, including diabetes, atherosclerosis, myocardial infarction, stroke, and peripheral vascular diseases (Schmid-Schonbein, 1995). The evidence suggests that activation of leukocytes in the circulation is accompanied by destructive side effects. Synovial fluid from patients with rheumatoid arthritis often contains large numbers of PMNs (Moore et al., 1993). The degradation of cartilage matrix in rheumatoid arthritis by stimulated PMNs, is due primarily to the release of proteolytic enzymes. Inflammatory mediators such as oxygen species which are released by PMNs may also be responsible for the tissue damage in acute ulcerative colitis (Kazi et al., 1995). An important feature of inflammatory lung disease is the accumulation of PMNs in the distal airspace. Other pulmonary processes such as idiopathic pulmonary fibrosis, asthma, bronchiolitis obliterans are also associated with increased PMNs in the airspaces (Hunninghake et al., 1981; Konstan et al., 1994). Epithelial lining does not seem to pose a significant obstacle to PMN trafficking. Fibrin degradation products (FDP) are potent chemotactic factors for human PMN. They induce transepithelial migration of PMN. Thus increased local accumulation of FDP combined with increased epithelial permeability may specifically recruit activated inflammatory cells to the distal airspaces (Leavell et al., 1996).
PMNs are also responsible for myocardial injury by adhering to the endothelium of the coronary microvasculature and releasing mediators (i.e. $\text{H}_2\text{O}_2$ and elastase) that directly lead to myocytes death without any direct contact between these two cell types (Buerke et al., 1994). Fibrin dissolution also involves leukocytes. After disintegration leukocytes produce lysis of plasminogen-rich as well as plasminogen-free fibrin. The activity is caused by protease directly and not by plasminogen activator. Still, the effect of leukocytes is weak compared to that produced by the plasminogen activator in capillary endothelial cells invading fibrin deposits in the body (Astrup et al., 1967).

Daudi et al. (1991) studied the degradation of fibronectin bound to denaturated collagen by inflammatory polymorphonuclear leukocytes (PMNL). The degradation of gelatine-bound fibronectin appeared to be due to the release of PMN-derived leukocyte elastase. The role of fibronectin in endothelial cell adhesion to a collagenous matrix is believed to be related to its binding affinity for both collagen and integrin receptors on cell surface. Fragmentation of matrix fibronectin may not only result in cell detachment leading to altered vascular integrity, but may also have the potential to competitively inhibit cell adhesion or matrix assembly. Fragments of fibronectin could potentially amplify vascular injury by further recruiting inflammatory cells. Fragmentation of fibronectin complexed to denatured collagen by leukocyte-derived elastase, support the concept that sequestration of activated PMNL into the lung microvascular and interstitial area could alter matrix and tissue integrity (Daudi et al., 1991). PMN and their products, such as elastase therefore mediate the lung and also the liver injury after intestinal ischemia-reperfusion (Simpson et al., 1993).
In conclusion prevention of PMN activation may result in improving the rate of healing in chronic systemic conditions as well as minimizing the recurrence rates (Schmid-Schonbein, 1995).
1.3.2. PMNs and periodontal health

Persistent periodontal inflammation leads to breakdown of connective tissue (Seymour, 1987). Prior to this connective tissue loss, PMN invasion of the junctional epithelium is evident. Even in health, the junctional epithelium is constantly being renewed and cells desquamated from the surface are replaced with cells of the lower layers (Skougaard, 1965). Acceleration of apical migration of the epithelial attachment is seen in the presence of clinically evident periodontal disease (Saxe et al., 1967, Suomi et al., 1971). PMNs are involved in the mechanisms of host defense and are considered to be the first line of defense against bacterial infections. In the control of bacterial periodontal disease, PMNs represent the first cellular line of defense (Van Dyke & Vaikuntam, 1994). Polymorphonuclear leukocytes are the predominant leukocytes in the exudate obtained in the gingival crevice (Attstrom, 1970). In histologic sections of normal or slightly inflamed gingivae, the dento-gingival leukocytes are found in the intercellular spaces of the junctional epithelium and sometimes along the tooth surface and they are usually more numerous at the sulcus base and they gradually decrease in number towards the apical end of the junctional epithelium ( Schroeder, 1973a). PMN isolated from the gingival crevice are capable of phagocytosis, which represents a liberation of enzymes (Attstrom, 1975). PMNs also use oxidative as well as non oxidative mechanisms and either kill bacteria, influence bacterial growth or modify bacterial colonization in the periodontium. Delivery of antimicrobial substances by PMNs involves respiratory bursts of activity, phagocytosis, secretion or cytolysis/apoptosis (Miyasaki, 1991). Lysosomal release from intact cells (PMNs) can be activated by bacterial products such as endotoxins, streptolysins and other substances (Weissman & Thomas, 1962, Weissman, 1965).
Monocytes which develop into tissue macrophage to digest antigens are also involved initially. They migrate less actively than PMNs into the gingival crevice but their number increases significantly in gingival tissue during chronic gingivitis and periodontitis (Attstrom, 1975). Macrophages involved in an inflammatory response may be rapidly primed by proteases released from degranulating PMNs. They can then mount an effective oxidative metabolic response to microorganisms or tumor cells but also can cause greater tissue damage (Speer et al., 1984). The junctional epithelium contains less desmosomes than oral epithelium and the intercellular spaces are wider. Absence of desmosomes between epithelial cells and inflammatory cells have also been noted (Geinsenheimer & Han, 1971). PMNs traverse the junctional epithelium and migrate into the gingival sulcus forming a protective barrier wall against bacterial plaque. Still, for PMNs to infiltrate epithelium some desmosal detachment has to occur (Neiders, 1972). The dynamics of PMN function can be divided in three distinct events; sensory, which involves detection of stimuli by the neutrophil membrane, transduction, the biochemical process by which signals cross the plasma membrane and the effector or function responses such as chemotaxis and phagocytosis (Van Dyke & Vaikuntam, 1994). Individuals with PMN defects or low numbers of PMN will most likely develop advanced forms of periodontal disease (Miyasaki, 1991) and abnormalities in the neutrophil/antibody/complement axis often will lead to increased periodontal susceptibility (Genco, 1992).

Diseases associated with leukocyte defects include Chediak-Higashi syndrome, leukocyte adhesion defect (LAD I & II), cyclic neutropenia, diabetes mellitus, trisomy 21 as well as the early onset forms of periodontitis (Hart, 1994). The function of crevicular fluid PMNs is believed to be significantly depressed in patients with localized juvenile periodontitis (LJP), post juvenile periodontitis and rapidly progressive periodontitis (Sigush et al., 1992, MacFarlane et al., 1992).
In particular in LJP patients, the PMN is capable of internalizing the pathogen (Aa), but a defect in the bactericidal activity and qualitative defect in the ability to neutralize Aa seems to exists (Van Dyke & Hoop, 1990). In patients with LJP where a minimal inflammatory reaction is present, the corresponding elastase activity is also low (Ingman et al. 1994). In patients with cyclic neutropenia, which is characterized by episodic fluctuations in the numbers of blood PMNs, monocytes, lymphocytes, platelets and eosinophils, severe generalized prepubertal onset periodontitis with rapid alveolar bone loss is seen (Prichard, 1984; Kirstila et al. 1993). The Chediak-Higashi syndrome is associated with a defective intracellular bactericidal activity in PMNs (Clark, 1971). PMN dysfunction is also said to be an explanation for oral and periodontal breakdown seen in patients with AIDS (Van Dyke & Hoop, 1990). In this particular form of periodontal disease also an increase in the PMN function has been reported (Ryder et al., 1988). The cell surface expression of glycoproteins Mac-1, LFA-1 and GP150, 95 are deficient in patients with LAD (an autosomal recessive disease). These surface glycoproteins are important in regulating the cellular adhesion reactions which permit the communciation of PMNs with vascular endothelial cells and complement components for the migration of PMNs from the peripheral circulation to the areas of inflammation (Van Dyke & Hoop, 1990).
1.4. Elastase activity in relation to various health disorders

1.4.1. Elastase and its inhibitors

In 1949 Balo and Banga were the first to establish the presence of elastase in the pancreas (Balo and Banga, 1949). The pure enzyme was obtained in 1956 from porcine pancreas (Lewis, 1956) and the presence of elastase activity in granules of human PMNs was reported later (Janoff and Scherer, 1968). Furthermore elastolytic activity has been demonstrated on the surface washings of human skin, in cultured normal and tumor cells and in the venom of rattlesnakes (Bieth, 1978). Macrophage elastase compared with other mammalian elastases (granulocyte and pancreatic) is catalytically distinct and its proteolytic cleavage differs. Macrophage elastase is a broad-range proteinase capable of degrading fibrinogen, immunoglobulin G subclasses, fibronectin among other tissue substrates. Finally the inhibitors of granulocyte elastase and pancreatic elastase are ineffective against macrophage elastase (Werb et al., 1982).

The elastase of human PMNs is a serine proteinase. It is a single chain polypeptide with a molecular weight of 33,000 and is active at neutral pH (Ohlsson & Olsson, 1974). Valine or alanine residues are preferred as substrates (Blow, 1977). Although the action of elastase is beneficial to the organism against invading pathogens, because it participates in their inactivation, the adverse effect of this enzyme is destruction of the host tissue. It is synthesized primarily in promyelocytes and stored in the cytoplasmic azurophilic granules of maturing PMNs. It is discharged in the tissues when the cell encounters objects to be phagocytized (Janoff, 1985).
Type III collagen and elastin (major supporting components of lung connective tissue and also blood vessels and gut) and type IV collagen (important for the integrity of epithelial and endothelial basement membranes) are important proteins and glycoproteins which can be attacked by PMN enzymes (Mainardi et al., 1980). The degradation products of elastin by the elastase can recruit even greater amounts of leukocytes through a chemotactic activity of the elastin fragments for leukocytes therefore causing even more destruction of the host tissues (Senior & Campbell, 1983). Core proteins of proteoglycan molecules in the connective tissues ground substance (Kaiser et al., 1976) and fibronectin (McDonald & Kelly, 1980) a major cell-adhesion molecule which is critical to the organization of many tissues are also degraded by elastase. The fragments of fibronectin produced could potentially amplify vascular injury by recruiting inflammatory cells (Daudi et al., 1991). PMN leukocytes participate in the pathogenesis of increased permeability of the lung microvasculature. The proteolysis of matrix fibronectin is one explanation for increased vascular permeability (McDonald et al., 1979). When stimulated, PMN bind to endothelial cells as well as matrix proteins (including fibronectin) which results in the release of proteases (elastase, cathepsin G) that have the potential to produce vascular injury (Senior & Campbell, 1984). Pulmonary epithelial injury also leads to increased permeability and plasma exudation. Fibrinolysis leads to high concentrations of airway fibrin degradation products which cross the injured epithelial barrier and recruit PMNs from the vascular space. The increased burden of activated PMNs leads to the release of elastase which then results in further digestion of fibrin and increase recruitment of PMNs (Leavell et al., 1996).
Many other plasma proteins (immunoglobulins and clotting factors) can also be hydrolysed by PMN elastase (Havemann & Gramse, 1984). IgG is fragmented into the Fc and Fab fragments by PMN elastase. These fragments regulate the functions of stimulated PMNs such as chemotaxis, oxidative bursts and enzyme release. The Fc fragments have an inhibitory effect on inflammation by decreasing chemotaxis and oxidative burst of stimulated PMNs while the release stimulating activity by the Fab fragments results in an upregulation of elastase induced IgG degradation (Eckle et al., 1992).

Some of the diseases in which PMN elastase and oxidants are thought to be involved include pulmonary emphysema, chronic bronchitis, cystic fibrosis, bronchiectasis, adult respiratory distress syndrome, artherosclerosis, rheumatoid arthritis, psoriasis, vasculitis, glomerulonephritis, and consumption coagulopathies associated with gram-negative sepsis, leukemias, pancreatitis, ulcerative colitis and colonic Crohn's disease (Janoff, 1985; Daudi et al., 1991; Finn et al., 1993; Uehara et al., 1992; Saitoh et al., 1995; Rudolphus et al., 1992; Leavell et al., 1996). Leukocyte activation with concomitant protease release has been implicated as a possible source of organ failure after cardiac surgery (Jacob et al., 1980). An increase in circulating elastase is seen during cardiopulmonary bypass surgery (CPB) (Faymonville et al., 1991; Finn et al., 1993). This continuing increase in leukocyte elastase levels after surgery may reflect an alteration in the mechanisms of elimination of this enzyme by its antiproteases (α1-PI and α2-macroglobulin) (Faymonville et al., 1991). Endothelial injury inflicted by adherent PMNs results in an acute post-operative leak in children who are undergoing this type of surgery (CPB), an increase proportion of PMN is seen in conjunction with a rise in the levels of CD11/CD18, which may sequester selectively in the lungs (Finn et al., 1993).
Following the treatment of bronchopulmonary dysplasia with disodium cryomoglycate, the levels of PMN elastase concentration gradually decreased (Yamamoto et al., 1992). PMN elastase may also contribute to cartilage glycosaminoglycan loss in rheumatoid arthritis making it a rational target for therapeutic intervention (Moore et al., 1993). PMN, monocyte and macrophage elastases have all been implicated as determinants of parenchymal lung injury (Janoff, 1985). Elastinolytic proteinases have also been identified in platelets, monocytes, macrophage-conditioned medium and smooth muscle cells (Janoff, 1985). Macrophages can also bind and internalize PMN elastase and release it latter in an active form contributing to further tissue injury especially if the cells undergo anoxic injury (Janoff, 1985).

The natural defense mechanisms preventing injury involve the circulating inhibitors. The most prominent and specific inhibitors of proteinases are serpins, which are glycoproteins of 50 to 100 kD (Stryer, 1988). α1 proteinase inhibitor (α1Pl) initially named α-antitrypsin because of its ability to inactivate pancreatic trypsin (Travis & Salvesen, 1983), α2-macroglobulin (α2M) and secretory leukocyte protease inhibitor (SLPI), present in large quantities in serum to neutralize the proteases. α1Pl is the main inhibitor of PMN elastase, which is also regulated by α2M and secretory leukoproteinase inhibitor (Weiss, 1989). α1Pl, main source is the liver but also it is synthesized and secreted locally in the tissues by macrophages (Koj et al., 1978). In normal conditions, α1-antitrypsin protects the lungs from destruction by the proteolytic PMN elastase (Van Steenbergen, 1993). Genetically some patients may be predisposed to higher levels of elastase activity due to deficiencies in antitrypsin (Carell, 1990; Gadek, 1992; Barker 1992).
This deficiency may lead to the development of emphysema due to the controlled action of proteases on lung tissue (Wewers et al., 1987), and is also associated with predisposition to developing other medical conditions such as liver cirrhosis in early childhood (Kalsheker, 1994; Blank, 1994). The local inactivation of elastase inhibitors is thought to lead to extensive elastin degradation. Macrophage elastase is not inhibited by α1PI and it is also able to degrade elastin. Intratracheal administration of lipopolysacharrides (LPS) results in a release of proteolytic enzymes from infiltrating PMNs. At least part of these released enzymes represent elastase, because proteolytic activity as detected by elastase selective-substrates can be inhibited by specific elastase inhibitors (Rudolphus et al., 1992). Removal of leukocyte elastase depends upon receptor-mediated clearance. Alveolar macrophages are capable of binding and internalizing elastase in two ways; elastase is first bound to a circulating proteinase inhibitor and then the macrophage will bind to the elastase-α2 macroglobulin complexes. The second way involves binding to elastase directly without its proteinase inhibitor. These mechanisms may be of importance for defense against proteolytic injury (Campbell, 1982).

The studies reported here indicate the direct implication of PMN and its elastase in the pathogenesis of several systemic diseases and points to the research being undertaken to understand and control the destruction caused by this enzyme during the inflammation process (Finn et al., 1993). Safe and effective inhibitors of elastinolytic proteases especially of PMN elastase are being developed to control these disease processes (Janoff, 1985).
1.4.2. Elastase and periodontal health

In the periodontium, proteases have a multiple role on both the normal remodeling of the tissue and in pathological conditions such as periodontal disease where the destruction of the tissue structure is likely to be associated with increased proteolytic activity (Uitto, 1987; Birkedal-Hansen, 1993). Initial studies involved mostly the detection of enzymes in the gingival crevicular fluids. The concentration of free lysosomal enzymes increases within the gingiva in the presence of increasing inflammation, and a number of reports indicate that lysosomal enzymes in the crevicular area tend to increase in gingivitis and periodontitis (Cimasoni et al., 1987). These enzymes detected in the gingival crevicular include acid and alkaline phosphatase, β-glucuronidase, lysozyme, hyaluronidase and proteolytic enzymes such as cathepsin D, neutral proteinases, collagenase, type IV collagenases (MMP-2 and MMP-9) and elastase (Makela et al., 1994; Overall et al., 1987; Overall et al., 1991). Asparate amino transferase (AST), a cytoplasmic enzyme released upon cell death into the extracellular space, has also been studied extensively (Chambers et al., 1991; Persson & Page, 1992). Elastase, collagenase and gelatinase particularly play a central role in periodontal attachment loss and disease progression (McCulloh, 1994). According to this paper, of all these enzymes, matrix metalloproteinases, AST and elastase show the most promise as markers for disease progression. Although a bacterial origin cannot be excluded, it is probable that most of this neutral proteolytic activity is due to elastase from migrating PMNs (Cergneux et al., 1982).
In fact elastase in the oral cavity is believed to be primarily of PMN origin because there is a clear preference of salivary elastase to valine-containing substrate while oral bacteria appears to prefer alanine substrate (Bieth et al., 1974). Elastase is stored in the primary granules (azurophilic granules) of the PMNs (Ingman et al., 1994; Shapiro et al., 1991). It is an endopeptidase and it cleaves natural substrates such as collagen (although not initially) and proteoglycans. Other sources include mast cells (Seppa & Jarvinen, 1979) fibroblasts and epithelial cells (Bourdillon et al., 1980). Elastase is active at neutral pH but only in the absence of circulating anti proteases such as alpha 2 macroglobulin (α2-macroglobulin) and alpha 1 antitrypsin (α1-antitrypsin) (Cimasoni et al., 1987). These inhibitors form a complex with the enzyme which is rapidly eliminated by the reticuloendothelial system, from the circulation by the liver and from the local inflammation by accumulated phagocytes (Travis & Salvesen, 1983). At least two studies have shown the presence of the α1-antitrypsin in the GCF (Giannopoulou et al., 1992; Schenkein & Genco, 1977) while other studies have shown increased concentration of α2-macroglobulin in the GCF from inflamed gingiva (Ohlsson et al., 1973). However other workers have shown decreasing concentration with increasing pocket depths and bone loss (Skaleric et al., 1986).

Elastase has several functions in degradation of structural proteins (Uitto, 1987). Elastase among other enzymes has been suggested as participating in collagen degradation by solubilizing the polymeric collagen fibers into individual tropocollagen molecules (Barret et al., 1978). This enzyme produced time and concentration dependent detachment of gingival epithelial cells.
Collectively these actions could have profound adverse effects on the function and integrity of the gingival epithelium (Altman, 1992). Elastase degrades elastin, fibronectin (Balian et al., 1979) and some basement membrane proteins (type IV collagen and laminin) (Mainardi, 1980; Ott et al., 1982). It is also believed that it may be partly responsible for the activation of at least one other enzyme, gelatinase (Makela et al., 1994). In that study a good correlation between the oral rinse elastase and gelatinase activity was found in patients with periodontitis although the elastase activity correlated better with the severity of the periodontitis compared with the activity of the gelatinase. Elastase is also said to inactivate tissue inhibitors of metalloproteinase (TIMPs) (Okada et al., 1988). PMN elastase is, therefore, an important player in the extracellular matrix degradation during periodontal inflammation. It's importance has been addressed in several studies (Cox et al., 1990; Zafiropoulos et al., 1991; Palcanis et al., 1992; Armitage et al., 1994). In microscopical studies elastase has been connected to degrade action of extracellular elements of both epithelium and connective tissue in gingiva. It is capable of widening the epithelial intercellular spaces, create a loss in the continuity of the lamina densa and collagen as well as ground substance in the connective tissue (Cergneux et al., 1982). Its presence can only be detected in the area of degradation. Crevicular fluid PMNs release up to five times more elastase than peripheral blood PMNs in patients with periodontitis (Lamster et al., 1991; Palcanis et al., 1992). Sulcular fluid elastase has been found to correlate positively to periodontal attachment loss (Palcanis et al., 1992; Gustafsson et al., 1992; Armitage et al., 1994.).
The presence of some putative periodontopathogenic bacteria in subgingival plaque is also found to correlate with sulcular fluid elastase levels (Zafiropoulos et al., 1991). Studies which involved measuring the activity levels of elastase to compare with periodontal severity were carried out by Nieminen et al. (1993). They found that the activity of salivary elastase correlated significantly with the number of deep gingival pockets (PPD> 6mm) and with gingival index and BOP. Other studies have also found a positive correlation between periodontal disease and the level of elastase activity in the GCF (Jin et al., 1995; Gustafsson et al., 1994; Gustafsson et al., 1994). Elastase activities have been shown to decrease following periodontal treatment (Zambon, 1985; Cox & Eley, 1992). A dramatic reduction of salivary elastase activity was seen following treatment in patients with periodontal diseases (Uitto et al., 1996). Salivary elastase appears to reflect periodontal destruction relatively well (Cox & Eley, 1992). The authors studied 20 patients with chronic periodontitis and again the GCF protease levels appeared to reflect the clinical status of periodontal lesions. Following treatment there was a reduction in all clinical parameters and all protease activities (elastase included). The authors proposed that measurements of protease levels may be of certain value in monitoring disease activity.

Elevated levels of PMN elastase and alpha 1-proteinase inhibitor (alpha-1-PI), its endogenous inhibitor, has been detected in the GCF of patients with AP but not in controls (Darany et al., 1992). Also elevated levels of crevicular elastase-alpha-1-PI complex levels in sites with clinically progressive lesions may reflect early manifestation of a progressive or potentially progressive
lesion (Huynh et al., 1992). Meyle et al. (1992), evaluated the GCF levels of elastase before and after oral hygiene measures. A significant improvement in clinical parameters was found with a decrease in the levels of elastase concentrations. Still healthy sites continued to show functional elastase. In the GCF of patients with periodontitis the levels of PMN elastase surpass that of their inhibitor (alpha 1 AT). This excess of free neutrophil elastase could explain the tissue destruction which occurs in adult periodontitis (Sugiyama et al., 1992). Another study which supports the theory of involvement of PMNs and their proteolytic enzymes in the mechanisms of periodontal destruction is one by Gustafsson et al. (1994), which showed that sites with tissue destruction had significantly higher elastase activity and concentration in GCF and lower levels of α-2-macroglobulin (its inhibitor) compared to sites without destruction (gingivitis or attachment loss). In a follow up study by Gustafsson et al. (1994), the levels of elastase and lactoferrin were measured in GCF of patients with gingivitis and in patients with periodontitis with inflamed sites with and without tissue destruction. The elastase activity was elevated in both patients with gingivitis and periodontitis but the activity was higher in patients with periodontitis. Higher levels of gingival crevicular fluid (GF) elastase activity at sites of intermediate probing depths than at healthy sites were also found in a study by Darany et al. (1992).

A study by Boutros et al. (1996) studied the GCF levels of specific enzymes in natural teeth and around well-integrated and failing endosseous implants. The results showed that the levels of neutrophil elastase, myeloperoxidase, and β-glucuronidase were significantly higher around failing implants compared to successful implants and therefore these enzymes could be used as risk markers of implant failure (Boutros et al., 1996).
Ratkay et al. (1995), studied volatile sulfur compound methyl mercaptan (CH$_3$SH) a byproduct of bacterial metabolism and a principal component of oral malodour. They examined the effects of this product on elastase activity. This compound was found to significantly enhance the secretion of PGE2, cAMP and procollagenase by human gingival fibroblasts and stimulate mononuclear cells to produce IL-1 and enhance the activity of cathepsin B. It did not however affect significantly elastase activity. Their conclusion was that CH$_3$SH was a contributing factor in the enzymatic and immunological cascade of events leading to the tissue degradation in periodontal diseases. Elastase levels appear to be also valid diagnostic markers of advanced dental pulp disease (Rauschenberger et al., 1991).

Saliva is a fluid which originate from several sources. More than 30 enzymes have been found in saliva. A number of these enzymes are produced by microorganisms which normally inhabit the oral cavity. Many are present in the leukocytes found in saliva and a large portion secreted generated by the salivary glands (Chauncey, 1961). Whole saliva contains only small quantities of serum enzymes which may imply that the serum like gingival crevicular fluid becomes diluted by saliva when entering the oral cavity (Nakamura & Slots, 1983).

Oral rinse fluid is a sum of enzyme activity released from all periodontal sites (Gangbar et al., 1990). It could therefore be used as a tool in general assessment of periodontal status. Also it could be used as an adjunct in the evaluation of treatment efficacy, especially in refractory periodontitis cases or in patients with increased susceptibility to periodontitis and when a non-surgical approach of therapy is contemplated.
Finally it could also be used for patient motivation as color changes are notable during enzymatic assay (Uitto et al., 1996). Patients with rapidly progressive and recurrent periodontitis would benefit from early diagnosis (McCulloh, 1994; Eyle & Cox, 1992). The levels of certain enzymes such as cathepsin B- and L-like activity, elastase, tryptase, trypsin and dipeptidyl peptidase IV all GCF proteases may provide useful information on the periodontal status of the patients with severe forms of periodontitis (Eyle & Cox, 1992). Increased levels of elastase in the gingival crevicular fluids, were found to be associated with disease progression. Measurement of levels were also recommended as a monitor of the response to longitudinal maintenance therapy (Jin et al., 1995). In another study by Gustafsson et al. (1992) the levels of elastase activity were higher in the periodontitis patients compared with the gingivitis patients despite the fact that both groups had the same degree of inflammation as expressed by both the gingival index and the antigenic elastase per microliter. Therefore it was concluded that elastase activity in the GCF could be used as an indicator of patients at risk for periodontitis.
1.5. Smoking and periodontal disease

1.5.1. Smoking and general health

Peripheral blood leukocyte counts are found to be higher in smokers than in nonsmokers (Hind et al., 1991). Among numerous other effects, smoking also increases the levels of elastase released by peripheral blood PMNs (Hind et al., 1991; Du By, 1989).

A great deal of experimental evidence suggests that emphysema in smokers is caused by action of PMN elastase on lung elastin. Cohen & Morris, (1961) showed that cigarette smoking could disturb the elastase-anti-elastase balance and aggravate lung disease through this mechanism (Van Steenbergen, 1993; Weitz et al., 1987). Cigarette smoke has been shown to inactivate the elastase inhibitor, alpha 1-proteinase (α1PI), which is believed to be the major anti-elastase in the lower respiratory tract (Trefz et al., 1989; Fera et al., 1987; Ogushi et al., 1991; Hubbard et al., 1987; Muley et al., 1994). Smoker lung lining fluid have also been shown to take approximately 1.5 times to 2 times longer to inhibit PMN elastase (Wewers et al., 1989; Ogushi et al., 1991). In fact, patients with deficiency of α1-antitrypsin are recommended to avoid or discontinue the smoking habit (Van Steenbergen, 1993). Several mechanisms for the tissue destruction observed have been proposed. Cigarette smoke may indirectly affect lung α1-antitrypsin by virtue of its ability to recruit and activate blood PMNs. Cigarette smoke induces alveolar macrophages to liberate a chemotactic factor that activates PMNs and attracts them to the lung which results in an increase of released proteases (elastase). Cigarette smoke also has a direct toxic effect on PMNs and this may result in further release of elastase (Gadek et al., 1979).
The major risk factor for developing emphysema is believed to be environmental rather than genetic and smoking is strongly implicated partly due to a local inactivation of lung α1-antitrypsin during smoking. These patients have a reduced ability to inhibit neutrophil elastase (Gadek, 1992; Janoff et al., 1979). Other studies have also discussed the possibility that cigarette smoke reduces the elastase inhibitory capacity by an oxidative process and may promote the development of pulmonary emphysema (Vagliasindi & Fregman, 1989; Kalsheker, 1994). This process involves the oxidation of a critical methionine residue at the active diseased site, rendering α1PI an inefficient inhibitor of PMN elastase. Smoking habit also plays an important role in cardiovascular diseases and cancer (Du BY, 1989).
1.5.2. Smoking and periodontal health

Early studies have shown that smoking is associated with increased risk of oral neoplasms (Bastiaan & Reade, 1967). Recently a number of studies have found that smokers are also a high risk group for periodontitis and it has been shown that a substantial proportion of periodontitis cases may be attributable to cigarette smoking (Haber & Kent, 1992; Haber et al., 1993; Horning et al., 1992; Macfarlane et al., 1992; Bergstrom & Blomlof, 1992). Pindborg also found smoking to be a risk factor for necrotizing ulcerative gingivitis (Pindborg, 1947; Pindborg, 1949). Interestingly, gingival bleeding as determined by probing (BOP) is found to be decreased in smokers (Preber & Bergstrom, 1986). This is attributed to a reduction in the gingival blood flow which ultimately alters the defense mechanisms of the tissues and impacts on the periodontal disease process (Clarke & Carey, 1985; McGuire et al., 1989). The irritation caused by the heat from this habit may also be responsible for the alterations in the vascular flow (Goldman & Cohen, 1980). In the study by McGuire et al. cotinine (a metabolite of nicotine) was found to be present in the saliva and the gingival crevicular fluid of all smoker subjects and completely absent in nonsmokers (McGuire et al., 1989). This reduction in BOP does not reflect the findings on oral hygiene in this group of patients. In general nonsmokers show less debris and calculus formation than smokers and the levels of oral cleanliness decreases gradually from nonsmokers to heavy smokers (Sheiham, 1971). Periodontitis seems to progress more rapidly in smokers (Haber & Kent, 1992; Haber et al., 1993). Probing depths, alveolar bone loss and mobilities were all found to be more advanced in patients who smoked (Feldman et al., 1983; Goultschin et al., 1990; Bergstrom & Eliasson, 1987; Bergstrom & Eliasson, 1991). In patients with generalized early onset (G-EOP) form of periodontitis, smoking had a significant effect on attachment loss.
In these patients the attachment loss was greater with more teeth being involved compared to patients with G-EOP who did not smoke (Schenkein et al., 1995). More recent studies using guided regeneration techniques for the treatment of infrabony defects and CI.II furcations, have found that healing responses were significantly impaired in smokers (Tonetti et al., 1995; Rosenberg et al., 1994).

Studies have also been showing that patients who smoke do not respond as well to periodontal therapy (Preber & Bergstrom, 1985; Preber & Bergstrom, 1990). Following the debridement of supra and subgingival deposits a more pronounced reduction in gingival bleeding was observed in nonsmokers than smokers. This was assessed by the use of a controlled standardized probing pressure (Preber & Bergstrom, 1986). The average reduction in probing depth attained was less in smokers for all regions of the dentition when these were compared to nonsmokers (Preber & Bergstrom, 1985). After 12 months post surgery (Widman flap surgery on 4 to 6mm pockets) the reduction of these pocket depths was significantly greater for nonsmokers compared to smokers, this difference persisted even after accounting for plaque accumulation (Preber & Bergstrom, 1990).

The potential mechanisms for the pathogenesis of smoking associated periodontitis have not been extensively investigated but immunosuppression, impaired soft tissue cell function and impaired bone cell function have been implicated. Cigarette smoking exerts both local effects such as decreased gingival bleeding and inflammation when compared to nonsmokers (Bergstrom & Floderus-Myrhed, 1983; Preber & Bergstrom, 1985; Feldman et al., 1983; Preber & Bergstrom, 1986) and systemic effects such as inhibition of peripheral blood and oral PMN function (Noble & Penney, 1975; Kenney et al.,1977).
Kenney et al., (1977) in particular found that PMN harvested from the oral cavities of smokers were less vital and less able to phagocytize particles. In this study smokers were closely matched to nonsmokers with respect to age, general and oral health. Smoking is believed to exert mostly a systemic influence. In the study by Bergstrom & Eliasson, (1991) periodontal bone loss occurred irrespective of plaque infection suggesting that smoking primarily alters the body response mechanisms rather than adding to the local destructive effects. Reduced antibody production (Bennet & Read, 1982; Haber et al., 1993) and alteration of peripheral blood immunoregulatory T-cell subset ratios (Ginns et al., 1982) are believed to be involved. Tobacco use also seems to contribute to the destruction seen in refractory periodontitis. These findings were reported in a retrospective study which showed that 90% of the refractory patients were smokers (MacFarlane et al., 1992). Earlier studies also reported interesting results as to the effects of smoking on PMN cells. Eichel & Sharik, (1969) found that the toxic materials of smoking were able to completely inhibit the function of oral salivary PMNs in situ. In a later study Kraal & Kenney, (1979) were however not able to show any differences in the chemotaxis activity of PMNs between smoker and nonsmokers (Eichel & Sharik, 1969; Kraal & Kenney, 1979).

Another possible area was also investigated to attempt to explain the differences seen in the periodontal health of smokers. Initial studies reported that the proportions of gram positive bacteria and anaerobes in smokers were elevated compared to the proportion of gram negative bacteria (Bardell, 1981; Bastien & Waite, 1978). Preber et al. compared the bacterial flora of smokers to nonsmokers but found no differences in the occurrence and relative frequency of certain periodontal pathogens; *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia* (Preber et al., 1992).
More recently a study by Quinn et al. showed that in patients with G-EOP, smoking was associated with depressed serum IgG2 concentration. IgG1 and IgG3 were not found to be affected. Race appeared to also have an impact on the results of the study and black subjects with this early onset form of periodontitis showed not only depressed IgG2 but also had depressed serum levels of IgG4. In this study the smoking status was determined from serum cotinine levels and the serum IgG subclass concentrations were determined by using radial immunodiffusion (Quinn et al., 1996). Interestingly, the smoking habit has also been associated with the presence of higher levels of elastase inhibitors in the saliva of smokers (Giannopolou et al., 1994). Thus, smoking status is believed to be a clinically useful predictor of future disease activity (Haber, 1994).
CHAPTER 2

Aims of the study

Oral rinse elastase activity has been shown to distinguish periodontitis from health in most patients. A subset of patients, however, appear to have low elastase activity although they have clinical periodontitis. The aims of this investigation were:

1) to review the medical conditions (diabetes...) and/or habits (smoking..) in patients with periodontitis and low elastase activity. In addition, determine whether patients with periodontitis and low oral rinse elastase have a systemic defect in PMN recruitment into the oral cavity or high levels of enzyme inhibitors.

2) to determine whether oral rinse elastase activity correlates with the PMN or epithelial cells numbers that are present in oral rinse samples. The hypothesis being that elastase levels reflect PMN counts in most patients. Some studies have shown a correlation between epithelial cells and the severity of periodontal disease.

3) to determine the relationship between epithelial cells and PMN cells. Our hypothesis being that with increasing severity of periodontal disease their will be an increasing number of PMN cells and epithelial cells that are present in an oral rinse samples. These numbers should therefore correlate as well.
CHAPTER 3

3. Materials and methods

3.1. Subjects

A total of 46 adult patients and 20 control subjects were included in the study (Table 1). 43 patients (20 females and 23 males) were diagnosed for adult periodontitis (aged 31 to 80 years, mean of 49.9 yr.) and 3 patients (1 female and 2 males) for early onset periodontitis (age 17 to 27, mean of 23.3 yr.). All patients were examined at the University of British Columbia by graduate periodontics students or at the Vancouver Community College by dental hygiene students. Twenty control patients were randomly selected among healthy hygiene students at the Vancouver Community College. These students had healthy periodontal status with no previous history of periodontal disease. As part of a screening appointment, periodontal examination and a review of the medical history and smoking habits were completed.

The percentage of probing depths of ≥5mm for each patient's dentition were calculated. Based on the percentage of probing pocket depths of ≥5mm present, the patients were divided in subgroups according to the disease severity:

- mild periodontitis <20%,
- moderate periodontitis 20-40%,
- advanced periodontitis >40%.

None of these patients had received any periodontal therapy for a period of at least one year.
The recordings, oral rinse samples and immediate preparation of the saliva smears for subsequent staining were done by three graduate periodontics students and by several dental hygiene students. No inter examiner calibration was done.

3.2. Collection of oral rinse samples and preparation of smears

Oral rinse samples were collected at least once before initial therapy for all patients and twice for twenty five patients before probing. Eleven of the patients gave oral rinse samples post-initial therapy. Initial therapy consisted of one or more sessions of scaling and root planing performed by dental hygiene students or graduate periodontics students.

In one experiment, oral rinse samples were collected from three patients at 9 AM, noon and 9 PM during the same day patients were following standard procedures (collection, freezing...). This was performed to evaluate possible fluctuation in the elastase activity for each patient.

One oral rinse samples was collected for 20 healthy subjects. Two oral rinse samples were taken on separate dates from 16 control subjects. Control patients were selected based on the fact that they did not have any probing pocket depths deeper than 4 mm in their entire dentition. None of these control subjects were smokers.

The collection of the oral rinse samples and the determination of elastase activity were done according to the technique previously described by Uitto et al. (1996). Briefly, the subjects rinsed their mouth with 20 ml of tap water for 5 s and expectorated. This was done to remove salivary food debris or any other material that might interfere with the test. Then 3 ml of tap water was given to the subjects so that they could rinse vigorously between their teeth for 30 s. The fluid was then collected in graduated tubes and frozen at -20° C for future experiments.
Smears from the oral rinse sample were made at the time of collection and prior to freezing of the samples. For counting the number of oral rinse leukocytes and epithelial cells after mixing, a fixed volume aliquot was taken from each sample and spread over a glass slide and immediately fixed with cytoprep fixative (Fisher scientific LTD, Ontario, Canada). Measurement of oral rinse elastase activity was done after thawing of the salivary samples. 100 ml of the fluid was transferred into a microtiter plate containing the elastase substrate in a buffer solution as described below. The plates were then incubated at 37° C. The elastase activity was measured utilizing the chromogenic substrate succinyl-alanyl-alanyl-valine-p-nitroanilide dissolved in a solution with the following final concentrations: 1mM substrate, 0.1% dimethyl sulfoxide, 0.2M NaCl and 0.05 M Tris-HCL buffer, pH 7.5. The colour was read with a spectrophometric microtiter plate reader at 405 nm immediately (0 h value) and following incubation of 1, 2 and 20 h at 37°C. The enzyme activity is expressed as optical density per time, usually 20 h.

3.3. Experiments with purified elastase

To evaluate whether inhibitors of oral rinse elastase could be present in the samples, purified pancreatic or leukocyte elastase was added to samples of subjects who showed repeated negative elastase activity.

3.3.1. Pancreatic elastase (Pancreatopeptidase E;EC 3.4.21.36, E-0258, Sigma, St-Louis) (Baumstark et al. 1976)

Thirteen periodontitis patients were selected based on negligible elastase activity of their oral rinse samples. Three of the patients had two oral rinse samples collected at the same visit. Both of these showed negative elastase and both were used in the experiments.
Six other patients had two oral rinse samples collected on two separate visits and were both used. Five of these samples had been collected post initial therapy and were also used in this first experiment. All other patients had only one salivary sample which showed low elastase activity. Samples from a patient with high elastase activity was selected as control. 100 µl from each sample was added to a vial containing 100 µl of PBS and 20 µl of SAAVNA or 100 µl of commercial purified pancreatic elastase 12.5 mU/ml (PE). Enzyme activity was then measured as above. In a second part, the experiment was then repeated this time adding 100 µl of 12.5 mU/ml PE to all oral rinse samples instead of the PBS.

3.3.2. Leukocyte elastase (Lysosomal elastase; EC 3.4.21.37, E-8140, Sigma, St-Louis) (Barrett, 1981)

In one experiment, seven oral rinse samples were selected based on the results of the above experiment. Samples with low elastase activity with added pancreatic elastase were selected. 100 µl of the samples was now mixed with 100 µl of purified leukocyte elastase 500 mU/ml (LE). The substrate was added and elastase activity measured as above.

Samples from patients who showed low elastase after mixing with both pancreatic and leukocyte elastase were then compared to the healthy control group (N=4).

3.4. Oral rinse leukocytes and epithelial cells

Following the collection of the oral fluid samples and prior to freezing, a fixed volume of sample was taken with a graduated pipette, placed on a glass slide and fixed with cytospray. The oral rinse cell smears were stained using a modification of the Wright-Giemsa technique (Sigma Diagnostics, St-Louis, MO, USA).
A few drops of 0.15% Wright-Giemsa stain was placed on the smears for 1 minute followed by a 30 s rinse under tap water. The numbers of polymorphonuclear neutrophil (PMN) and epithelial cells in the smears were then counted. Briefly, forty randomly selected squares of 0.5 cm$^2$ were counted using light microscopy (Zeiss, Axioskop, HBO 100 w/2, Oberkochen, Germany). Analysis of accuracy and reproducibility of the measurements was assessed by repeated counting of ten randomly selected slides.

3.5. Statistics

The coefficient of correlation ($r$) was calculated for the PMN and epithelial cell numbers to the elastase activity and for the PMN to epithelial cell numbers for all subjects. The coefficient of correlation was also calculated for the percentage of probing pocket depths to the number of cells (PMN and epithelial cells). The P value was determined for all these correlations. The analysis of covariance (Anova) was calculated for the number of epithelial cells and PMN in healthy subjects compared to nonsmoking and smoking periodontitis patients. Finally the coefficient of correlation and the P value were also calculated for the elastase activity between first and second oral rinse samples for all subjects (healthy and disease). Statistics were done using the Excel Window's program.
CHAPTER 4

4. Results

4.1. Description of patients

Table 1 describes the collection of oral rinse sample for all patients and controls and Fig. 1 represents the typical curves for the variation in the levels of elastase activity in the oral rinse samples of both periodontitis patient and healthy subjects. Healthy subjects had activity levels below 0.5U after 20 hours incubation period 0.5U optical density. Periodontitis patients typically had elastase activity above 0.5U after 2 hours of incubation and a dramatic increase was observed between the two and 20 hour periods of incubation.

As presented in Table 2 forty two percent of patients with adult periodontitis who had oral rinse samples taken only once prior to initial therapy had low elastase activity in their samples. This percentage dropped to 26% when oral rinse samples were taken on more than one occasion prior to initial therapy. The patients with low levels of elastase activity had only mild to moderate periodontitis as expressed by the percentage of probing pocket depths ≥ 5mm in their dentition. A high percentage of patients with early onset periodontitis (67% and 100%) showed low elastase activity when these patients had oral rinse samples collection taken once or twice prior to initial therapy.

A majority of control subjects showed low elastase activity in their oral rinse samples taken once or twice prior to initial therapy (85% and 81% respectively) (Fig. 2a).
Six adult periodontitis and two early onset periodontitis patients repeatedly showed low elastase activity levels at different oral rinse sampling period prior to initial therapy (Table 2) but 68% patients with periodontal disease presented at least on one occasion high elastase activity, above 0.5U (Fig. 2b).

4.2. Effects of the time of collection of oral rinse samples on the elastase activity

To assess if the time of collection of samples could affect the elastase activity levels, three patients were asked to collect oral rinse samples at different time period during the same day (Fig. 3). All three patients showed elevated elastase activity after an incubation period of 20 hours but variation was observed between two different sampling times. When oral rinse samples were collected in the evenings (9 PM) the elastase activity levels tended to remain low (below 0.5 U).

4.2.1. Effects of smoking on the oral rinse elastase activity

Table 3 presents those patients who included smoking in their medical history. A significant increase was seen in the percentage of patients who showed low levels of elastase activity in their oral rinse samples; 67% of patients who had only one oral rinse sample collected prior to initial therapy and 60% of patients who had more than one sample prior to initial therapy. 100% of patients with early onset periodontitis who included smoking as a habit, showed repeatedly low elastase activity in their samples. A brief description of the patients who reported being smokers (4 smokers and 1 chewing tobacco of 8 patients) and who had low levels of elastase activity on more than one occasion prior to initial therapy is presented (Table 4).
4.3. Correlation of the numbers of epithelial and PMN cells to the elastase activity

Fig 4a to 5b examines the correlation between the elastase activity and the numbers of epithelial cells or PMN cells found in a smear of oral rinse samples of patients with adult periodontitis and controls after an incubation period of 20 hours. A good correlation (r=0.3583, P=0.001) was detected between the number of PMN cells and the elastase activity in patients with periodontitis compared with the lack of correlation for healthy subjects (r=0.1806, P=0.15) (Fig 4b and 5b). No correlation was found between the number of epithelial cells and the elastase activity in the samples of healthy subjects (r=0.0405, P=0.4) (Fig. 5a) or for periodontitis patients (r=0.1541, P=0.1) (Fig. 4a).

4.3.1. Correlation between cell numbers (PMN and epithelial cells)

A very strong correlation was found between the number of PMN and epithelial cells in the healthy group (r=0.6018, P=0.0001). The correlation was also relatively high for periodontitis patients (r=0.3996, P=0.002) (Fig. 6a and 6b). Epithelial and PMN cells were more numerous in the smears of rinse samples of periodontitis patients compared to those of healthy subjects. Patients with periodontitis had 1.75-fold increase in the numbers of PMNs and 1.89-fold increase in epithelial cells compared to healthy individuals, respectively (Table 5).

4.3.2. Correlation between disease severity and number of cells (PMN and epithelial) in oral rinse samples of periodontitis patients.

The correlation of the percentage of deep pockets ≥ 5mm to the number of PMN cells and epithelial cells found in the oral rinse samples of periodontitis patients was determined.
No correlation was found between the number of epithelial cells and the percentage of deep PPD. Similarly, no correlation was found between PMN cells and the presence of a higher number of deep pocket depths (Fig. 7a and 7b).

4.3.3. Effects of periodontal disease on PMN and epithelial cells counts

Fig. 8. shows oral rinse smears representative for healthy subjects and periodontitis patients. The epithelial cells dominated in the smears of healthy subjects and PMN cells in the smears of periodontitis patients.

4.4. Effects of periodontal therapy on the elastase activity

Periodontal therapy showed a decrease in the normal elastase activity in patients who had samples taken following initial therapy. As seen in Fig. 9, 82% of patients showed a net decrease in the elastase activity levels in the oral rinse samples taken after periodontal therapy. One patient showed only a slight decrease and one patient showed an actual increase in the activity. This latter patient had low elastase activity in his pre-initial therapy samples.

4.5. Effects of smoking on epithelial and PMN cell counts

Smokers with periodontitis had less PMN cells in their oral rinse samples (Table 5) (1.760 ± 1.567)* compared with nonsmokers (2.744 ± 3.629)*. These numbers of PMN cells in smokers were in fact close to those of healthy controls (1.420 ± 1.718)*. Epithelial cells were slightly more numerous in oral rinse of smokers (0.937 ± 1.073)* compared with nonsmokers periodontitis patients (0.870 ± 0.918)* but much higher than controls.

* mean + SD; values X 10^5.
4.6. Effects of addition of leukocyte elastase and pancreatic elastase to oral rinse samples of selected subjects.

Fig. 10, Fig. 11 and Fig. 12 Pancreatic elastase (12.5 mU/ml) (PE) or leukocyte elastase (500 mU/ml) (LE) were added to selected oral rinse samples to demonstrate the presence of elastase inhibitors in oral rinse samples. About half of the samples showed inhibitory effect on elastase activity. The samples showing the strongest effect were tested on their effect on leukocyte elastase. Four samples that demonstrated inhibition of pancreatic elastase (3 patients) also had low elastase activity in the presence of LE. These three patients were further described in Table 6. The common feature observed was that all these patients had only mild periodontal disease. One of these patients (18) who also showed the least response to the addition of LE, reported being a smoker (Fig 11).

Patients 2 and 14 (Fig. 10 and Fig. 11) had as expected low elastase activity in samples which had been taken after initial therapy. The addition of LE and PE resulted in normal elastase activity (increase of the activity) for both these samples.

Healthy patients who had low elastase activity in their samples, retained these low levels of activity despite addition of leukocyte elastase. Less than 25% of the elastase activity was present when leukocyte elastase was mixed with control oral rinse samples (Fig. 12)
4.7. Sensitivity and specificity of the oral rinse test for elastase activity

The sensitivity and specificity of the oral rinse test for elastase activity was calculated and presented in Table 7. When all periodontitis patients were considered the sensitivity of the test was 70%. If only smokers were considered the sensitivity decreased to 46%. The sensitivity of the test was the highest, 77% when all the smokers were excluded from the calculations. The specificity of the test was 85%.
A simple enzyme test would be particularly helpful as a diagnostic marker for periodontal disease. Host cell-derived enzyme activity has been measured in whole saliva (Chauncey, 1961), GCF (Cimasoni et al., 1977), pure glandular secretion (Makinen, 1966) and inflamed gingival tissue (Uitto, 1987). As GCF may be a possible diagnostic indicator for detection of active versus inactive disease processes at the site level, whole saliva may reflect the periodontal status of the whole dentition (Wilton et al., 1989). Whole saliva contains a variety of enzymes including esterases, transferases, glycosidases and proteases (Chauncey, 1961; Nakamura & Slots, 1983; Makinen, 1989). Salivary glands, serum, leukocytes, epithelial cells, fibroblasts, macrophages and oral bacteria are all potential sources of these enzymes. Elevated GCF and/or oral fluid levels of certain enzymes, e.g., peptidases and glycosidases (Zambon et al., 1985), myeloperoxidases (Smith et al. 1986) collagenases (Uitto et al., 1990), elastases (Gustafsson et al., 1992; Palcanis et al., Armitage et al., 1994) and gelatinases (Gangbar et al., 1990; Ingman et al., 1994; Makela et al., 1995) have been found in periodontal diseases. Other factors that have been investigated as possible markers for disease progression include tissue breakdown products such as hydroxyproline (Svanberg, 1987) and glycosaminoglycans (Last et al., 1985) and inflammatory mediators such as prostaglandins E2 (Offenbacher et al., 1986), tumor necrosis factor-α (Rossomando & White, 1993) and interleukin 1-β (Masada et al., 1990). However, no commercial tests are available for measuring these compounds.

PMN elastase is known to play an important role in extracellular matrix degradation during periodontal inflammation.
It degrades primarily elastin but can also degrade other connective tissue proteins such as collagens and proteoglycans (Van Dyke & Vaikuntam, 1994). Elastase in the GCF appears to be primarily of PMN origin, since there is a clear preference of salivary elastase to valine-containing substrates, while elastase from bacteria has a strong effect on alanine substrates (Bieth et al., 1974; Uitto & Haapasalo, 1986).

The results have shown that a correlation does exist between the numbers of PMNs and elastase activity in patients with periodontitis (P=0.001). Recent site specific studies with fluorogenic substrates (Cox & Eley, 1989), peptide substrates (Darany et al., 1992), immunoreactive assays (Gustafsson et al., 1992) or a chairside kit based on an elastase substrate embedded in a paper strip (Palcanis et al., 1992) have also demonstrated a positive relationship of the amount and activity of GCF PMN elastase with periodontitis and deep pockets with attachment loss. Higher levels of gingival crevicular fluid (GF) elastase activity were found at sites of intermediate probing depths than at healthy sites (Darany et al., 1992). According to our calculation presented in Table 1 there is a tendency towards an increase in elastase activity as the level of periodontal disease increases from healthy subjects to patients with severe periodontal diseases. In a follow up study by Gustafsson et al. (1992) the levels of elastase were measured in GCF of patients with gingivitis and in patients with periodontitis with inflamed sites with and without tissue destruction. The elastase activity was elevated in both patients with gingivitis and periodontitis but the activity was higher in patients with periodontitis.

The specificity and sensitivity of the oral rinse elastase data of the present study agrees with those of Uitto et al. (1996). However, in our study a lower percentage of all periodontitis patients (70%) showed positive elevated salivary elastase activity. In the previous study 15% of patients had false negative results and our results demonstrated that this percentage was 30% when all patients were considered.
It was also shown that 26% of our patients (6 patients) had repeatedly low elastase activity in their oral rinse samples (Table 2). An attempt at explaining the reasons behind such false negative results was carried out. Patients with only moderate or very localized periodontitis reduce the sensitivity of the test. The sensitivity of the elastase activity test in our results then approached the values found in Uitto's study (1996). In their study it was found that if patients had minimal periodontitis and mostly gingival inflammation then the sensitivity of the test also decreased with only 45% of patients showing positive elastase activity (Uitto et al., 1996). In our study when all periodontitis patients were considered then 58% of the patients showed positive elastase activity, but when only the patients with mild periodontitis were considered only 47% were showing positive elastase activity. Studies by Wright (1964) also show that the number of migrating leukocytes to the oral cavity is related more to the surface area of periodontal pockets than the extent of gingival inflammation. As shown in figures 7a and 7b we did not find a correlation between the elastase activity and the percentage of deeper pocket depths. This may be a reflection of the small number of patients in the deeper pocket depths group. Also in our study we used oral rinse samples and not GCF samples, therefore this could have affected the results. In our patients, those who showed false negative results repeatedly, two common features were almost always observed. These patients were affected only with mild periodontal disease (less than 20% of deep ≥ 5mm probing pocket depths, present in the dentition) (Table 2). It has been suggested that in edentulous persons and patients with periodontal disease with only minimal inflammation such as localized juvenile periodontitis, the corresponding elastase activity is also low (Uitto et al., 1996). Interestingly, a majority of patients with early onset periodontitis had low elastase levels (67% when only one pre-initial therapy sample was taken and all of those patients who had repeated sampling). Another common finding in this group of patients was that a majority of them were smokers (67%). However the sample size in this category of periodontal disease was very small (3 and 2 respectively), therefore these results must be verified with larger patient population.
However the finding raises a question about elastase/PMN defect as a possible factor in early onset periodontitis.

The presence of a smoking habit is another factor found among the patients who showed false negative results, 67% of patients who reported a smoking habit had false negative results if only one sample was taken prior to initial therapy and 60% were negative at two or more sampling occasions (Table 3). The mechanism how tobacco smoking affects the elastase activity is not known in detail.

Early studies by Eichel & Shahrik (1969) concluded that the volatile gaseous phase of tobacco smoke (not the nicotine) contains most of the undesirable substances toxic to the human oral leukocyte by seriously impairment of its functions. Similar effects may be involved with PMNs. Alavi et al. (1995) concluded that the GCF concentration of elastase was lower in smoker patients compared to nonsmokers. These patients were matched for gender, age, background as well as the clinical and radiographic extent of disease and had moderate to advanced periodontal disease. The samples were also analyzed for PMN cell counts but showed no differences between the groups. Our findings with a relatively small group of smokers, showed similar results as the majority of smokers had low elastase activity in their pre-initial therapy samples. Most of the leukocytes found in the oral fluids migrate into the oral cavity along with gingival crevicular fluids. Numerous studies have shown that smokers have less gingival inflammation and bleeding compared with nonsmokers (Bergstrom & Floderus-Myrhed., 1983; Feldman et al., 1983; Preber & Bergstrom., 1985; Preber & Bergstrom, 1986). We found that smokers had PMN counts in oral rinse that were comparable to health. An analysis of variance (Anova) indicated that there was a significant difference between the epithelial and PMN cell counts in healthy controls compared with periodontitis patients who were smokers and those who were non smoker, although this test is not capable of detecting where and why this difference exists (Table 5). This may in part explain the low elastase levels found in the samples of smoking patients. The role of cellular adhesion molecules (CAMs) in the inflammatory and immune responses, particularly in leukocyte migration into the tissues in inflamed gingiva has been demonstrated (Gemmell et al., 1996).
A recent study by Koundouros et al. (1996) discusses the increased levels of soluble ICAM-1 in serum of smokers compared to nonsmokers. This soluble form of ICAM could compete with endothelial and junctional epithelial membrane-bound ICAM-1 for its leukocyte ligand and prevent their attachment and subsequent migration into the tissues. Direct inhibitory effects of tobacco smoke on PMNs are probably also involved (Eickel, 1969). Salivary constituents may modulate either positively or negatively the elastase activity. Saliva contains several types of enzymes inhibitors which may impede the value of salivary enzyme activities for diagnostic purposes. Some of the inhibitors originate from serum/GCF, others appear to be secreted by the salivary glands (Schiessler et al., 1978; Drouin et al., 1988). Elastase inhibitors alpha 2-macroglobulin and alpha1-antitrypsin (α1PI) are serum/sulcular fluid derived (Uitto et al., 1996), tissue inhibitor of matrix metalloproteinases (Drouin et al., 1988), cysteine proteinase inhibitors and low molecular weight PMN serine proteinase inhibitors appear to be secreted by the salivary glands (Schiessler et al., 1978). Destruction of mature elastin by other mammalian elastases is probably the result of an imbalance in the normal inhibitor-proteinase ratio. α1PI is the primary regulator of granulocyte and pancreatic elastase activities in vivo. Deficiencies in α1PI have been implicated in the early onset of emphysema and childhood cirrhosis (Kalsheker, 1994). The local inactivation is thought to lead to extensive elastin degradation. In periodontal diseases, the GCF level of PMN elastase available could surpass that of their inhibitor. This excess of at least locally transient free PMN elastase explains the tissue destruction which occurs in adult periodontitis (Sugiyama et al., 1992). The question remains, why do some periodontitis patients show low elastase activity in their samples?
Could it be inactivated by this inhibitor which would be present in greater concentration in some patients or could other enzymes be mostly responsible for the tissue destruction noted (peptidases, glycosidases, myeloperoxidases, collagenases and gelatinases have also been found to be elevated in periodontal diseases) (Zambon et al., 1985; Smith et al., 1986; Uitto et al., 1990; Gangbar et al., 1990; Ingman et al., 1994; Makela et al., 1995). Alternatively, low elastase could be the result of active tissue destruction and release of elastase from PMNs before they enter the oral cavity.

Our study showed that oral rinse samples of many of the patients with low elastase activity inhibited pancreatic and leukocyte elastase. A study by Schiessler et al. (1978) discuss that these inhibitors of PMN elastase are also active against pancreatic elastase. This raises another question, could it be that most of these patients with low elastase also have high levels of inhibitors in their samples? This finding appears to be in disagreement with findings regarding other diseases. Studies in the medical field have shown that tobacco increases the release of elastase by PMNs and also inactivates its inhibitors (Janoff et al., 1979; Hubbard et al., 1987; Fera et al., 1987; Trefz et al., 1989; Ogushi et al., 1991; Gadek, 1992; Muley et al., 1994). Again it is possible that elastase was released prior to PMNs entering the oral cavity and elastase inhibitors were also utilized prior to leakage to the oral environment.

The study also demonstrated a strong correlation between the number of PMN cells and the elastase activity in patients with periodontitis when compared with the same correlation for healthy subjects who did not show a significant correlation (P=0.001 and P=0.15 respectively). It has been found that PMNs can cause non-lytic detachment of keratinocytes when interacting with certain bacteria (Smith et al., 1994).
Studies have shown that the migration rate of leukocytes to the oral cavity may be increased as much as 150 times in periodontitis (Klinkhamer, 1968). The present study has shown a significant correlation between epithelial cell and PMN cell in both periodontitis patients and healthy subjects (P=0.002 and P=0.0001 respectively). Oral leukocytes migrate to the oral cavity with sulcular fluid (Wright 1964; Schiott & Loe 1970), therefore measurements of salivary elastase would also provide a simple measurement of the PMN count. The relation of elastase and PMN count is however not clear in all patients. The number of PMN and epithelial cells were found to be twice as numerous in periodontitis patients compared with amounts found in the salivary samples of healthy subjects.

A study by Watanabe et al. (1981) has shown that the numbers of epithelial cells in saliva were highly correlated to the protease activity. Our study also did not show any correlation between the numbers of epithelial cells and elastase activity in either healthy or periodontitis subjects (r=0.0405, P=0.4 and r=0.1541, P=0.1 respectively). Finally, the percentage of deep pockets (PPD) ≥ 5mm was also correlated to the number of PMN cells and epithelial cells found in the oral rinse samples of periodontitis patients. Again no correlation were found (r=0.1155, P=0.16 and r=-0.052, P>0.3 respectively).
CHAPTER 6

6. Conclusion

The oral fluid elastase test was shown to have a high specificity and sensitivity. The sensitivity was improved when smokers were excluded from the periodontitis patient group. A small percentage of patients still remains who did not smoke and still show low salivary elastase levels. We have attempted to explain the reasons behind the false negative results found in periodontitis patients who took part in our study and those reported previously (Uitto et al., 1996). This was done in hope of directing future research for the development of a simple enzymatic test which could help in the detection of periodontal disease. This test could be devised as "home test" and help direct patients in need of periodontal examination towards seeking care or alternatively help to recognize customized recall intervals. The results show that a small group of patients had repeatedly low elastase activity in their samples. Smoking, mild periodontitis, specific types of periodontitis with minimal inflammation such as localized periodontitis, all seem to be partly associated with these false negative results. Presence of elastase inhibitors in the samples were found to be one contributory factor. Inaccuracies in the sampling techniques may also have accounted for the results. The groups of patients representing smokers and early onset periodontitis were, however, small.

Future studies on oral rinse elastase should involve a larger sample of smokers with advanced periodontal disease. A larger group of patients with early onset periodontitis would be also interesting. In this subgroup of patients, oral rinse elastase could perhaps be used as an adjunctive diagnostic aid since diagnosis between adult periodontitis and early onset periodontitis is sometimes difficult.
Should the result of these proposed studies support our results, a test could be developed and its use promoted in patients who are at risk for periodontal disease and who do not smoke. We have shown albeit with a limited numbers of patients, that elastase activity may vary depending on the time of collection of the sample. This test could be repeated at several occasions in patients who may suspect periodontal disease and show low levels of elastase activity in first sampling. Should the test become positive at one occasion than the patients would be advised to visit dental care professionals to rule out the possibility of periodontal disease. Since previous studies have also shown a decrease in elastase activity following successful periodontal therapy whether it be scaling and root planing or surgical therapy, this test could also be used in the intervals between periodontal supportive therapy to determine the frequency of periodontal appointments as well as for patients motivation. The validity of the test might also be questioned in patients with certain medical conditions such as agranulocytosis, neutropenia, diabetes mellitus and acquired immune deficiency syndrome (Van Dyke & Hoop, 1990).

In summary the oral elastase test appears to have value in periodontal diagnosis and treatment. Its sensitivity and specificity values are better than many of the test currently in routine use in dentistry and medicine. However more studies are needed to find out its limitations and hopefully develop the method which would be applicable for most subjects.
BIBLIOGRAPHY


Hubbard RC., Ogushi F., Fells GA., Cantin AM., Jallat S., Courtney M., Crystal RG. Oxidants spontaneously released by alveolar macrophages of cigarette smokers can inactivate the active site of alpha 1-antitrypsin, rendering it ineffective as an inhibitor of neutrophil elastase. J Clin Invest 1987;80:1289-1295.


-McDonald JA., Baum BJ., Rosenberg DM., Kellman JA., Brin SC., Crystal RG. Destruction of major extracellular adhesive glycoprotein (fibronectin) by neutral proteases from polymorphonuclear leukocyte granules. Lab Invest 1979;40:353.


-Okada Y., Nakanishi I. Activation of matrix metalloproteinase 3 (stromelysin) and matrix metalloproteinase 2 (gelatinase) by human neutrophil elastase and cathepsin G. FEBS lett 1989;249-353-356.


Table 1. Patient population of the study

<table>
<thead>
<tr>
<th>Patients/Diagnosis</th>
<th>T=3</th>
<th>N=1</th>
<th>N≥2</th>
<th>Post Tx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Periodontitis</td>
<td>43</td>
<td>0.88</td>
<td>43</td>
<td>23</td>
</tr>
<tr>
<td>Mild</td>
<td>15</td>
<td>0.57</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Moderate</td>
<td>21</td>
<td>1.10</td>
<td>21</td>
<td>15</td>
</tr>
<tr>
<td>Advanced</td>
<td>7</td>
<td>1.00</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>EOP</td>
<td>3</td>
<td>0.64</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Controls/healthy</td>
<td>20</td>
<td>0.20</td>
<td>20</td>
<td>16</td>
</tr>
</tbody>
</table>

N; total number of patients.
N=1; oral rinse samples collected once prior to intial therapy.
N≥2; two or more oral rinse samples collected prior to intial therapy.
Post Tx; oral rinse collected post intial therapy.
T=3; mean elastase activity as a change of optical density 405nm at 20 hours incubation period.

Mild adult periodontitis; 0-20% of probing pocket depths ≥5mm.
Moderate adult periodontitis; 20-40% of probing pocket depths ≥5mm.
Advanced adult periodontitis; > 40% of probing pocket depths ≥5mm.
EOP; Early Onset Periodontitis; the disease detected prior to age 30.
Controls; Dental hygenist students with healthy periodontium.
Table 2. Patients with low elastase activity.

<table>
<thead>
<tr>
<th></th>
<th>N=1</th>
<th>(-)</th>
<th>%</th>
<th>N≥2</th>
<th>(-)*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult Periodontitis</td>
<td>43</td>
<td>18</td>
<td>42</td>
<td>23</td>
<td>6</td>
<td>26</td>
</tr>
<tr>
<td>Mild</td>
<td>15</td>
<td>8</td>
<td>53</td>
<td>6</td>
<td>2</td>
<td>33</td>
</tr>
<tr>
<td>Moderate</td>
<td>21</td>
<td>10</td>
<td>48</td>
<td>15</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>Advanced</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EOP</td>
<td>3</td>
<td>2</td>
<td>67</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Controls</td>
<td>20</td>
<td>17</td>
<td>85</td>
<td>16</td>
<td>13</td>
<td>81</td>
</tr>
</tbody>
</table>

N=1; number of patients sampled for elastase prior to intial therapy.  
N≥2; number of patients sampled twice or more prior to intial therapy.  
(−); patients who had low elastase readings (elastase absorbance ≤0.5).  
%; percentage of patients who were negative according to number of patients sampled.

* (−) applies to patients who showed low elastase reading in two samples or more at different time periods.
Table 3. Elastase activity in oral rinse samples of patients who reported smoking in their health history.

<table>
<thead>
<tr>
<th></th>
<th>N=1</th>
<th>(-)</th>
<th>%</th>
<th>N≥2</th>
<th>(-)*</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult periodontitis</td>
<td>9</td>
<td>6</td>
<td>67</td>
<td>5</td>
<td>3</td>
<td>60</td>
</tr>
<tr>
<td>Mild</td>
<td>3</td>
<td>2</td>
<td>67</td>
<td>1</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>4</td>
<td>100</td>
<td>3</td>
<td>2</td>
<td>67</td>
</tr>
<tr>
<td>Advanced</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>EOP</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Controls</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

N=1; Smokers who had salivary samples taken prior to intitial therapy.  
N≥2; smokers who had salivary samples taken prior to initial therapy more than once.  
(-); smokers who showed low elastase activity (elastase absorbance ≤0.5).  
%; percentage of patients who had low elastase.  
*(-) applies to the patients who showed low elastase (elastase absorbance ≤0.5) in at least two or more samples.
Table 4. History of all patients who showed low elastase activity at two or more time periods.

<table>
<thead>
<tr>
<th>Periodontal disease</th>
<th>% of PPD</th>
<th>Medical History</th>
<th>PMN numbers X 10^5/ml</th>
<th>Teeth Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>19.2</td>
<td>Diabetes</td>
<td>0.37</td>
<td>26</td>
</tr>
<tr>
<td>AP</td>
<td>24.4</td>
<td>Smoker</td>
<td>2.30</td>
<td>13</td>
</tr>
<tr>
<td>AP</td>
<td>36.2</td>
<td>Smoker</td>
<td>0.24</td>
<td>29</td>
</tr>
<tr>
<td>EOP</td>
<td>15.10</td>
<td>Smoker/Immunosuppressants</td>
<td>2.90</td>
<td>32</td>
</tr>
<tr>
<td>AP</td>
<td>30.4</td>
<td>Prozac</td>
<td>0.48</td>
<td>23</td>
</tr>
<tr>
<td>AP</td>
<td>11.9</td>
<td>Chewing Tobacco</td>
<td>0.70</td>
<td>32</td>
</tr>
<tr>
<td>AP</td>
<td>16.6</td>
<td>Non contributory</td>
<td>0.45</td>
<td>26</td>
</tr>
<tr>
<td>EOP</td>
<td>7.8</td>
<td>Smoker</td>
<td>0.64</td>
<td>32</td>
</tr>
</tbody>
</table>

AP; adult periodontitis
EOP; early onset periodontitis
%PPD; % of probing pocket depths of ≥ 5mm.
Teeth present; number of teeth present for each patient.
Table 5. Leukocyte and epithelial cell counts in all subjects.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>PMN cells</th>
<th>Epithelial cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>32</td>
<td>1.420 ± 1.718</td>
<td>0.458 ± 0.505</td>
</tr>
<tr>
<td>Periodontitis All</td>
<td>70</td>
<td>2.492 ± 3.267</td>
<td>0.865 ± 0.947</td>
</tr>
<tr>
<td>Periodontitis/Non-smokers</td>
<td>54</td>
<td>2.744 ± 3.629</td>
<td>0.870 ± 0.918</td>
</tr>
<tr>
<td>Periodontitis/Smokers</td>
<td>19</td>
<td>1.760 ± 1.567</td>
<td>0.937 ± 1.073</td>
</tr>
</tbody>
</table>

Mean ± SD; all values should be X 10^5

Smear samples were made after collection of two oral rinse samples per subjects.

An analysis of variance was calculated (Anova) and showed a significant variability between the groups (healthy, periodontitis patients who smoke and periodontitis patients who do not smoke) for epithelial cell counts (F=13.4 and P=0.000007) and PMN cell counts (F=10.9 and P= 0.00005)
Table 6. Analysis of patients in fig. 10 and 11 who showed low elastase activity despite additions of leukocyte and pancreatic elastase.

<table>
<thead>
<tr>
<th>Fig. 10</th>
<th>Fig. 11</th>
<th>Periodontitis</th>
<th>Health Status</th>
<th>Teeth present</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a-3b</td>
<td>3a-3b</td>
<td>Mild</td>
<td>Diabetes +HBP</td>
<td>26</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Mild</td>
<td>Cancer in 1978, chemo and radiotherapy</td>
<td>26</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>Mild</td>
<td>Smoker</td>
<td>27</td>
</tr>
</tbody>
</table>

HBP; high blood pressure.
Teeth present; number of teeth present for each patient.
Table 7. Specificity and sensitivity of the oral elastase test

<table>
<thead>
<tr>
<th>Specificity</th>
<th>85%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>70%</td>
</tr>
<tr>
<td>Sensitivity smokers</td>
<td>46%</td>
</tr>
<tr>
<td>Sensitivity non smokers</td>
<td>77%</td>
</tr>
</tbody>
</table>

A positive value was defined as a change of optical density at 405 nm of at least 0.5 units in 20 hr incubation.

* includes all periodontitis patients (n=46).
** periodontitis patients who did not report a smoking habit (n=35).
*** periodontitis patients who reported a smoking habit (n=11).
Fig. 1. Elastase activity in selected oral rinse samples of periodontitis patients and healthy controls. The samples were incubated at 37°C with 1mM SAAVNA substrate and color read spectrophotometrically at 0, 1, 2 and 20 hours (t=3; average and standard deviation for disease and healthy patients, 0.91 ± 0.60 and 0.20 ± 0.31 respectively). A positive test results was defined as a change of optical density 405nm of at least 0.5 units. Generally controls had elastase activity below 0.5 units whereas adult periodontitis patients had elastase activity above 0.5 units.
Fig. 2a. Elastase activity of two different oral rinse samples for control subjects. The samples were incubated at 37°C with 1 mM SAAVNA substrate and color read spectrophotometrically at 20 hours. A positive test result was defined as a change of optical density 405nm of at least 0.5 units. Generally all control subjects were below the 0.5 unit value for both sample periods. Three subjects had high elastase activity at only one sample period.
Fig. 2b. Elastase activity of two or three oral rinse samples for diseased patients. The samples were incubated at 37°C with 1 mM SAAVNA substrate and color read spectrophotometrically at 20 hours. A positive test result was defined as a change of optical density 405nm of at least 0.5 units. Some patients showed low elastase activity repeatedly (below 0.5 units) and were thereafter considered false negative.
Fig. 3. Elastase activity of oral rinse samples of three patients at three times of the same day. The samples were incubated at 37°C with 1mM SAAVNA substrate and color read spectrophotometrically at 20 hours. A positive test results was defined as a change of optical density 405nm of at least 0.5 units. Variation at the different time points in the elastase activity can be observed.
Fig. 4a. Number of epithelial cells/ml in oral rinse samples of patients with periodontitis in relation to elastase activity in their samples after a period of incubation of 20 hours (one patient who had more than $0.75 \times 10^6$ epithelial cells in his sample was deleted from the graph).

Correlation between epithelial cells numbers and elastase activity; $r=0.1541, P=0.1$
Fig. 4b. Number of PMN cells/ml in oral rinse samples of patients with periodontitis in relation to elastase activity in their samples after a period of incubation of 20 hours (one patient who had more than 3 X 10^6 PMN cells in his sample was deleted from the graph). Correlation between PMN cells numbers and elastase activity; r=0.3583. P=0.001.
Fig. 5a. Number of epithelial cells/ml in oral rinse samples of healthy subjects in relation to elastase activity in their samples after a period of incubation of 20 hours. Three subjects who had more than $0.2 \times 10^6$ epithelial cells in their samples were deleted from the graph. Correlation between the number of epithelial cells and elastase activity; $r=0.0405$. $P=0.4$
Fig. 5b. Number of PMN cells/ml in oral rinse samples of healthy subjects in relation to elastase activity in their samples after a period of incubation of 20 hours (one subject who had more than 4 X10⁶ PMN cells in his sample was deleted from the graph). Correlation between PMN cell numbers and elastase activity; r=0.1806. P=0.15.
Fig. 6a. Correlation between the number of PMN cells to epithelial cells in oral rinse samples of patients with periodontitis (one patient who had more than $3 \times 10^6$ PMN cells and more than $0.75 \times 10^6$ epithelial cells in his sample was deleted from the graph).
Correlation between epithelial cell numbers and PMN cell numbers; $r=0.3996$. $P=0.002$. 
Fig. 6b. Correlation between the number of PMN cells and epithelial cells in oral rinse samples of healthy subjects (three subjects who had more than 0.2 $\times$ 10$^6$ epithelial cells and one subject who had more than 4 $\times$ 10$^6$ PMN cells in their samples were deleted from the graph).

Correlation between epithelial cell numbers and PMN cell numbers; $r=0.6018$. $P=0.0001$. 
Fig. 7a. Number of epithelial cells/ml in oral rinse sample of patients with periodontitis in relation to the percentage of probing pocket depths ≥ 5mm in their dentition (one patient who had more than $0.75 \times 10^6$ epithelial cells was deleted from the graph). Correlation between epithelial cell numbers and the percentage of probing pocket depths; $r=-0.052$. $P>0.3$. 
Fig. 7b. Number of PMN cells/ml in oral rinse samples of patients with periodontitis in relation to the percentage of probing pocket depths ≥ 5mm in their dentition (one patient who had more than 0.75 \times 10^6 PMN cells was deleted from the graph). Correlation between PMN cell numbers and the percentage of probing pocket depths; \( r = 0.1155 \). \( P = 0.16 \).
Fig. 8. Wright-Giemsa staining of oral rinse smears of healthy subjects (A) and periodontitis patient (B). Arrow head point to PMN cells and arrows point to epithelial cells.
Fig. 9. Pre and post treatment oral elastase activity for eleven patients.
Incubation period, 20 hr.
2,3,4,7,8,5,6,9,10: adult periodontitis patients
1,11: early onset periodontitis patients
Fig. 10. Effect of oral rinse samples on pancreatic elastase activity (12.5 mU/ml). The periodontitis patients selected showed low elastase activity in all their pre-initial therapy oral rinse samples. Incubation period 20 hr.

1; patient who had (+) elastase activity used as control.
2, 7b, 12, 13, 14; post treatment samples. 3a&b, 4a&b, 5a&b; same patients 2 samples same day.
7a&b, 10a&b, 11a&b, 16a&b, 17a&b, 19a&b&c; same patients samples from different days.
Fig. 11. Effect of oral rinse samples on periodontitis patients on leukocyte elastase activity (500mU/ml) 2, 3a, 3b, 6, 14, 17b, 18 and conditions are the same as in Fig. 10.
Fig. 12. Effect of oral rinse sample of healthy subjects on leukocyte elastase activity (500mU/ml) and one selected periodontitis patient in Fig 9.
1,3,4,5; healthy controls. 9; same patient as in Fig.9.