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**TOBACCO SMOKING, VASCULAR REACTION AND NEUTROPHIL ACTIVITY
IN PERIODONTAL HEALTH AND DISEASE**

by

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To Jan, Erik and Ola

ABSTRACT

Tobacco smoking has considerable negative effects on periodontal health. The mechanisms behind these effects may be related to the inflammatory response.

In this thesis, the main aim was to study the influence of tobacco smoking on the inflammatory response in periodontal health and disease.

The specific aims were to study the influence of tobacco smoking on the gingival vasculature in periodontal health and in experimentally induced gingivitis (*Studies I, II*). Secondly, the aim was to study the influence of tobacco smoking on the presence and activity of neutrophil derived protease in periodontal health and disease (*Studies III-IV*). Finally, the purpose was to investigate the influence of tobacco smoking on neutrophil derived protease and collagenase following periodontal treatment (*Study V*).

In *Studies I and II*, the influence of tobacco smoking on the vascular reaction, in terms of the number of visible vessels was studied. During the experimental plaque accumulation the number of visible vessels increased over time in both smokers and non-smokers, however, this was less pronounced in smokers. Under clinically healthy periodontal conditions, the number of visible vessels was similar in both smokers and non-smokers.

In *Studies III and IV*, the influence of tobacco smoking on the presence, in GCF, of neutrophil derived elastase and LF and the protease inhibitors α -1-AT and α -2-MG was studied. It was found that under clinically healthy periodontal conditions, there was no difference between smokers and non-smokers, whereas under periodontal diseased conditions, smokers were found to have lower α -2-MG concentration compared to non-smokers.

In *Study V*, following periodontal surgery, the influence of tobacco smoking on the presence, in GCF, of elastase and MMP-8 and the protease inhibitors α -1-AT and α -2-MG, was studied. In smokers, it was found that the concentrations of elastase, MMP-8, α -1-AT and α -2-MG remained unchanged during the study period, whereas in non-smokers there was a change toward increased concentrations of α -1-AT and α -2-MG and decreased concentration of MMP-8.

Conclusion

The present observations indicate that tobacco smoking exerts an effect on the inflammatory response resulting in suppressed vascular reaction, decreased level of α -2-MG and, following surgery, unchanged levels of MMP-8 and α -1-AT and α -2-MG compared to non-smokers.

Key words: tobacco smoking, vascular reaction, neutrophil activity, gingival crevicular fluid, periodontal disease, periodontal health, periodontal treatment.

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Tobacco smoking

PREFACE

This thesis is based on the following studies and will be referred to in the text as *Studies I-V*. Reproduced with the permission of the publishers.

Study I

Bergström J, Persson L, Preber H. Influence of cigarette smoking on vascular reaction during experimental gingivitis. *Scand J Dent Res* 1988;96:34-39.

Study II

Persson L, Bergström J. Smoking and vascular density of healthy marginal gingiva. *Eur J Oral Sci* 1998;106:953-957.

Study III

Persson L, Bergström J, Gustafsson A, Åsman B. Tobacco smoking and gingival neutrophil activity in young adults. *J Clin Periodontol* 1999;26:9-13.

Study IV

Persson L, Bergström J, Ito H, Gustafsson J. Tobacco smoking and neutrophil activity in patients with periodontal disease. *J Periodontol* 2001;72:90-95:

Study V

Persson L, Bergström J, Gustafsson A. The effect of tobacco smoking on neutrophil activity following periodontal surgery. Submitted.

ABBREVIATIONS

ANOVA	analysis of variance
α -1-AT	α -1-antitrypsin
α -2-MG	α -2-macroglobulin
ELISA	enzyme linked immunosorbent assay
GBI	gingival bleeding index
GCF	gingival crevicular fluid
kDa	kilodalton
GI	gingival index
IR	interquartile range
LF	lactoferrin
Mabs	milliabsorbance
MMP	matrix metalloproteinase
PBS	phosphate buffered saline
PI	plaque index
SD	standard deviation
SEM	standard error of the mean
TIMP	tissue inhibitor matrix metalloproteinase

INTRODUCTION

The consequences of tobacco smoking on health

According to the World Health Organization, there are currently 1.1 billion smokers. This equates to approximately 1/3 of the world's entire population over 15 years of age. Although smoking prevalence has dropped among adults over 30% of adults in the European Region are still regular daily smokers (in Sweden about 19 %). By contrast, tobacco consumption is increasing in developing countries and the prevalence among men is about 48% (World Health Organization, The World Health Report 1999, Tobaksfakta.org 2002).

Evidence that smoking tobacco harms health was largely ignored until 1950, when five case-control studies associated smoking with the development of lung cancer (Doll 1950, Levin et al 1950, Mills & Porter 1950, Schrek et al 1950, Wynder & Graham 1950).

Since then, more than 70,000 scientific articles have shown that tobacco smoking is directly related to the incidence and prevalence of a variety of health problems including cancer and cardiovascular, pulmonary and renal diseases. (Bartecchi et al 1994, Yuan et al 1998, Woodward et al 1999, World Health Organization, The World health report 1999, Bleyer et al 2000, Patel & Homnick 2000, Villablanca et al 2000).

Tobacco smoking and periodontal disease

The influence of tobacco smoking as an etiological factor in periodontal disease has been the subject of many studies. In the late 1940s, Pindborg (1951) found a correlation between the consumption of tobacco and ulceromembranous gingivitis. Also Arnö et al (1958) found a significant correlation between tobacco smoking and gingivitis when hygiene and age were kept constant. This correlation was later confirmed by Kristoffersen (1971) and Preber & Kant (1973) when they demonstrated that gingival inflammation is more frequent among smokers than non-smokers. Ainamo (1971), however, suggested that gingivitis might be less prevalent in smokers. After these and other early studies (Feldman et al 1983, Bergström & Floderus-Myrhed 1983, Ismail et al 1983), the investigation of the relationship between tobacco smoking and periodontal disease has continued. From when cigarette smoking was first mentioned as a risk factor for periodontal disease (Preber & Bergström 1986), smoking has now been accepted as a risk factor for the prevalence and severity of periodontitis (Bergström 1989, Stoltenberg et al 1993, Haber et al 1993, Holm 1994, Grossi et al 1995).

Although the majority of investigations into the relationship between smoking and periodontal disease are cross-sectional, longitudinal investigations have also been conducted (Burt et al 1990, Holm 1994, Norderyd & Hugosson 1999, Bergström et al 2000).

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Depending on the study design, studies have shown that tobacco users are more likely to develop periodontal disease than non-smokers with an odds ratio ranging from 2.5 to 11.8 (Bergström 1989, Haber & Kent 1992, Stoltenberg et al 1993, Haber et al 1993, Grossi et al 1995, Norderyd & Hugosson 1998).

The prevalence of periodontal disease is lower in former smokers than in those who continue to smoke, which suggests that smoking cessation is beneficial to periodontal health (Haber & Kent 1992, Bolin 1993, Krall et al 1997, Grossi et al 1997, Bergström et al 2000).

Tobacco smoking and vascular response

Several studies have used the morphology, topography and function of the gingival vasculature to evaluate gingival inflammation. In healthy conditions the vasculature is characterized by vessels connected in a network with hairpin-like capillaries, so-called loops running largely perpendicular to the gingival margin. In inflamed gingiva, the vasculature is characterized by a proliferation of vessels. Furthermore, the width and length of the vessels are increased and the course is changed resulting in a more pronounced formation of vessel loops (Forslund 1959, Egelberg 1966, Folke 1967, Hansson et al 1968, Kindlova 1968, Hock & Nuki 1971, Hock 1974, Nuki 1974, Hock 1979, Weeks 1986).

After early studies showing that gingival inflammation is more frequent among smokers than non-smokers, clinical and epidemiological studies have shown that gingival inflammation is suppressed in smokers i.a., (Ainamo 1971, Bergström & Floderus-Myrhed 1983, Preber & Bergström 1985, Bergström & Preber 1986,

Danielsen et al 1990, Lie et al 1998) and that smoking exerts a constrictive effect on microcirculatory vessels and decreases the microcirculatory blood flow rate in humans (Lampson 1935, Asano & Brånemark 1970). Tobacco smoking also effects the vasculature in periodontal tissue. In an animal model, gingival blood flow was monitored while nicotine was repeatedly infused intra-arterially. With each injection, blood flow increased for a few minutes then dropped to below baseline levels (Clarke et al 1981, Clarke & Sheppard 1984). Using the laser Doppler flowmeter technique gingival blood flow was measured in healthy young smokers (Baab & Öberg 1987). The results showed an immediate but transient increase in relative blood flow during smoking, compared to pre-smoking or resting measurements. In humans, with healthy gingiva Meekin et al (2000) could not observe any local vasoconstriction in periodontal tissues in either light or in heavy smokers.

Systemic nicotine administration leads to increased blood pressure, increased heart rate, increased respiratory rate and decreased skin temperature. In the peripheral nervous system, nicotine affects the release of catecholamines, adrenalin and noradrenalin. This release of catecholamines has important effects on cardiac function, vascular tonus and lipid metabolism (Benowitz 1986). Smoking-induced vasoconstriction may contribute to impaired gingival blood flow and decrease the amount of oxygen leading to tissue damage. When comparing the pocket oxygen tension in smokers and non-smokers with periodontal disease, it was found that pocket oxygen tension was lower in smokers and that there was a correlation between oxygen tension and pocket depth (Hanioka et al 2000).

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GCF

GCF is a serum transudate, or an inflammatory exudate. The fluid portion of the exudate is derived from leakage of the subgingival microvasculature. The constituents in GCF can be derived from a number of sources, including serum and periodontal tissue (Glynn 1983, Lindhe 1997). Analysis of GCF constituents provides information about periodontal inflammation and the sampling of GCF was introduced by Brill & Krasse (1958). The GCF volume is reportedly reduced in smokers compared to non-smokers (Bergström & Preber 1986, Holmes 1990). McLaughlin et al (1993) observed an immediate but transient increase in the volume of GCF in healthy individuals after smoking 1 cigarette. No comparison to matched non-smokers was made. This could be comparable to the immediate transient increase in gingival blood flow (Clarke et al 1981, Clarke & Shepphard 1984, Baab & Öberg 1987).

Neutrophil elastase

Neutrophil elastase is a protease, (33kDa), that is stored in the primary granule of the neutrophils in amounts of up to 3 picograms per cell and released extracellularly during phagocytosis. Elastase can degrade a variety of molecules such as elastin, laminin, fibronectin and collagens (Janoff & Zeligs 1968, Janoff 1985). Excessive release or inadequate inhibition can lead to tissue damage in a number of diseases, such as pulmonary emphysema, chronic bronchitis, rheumatoid arthritis and periodontitis (Janoff 1985, Fujita et al 1990, Moore et al 1999). During inflammation in

the periodontal tissues, elastase is released into the tissue and increased levels are found in the gingival crevicular fluid. Increased amounts of GCF elastase have been found during gingival inflammation (Giannopoulos et al 1992, Gustafsson et al 1992). An increased release of elastase may contribute to tissue destruction and the development of periodontitis (Palcanis et al 1992, Armitage et al 1994, Alpagot et al 2001). Following non-surgical periodontal therapy in subjects with chronic periodontitis, lower levels of elastase have been observed compared to pre-treatment levels (Jin et al 2002). The influence of tobacco smoking on the GCF elastase levels in periodontitis patients is controversial. Increased (Söder 1999), decreased (Alavi et al 1995) and similar (Alpagot et al 1996) levels of GCF elastase in smokers compared to non-smokers have been reported.

LF

LF is an iron binding protein (72.5 kDa), that is stored in the secondary granules of neutrophils in amounts of 3 picogram/cell (Querineau et al 1971, Spitznagel et al 1974). Together with other lysosomal proteins, LF is released during phagocytosis and influences neutrophil functions, such as adhesion and chemotaxis (Wright and Gallin 1979, Gahr et al 1991). A degranulation of specific granules, with a momentary release of LF and a prolonged release of elastase after activation by chemotactic stimuli, has been demonstrated (Beentwood & Henson 1980).

In periodontal tissues, levels of LF are higher in GCF from gingivitis and periodontitis sites compared to healthy sites (Adonogianaki et al 1993, Tsai et al 1998). Other

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studies, however, have failed to associate LF with disease severity (Adonogianaki et al 1996, Figueredo et al 1999).

α -1-AT and α -2-MG

The activity of neutrophil proteases is mainly modulated by the plasma proteinase inhibitors α -1-AT and α -2-MG. These plasma proteins constitute, by weight, the third largest group (after albumin and immunoglobulins) of functional proteins in humans. (Travis & Salvesen 1983). Both are glycoproteins, while α -1-AT is the smaller molecule (53kDa) with a number of biochemical variants (Hoffman & Van der Broeck 1977), and α -2-MG is the larger molecule (725 kDa). An important function of α -1-AT and α -2-MG is to control the activity of neutrophil elastase. The half time of association of α -1-AT and α -2-MG with elastase is 0.61 milliseconds and 7.2 milliseconds, respectively (Travis & Salvesen 1983). In individuals with normal plasma protein levels, there are sufficient numbers of active inhibitors to control proteolysis. Plasma proteins, however, are reported to be congenitally deficient in specific individuals. When this occurs, development of specific disease states become manifest, for example familial pulmonary emphysema due to α -1-AT deficiency (Cox 1993). A decreased α -1-AT activity is considered responsible for the development of emphysema in smokers (Gadek et al 1979). Both pulmonary emphysema and periodontal disease are characterized by uncontrolled proteolysis of connective tissue by proteinases derived from human neutrophils (Travis et al 1994).

In a study by Fokkema et al (1998), α -1-AT deficient patients were found to have worse periodontal conditions than healthy controls.

Decreased levels α -2-MG indicate tissue destruction since lower concentrations of α -2-MG have been found in periodontal diseased sites compared to periodontal healthy sites (Condacci et al 1982, Gustafsson et al 1994).

In addition, in periodontal diseased patients, levels of elastase complexed with α -1-AT or α -2-MG have been found to be lower in smokers compared to non-smokers (Alavi et al 1995). However, in a recent study, Söder et al (2002) found higher levels of elastase complexed with α -1-AT in smokers compared to non-smokers. Following surgical periodontal therapy, lower levels of elastase complexed with α -1-AT compared to pre-treatment has been found. Smoking, however, was not taken into account (Buchmann et al 2002).

MMP-8

MMPs is a family of proteolytic enzymes that is involved in normal tissue remodeling as well as collagen degradation and tissue destruction in periodontal disease (Birkedal-Hansen 1993, Woessner 1991, Golub et al 1997, Chen et al 2000). MMP-8 is a collagenase released in a latent form from secretory granules stored in the neutrophils (Hasty et al 1986). Activation of the proenzyme occurs in the extracellular space after secretion, resulting in removal of a part of the pro-domain. MMP-8 in a latent form has a molecular weight of 80-90 kDa while the active enzyme has a weight of 60-70 kDa.

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The inhibition of MMP-8 is mainly regulated by TIMP but also by α -2-MG. (Birkedal-Hansen 1993, Romanelli et al 1999).

MMP-8 is reportedly the main type of interstitial collagenase in GCF of periodontal patients (Sorsa et al 1988, Ingman et al 1996). The relationship between tobacco smoking, MMP-8 levels and periodontal status has only been investigated in a few studies. The results are ambiguous (Liede et al 1999, Söder 1999).

Cotinine

Nicotine is mainly metabolized by oxidative metabolism to cotinine (Benowitz & Jacob 1993). Cotinine is cleared primarily by oxidation and is excreted unchanged in urine (Byrd et al 1994). It has a longer half-life, approximately 20 hours, compared to nicotine (half-life 1-2 hours) and is used as a marker of nicotine exposure both in smokers and non-smokers (Pilotti 1980, Benowitz 1996).

Cotinine can be detected in serum, saliva and GCF (McGuire et al 1989, González et al 1996, Machtei et al 1997). The advantages of measuring cotinine are that its levels remain relatively constant over long periods of time and that it is found in higher concentrations than nicotine.

AIMS

General aim

The general objective of this thesis was to study the influence of tobacco smoking on the inflammatory response in periodontal health and disease.

Specific aims

- To study the influence of tobacco smoking on the marginal gingival vasculature in periodontal health (*Study II*)
- To study the influence of tobacco smoking on the marginal gingival vasculature in experimental gingivitis (*Study I*)
- To study the influence of tobacco smoking on the presence and activity of neutrophil derived elastase and the inhibitors α -1-AT and α -2-MG in periodontal health and disease (*Studies III, IV*)
- To study the influence of tobacco smoking on the presence and activity of neutrophil derived elastase and collagenase, and the inhibitors α -1-AT and α -2-MG following periodontal treatment (*Study V*)

MATERIAL

Study populations

A total of 60 healthy individuals, 30 smokers and 30 non-smokers, and 62 patients, 30 smokers and 32 non-smokers, participated in the various studies of this thesis.

In *Study I*, the population consisted of 16 healthy dental students, 8 smokers (5 women and 3 men) aged between 23-38 years, and 8 non-smokers (5 women and 3 men) aged between 19-42 years. The smokers were regular daily cigarette smokers with a current consumption of between 10-20 (mean 13.4) cigarettes per day. They had been smokers for at least 4 years.

Study II included 14 female dental students. Seven of the subjects were regular cigarette smokers with a current consumption of between 10-25 (mean 16.1) cigarettes per day and a smoking duration ranging from 8 to 18 (mean 13.1) years. Seven subjects were non-smokers with no previous history of smoking. The age of the smokers ranged between 25-38 (mean (SD) 33.6 (4.4)) and the age of the non-smokers ranged between 25-34 (mean (SD) 27.3 (4.0)) years. All subjects were in general good health according to their own statements and their gingival conditions were clinically healthy (Løe & Silness 1963). In the area to be treated GI as well as PI (Silness & Løe 1964) were equal to 0. The participants were free to maintain their usual dental hygiene habits during the study period.

In *Study III*, 30 healthy dental students with no clinical signs of periodontitis volunteered. 15 were smokers (8 women and 7 men) aged between 20-32 years (mean (SD) 27.5 (4.1)) and 15 were non-smokers (7 women and 8 men)) aged

between 22-31 years (mean (SD) 25.5 (3.1)). The smokers were regular daily cigarette smokers with a current consumption of between 7-20 (mean (SD) 12.3 (3.8)) cigarettes per day and a smoking duration ranging from 6 to 17 (mean (SD) 10.4 (4.5)) years.

In *Study IV*, 32 patients, with moderate to severe periodontitis participated, 15 smokers (5 women and 10 men) aged between 34-69 years and 17 non-smokers (5 women and 12 men) aged between 31-81 years. The mean (SD) ages of the smokers and non-smokers were 54 (11.4) years and 63 (13.1) years respectively. The age difference was significant ($p=0.02$). The patients had moderate to severe periodontal disease with at least 3 sites with a probing depth of 4 mm or more (mean (SD) 5.8 (1.0)) classified as severe lesions and at least 3 sites with a probing depth of less than 4 mm (mean (SD) 2.8 (0.7)) classified as moderate lesions. The smokers were regular daily cigarettes smokers with a current consumption of between 10-40 cigarettes per day (mean (SD) 16.4 (7.6)) and a smoking duration ranging from 10 to 50 years (mean (SD) 31.9 (10.9)).

Study V included 30 patients with moderate to severe periodontitis as evidenced by multiple sites with a probing depth of 5 mm or more.

15 were smokers (10 women and 5 men) in the age range 42-60 years and 15 were non-smokers (11 women and 4 men) in the age range 39-76 years. The median (IR) ages of smokers and non-smokers were 52.0 (47-58) years and 57.0 (53-66) years respectively. The age difference was significant ($p=0.03$).

The smokers were regular daily cigarette smokers with a current consumption of between 10-20 cigarettes per day (median 12.5 (IR 10-15)) and a smoking duration ranging from 16 to 43 years (median 30.0 (IR 26-35)).

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The non-smokers had never smoked or had stopped using any form of tobacco at least 10 years ago. Twelve smokers were in general good health according to their own statement and not using any medications. Three smokers reported a medical history of hypertension or bronchitis. Six non-smokers were healthy and 9 non-smokers suffered from hypertension and/or other diseases such as diabetes and respiratory diseases.

Clinical characteristics in the study populations

The gingival inflammation and amount of plaque in the area to be treated were similar in both smokers and non-smokers, in both clinically healthy (*Studies I-III*) and in periodontal diseased (*Studies IV and V*) individuals.

When comparing moderate and severe lesions (*Study IV*) the gingival inflammation was more pronounced in severe lesions compared to moderate lesions in both smokers and non-smokers. Smokers, however, were found to have more plaque in severe lesions than in moderate lesions. Furthermore, in the area to be treated the pocket depth was similar in both smokers and non-smokers (*Studies IV and V*).

However, the total number of sites with pocket depths of 6 mm or more and the total number of diseased sites were significantly greater in smokers (*Study V*) (Tables 1, 2).

Table 1

Clinical characteristics. Mean (SD) and Range according to smoking for moderate and severe lesions (*Study IV*)

Variable	Smoker			Non-smoker			P value
	Mean	(SD)	Range	Mean	(SD)	Range	
Probing depth (mm)							
Moderate lesions	2.8	(0.75)	1.7-4.0	2.8	(0.67)	1.7-4.0	0.83
Severe lesions	5.7	(1.12)	4.0-8.3	5.9	(0.87)	5.0-8.0	0.36
P value	0.001			0.001			
Gingival mean score							
Moderate lesions	1.5	(0.79)	0.3-3.0	1.6	(0.73)	1.0-3.0	0.37
Severe lesions	2.0	(0.68)	1.0-3.0	2.1	(0.67)	1.0-3.0	0.65
P value	0.002			0.021			
Plaque mean score							
Moderate lesions	0.3	(0.42)	0-1.0	0.4	(0.47)	0-1.0	0.82
Severe lesions	0.6	(0.57)	0-1.0	0.5	(0.48)	0-1.0	0.89
P value	0.047			0.063			

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Table 2

Clinical characteristics at baseline. Median and IR according to smoking (*Study V*)

Variable	Unit	Smoker		Non-smoker		P value
		Median	IR	Median	IR	
4-5 mm sites	N	19	17-24	15	13-22	NS
6+ mm sites	N	9	7-16	6	4-9	0.04
4+ mm sites	N	30	27-41	22	16-31	0.03
Teeth	N	23	20-27	24	23-27	NS
GBI	%	28	20-35	25	13-30	NS
PI	%	9	6-18	10	4-14	NS
Mean pocket depth in the area to be treated	Mm	5.7	5.3-6.0	6.0	5.3-6.7	NS
Mean proportion of bleeding sites in the area to be treated		0.0	0.0-0.3	0.3	0.0-0.3	NS

METHODS

Experimental gingivitis

Gingivitis (*Study I*) was induced in the subjects by refraining from all oral hygiene measures for a period of 28 days. The gingivitis induction was limited to the mandibular canines and incisors. Seven days before the experiment started, clinically

healthy gingiva was obtained by scaling and cleaning with rubber cups and pumice. At the termination of the experiment the teeth were cleaned by scaling and polishing and habitual dental hygiene routines were reinstated. Gingiva and teeth were examined prior to the start of the experiment (day 0), at days 14 and 28 of the experiment and finally, at days 35 and 42, i.e. 7 and 14 days after the termination of the experiment.

Stereophotographic evaluation of vascular characteristics

The photographic documentation (*Studies I, II*) of the gingival area to be investigated was performed with a Zeiss stereomicroscope connected to a camera system for synchronous exposure of two films (Berghagen et al 1968, Bergström & Jonasson 1974). With this method, stereo pairs of photographs are obtained for subsequent stereoscopic evaluation. Photography was performed under standardized conditions, and the same area was followed throughout the total experiment.

Color film of format 24x36 mm, Agfachrome 50 L (*Study I*) or Kodak Ektachrome 160T (*Study II*) was used. The negative scale was 1:0.22. The evaluation of the stereo pairs of photographs was made with a mirror stereoscope under a 10-x magnification. The labial aspect of the marginal gingiva of two mandibular incisors (*Study I*) or one mandibular or maxillary incisor (*Study II*) constituted the area to be investigated. Photodocumentation was performed on 5 occasions, similar to the experimental gingivitis, (*Study I*) or on 4 occasions at 4 weekly intervals (*Study II*). The marginal gingiva was subdivided into 6 zones, 3 basal and 3 crestal (*Study I*) within which the vessels were counted (Figure 1, p.36, *Study 1*). Every clearly visible vessel that appeared as a distinct point compared to surrounding tissue was defined

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as a vessel. The degree of vascular inflammation was expressed as the total sum of visible vascular points within the 6 zones. For the material, observer reproducibility was evaluated from immediately repeated measurements in images from all subjects and all observations. The number of vessels was counted in all the above 6 zones and the enumeration was then repeated. The correlation between repeated series was $r=0.99$ (Pearson correlation coefficient).

In *Study II*, this method was improved, allowing an approximately 2-fold greater magnification than was previously possible. The increased power, however, was counteracted by an utterly small depth of field and a reduced area for reproduction. The area to be investigated was subdivided into one crestal and one basal zone. The counting of the vessels was done in the basal zone of the marginal gingiva as illustrated (Figure 1, p.954, *Study II*). The reason for this was that the basal zone exhibits a greater number of vessel structures than the crestal zone (Bergström 1992). The basal zone was further subdivided into a mesial and distal part on either side of the midline (B1 and B2) and the counting was done in either B1 or B2. The width of this measurement zone was 0.55 mm and the length 4 mm, corresponding to a total area of approximately 2.20 mm² in the film scale. Within the measurement zone, distinct vascular structure that contrasted with the surrounding tissue was counted and evaluated in regard to form and structure according to a semi quantitative index method. Three types of vascular structure were observed: (1) a point, (2) a single-limbed loop and (3) a double-limbed loop (Figure 2, p 955, *Study II*). Each type was given a code and the sum of the individual vessel codes within the measurement zone was labeled as the vascular density score.

It is supposed that the vascular density score yields a superficial representation of complex microcirculatory network. The counting and evaluation procedure in the

subjects was always related to the same zone throughout the observation period. The identification of vessels is dependent on factors such as anatomical variations and the capacity of the observer. The variation in the identification of vessels was studied by means of (1) a comparison of repeated readings in the image pairs before and after an interval of one week; and (2) a comparison of readings in image pairs from two immediately repeated exposures (intraobserver variation). In addition, the variation was studied by means of (3) a comparison of image pairs read by two observers independently (interobserver variation).

Error of stereophotographic method

The results of the intraobserver variation as studied by repeated readings in the same image pairs with an interval of one week, are presented in Table 3. There were no statistically significant differences between time points. The variation in identification and refocusing was studied by comparing the image pairs obtained from two sets of immediately repeated exposures (Table 4). There were no statistically significant differences between the time points. The interobserver variation in identification was studied by comparing 24 image pairs in 12 subjects read by two observers independently. The mean (SD) difference between observers was 0.68 (2.29). The difference was not statistically significant ($t=1.03$, $P>0.05$), suggesting that there was no systematic interobserver variation.

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Table 3

Variation in vascular density score following measurements in the same image pairs repeated after an interval of one week. Intraobserver differences based on the 12 subjects at 3 time points

Time point	Difference		
	Mean	SD	Standard error of Measurements (SD/ $\sqrt{2}$)
1	0.417	2.429	1.717
2	0.416	1.083	0.765
3	0.500	1.783	1.260

Table 4

Variation in vascular density score following measurements in image pairs from 2 immediately repeated exposures. Differences between exposures based on 12 subjects at 3 time points

Time point	Difference		
	Mean	SD	Standard error of Measurements (SD/ $\sqrt{2}$)
1	0.583	0.900	0.636
2	0.417	0.900	0.636
3	0.417	0.900	0.636

Gingival inflammation and plaque

In *Studies III and IV*, the gingival condition was assessed by evaluating redness, swelling and bleeding according to the Gingival Index by Löe & Silness (1963). Finally, in *Study V*, the gingival inflammation was dichotomously scored as bleeding or no bleeding, within 15 seconds in response to the probing (Ainamo & Bay 1975). The amount of plaque accumulation (*Study I*) on the labial surfaces of the teeth was evaluated from the stereo pairs of photographs. The amount of plaque was measured for each tooth and a mean was calculated for the subjects (Bergström (1981). In addition, supragingival plaque was evaluated (*Study II*) according to the criteria of Silness and Löe (1964). In *Studies IV and V*, plaque was dichotomously scored as present or absent on the teeth surfaces (Ainamo & Bay 1975).

Pocket depth

The depth of periodontal pockets was measured using a graduated metal probe with a diameter of 0.55 mm (Hu-Friedy Inc., Chicago, IL) (*Study IV*) or a periodontal metal probe, 0.5 mm in diameter with an approximately probing force of 20 g (Vivacare TPS, Vivadent Ets., Shaan, Lichtenstein) (*Study V*).

Tobacco smoking

GCF

GCF was collected with prefabricated paperstrips (Periopaper GCF strips, Oraflow Inc., Plainview, NY, USA). The strips were inserted into the crevice until mild resistance was felt and held there for 30 seconds. Samples contaminated with blood or saliva were discarded. The fluid volume collected was measured immediately after sampling with a calibrated Periotron® 6000 (*Study III*) or with a Periotron® 8000 (*Studies IV, V*) (IDE Interstate, Amityville, NY, USA).

The 6 strips (*Study III*) or the 3 strips (*Studies IV, V*) from each individual were pooled and placed in 1000 or 500 µl PBS, pH 7.4, and shaken for 15 or 45 minutes at room temperature. After removing the strips, the samples were centrifuged at 3,000 g for 5 minutes. The supernatants were then stored in sealed containers at 70°C, pending analysis. Before each study, the Periotron was calibrated, using saline administered with a Hamilton syringe. The calibration curve included the range 0.05 to 0.8 µl (*Study III*) or 0.1 to 1.0 µl (*Studies IV, V*).

Protein

The total protein concentration (*Study III*) in GCF was measured with the Bradford protein staining method (Bradford 1976), using Bio Rad's protein assay. The protein reagent was added to the samples and its color intensity was measured in a spectrophotometer at 500 nm. The samples were compared to a standard curve obtained by serial dilution of a standard serum with PBS on the same plate. The protein composition of the serum is similar to that of GCF.

LF

The LF concentration was determined with an ELISA. The wells of the microtiter plate (Nunc Maxisorb, Nunc, Roskilde, Denmark) were coated with a monoclonal mouse antibody against LF (Hy-Test, Turku, Finland) and incubated overnight at 4°C. After 4 washes with PBS containing 0.05% Tween®, samples and standards diluted 40 times were added to the wells and incubated at +37°C for 1 hour, washed again, and the second antibody, a polyclonal rabbit anti-LF (Dako-immunoglobulins a/s, Glostrup, Denmark) was added. The plates were incubated for 1 hour at +37°C, washed 4 times before adding the third antibody, an alkaline phosphatase-conjugated polyclonal goat antibody against rabbit (Boehringer, Mannheim, Germany) and incubated for 1 hour. After a final wash, the substrate was added and the absorbency at 405 nm was then read.

Elastase

Elastase activity was measured with a low molecular weight substrate S-2484 (L-pyro-glutamyl-L-propyl-L-valine-nitroaniline, Hemachrome Diagnostica, Mölndal, Sweden) that is specific for neutrophil elastase (Kramps et al 1983). The substrate was added to the sample. The mixture was incubated at 37° C and after 5 or 3 hours (*Studies III-V*) the absorbance at 405 nm was measured. The elastase activity collected with strips mainly originates from elastase complexed with α -2-MG, and the recovery of uncomplexed free elastase is very low (Gustafsson 1996).

Tobacco smoking

α -1-AT and α -2-MG

The concentrations of α -1-AT and α -2-MG were determined with ELISA. The samples and standards were diluted 40 times. The wells of the microtiter plates (Nunc Maxisorb, Nunc, Roskilde, Denmark) were coated with standards and samples overnight at 4°C. After 4 washes with PBS containing 0.05% Tween, the polyclonal rabbit antibody against α -1-AT and α -2-MG (Dakopatts a/s, Roskilde, Denmark) was added. The plates were incubated at 37°C for 1 hour and washed 4 times before the alkaline phosphatase conjugated polyclonal goat antibody against rabbit IgG were added. After incubation at 37°C for 1 hour and another 4 washes, the substrate 4-p-nitrophenyl-phosphate was added. The absorbance at 405 nm was measured after approximately 30 min in a spectrophotometer. The samples were compared to a standard curve obtained by serial dilution of a human standard serum on the same plate.

MMP-8

The MMP-8 was determined with a commercial ELISA kit. The kit instructions were followed. Polystyrene microplates was pre-coated with a murine monoclonal antibody specific for MMP-8. The samples, diluted in a buffered protein base, or the standards were added to each well. The plates were incubated at room temperature, shaken for 2 hours and washed 4 times with PBS containing 0.05% Tween. After washings, a polyclonal antibody conjugated with horseradish peroxidase was added.

The plates were incubated for 30 min, shaken and washed again as described above before adding the substrate solution. The color development was stopped with sulfuric acid after 30 min and the absorbance at 450 nm was read in a spectrophotometer.

Cotinine

The saliva cotinine concentration was determined with a commercial ELISA kit. The kit instructions were followed. Anti-cotinine coated-plates were labeled with samples, calibrators or controls before adding enzyme conjugate. After incubation for 30 minutes at room temperature and 4 washes with diluted wash buffer, substrate reagent was added. The plates were then incubated as above and the color development was stopped with sulfuric acid. The absorbance at 450 nm was read in a spectrophotometer within 30 minutes after stopping the reaction. The measured absorbance was inversely proportional to the amount of free cotinine in the sample.

Statistical methods

In *Study I*, a two-tailed significant analysis of differences between smokers and non-smokers was performed using Student's t-test. Data was expressed as mean and SEM.

In *Study II*, the results were analyzed using a three-way ANOVA with repeated measures on three factors. The factors were time with three levels, exposure with two levels and reading with two levels. Data was expressed as mean, SD and standard error of measurements ($SD/\sqrt{2}$).

Tobacco smoking

In *Study III*, data was expressed as median, mean and standard deviation. The statistical significance of differences between smokers and non-smokers was calculated according to the Mann-Whitney U-test.

In *Study IV*, the statistical significance of differences between smokers and non-smokers was calculated according to the Mann-Whitney U-test. The Wilcoxon signed rank test was used to compare data within groups. As age was different significantly in the smoking groups 2-factor ANOVA was run with α -2-MG, α -1-AT, LF or elastase as the dependent variables, and smoking and age as independent variables. Data was expressed as mean, SD and range.

In *Study V*, data was expressed as median and interquartile range (IR). The statistical significance of the differences between smokers and non-smokers was calculated according to the Mann-Whitney U-test. The Wilcoxon matched-pairs test was used to compare data within groups over time. The Spearman rank order correlation was used to determine the correlation coefficient (r) for the correlation between cotinine concentration and the number of cigarettes smoked per day. The subjects throughout constituted the unit in the analysis, and the statistical significance was accepted at the probability level of $P \leq 0.05$.

RESULTS

Vascular reaction during experimental gingivitis

In *Study I*, gingivitis was experimentally induced by the subjects refraining from all oral hygiene measurements for a period of 28 days. There were no significant differences in plaque accumulation between smokers and non-smokers during the experiment (Table 5, Figure 1).

The gingival inflammation was expressed as the number of visible vessels in the marginal gingiva. Before the start of the experiment, the number of visible vessels did not significantly differ between smokers and non-smokers (Table 6, Figure 2). After 28 days of plaque accumulation, the number of vessels had increased compared to day 0 in both smokers and non-smokers. The increase, however, was less pronounced in smokers and the differences between the smoking groups were significant at day 14 and day 28 ($t=2.41$ and $t=2.69$, respectively $p < 0.05$). After removing plaque and resuming oral hygiene, a return towards pre-experimental values was observed in both groups (day 35).

Tobacco smoking

Table 5

Plaque accumulation (%) in smokers and non-smokers during the experiment. Mean and SEM according to smoking

Day	Smoker Mean	SEM	Non-smoker Mean	SEM	P value
0	0	0	0	0	NS
14	49	8	48	9	NS
28	56	5	53	7	NS
42	0	0	0	0	NS

Figure 1

Plaque accumulation in 8 smokers and 8 non-smokers during the experiment

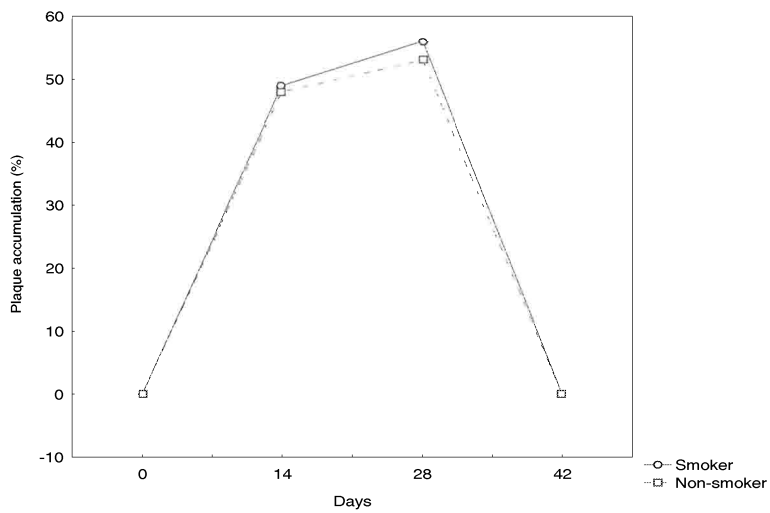


Table 6

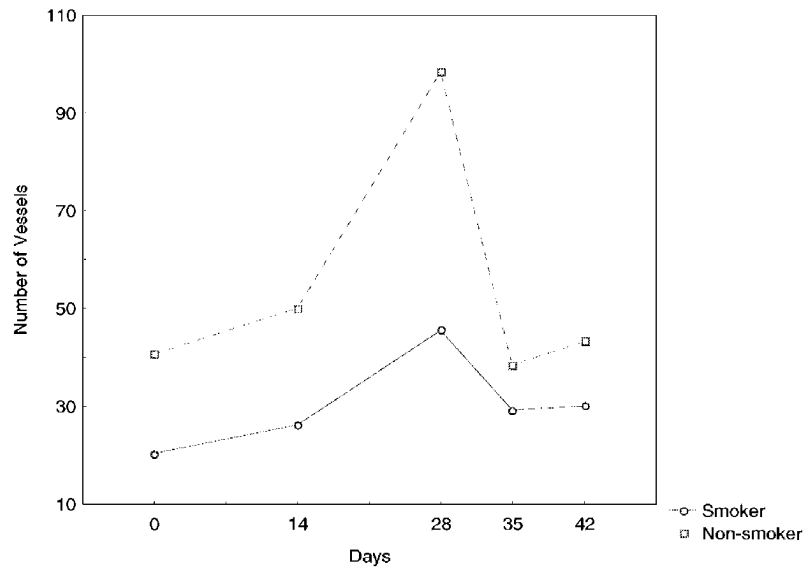
Vascular changes (number of visible vessels) in smokers and non-smokers during the experiment. Mean and SEM according to smoking

Day	Smoker		Non-smoker		P value
	Mean	SEM	Mean	SEM	
0	20.4	6.00	40.7	7.96	NS
14	26.2	5.06	50.1	8.55	< 0.05
28	45.5*	7.50	98.4*	18.23	< 0.05
35	29.3	7.71	38.4	11.27	NS

* Significantly different from day 0

Figure 2

Vascular changes during 28 days of experimentally induced gingivitis and 14 days after termination of the experiment. Number of visible vessels in 8 smokers and 8 non-smokers



Tobacco smoking

Vascular density in healthy marginal gingiva

The results of the influence of tobacco smoking on the vascular density in healthy marginal gingiva during the 11 weeks of investigation (*Study II*) are presented in Table 7.

The differences between time points for non-smokers were very small, indicating great stability of vascular characteristics over time. For smokers, the differences between time points were somewhat greater but there were no significant differences. Furthermore, there were no significant differences between smokers and non-smokers over time.

The predominant vascular characteristic was the “point” (91 %).

Table 7

Vascular density score in smokers and non-smokers (n). Mean and SD.

Time point	Smoker		Non-smoker		P value
	Mean	SD	Mean	SD	
1	7.57	3.78	11.36	6.80	NS
2	10.36	5.54	11.21	7.38	NS
3	10.36	4.85	11.71	8.12	NS
4	12.21	8.62	10.71	8.54	NS
Total	10.13	4.39	11.25	7.32	NS

α -1-AT and α -2-MG

The GCF concentrations of α -1-AT and α -2-MG according to smoking for healthy and diseased periodontal conditions are presented in Tables 8 and 9. In young adults (*Study III*), with healthy or slightly inflamed gingival conditions, no significant differences were found in the GCF concentrations of α -1-AT and α -2-MG between smokers and non-smokers (Table 8).

In periodontal patients (*Study IV*), both moderately and severely inflamed lesions were evaluated. With regard to moderate lesions, no significant differences in α -1-AT and α -2-MG concentrations between the smoking groups were found. With regard to severe lesions, however, smokers had lower α -2-MG concentrations as well as lower total amounts of α -1-AT and α -2-MG. When comparing moderate and severe lesions, smokers exhibited no gradual increase of protease inhibitors with disease severity in contrast to non-smokers who showed significantly increased α -2-MG concentrations compared to moderate lesions (Table 9).

Following periodontal surgery (*Study V*), the GCF concentrations of α -1-AT and α -2-MG were significantly lower in smokers compared to non-smokers at the 5-week observation. In non-smokers, the concentrations of both α -1-AT and α -2-MG increased over time, whereas the concentrations in smokers did not differ significantly during the study period. For both inhibitors, the 5-week change from baseline differed significantly in smokers and non-smokers ($p=0.013$ and $p=0.012$, respectively) (Tables 10, 11, Figures 3, 4).

The total amount of α -1-AT in smokers at baseline and at the 5-week observation was significantly reduced compared to non-smokers.

Tobacco smoking

Regarding α -2-MG, this was only true at the 5-week observation. At the end of the study, the total amount of α -1-AT and α -2-MG did not differ significantly compared to the baseline of either smokers or non-smokers (Tables 10, 11).

Protein

The GCF concentration of protein in healthy individuals (*Study III*) did not differ significantly between smokers and non-smokers (Table 8).

Table 8

α -1-AT, α -2-MG and protein. Mean and (SD) according to smoking for healthy periodontal conditions

Variable	Units	Smoker		Non-smoker		P value
		Mean	SD	Mean	SD	
α -1-AT	ng/ μ l	155.5	63.4	174.2	81.5	NS
α -2-MG	ng/ μ l	262.1	125.4	391.2	275.1	NS
Protein	μ l/ μ l	35.6	20.6	29.3	19.4	NS

Table 9

Laboratory variables. Mean (SD) and range according to smoking for moderate and severe lesions

Variable	Units	Smoker		Non-smoker		P value
		Mean (SD)	Range	Mean (SD)	Range	
α -1-AT	ng/ μ l					
Moderate lesions		557.2 (459.9)	147.6-2003.7	547.5 (290.7)	172.4-1130.7	NS
Severe lesions		540.9 (285.3)	92.6-1031.8	690.3 (307.8)	214.9-1346.9	NS
P value		NS		NS		
α -2-MG	ng/ μ l					
Moderate lesions		278.4 (286.2)	99.0-1086.5	310.7 (222.9)	82.3-822.7	NS
Severe lesions		295.7 (193.9)	48.1-741.3	413.3 (174.0)	175.7-774.9	0.05
P value		NS		0.05		

Table 10

α -1-antitrypsin in GCF. Median and IR according to smoking and observation time point following treatment

(a) Concentration (ng/ μ l)

	Baseline		Week 1		Week 5	
	Median	IR	Median	IR	Median	IR
Smoker	2680	1258-3453	2449	1576-2894	1395	743-2380
Non-smoker	1829	1495-2465	2414	1578-3488	3532	2140-5052*
P value	NS		NS		0.03	

(b) Total Amount (ng)

	Median	IR	Median	IR	Median	IR
Smoker	1528	606-2202	2504	1546-3049*	656	618-1427^
Non-smoker	2262	1927-3189	2966	1619-3288	1998	891-3121^
P value	0.05		NS		0.05	

*Significantly different from baseline

^Significantly different from week1

Tobacco smoking

Table 11

α -2-macroglobulin in GCF. Median and IR according to smoking and observation time point following treatment

(a) Concentration (ng/ μ l)

	Baseline		Week 1		Week 5	
	Median	IR	Median	IR	Median	IR
Smoker	1980	1030-3036	1618	856-2293	869	731-1728
Non-smoker	1421	1250-1716	1547	911-1998	2346	1547-3438*^
P value	NS		NS		0.02	

(b) Total Amount (ng)

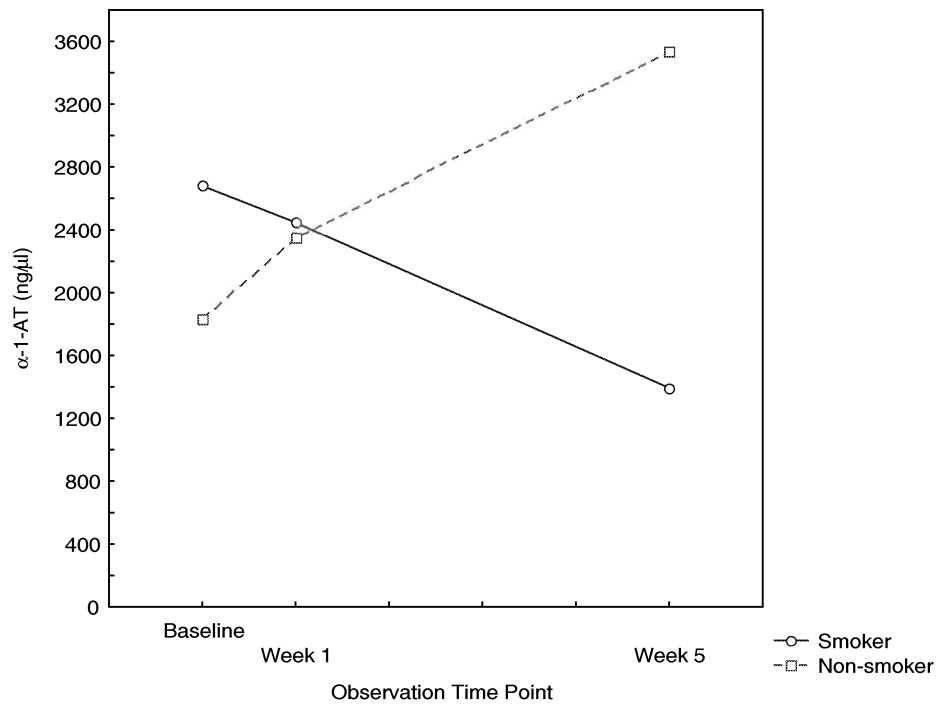
	Median	IR	Median	IR	Median	IR
Smoker	1312	869-1860	1581	1163-2016	868	438-1024^
Non-smoker	1803	1410-2137	1554	1338-2191	1227	1129-1973
P value	NS		NS		0.01	

*Significantly different from baseline

^Significantly different from week1

Figure 3

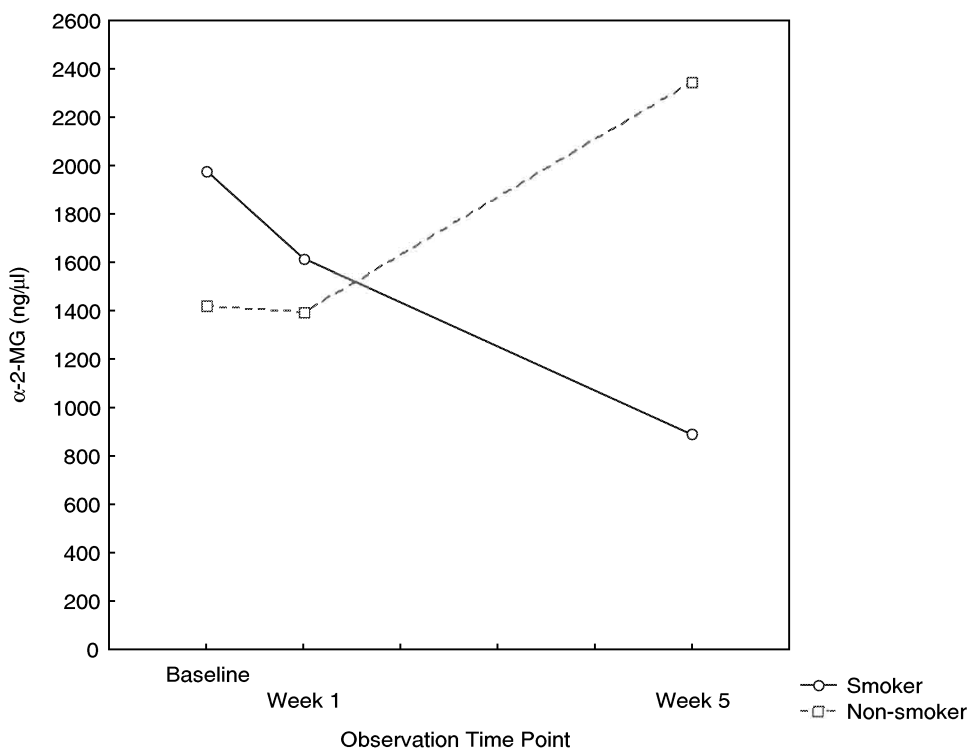
The α -1-AT concentration during the study period in smokers and non-smokers



Tobacco smoking

Figure 4

The α -2-MG concentration during the study period in smokers and non-smokers



Elastase

The elastase concentration (Studies III-IV) did not differ significantly between smokers and non-smokers in either healthy or periodontal diseased individuals (Tables 12, 13).

In surgically treated patients (Study V), there were no significant differences either in or between smokers and non-smokers during the course of the study. The overall levels, however, were numerically greater in smokers compared to non-smokers. This was also true for the total amount of elastase (Table 14, Figure 5).

LF

The LF concentration (*Study III and IV*) did not differ significantly between smokers and non-smokers in either healthy or periodontally diseased conditions (Tables 12,13).

MMP-8

The MMP-8 concentration (*Study V*) in surgically treated patients did not differ significantly between the smoking groups at any of the observation time points (Table 15, Figure 6). However, there was a tendency towards significantly lower levels in smokers at baseline. This was also observed with regard to the total amount (Table 15). In smokers, neither the concentration nor the total amount of MMP-8 differed significantly over time. In non-smokers, however, the concentration as well as the

Tobacco smoking

total amount decreased over time. The 5-week change in MMP-8 concentration from baseline tended to be different in smokers and non-smokers (p=0.07).

Table 12

Elastase and LF concentrations. Mean and (SD) according to smoking for healthy periodontal conditions

Variable	Units	Smoker		Non-smoker		P value
		Mean	SD	mean	SD	
Elastase	MAbs/ μ l	3690.8	5230.0	2228.5	2102.3	NS
Lactoferrin	ng/ μ l	169.5	172.3	151.1	139.1	NS

Table 13

Laboratory variables. Mean (SD) and range according to smoking for moderate and severe lesions

Variable	Units	Smoker		Non-smoker		P value
		Mean (SD)	Range	Mean (SD)	Range	
Elastase	MAbs/ μ l					
Moderate lesions		1306.2 (2202.4)	23.0-8454.6	759.4 (1322.4)	0-4531.5	NS
Severe lesions		3079.6 (5302.2)	32.5-20169.6	2287.0 (3120.5)	72.5-12351.5	NS
P value		NS		NS		
Lactoferrin	ng/ μ l					
Moderate lesions		44.4 (35.2)	0.9-133.1	53.3 (69.2)	3.5-295.3	NS
Severe lesions		49.6 (43.0)	13.7-166.5	72.8 (75.8)	8.4-299.1	NS
P value		NS		NS		

Table 14

Elastase in GCF. Median and IR according to smoking and observation time point following treatment

(a) Concentration (mAbs/ μ l)

	Baseline		Week 1		Week 5	
	Median	IR	Median	IR	Median	IR
Smoker	650	154-1460	370	106-1087	463	92-681
Non-smoker	116	37-1736	110	10-79	153	54-338
P value	NS		NS		NS	

(b) Total Amount (mAbs)

	Baseline		Week 1		Week 5	
	Median	IR	Median	IR	Median	IR
Smoker	73	11-69	71	8-232	57	13-119
Non-smoker	27	8-733	20	2-405	13	7-47
P- value	NS		NS		NS	

Tobacco smoking

Table 15

MMP-8 in GCF. Median and IR according to smoking and observation time point following treatment

(a) Concentration (ng/μl)

	Baseline		Week 1		Week 5	
	Median	IR	Median	IR	Median	IR
Smoker	0.57	0.26-1.59	0.20	0.00-4.28	0.24	0.00-0.72
Non-smoker	2.30	0.58-6.06	1.28	0.32-5.30	0.19	0.00-2.42*
P value	NS		NS		NS	

(b) Total Amount (ng)

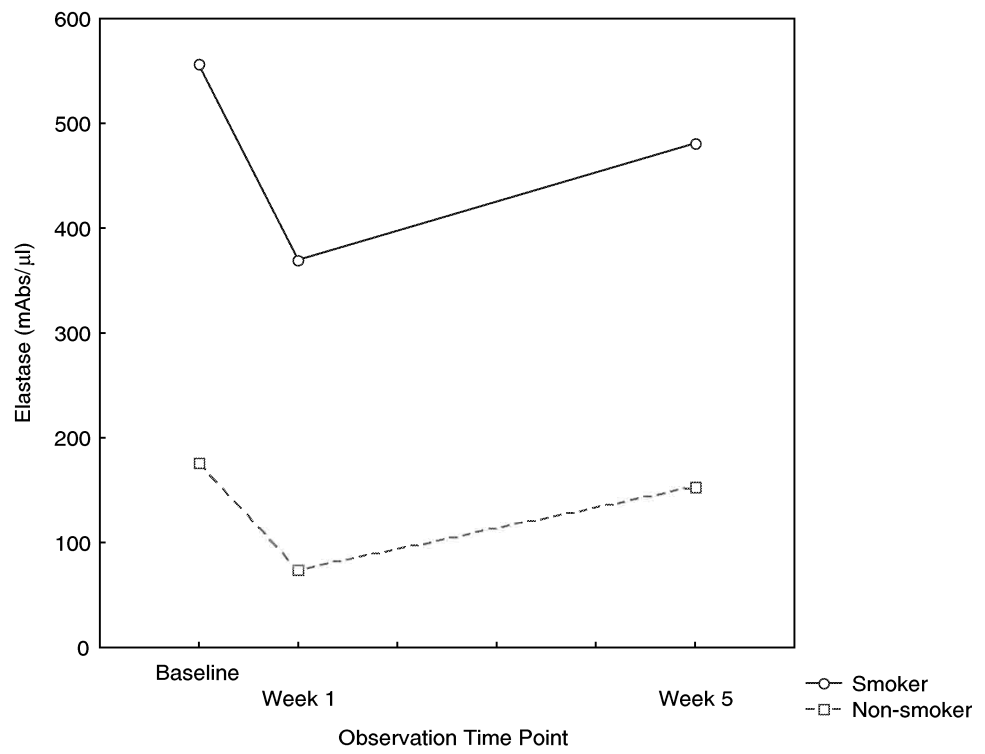
	Median	IR	Median	IR	Median	IR
Smoker	0.29	0.12-2.32	0.20	0.00-3.77	0.32	0.00-0.45
Non-smoker	2.65	0.40-8.90	2.28	0.12-6.75	0.19	0.00-1.30*^
P value	0.02		NS		NS	

* Significantly different from baseline

^ Significantly different from week 1

Figure 5

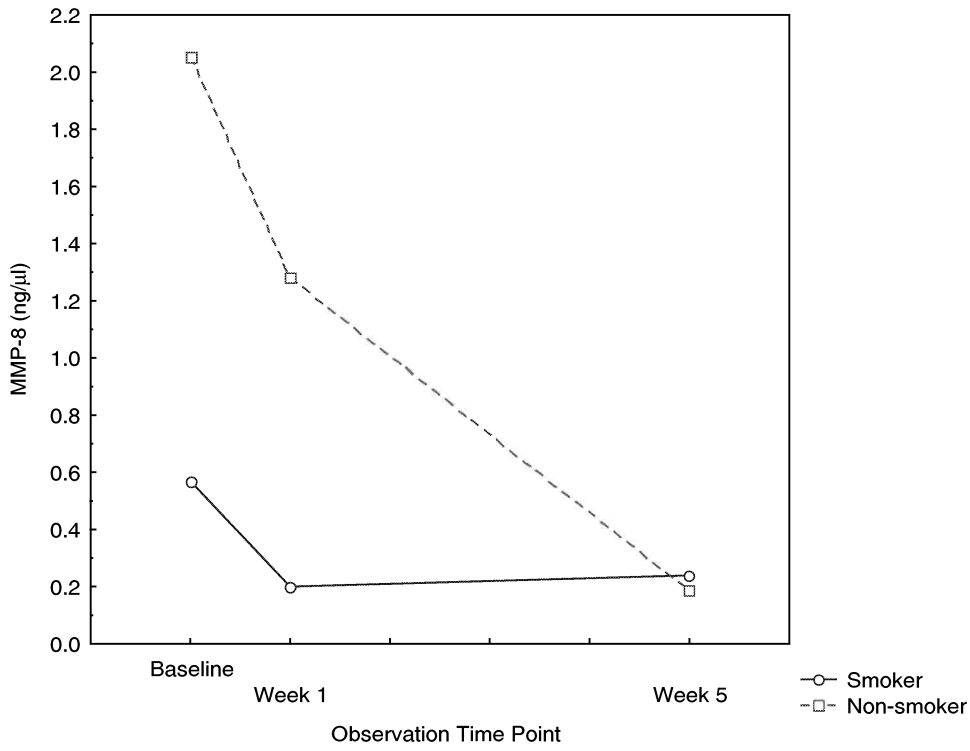
The elastase concentration during the study period in smokers and non-smokers



Tobacco smoking

Figure 6

The MMP-8 concentration during the study period in smokers and non-smokers



GCF

The mean (SD) GCF volume in healthy periodontal conditions (*Study III*) was significantly less in smokers compared to non-smokers (0.15 (0.04) μ l/site and 0.19 (0.06) μ l/site respectively. $P=0.044$). In diseased periodontal conditions (*Study IV*), however, the GCF volume did not differ significantly between smokers and non-smokers. Furthermore, in severe lesions, the GCF volume was significantly increased compared to moderate lesions in both smoking groups.

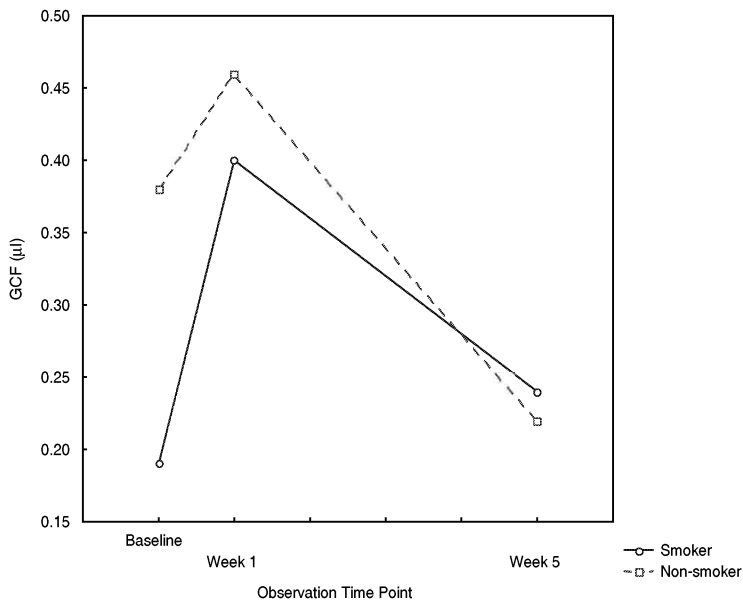
In surgically treated patients (*Study V*), the median (IR) GCF volume (Fig 7) at baseline was significantly lower in smokers compared to non-smokers ($P=0.005$).

One week after surgical treatment, the GCF volume increased in both smokers and non-smokers. By the end of the study, the volume had returned to the baseline levels in smokers. In non-smokers, the decrease in GCF volume was significantly lower than baseline level. ($P=0.019$).

Tobacco smoking

Figure 7

The GCF volume during the course of the study

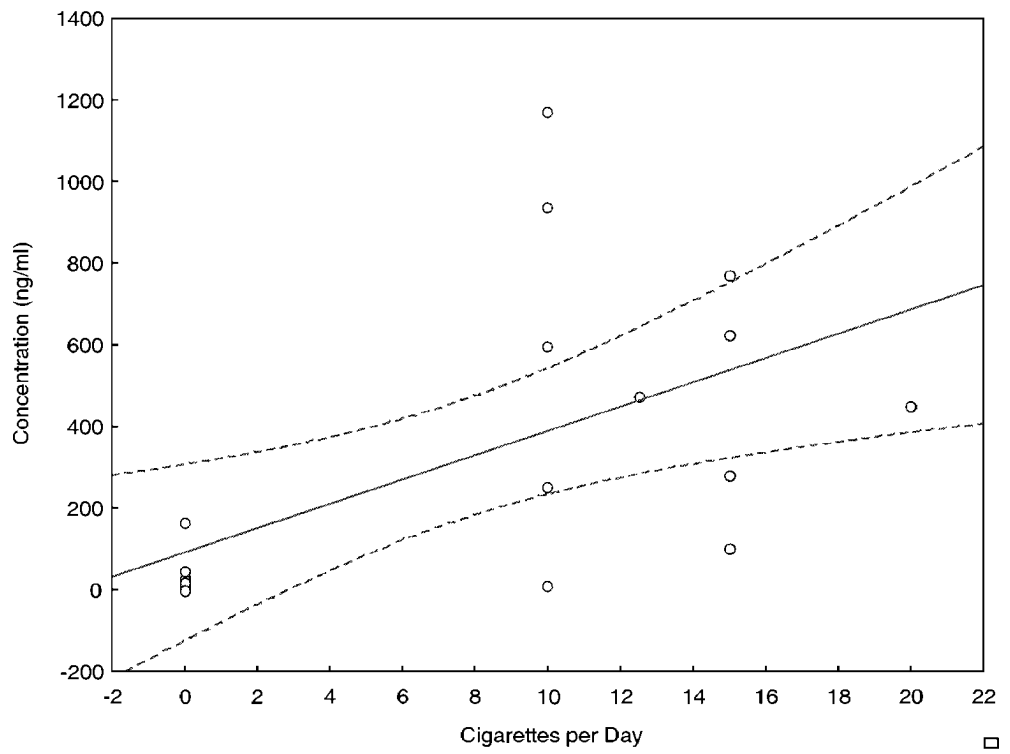


Cotinine

In *Study V* saliva samples were collected for analysis of cotinine concentration. The median (IR) saliva cotinine concentration (Figure 8) in 11 smokers and 9 non-smokers was 472 (51-71) ng/ml and 23 (2-7) ng/ml, respectively. The difference was statistically significant ($P=0.002$).

Figure 8

Scatterplot of the correlation between the cotinine concentration and the number of cigarettes smoked per day. Regression line and 95 % confidence intervals



DISCUSSION

Vascular reaction and gingival inflammation

Studies have shown that gingival inflammation is suppressed in smokers i.a., (Ainamo 1971, Bergström & Floderus-Myrhed 1983, Preber & Bergström 1985, Bergström & Preber 1986, Danielsen et al 1990, Lie et al 1998) and that tobacco smoking and or nicotine have an influence on gingival vasculature (Clark et al 1981, Baab & Öberg 1987). In *Studies I and II*, the gingival inflammation was expressed as the vascular reaction in terms of the number of visible vessels. During the experimental plaque accumulation, the number of visible vessels increased over time in both smokers and non-smokers. This is in general agreement with other studies, which suggest that the number of vessels is increased in inflamed gingiva as compared to non-inflamed gingiva (Egelberg 1966, Hansson et al 1968, Hock & Nuki 1971, Söderholm & Egelberg 1973). In smokers, however, the increase in gingival vascular reaction was less pronounced, especially during the latter part of the experiment, although the plaque accumulation rate was equivalent in both groups. The results indicate that the clinically observed reduced level of gingival inflammation in smokers can be explained by a suppressive effect of smoking on the gingival vasculature. Such an inflammatory reaction may have a negative impact on periodontal conditions and predispose to disease development.

Under clinically healthy periodontal conditions in young healthy individuals (*Studies II,III*), the gingival inflammation was similar in both smokers and non-smokers expressed as either the number of visible vessels or clinical index. This similarity in gingival inflammation between smokers and non-smokers was also true for periodontal diseased patients with slightly inflamed gingiva (*Studies IV,V*). Also in

other studies, no difference in gingival inflammation between smokers and non-smokers have been observed (Danielsen et al 1990, Lie et al 1998, Ah et al 1994, Scabia et al 2001, Söder et al 2002). While tobacco smoking may influence the gingival vasculature under healthy or slightly inflamed periodontal conditions, this effect may be difficult to disclose with clinical indices or even with the stereophotographic method.

Vascular reaction, in terms of vascular permeability, reflects the increased leakage of fluid or GCF from intravascular compartment into extravascular spaces during inflammatory response (Glynn 1983, Lindhe 1997). Although there was no difference in gingival index between smokers and non-smokers under clinically healthy periodontal conditions (*Study III*), the GCF volume was comparably reduced in smokers. This result confirms previous observations by Bergström & Preber (1986) and Holmes (1990) where smokers compared to non-smokers were found to have less GCF volume. Also in *Study V*, at baseline, the GCF volume was significantly reduced in smokers, despite the fact that gingival index levels were similar. Following surgical treatment, there was an immediate increase in GCF volume, particularly in smoker patients. One week following treatment, the GCF volume in smokers had increased to a similar level as that in non-smokers. This increase in GCF volume was followed by a decrease in volume, in both smokers and non-smokers.

In non-smokers, however, the GCF volume fell below the pre-surgical treatment level. This is in general accordance with the observations of Talonpoika & Hämäläinen (1993) that show an increased GCF volume immediately after non-surgical treatment followed by a decreased GCF volume during the recovery period.

Tobacco smoking

Neutrophil activity

Analysis of the constituents of the GCF, such as neutrophil proteases and their inhibitors, gives information about periodontal inflammation i.a., (Giannopoulou et al 1992).

Elastase is one of the major proteases released by neutrophils (Janoff 1985). The activity of the elastase is mainly modulated by the plasma protease inhibitors α -1-AT and α -2-MG (Travis & Salvesen 1983). An increased release of elastase may contribute to tissue destruction and the development of periodontitis (Palcanis et al 1992, Armitage et al 1994, Smith et al 1995, Alpagot et al 2001). Observations of tobacco smoking on GCF elastase levels in periodontitis patients are controversial. Both decreased, increased and similar levels of elastase in smokers compared to non-smokers have been observed (Alavi et al 1995, Alpagot et al 1996, Söder et al 1999). We found that the elastase concentrations were numerically greater in smokers compared to non-smokers in both healthy and periodontal diseased individuals (*Studies III-V*). The comparably increased concentration in smokers, however, was not significant.

The elastase activity is modulated by α -1-AT and α -2-MG. Under clinically healthy periodontal conditions in young healthy individuals (*Study III*), the concentrations of α -1-AT and α -2-MG were similar in both smokers and non-smokers. In *Study IV*, patients with moderate to severe periodontitis were investigated. The smokers and non-smokers were similar regarding clinical characteristics, i.e. pocket depth, gingival inflammation, plaque amount and GCF volume. In spite of the clinical similarity smoking patients with severe lesions had a lower concentration of α -2-MG and lower total amounts of α -2-MG and α -1-AT than non-smokers. This may indicate an

imbalance between elastase and α -1-AT and α -2-MG and an increased risk for periodontal tissue destruction in smoking patients.

Following surgical treatment (*Study V*), the concentrations of α -1-AT and α -2-MG in smokers, did not change during the study period, whereas the concentration of these protease inhibitors in non-smokers increased over time.

In order to further elucidate possible associations of smoking with neutrophil activity and tissue destruction, MMP-8 was studied (*Study V*). MMP-8 is a neutrophil collagenase that is involved in normal tissue remodelling as well as collagen degradation and tissue destruction in periodontal disease (Birkedal & Hansen 1993, Woessner 1991, Golub et al 1997, Chen et al 2000). The activity of MMP-8 is mainly modulated by TIMP but also by α -2-MG (Romanelli et al 1999).

In *Study V*, at baseline, there was a tendency towards lower MMP-8 concentration in smokers compared to non-smokers. Lower levels of MMP-8 in saliva in current smokers compared to former smokers have been reported by Liede et al (1999). Following surgical treatment, the MMP-8 concentration decreased in non-smokers, whereas in smokers the concentration remained unchanged over time. In non-smoking patients, there was a reduction in MMP-8 concentration together with an increase in the concentration of α -2-MG. In smokers, however, both α -2-MG concentration and MMP-8 concentration remained unaffected. In addition, following surgery, no change in the elastase concentration was found in either smokers or non-smokers. This is in general accordance with Chen et al (2000), observing unchanged levels of elastase and decreased levels of MMP-8 after non-surgical treatment.

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In *Study IV*, patients with moderate to severe periodontitis were observed. In the area investigated smokers and non-smokers were similar regarding clinical characteristics, i.e. pocket depth, gingival inflammation, plaque amount and GCF. For severe lesions, in both smokers and non-smokers, there was an increase in clinical characteristics compared to moderate lesions. Regarding laboratory variables, there were no differences between moderate and severe lesions, except for lower concentrations of α -2-MG and LF in moderate lesions in non-smoking patients. This may, to some extent, reflect an altered inflammatory response in smokers compared to non-smokers. In *Study V*, the clinical characteristics, except for GCF volume, were similar in both smokers and non-smokers. The total number of diseased sites, however, was greater in smokers than non-smokers. It should also be noted that smoker patients were younger than non-smoking patients. A comparably younger age and greater disease severity among smokers have previously been observed by Bergström & Boström (2001). This may imply a greater disease severity in smokers.

Methodological aspects

Photographic documentation

The photographic documentation (*Studies I and II*) of the gingival area to be investigated was performed with a camera system allowing synchronous exposure of two films. Photography was performed under standardized conditions, and the same area was followed throughout the experiment. The counting and identification of

vessels in the gingival area was always related to the same zone. The identification of vessels is dependent on several factors such as anatomical variations and the capacity of the observer.

The intraobserver variation was studied by repeated readings in the same image pairs with an interval of 1 week. The error associated with the readings of one observer was insignificant. The variation in identification and refocusing was studied by comparing the image pairs obtained from two sets of immediately repeated exposures. There was no statistical difference between pictures obtained from immediately repeated exposures. The interobserver variation in identification was studied by comparing the image pairs read by two observers independently. The difference in reading between two observers was not significant.

The measurements regarding variations in identification demonstrated that the methodological error was smaller than the biological variation.

GCF

GCF was collected with paperstrips. The fluid volume collected was measured immediately after sampling with a calibrated GCF meter. Before each study, the GCF meter was calibrated. The calibration curve included the range of 0.05 to 0.8 μl (*Study III*) or 0.1 to 1.0 μl (*Studies IV, V*). GCF values close to either the lowest or highest value on the calibration curve may be less secure as the capacity of the GCF meter will be touched.

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Elastase

The low molecular weight substrate, used to determine elastase (*Studies III-V*), is hydrolysed either by free elastase or elastase in complex with α -2-MG (Travis & Salvesen 1983). Since the elastase activity collected with strips originates mainly from elastase complexed with α -2-MG (Gustafsson 1992), the elastase activity found indicates a higher concentration of elastase in complex with α -2-MG than free elastase.

Cotinine

The saliva cotinine was used for evaluating tobacco exposure (*Study V*). Analysis of cotinine permits the degree of passive and active exposure to tobacco to be established. Our results found a cotinine concentration of 472 ng/ml in smokers, compared to 23 ng/ml found in non-smokers. The level of cotinine concentration varied within smokers, especially in individuals who reportedly smoked 10 cig/day. Studies in serum cotinine levels have demonstrated that some individuals obtain a much higher dose of tobacco smoke than others who reportedly smoked the same number of cigarettes per day (González et al 1996, Scott et al 2000, Chen et al 2001). Factors that may influence this variation are differences in individual metabolism and variation in smoking habits (Benowitz et al 1999, Palmer et al 1999).

CONCLUSIONS

Specific conclusions

The marginal gingival vasculature density in periodontal health did not differ between smokers and non-smokers. (*Study II*).

The marginal gingival vasculature reaction in response to plaque formation was suppressed in smokers compared to non-smokers. (*Study I*).

The influence of tobacco smoking on the presence and activity of neutrophil derived elastase, and its inhibitors α -1-AT and α -2-MG was limited in periodontal health. In periodontal disease, however, the concentration of α -2-MG was lower in smokers compared to non-smokers, whereas the concentrations of α -1-AT and elastase were similar in both smokers and non-smokers (*Studies III-IV*).

In smokers, following surgery, the levels of neutrophil derived elastase and collagenase, and the inhibitors α -1-AT and α -2-MG did not change over time whereas in non-smokers, there was a change towards decreased concentration of collagenase and increased concentrations of α -1-AT and α -2-MG (*Study V*).

General conclusion

Under clinically healthy periodontal conditions the influence of tobacco smoking on the vascular density and neutrophil derived elastase and its inhibitors, α -1-AT and α -

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2-MG, was very limited. In response to plaque accumulation, the vascular reaction was suppressed by smoking. Under clinically periodontally diseased conditions the α -2-MG was suppressed by smoking. Following surgical intervention, smoking seems to exert a suppressive influence on MMP-8, and α -1-AT and α -2-MG.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Att tobaksbruk har en skadlig inverkan på såväl allmänhälsan som tandhälsan är sedan länge känt. Redan i slutet av 1940-talet kunde forskare konstatera att tobak har en negativ påverkan på tandhälsan. Senare forskning inom området har försökt finna bakomliggande orsaker till denna skadliga inverkan på tänderna och den omgivande stödjevåvnaden. Avsikten med denna avhandling har varit att studera orsaker till att tandlossning är mera utbredd hos rökare jämfört med icke rökare. Frågeställningen har varit om kroppens försvar vid en inflammation i tandens stödjevåvnader är annorlunda hos en som röker jämfört med en som inte röker. I ett par studier har undersökts om de vid en vävnadsinflammation normalt förekommande kärlförändringarna i tandköttets blodkärl skulle vara annorlunda hos en rökare. Dessutom har i ett par andra studier undersökts om det i tandköttet hos en rökare, med eller utan tandlossningsbesvär, finns en skillnad i mängden vävnadsnedbrytande ämnen och deras hämmare. Slutligen har patienter som opererats i tandköttet på grund av tandlossningsbesvär undersökts. Resultaten visar att vid en inflammation i tandköttet har rökare färre blodkärl i vävnaden jämfört med en icke rökare och hos en patient med tandlossning är mängden ämnen som har en hämmande inverkan på vävnadsnedbrytningen lägre hos en rökare. Efter en tandköttsoperation, jämfört med innan behandlingen, påvisade icke rökare en mindre mängd vävnadsnedbrytande ämnen och en större mängd hämmare. Hos rökarna däremot sågs ingen skillnad över tiden. Slutsatsen är att rökning har en effekt på inflammationssvaret vilket kan leda till ökad risk för tandlossningssjukdom och ett försämrat behandlingsresultat hos en som röker jämfört med en som inte röker.

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