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# Cytokines du fluide gingival après accumulation de plaque chez des fumeurs et des non-fumeurs

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**How to cite**

CAPPUYNS, Isabelle. Cytokines du fluide gingival après accumulation de plaque chez des fumeurs et des non-fumeurs. Doctoral Thesis, 2003. doi: 10.13097/archive-ouverte/unige:564

This publication URL: https://archive-ouverte.unige.ch/unige:564
Publication DOI: 10.13097/archive-ouverte/unige:564

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UNIVERSITE DE GENEVE

FACULTE DE MEDECINE
Section de Médecine dentaire
Département de Thérapeutique buccale et Orthodontie
Division de Physiopathologie buccale et Parodontie

Thèse préparée sous la direction du Professeur Andrea MOMBELLI

# CYTOKINES DU FLUIDE GINGIVAL APRES ACCUMULATION DE PLAQUE CHEZ DES FUMEURS ET DES NON-FUMEURS

## Thèse

présentée à la Faculté de Médecine
de l'Université de Genève
pour obtenir le grade de docteur en médecine dentaire

par

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**de**

**Louvain / Belgique**

**Thèse n° 629**

**GENEVE**

**2003**

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# 1 Summaries

## *1.1 Abstracts*


CYTOKINE RESPONSE TO PLAQUE ACCUMULATION IN SMOKERS AND NON-SMOKERS.

Cigarette smoking is a significant risk factor in the pathogenesis of periodontal disease, able to influence both subgingival microbiota and host responses. The aim of the present study was to determine the influence of smoking on the expression of IL-1β, IL-4 and IL-8 in GCF during experimental gingivitis in humans.

Twenty-two healthy subjects, 10 smokers (S) and 12 non-smokers (NS), participated in the study. After professional cleaning they performed optimal hygiene to reach perfect clinical gingival health. Oral hygiene measures were ceased for a period of 10 days. Clinical indices, including Plaque Index (PI), Gingival Index (GI), Probing Pocket Depth (PPD) and Bleeding on Probing (BOP), were assessed 2 days before (day –2), at the beginning (day 0), and at the end of the experimental gingivitis period (day 10). At the same time, GCF was collected from 12 sites in each patient, and the content of IL-1β, IL-4 and IL-8 was determined by ELISA.

Clinical data revealed that both S and NS showed an increase in inflammation during the experiment. However, this increase was significantly less pronounced in S. At baseline, NS had higher total amounts of IL-4 but lower amounts of IL-8 than S, and this pattern remained similar throughout the experiment. Total amounts of IL-1β and IL-8 increased significantly during plaque accumulation in both groups. IL-4 remained stable for the S group and decreased for the NS group.

The present results indicate that smoking interferes with cytokine production, thus affecting the inflammatory response**.**

CYTOKINES DU FLUIDE GINGIVAL APRES ACCUMULATION DE PLAQUE CHEZ DES FUMEURS ET NON-FUMEURS

Le but de cette étude était de déterminer les niveaux de IL-1β, IL-4 et IL-8 dans le fluide gingival chez 10 fumeurs et 12 non-fumeurs lors d'une période de gingivite expérimentale. Des échantillons de fluide ont été prélevés de 12 sites par sujet, 2 jours avant, au début et à la fin de la gingivite expérimentale. La production des cytokines a été mesurée par ELISA.

Cliniquement les fumeurs montraient moins d'inflammation et moins de saignement au sondage que les non-fumeurs. A l'examen initial, les non-fumeurs montraient des quantités plus élevées de IL-4 et plus faibles de IL-8 que les fumeurs. La quantité totale de IL-1β et de IL-8 a augmenté de façon significative dans les 2 groupes après accumulation de plaque. Le niveau de IL-4 a diminué de façon significative seulement chez les non-fumeurs.

Les résultats de cette étude ont montré que le tabac peut influencer la réponse inflammatoire de l'hôte en s'ingérant avec la production des cytokines.

## *1.2 Résumé en français*

### 1.2.1 Introduction

On admet généralement que la maladie parodontale débute par une inflammation des gencives (gingivite), due à une accumulation bactérienne importante à la surface des dents. Toutefois, d'autres facteurs sont également incriminés, notamment le tabagisme qui joue un rôle important dans la pathogénèse de la maladie parodontale, documenté par plusieurs chercheurs. Le tabagisme est associé à une perte d'os alvéolaire, à une augmentation de la profondeur de sondage et de la perte d'attache, ainsi qu'à la perte des dents. De plus, chez un fumeur, la capacité de défense et de réparation des gencives est affaiblie.

Le diagnostic parodontal conventionnel est basé principalement sur l'évolution des niveaux osseux analysés au moyen de radiographies et de mesures cliniques, comme la profondeur de poche parodontale et le niveau d'attache. Malheureusement, ces paramètres ne nous révèlent rien sur l'activité de la maladie parodontale. Aujourd'hui, le fluide gingival est considéré comme l'un des moyens les plus prometteurs pour indiquer l'activité de la maladie. Il s'agit d'un exsudat inflammatoire qu'on retrouve dans le sillon gingival ou dans la poche parodontale et qu'on peut facilement prélever avec des strips de papier filtre ou des tubes capillaires, ce fluide passant la barrière de l'épithélium jonctionnel et arrivant dans le sillon en cas de perméabilité capillaire augmentée, par exemple lors d'inflammation. Parmi les nombreux médiateurs d'inflammation présents dans le fluide gingival, les cytokines semblent impliquées aussi bien dans les changements dus à l'inflammation que dans la réparation des tissus parodontaux. Certaines cytokines, interleukine-1$\beta$ (IL-1$\beta$), interleukine-4 (IL-4) et interleukine-8 (IL-8), ont même été proposées en tant qu'indicateurs potentiels pour le pronostic ou le diagnostic de la destruction parodontale.
L'analyse de ces paramètres biochimiques dans le fluide gingival nous offre de nouvelles perspectives pour le diagnostic prématuré de la maladie parodontale ou pour prédire l'évolution d'une lésion parodontale. Il a été possible de

démontrer l'existence d'une corrélation entre l'un de ces marqueurs (IL-1β) et la progression de la maladie parodontale.

Le but de cette étude était d'examiner dans un premier temps les changements survenant au niveau de IL-1β, IL-4 et IL-8 dans le fluide gingival au cours d'une gingivite expérimentale chez des humains. On a cherché ensuite à déterminer l'influence du tabagisme sur l'expression de ces médiateurs d'inflammation, au début et à la fin de la gingivite expérimentale.

### 1.2.2 Matériel et méthodes

Vingt-deux volontaires, 10 fumeurs et 12 non-fumeurs, ont participé à l'étude. Les sujets ne devaient présenter ni inflammation gingivale sévère (GI>1), ni poches parodontales et/ou perte d'attache dans les sites interproximaux. Douze jours avant la phase de gingivite expérimentale, tous les sujets ont reçu des instructions d'hygiène bucco-dentaire ainsi qu'un nettoyage et un polissage de toutes les surfaces dentaires, dans le but de réduire à moins de 20% le saignement dans les sites interproximaux avant le jour 0 (=baseline). Du jour 0 au jour 10, les sujets ont arrêté toute mesure d'hygiène bucco-dentaire permettant ainsi l'accumulation de dépôts bactériens. Après cette phase de gingivite expérimentale, la denture entière a été nettoyée professionnellement puis fluorée ; les sujets ont ensuite repris le brossage de leurs dents comme avant l'expérimentation.

Les paramètres cliniques (indice de plaque, indice gingival et profondeur de poche) ont été relevés aux jours –2, 0 et 10, dans 12 sites interproximaux chez chaque sujet. Simultanément, on a collecté le fluide gingival au moyen de strips en papier filtre Durapore, et la présense d'IL-1β, d'IL-4 et d'IL-8 a été déterminée avec la méthode ELISA.

### 1.2.3 Résultats

- Paramètres cliniques

Les résultats cliniques n'ont montré aucune différence significative entre fumeurs et non-fumeurs au début de l'expérience. A l'exception de la profondeur de

poche, tous les paramètres cliniques ont augmenté de façon significative durant la gingivite expérimentale (10 jours). Au jour 10, toutefois, l'inflammation gingivale et le saignement au sondage étaient significativement moins prononcés chez les fumeurs.

- Echantillons de fluide gingival

Dans les deux groupes, on a détecté IL-1β, IL-4 et IL-8 dans tous les échantillons de fluide gingival. Au début, le niveau de IL-1β dans le fluide était similaire pour les deux groupes (fumeurs et non-fumeurs). Après 10 jours de gingivite expérimentale, on a observé une augmentation significative de IL-1β dans les deux groupes.

IL-4 présentait une corrélation inverse avec l'état parodontal ainsi qu'avec le tabagisme : on observait initialement un niveau de IL-4 plus élevé dans le fluide gingival des non-fumeurs par rapport à celui des fumeurs. A la fin de la phase expérimentale, les valeurs de IL-4 restaient inchangées dans le groupe des fumeurs, alors qu'on observait une baisse significative dans le groupe des non-fumeurs.

A la fin, le niveau de IL-8 dans le fluide montrait une corrélation positive avec l'accumulation de plaque et le tabagisme : les fumeurs présentaient initialement un niveau de IL-8 beaucoup plus élevé que celui des non-fumeurs. Au jour 10, le niveau de IL-8 s'était multiplié par 2,5 dans les deux groupes (fumeurs et non-fumeurs) ; par conséquent, les fumeurs présentaient toujours un taux de IL-8 beaucoup plus élevé que celui des non-fumeurs.

### 1.2.4 Discussion

Le but de cette étude était d'évaluer l'influence du tabagisme sur l'état clinique et le profil des cytokines du fluide gingival lors d'une gingivite expérimentale.
Dans la phase pré-expérimentale (jour –2) et au début de l'expérience (jour 0) on n'a observé aucune différence clinique entre fumeurs et non-fumeurs.
L'abstention de toute mesure d'hygiène bucco-dentaire dans les deux groupes a provoqué une accumulation de plaque similaire pendant la période

expérimentale de 10 jours. Ceci a également été confirmé par plusieurs autres études, basées sur le même modèle expérimental.

Dans notre étude, la tendance au saignement et l'inflammation gingivale étaient moins prononcées chez les fumeurs après 10 jours d'accumulation de plaque. Il semblerait donc que le tabagisme ait un effet vasoactif et qu'une augmentation moins importante des vaisseaux gingivaux soit observée chez les fumeurs par rapport aux non-fumeurs.

Au début de l'étude, le niveau de IL-1β, qui était similaire pour les fumeurs et les non-fumeurs, a augmenté de façon significative dans les deux groupes après accumulation de plaque. Plusieurs auteurs ont montré qu'il existait une corrélation entre l'augmentation de IL-1β et l'accumulation de plaque et ils ont suggéré que IL-1β pouvait être un indicateur du début de l'inflammation gingivale avant même tout autre indice clinique. L'association du niveau de IL-1β et de facteurs environnementaux dont le tabagisme a fait l'objet de plusieurs études qui, comme la nôtre, n'ont pu établir de relation entre IL-1β et le tabagisme. Dans notre étude IL-4 était inversement corrélé avec le tabagisme et l'état parodontal. Au début de l'expérience, on a observé des quantités élevés dans le groupe des non-fumeurs, puis une baisse importante s'est manifestée après 10 jours d'accumulation de plaque. Chez les fumeurs, en revanche, le niveau de IL-4 est resté inchangé à la fin de la phase expérimentale. Plusieurs chercheurs ont décrit l'absence de IL-4 dans des lésions parodontales sévères, la considérant associée à la maladie parodontale. Jusqu'à présent, peu de recherches ont étudié l'effet du tabagisme sur la production d'IL-4.

La quantité totale de IL-8 dans le fluide était de loin supérieure à celle des autres cytokines décrites, et on a pu démontrer une corrélation importante entre le niveau de IL-8 et le tabagisme, ainsi que l'état parodontal. Au début de l'étude déjà, la quantité de IL-8 était nettement supérieure dans le groupe des fumeurs. Après 10 jours de gingivite expérimentale, les différences se sont maintenues de façon significative entre les deux groupes. Des résultats contradictoires ont été rapportés quant à l'association de IL-8 avec le degré d'inflammation et le tabagisme. On peut avancer que la fumée favoriserait la présence de IL-8 dans les cellules du parodonte, provoquant ainsi une accumulation locale de

neutrophiles. Dans des conditions normales, la présence de IL-8 contribue à ce que les neutrophiles éliminent les bactéries infectieuses, alors qu'une libération excessive de IL-8 provoque une hyperactivité des neutrophiles conduisant à une destruction des tissus.

### 1.2.5 Conclusions

Les résultats obtenus dans cette étude nous font penser que les fumeurs et les non-fumeurs réagissent de façon différente au développement d'une gingivite expérimentale. Comme il a été démontré que le tabagisme provoquait différents effets sur les cytokines du fluide gingival, il est important de prendre en considération l'état « fumeur » des participants au cours d' études sur la pathogenèse de la maladie parodontale.

# 2 Introduction

It is generaly believed that periodontal disease starts with an inflammation of the marginal gingiva (gingivitis), caused by an accumulation of an unspecific bacterial mass on the tooth surface (Theilade 1971). Normal subjects maintaining a high standard of oral hygiene are not likely to develop gingival or periodontal disease. However, experimental, short-term clinical studies have shown that microorganisms quickly start to colonize clean tooth surfaces once an individual abstains from mechanical toothcleaning ; within a few days, microscopical and clinical signs of gingivitis are apparent. These inflammatory alterations are resolved or reversed when adequate toothcleaning is resumed (Loe et al. 1965). Thus microorganisms which form dental plaque contain or release components which induce gingivitis and/or periodontitis. Nevertheless, many factors other than bacteria can contribute to the etiology of periodontitis (i.e. local oral factors, host susceptibility, risk factors like smoking, diabetes and stress).

## *2.1 The role of cigarette smoking in the pathogenesis of periodontal disease*

The role of cigarette smoking in the pathogenesis of periodontal disease has been well documented by several investigators (for review, see Brochut & Cimasoni 1997a, 1997b). Smoking has been associated with alveolar bone loss (Bergström et al. 1991 ; Grossi et al. 1994), tooth mobility (Feldman et al. 1987), increased probing depth (Bergström 1989 ; Goultschin et al. 1990), greater % of sites with attachment loss (Grossi et al. 1994 ; Schenkein et al. 1995) as well as tooth loss (Heckert et al. 1986 ; Osterberg & Mellström 1986). Furthermore, cigarette smokers are more susceptible and develop severe disease at a younger age (Haber & Kent 1992). Cigarette smoke contains more than 1015 organic free radicals in the gas phase of each inhalation, which are able to cause widespread biological damage (Pryor & Stone 1993). Nicotine, the main constituent of the particulate phase of tobacco smoke, has been shown to adversely affect several major functions of the cells of the periodontium (Seymour 1991 ; Lannan et al.

1992 ; Tipton & Dabbous 1995 ; Giannopoulou et al. 1999). Locally, smoking causes a reduction in the gingival blood flow with a decreased number of circulating cells and less oxygen reaching the gingiva, thus weakening its defense-reparative posture (Bergström & Preber 1986 ; Bergström et al. 1988).

The underlying mechanisms by which smoking is associated with the pathological conditions of the periodontium are still not fully understood. For example, conflicting results have been reported on the subgingival microbiota in smoker and non-smoker patients with periodontal disease (Stoltenberg et al. 1993 ; Boström et al. 1998a ; Renvert et al. 1998 ; Darby et al. 2000 ; Zambon et al. 1996 ; Kamma et al. 1999). In order to further elucidate the role of smoking in periodontal disease, investigations of its influence on host response are needed.

### *2.2 Diagnosis of periodontal disease activity*

Conventional periodontal diagnosis relies mainly on the evaluation of bone levels on radiographs and on clinical assessments of pocket depths and attachment levels. These parameters demonstrate that the damage of the periodontal tissues already occured and thus reflect the history, rather than the current activity of the disease. In fact, these parameters are unable to detect initial stages of the disease. In an attempt to develop objective measures, a wide variety of studies have been undertaken using saliva, blood, plaque and gingival crevicular fluid (GCF) as the specimen source. GCF is a promising source of markers of disease activity because it contains components derived from plasma, connective tissue, host cells and from the subgingival microbial plaque (Cimasoni, 1983 ; Fine and Mandel, 1986).

GCF is a serum-like exudate which bathes the gingival sulcus or periodontal pocket, and which follows an osmotic gradient in local tissues. As this fluid traverses from the host microcirculation through inflamed tissues, and into the periodontal pocket, it captures mediators involved in the destructive host response, and products of local tissue metabolism. These constituents in crevicular fluid may be noninvasively harvested on filter paperstrips or in capillary tubes, and quantified or qualified with specific assays.

## *2.3 Cytokines*

Among many inflammatory and immune mediators identified in GCF, cytokines have attracted particular attention and are suspected to be involved in both inflammation-related alteration and repair of the periodontal tissues. Certain cytokines have been proposed as potentially useful diagnostic or prognostic markers of periodontal destruction (Birkedal-Hansen 1993 ; Genco 1992).
Interleukin-1β (IL-1β) is a multifunctional inflammatory mediator, able to modulate bone resorption by activating osteoclasts (Dewhirst et al. 1985) and by stimulating prostaglandin $E_2$ synthesis (Tatakis et al. 1988). Although IL-1β was originally considered to be a product of mononuclear phagocytes, evidence suggested that both keratinocytes and gingival fibroblasts can also produce IL-1β in response to stimulation by bacterial products (Dinarello 1988).The observation that IL-1β can act on a large number of cells, like fibroblasts, chondrocytes, bone cells, neutrophils and lymphocytes, suggests that periodontal destruction and repair in periodontitis may in part be associated with IL-1β (Jandinski 1988).
In 1982, Charon et al. were the first to demonstrate that GCF from inflamed sites of gingivitis patients had increased IL-1 β activity levels. Since then, several studies have shown increasing levels of IL-1 β in the GCF with increasing gingival inflammation and probing depth (Hou et al. 1995; Ishiara et al. 1997). Site specific increases were also observed in untreated periodontitis (Preiss & Meyle1994) and in experimental gingivitis models (Kinane et al. 1992).
Treatment of periodontitis resulted in dramatic local decrease of IL-1β, suggesting that this molecule is crucial in periodontal tissue destruction (Masada et al. 1990, Reinhardt et al. 1993). Diabetic patients with periodontitis showed higher levels of IL-1 β in GCF compared to non-diabetic controls with a similar degree of periodontal disease (Salvi et al. 1997).
Recently efforts have been made to study genetic associations with periodontal disease in particular that of the IL-1 genotype. This genetic marker includes two polymorphisms of the IL-1 gene cluster on chromosome 2. Kornman and co-workers have demonstrated an association between a specific composite genotype of the IL-1 gene cluster and periodontal disease severity (Kornman et al. 1997).

Engebretson demonstrated that carriage of the specific IL-1 gene cluster composite polymorphism is also related to increased IL-1 β expression in vivo. Among patients of similar disease severity, those with the peridontitis associated genotype (PAG) demonstrated elevated levels of IL-1 β in GCF and gingival tissues (Engebretson et al. 1999).

Interleukin-4 (IL-4), originally described as B-cell growth factor, is a potent down-regulator of macrophage function. IL-4 downregulates the CD14 lipopolysaccharide membane receptor of macrophages (Lauener et al. 1990) subsequently diminishing the production of cytokines by the macrophages and inducing apoptosis to monocytes (Mangan et al. 1992). It also inhibits the secretion of $PGE_2$ by human monocytes, which leads to bone resorption (Shapira et al. 1992, Corcoran et al. 1992). Furthermore, localized absence of IL-4 in periodontitis is associated with periodontal disease activity and progression (Kabashima et al. 1996; Shapira et al. 1992). These observations led to the hypothesis that the absence of IL-4 triggers periodontal disease (Shapira et al. 1992).

Finally, interleukin-8 (IL-8) is produced by a wide variety of cells including polymorphonuclear leukocytes (PMN's), monocytes, macrophages and fibroblasts. IL-8 has attracted particular interest because of its crucial role in the selective recruitment and activation of neutrophils (Bickel 1993; Baggiolini 1998), and in routing them to the gingival sulcus. The concentrations and total amounts of IL-8 in gingival crevicular fluid have been shown to correlate with the degree of inflammation (Tsai et al. 1995; Mathur et al. 1996; Giannopoulou et al.2003).

### *2.4 Experimental gingivitis*

The model of experimental gingivitis is a tool to study the development of gingival inflammation as a consequence of bacterial plaque accumulation in humans. Introduced by Loe and co-workers in 1965, this model has subsequently become a standard technique for investigating the initiation of gingivitis. In the first phase of this experiment, performed in subjects without periodontal disease, all bacterial deposits are removed from the teeth, and optimal oral hygiene procedures are

introduced to obtain a status of perfect gingival health. Next, the participants refrain from all oral hygiene measures for up to three weeks, during which a bacterial biofilm forms on the supragingival tooth surfaces, and as a consequence an inflammation of the adjacent gingival develops. Following the removal of the biofilm and reinstatement of oral hygiene measures the gingival inflammation subsides and gingival health is established again in a few days.

### *2.5 Aim of the study*

The aim of the present study was to examine changes of IL-1β, IL-4 and IL-8 in GCF during experimental gingivitis in humans and to determine the influence of smoking on the expression of these mediators at the beginning and the end of an experimental gingivitis.

# 3 Material and Methods

## 3.1 Subject population

Twenty-two healthy volunteers (14 male and 8 female, mean age of 26 years, range 19-33 years) were recruited. They were selected based on the following critera : presence of at least 24 teeth, absence of severe gingival inflammation (GI >1), absence of pockets > 4 mm, no attachment loss on approximal sites. 12 subjects were non-smokers and 10 were smokers. Smokers consumed on average 15 cigarettes a day (range 10-20) and had been smoking for at least 2 years. All participants were systemically healthy and had not received antibiotics during the 6 months prior to entering the study. Individuals who were pregnant or required premedication with a systemic antibiotic were not included. After a thorough instruction about the purpose and protocol of the study, participants were asked to give their consent in written form. The study protocol was approved by the Medical Ethics Committee of the School of Dental Medecine of the University of Geneva. Table 1 shows gender, age and smoking characteristics of the 22 participants.

***Table 1.*** **Demographic and behavioural variables**

| | Non-smokers | Smokers |
|---|---|---|
| **N** | 12 | 10 |
| **Men/ Women** | 7 / 5 | 7 / 3 |
| **Mean age** | 27.5 ± 3.1 | 24.9 ± 4.5 |
| **Cigarettes/day** | 0 | 15 ± 4 |
| **N smoking years** | 0 | 7 ±3 |

### *3.2 Outline of the study*

Twelve days prior to the experimental gingivitis period, patients received oral hygiene instructions, and all tooth surfaces were cleaned and polished with the goal of reaching less than 20% approximal bleeding sites before day 0. Between day 0 and day 10, the subjects refrained from all oral hygiene measures to allow for an undisturbed accumulation of bacterial deposits. After this phase the whole dentition was cleaned professionally, the tooth surfaces were treated with fluoride, and the subjects resumed their regular oral hygiene procedures. Clinical parameters were assessed and GCF samples were obtained twice before the non-brushing period (day –2 and day 0) as well as at the end of the experimental gingivitis period (day 10).

### *3.3 Site selection*

Clinical parameters were measured and GCF samples obtained at 12 sites in each subject. The mesio-buccal aspect of the first 2 upper molars, the mesio-buccal and disto-buccal aspect of the 4 upper premolars and the disto-buccal aspect of the 2 upper canines were used as experimental units.

### *3.4 Clinical examination*

The clinical examination included the Plaque Index (PII, Silness & Loe 1964), the Gingival Index (GI, Loe 1967), Probing Pocket Depth (PPD), measured with a force controlled probe (Paro Audio Probe, Esro AG, Thalwil, Switzerland) and Bleeding on Probing (BOP). Bleeding was scored dichotomously within 30s after probing.

#### 3.4.1 Criteria for the Plaque Index system (Silness & Loe 1964)

Each site is given a score from 0 to 3, as follows :

0 = No plaque in the gingival area

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

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

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

3.4.2 Criteria for the Gingival Index system (Loe 1967)

Each site is given a score from 0 to 3, as follows :

0 = Normal gingiva

1 = Mild inflammation – slight change in color, slight oedema. *No bleeding on probing.*

2 = Moderate inflammation – redness, oedema and glazing. *Bleeding on probing.*

3 = Severe inflammation – marked redness and oedema. Ulceration. *Tendency to spontaneous bleeding.*

## *3.5 Gingival crevicular fluid sampling*

The GCF was collected by means of Durapore filter membranes (pore size : 0.22 µm ; Millipore Corp., Bedford, MA, USA). Three minutes after isolation of the sites from saliva, a Durapore strip was inserted 1 mm into the sulcus and left in place for 20 sec. The strip was then placed into a microcentrifuge tube and immediately frozen at $-70^0$C until the day of the analysis. In case of visible contamination with blood, the strips were discarded. The GCF sampling procedure was performed after the assessment of the PII, and before the GI, PPD and BOP recordings. At day 10, supragingival plaque was removed gently with cotton rolls before the GCF sample was obtained.

### *3.6 Analysis of cytokine production*

The day of the analysis, 350 μl of phosphate-buffered saline (PBS, pH 7.2) was added to the tubes containing the strips. The tubes were gently shaken 1 min and then centrifuged at 2000 g for 5 min, with the strips kept at the collar of the tube in order to elute GCF components completely. After strip removal, the supernatant was divided into 3 aliquots for the determination of each of the three biochemical compounds. The amount of IL-1β, IL-4 and IL-8 was determined by enzyme-linked immunoadsorbent assays (ELISA) specific for each cytokine (Ruwag Diagnostics, Zurich, Switzerland). The assays were carried out in accordance with the manufacturer's instructions.
The levels of the biochemical compounds were reported as total amount per 20sec sample. This is in accordance with the suggestion of others who used total amounts rather than concentrations of biochemical parameters. Normally, prior to sampling, saliva and supragingival plaque are removed. However, contamination with these fluids is possible, particularly in inaccessible areas (e.g. mandibular molars). Small errors in the volume determination can lead to 50% or greater errors in the calculation of concentration, especially when the fluid volume is small, but a measurable amount of a biochemical compound is detected.
The assays used have a sensitivity of 0.4 pg/ml for IL-1β, of 0.6 pg/ml for IL-4 and of 1 pg/ml for IL-8 as minimum detectable dose.

#### 3.6.1 The ELISA method (Voller et al. 1976)

The ELISA test depends on two assumptions :

- that the antigen or the antibody can be attached to a solid-phase support retaining immunological activity and
- that either the antigen or the antibody can be linked to an enzyme and the complex retains both immunological and enzymatic activity.

Experience has shown that these assumptions are true for many antigen-antibody systems. Antibodies and many antigens can be readily attached to paper disks or to plastic surfaces, such as polyvinyl or polystyrene, either chemically or by passive adsorption, and still retain their activity. Antibodies and

antigens have been linked to a variety of enzymes including peroxidase, glucose oxidase, β-galactosidase and alkaline phosphatase, yielding stable, highly reactive reagents. Briefly, the double sandwich method for detection and measurement of antigen is described as follows :

a) The wells in polystyrene plates are coated with immunoglobulin containing specific antibody to the antigen, and than washed with phosphate buffered saliva, at pH 7.2.
b) The test solutions thought to contain antigen are incubated to the sensitized wells. Washing removes unreacted material and any antigen remains attached to the immobilized antibody on the plastic surface.
c) The conjugate consisting of enzyme-labeled specific antibody is then incubated in each well. This will react with any antigen already captured by the antibody on the well surface. A further washing removes excess conjugate.
d) Finally, the enzyme substrate is added ; its rate of degradation depends on the amount of enzyme-labeled antibody present. This, in turn, depends on the amount of antigen in the test sample. The enzyme substrate is chosen to give a color change upon the degradation. This can be assessed visually or measured in a spectro-photometer.

### *3.7 Data analysis*

A subject mean value was calculated for each clinical and biochemical parameter. To evaluate the reproducibility of the method, mean values and means of the differences of the evaluations between day –2 and day 0 were calculated for all parameters. To inspect the relation of mean to variance, the difference of each pair of values, corresponding to day –2 and day 0, was related to their respective mean.

Differences in both clinical and biochemical parameters between smokers and non-smokers were assessed using a Mann-Whitney U test. Differences in clinical and biochemical parameters between baseline (day –2) and day 10 were assessed by the Wilcoxon signed rank test. Multiple linear regression analysis

was performed to reveal correlations between cytokine levels and smoking status.

# 4 Results

## *4.1 Reproducibility*

Table 2 shows the mean values of the measurements at day –2, the mean difference between first and second measurement (corresponding to day –2 and day 0, respectively), the standard deviation of the mean difference and the corresponding coefficient of variation of all parameters.

***Table 2.*** **Clinical and biochemical parameters. Number of observations, mean of first values (day –2), and means of the differences (day –2 minus day 0), expressed as pg/20s sample. The standard deviation of the mean of the differences and the corresponding coefficient of variation are also shown.**

| | N | Mean values day –2 | Means of diff. (day –2, day 0) | SD of the means of diff. | Coefficient of variation |
|---|---|---|---|---|---|
| *Clinical data* | | | | | |
| PII | 22 | 0.09 | -0.06 | 0.22 | 0.05 |
| GI | 22 | 0.21 | 0.03 | 0.11 | 0.01 |
| PPD | 22 | 2.41 | 0.01 | 0.22 | 0.05 |
| BOP | 22 | 0.09 | -0.03 | 0.12 | 0.01 |
| *Biochemical data* | | | | | |
| IL-1β | 22 | 4.96 | -0.05 | 1.22 | 1.50 |
| IL-4 | 22 | 7.54 | 0.90 | 2.90 | 8.42 |
| IL-8 | 22 | 17.8 | 0.75 | 1.63 | 2.65 |

### *4.2 Clinical characteristics*

Table 3 shows the mean values of PII, GI, PPD and BOP for the 10 smoking and 12 non-smoking subjects before and after the 10-day experimental gingivitis.

***Table 3. Clinical parameters at the beginning (day –2) and the end of the experimental gingivitis trial (day 10) in smokers and non-smokers (mean±SE)***

| Data | Day -2 | | Day 10 | |
|---|---|---|---|---|
| | **Smokers** | **Non-smokers** | **Smokers** | **Non-smokers** |
| **PII** | 0.2[a]±0.4 | 0.05[a]±0.20 | 1.8±0.5 | 1.6±0.6 |
| **GI** | 0.2[a]±0.4 | 0.2[a]±0.4 | 1.3±0.4 | 1.5[b]±0.5 |
| **PPD(mm)** | 2.3±0.5 | 2.5±0.5 | 2.4±0.5 | 2.4±0.5 |
| **BOP** | 0.06[a]±0.23 | 0.12[a]±0.33 | 0.37±0.48 | 0.57[b]±0.49 |

PII : Plaque Index scores ; GI : Gingival Inflammation ; PPD : Periodontal probing depth ; BOP : Bleeding on Probing
a : significant difference between baseline and day 10
b : significant difference between smokers and non-smokers

No significant differences were observed at baseline for any of the clinical parameters between smokers and non-smokers. With the exception of PPD, all clinical parameters increased significantly during the 10-day period of experimental gingivitis in both groups ($p<0.01$ ; $p<0.05$). However, smokers presented significantly less gingival inflammation and less bleeding on probing as compared to non-smokers at day 10 ($p<0.05$).

### *4.3 Gingival crevicular fluid mediator levels*

IL-1β, IL-4 and IL-8 were detected in all GCF samples of both groups. Table 4 presents the mean total amounts/20 sec (±SD) of each mediator before and after the period of undisturbed plaque accumulation for smokers and non-smokers.

***Table 4. Total amounts of IL-1β, IL-4 and IL-8 in the GCF of smokers and non-smokers during the 10-days experimental gingivitis study (means±SE)***

| Mediator | Day -2 | | Day 10 | |
|---|---|---|---|---|
| | Smokers | Non-smokers | Smokers | Non-smokers |
| **IL-1β** | 6.2[a]± 4.60 | 3.99[a]±2.7 | 13.6±5.6 | 9.8±4.5 |
| **IL-4** | 4.4[b]±2.2 | 10.2[a]±7.0 | 4.1[b]±2.2 | 5.5±2.6 |
| **IL-8** | 21.1[a,b]±4.8 | 15.1[a]±4.2 | 56.1[b]±12.4 | 36.5±13.3 |

a : significant difference between baseline and day 10
b : significant difference between smokers and non-smokers

At baseline, GCF IL-1β levels were not different between smokers and non-smokers. After 10 days of experimental gingivitis, a significant increase of IL-1β levels could be observed for both groups. IL-1β increased from 6.2±4.6 pg to 13.6±5.6 pg in smokers, and from 3.99±2.7pg to 9.8±4.5 pg in non-smokers (Figure 3).

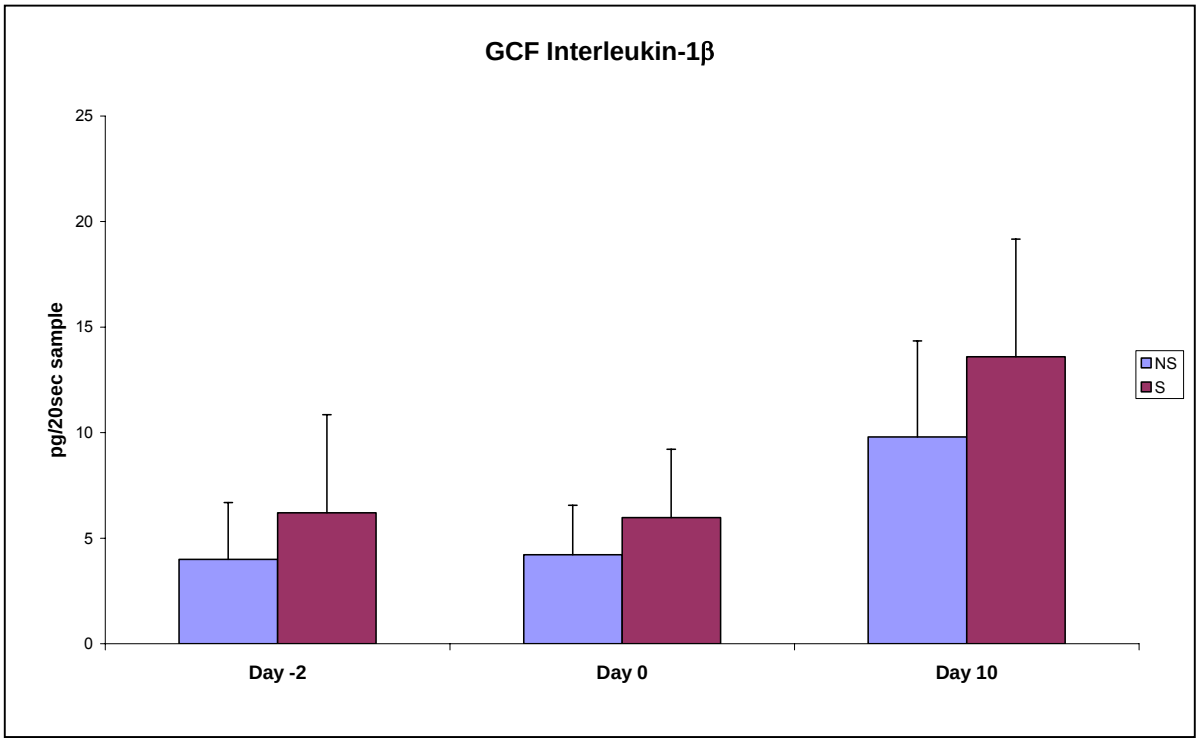


***Figure 3. Total amounts/20 sec sample (±SD) of GCF IL-1β at day –2, day 0 and day 10, in smokers (S) and non-smokers (NS).***

IL-4 showed an inverse association with  periodontal status, as well as with the smoking status. Higher amounts were observed at baseline in the GCF of non-smokers compared to smokers (10.2±7.0 pg vs 4.4±2.2 pg, p<0.005). From day 0 to day 10, IL-4 levels did not change in the smoking group while they decreased significantly in the non-smoking group (p<0.005)(Figure 4).

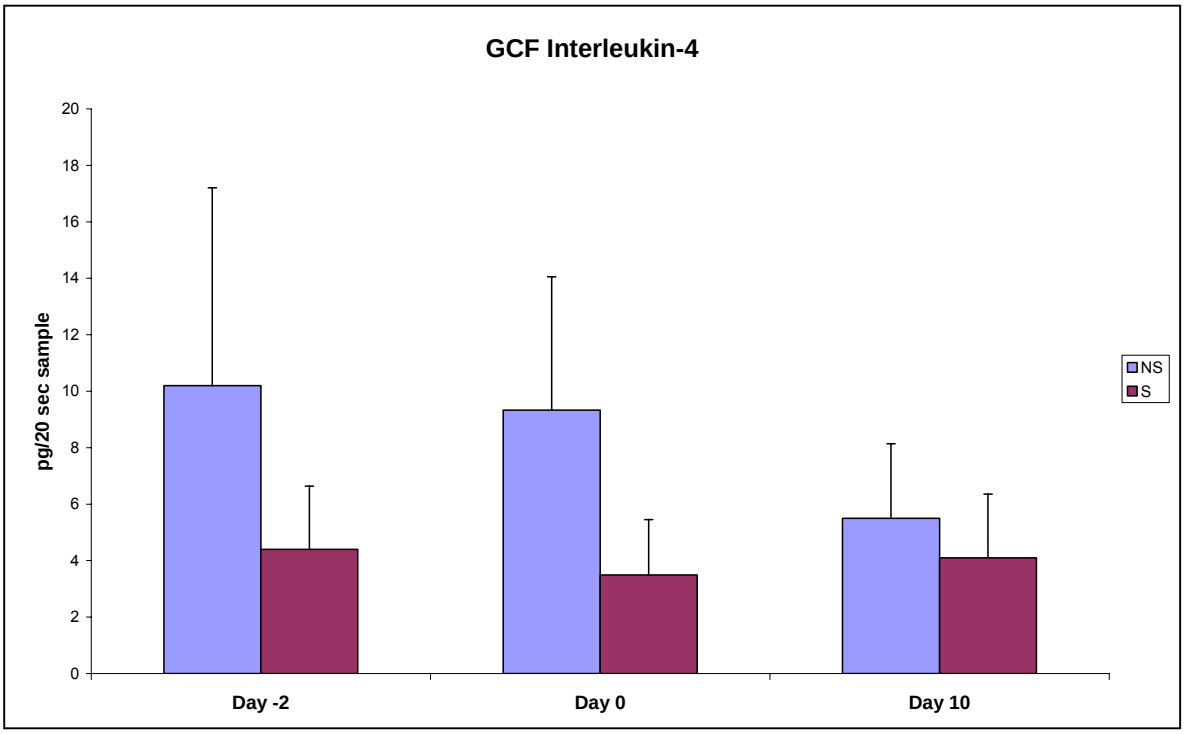


***Figure 4. IL-4 amounts (pg/20 sec sample) at day –2, day 0 and day 10, in smokers (S) and non-smokers (NS).***

In contrast to IL-4,  IL-8 levels were positively correlated with both smoking status and plaque accumulation : as shown in Table 4, smokers had significantly higher amounts of IL-8 at baseline than non-smokers (21.1±4.8 pg vs 15.1±4.2 pg, p<0.003) ; at day 10, a 2.5-fold increase was observed in both groups. However, at the end of the experiment, smokers had still significantly higher amounts of IL-8 compared to non-smokers (56.2±12.4 pg vs 36.5±13.3 pg, p<0.001) (Figure 5).

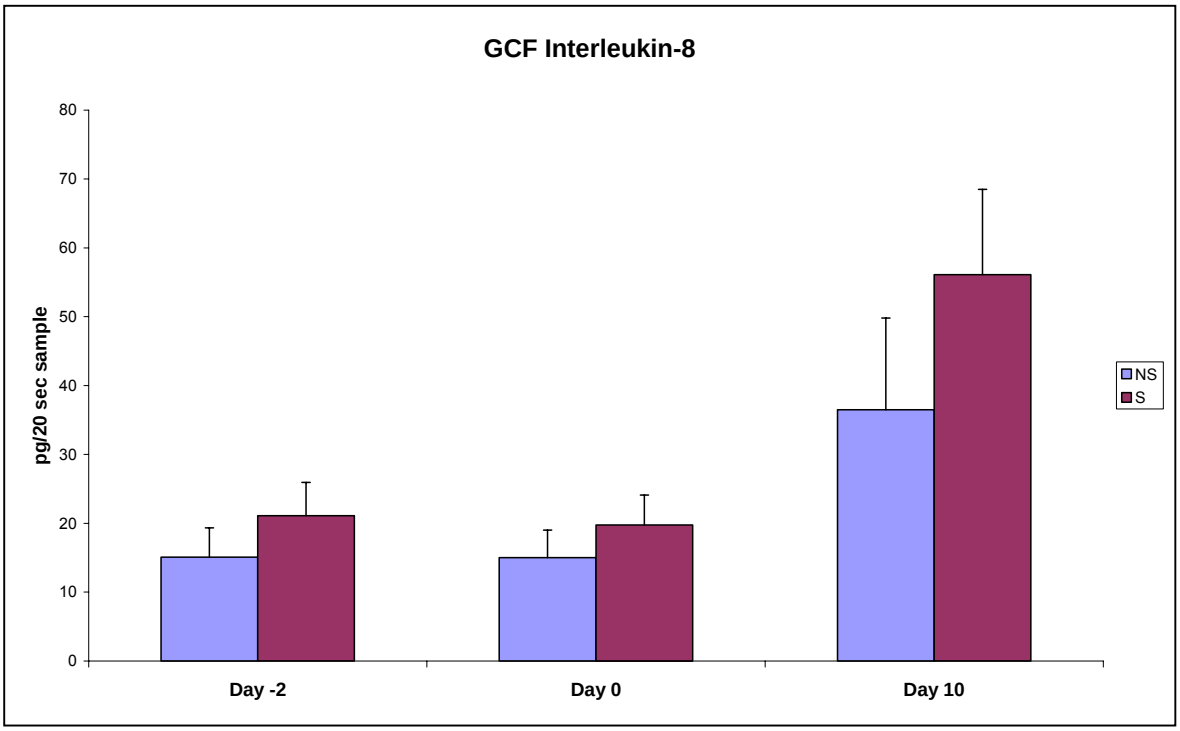


***Figure 5. IL-8 amounts (pg/20 sec sample) at day –2, day 0 and day 10, in smokers and non-smokers.***

Multiple linear regression analysis was performed to study associations between GCF cytokine levels and smoking status. Results revealed no association between any of the monitored biochemical parameters and smoking characteristics of the subjects (number of cigarettes/day and/or number of smoking years).

Stepwise multiple regression analysis was furthermore used to study the association between the levels of IL-1β, IL-4 and IL-8 and the clinical parameters PII, GI, PPD and BOP at day 10. These analyses showed a significant relationship between the levels of IL-1β and IL-4 and the PII (IL-1β: p=0.011, IL-4: p=0.018). In addition the levels of IL-8 were correlated to the PII (p=0.021) and the BOP (p=0.026).

## 5 Discussion

The objective of this study was to evaluate the influence of smoking on the clinical status and gingival crevicular fluid cytokine profile during experimental gingivitis. The standard deviations of the mean differences between measurements at day –2 and at day 0 (Table 2), indicated that the reproducibility between day –2 and day 0 was sufficient to evaluate changes occuring during the experimental gingivitis trial.

In the present study smokers showed less bleeding on probing and less gingival redness than non-smokers, after 10 days of plaque accumulation. At day –2, and at baseline (day 0), no significant differences were observed between smokers and non-smokers for any of the clinical parameters. This is in accordance with several other authors who, using the same model, showed that plaque and gingival status is almost the same in smokers and non-smokers (Bergström & Preber 1986 ; Danielsen et al. 1990 ; Lie et al. 1998). Since a score of <20% bleeding was necessary to enter into the experimental gingivitis trial, similar clinical characteristics were recorded in both groups.

The abstention from oral hygiene resulted in a similar accumulation of plaque during the 10-day experimental period in both smokers and non-smokers. Absence of a difference in PII between groups, indicating a similar plaque accumulation rate in smokers and non-smokers, is consistent with findings from other studies (Bastiaan & Waite 1978 ; Bergstöm 1981 ; Mac Gregor et al. 1985 ; Danielsen et al. 1990 ; Lie et al. 1998). Contradictory results have been reported between smokers and non-smokers concerning the microbial composition of both supragingival (Kenney et al. 1975 ; Bastiaan & Waite 1978) and subgingival plaque (Stoltenberg et al. 1993 ; Boström et al. 1998a ; Renvert et al. 1998). After 10 days of plaque accumulation, smokers showed significantly less bleeding on probing and gingival inflammation, compared to non-smokers (Table 3). The reason for this phenomenon, which has been noticed in other studies as well, is not fully understood. It has been suggested that smoking exerts local effects because of the vasoactive substances, including nicotine, present in

tobacco smoke. Earlier studies demonstrated a vasoconstructive effect of nicotine on gingival blood vessels, thus reducing gingival blood flow (Clarke et al. 1981). According to Bergström et al. (1988), the reduced inflammatory response is considered to be caused by a lower increase of the number of gingival blood vessels in smokers as compared to non-smokers. Several studies have reported that in smokers the gingival reaction to plaque accumulation was less pronounced as compared to non-smokers. Using the experimental gingivitis model, Bergström & Preber (1986) examined the development of gingivitis in smokers and non-smokers, by assessing the level of BOP at 5 time points during a 28-day period of no hygiene. As expected, both groups showed an increase in inflammation during the experiment, but the increase was less pronounced in smokers. Danielsen et al. (1990) observed already after 5 days of no-brushing, that smokers exhibited less inflammation as compared to non-smokers, and suggested that the inflammatory response in smokers during experimental gingivitis is delayed when measured clinically. Similarly, Lie et al. (1998) observed a suppressed inflammatory response to plaque accumulation in smokers and proposed that smokers should be identified as a distinct group in experimentally induced gingivitis studies.

At baseline, IL-1β total amounts were similar between smokers and non-smokers and a 2.5-fold increase was observed after the 10 days period of plaque accumulation in both groups. The presence of IL-1β in GCF from clinically healthy sites has been reported previously (Preiss & Meyle 1994 ; Giannopoulou et al. 2003), and the experimental gingivitis model has been already used in order to study associations of cytokines with inflammatory status of the periodontium. Kinane et al. (1992) observed that IL-1β increased rapidly with plaque accumulation and in advance to the subsequent visible gingival inflammation, suggesting that IL-1β may have potential as an early marker of gingival inflammatory changes. A significant increase of IL-1β levels from baseline to week 1 has been reported by Heasman et al. (1993) in a 4-week experimental gingivitis study. The levels of the cytokine remained stable throughout the experiment, without any further increase. Gonzales et al. (2001) observed significant changes in IL-1β levels during experimental gingivitis, and these changes were correlated with elastase activity in the same sample. A

possible association between IL-1β levels and environmental risk factors, such as smoking, has been the subject of several investigations. No association was observed In the present study between IL-1β and smoking status, eventhough smokers seemed to secrete slightly higher amounts of IL-1β as compared to non-smokers. The influence of smoking on IL-1β and IL-1ra in GCF was studied in a group of patients with moderate to severe periodontal disease (Boström et al. 2000). The authors observed that smoking had no effect on the expression of the above molecules. This finding was contrary to the results of previous studies of the same group (Boström et al.1998b, 1999) reporting that TNF-α was elevated in smokers. Recently, Giannopoulou et al. (2003) have shown that IL-1β levels in GCF increased gradually with the degree of inflammation when comparing periodontally healthy, gingivitis and chronic periodontitis subjects. Associations were observed between smoking and total amounts of IL-4, IL-6 and IL-8, but not with IL-1β.

In the present study, IL-4 showed an inverse relationship with the periodontal and smoking status: the highest amounts were observed in the non-smoking group at baseline, and a significant decrease was obtained after 10 days of plaque accumulation. No significant change was observed during plaque accumulation in the smoking group. The lack of IL-4 in severe periodontal lesions has been described by several investigators (Kabashima et al. 1996 ; Fujihashi et al. 1993a , 1993b ; Giannopoulou et al. 2003), confirming the hypothesis that the absence of IL-4 triggers periodontal disease (Shapira et al. 1992). Until now, very few investigations have been performed in order to study the effect of smoking on IL-4 production. IL-4 production by peripheral blood mononuclear cells of smokers was significantly higher than that of non-smokers (Byron et al. 1994).

IL-8 is considered to be the most important mediator for the recruitment and activation of PMN's. In the present study, total amounts of IL-8 were much higher as compared to the other cytokines described above, and showed significant associations with both periodontal and smoking status. Smokers expressed higher amounts of IL-8 than non-smokers already at baseline. After 10 days of plaque accumulation a 2.5-fold increase was observed in both groups. The differences remained significant between smokers and non-smokers. Conflicting

results have been reported on the association of IL-8 and the degree of inflammation and for smoking status of a subject. On one hand, an inverse relationship between IL-8 activity and PMN recruitment has been observed, as the concentrations of IL-8 in GCF were found to be significantly lower in patients with periodontitis (Jin et al. 2000 ;  Chung et al. 1997). Furthermore, lower GCF levels of IL-8 were found in localized periodontitis patients as compared to healthy subjects (Ozmeric et al. 1998). On the other hand, several studies have suggested a positive relationship between GCF IL-8 activity and periodontal disease, as total amounts were found to be significantly higher in GCF from diseased than healthy sites with a tendency to decrease after periodontal therapy (Mathur et al. 1996 ; Tsai et al. 1995 ; Giannopoulou et al. 2003).

Chronic smoking has been shown to cause changes in the cytokine profiles from bronchoalveolar lavage fluid (BALF) macrophages (Mikuniya et al. 1999).  IL-8 release was found to be higher in the BALF of smokers compared to that of non-smokers (Morrison et al. 1998). Exposure to cigarette smoke induced bronchial epithelial cells to release IL-8, thus contributing to airway inflammation (Moi et al.1997). However, in a recent study comparing smoking with non-smoking patients, smoking was found to reduce the sensitivity of peripheral neutrophils to stimulation of IL-8 in smokers (Fredriksson et al. 2002). Furthermore, smoking lowered IL-8 secretion by alveolar macrophages, thus modifying and /or decreasing the inflammatory response in the lung (Ohta et al. 1998).

It may be suggested that tobacco smoke activates more cells of the periodontium to express IL-8, thereby resulting in a local accumulation of PMN’s. Under normal conditions, IL-8 expression contributes to the elimination of the infecting bacteria by neutrophils. Conversely, an excessive release of IL-8 invokes hyperactivity of PMN’s, thus leading to tissue destruction (Yoshimura et al. 1997).

## 6 Conclusion

Our results suggest that smokers respond differently to the development of experimental gingivitis compared to non-smokers. Since cigarette smoking has been shown to have several effects on GCF cytokines, when performing studies regarding the pathogenesis of periodontitis, the smoking status of participating subjects needs to be taken in consideration.

## 7 Acknowledgements

Sincere thanks are due to Professor Andrea Mombelli and Catherine Giannopoulou who helped me with various aspects of my thesis project, including laboratory procedures, the analysis and the presentation of the results. I would also like to express my gratitude to all the students that participated in the experimental gingivitis trial.

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# 9 Appendix

**Legends**

Fig 1 : Clinical situation in a non-smoker at baseline (Fig 1a) and after 10 days of plaque accumulation (Fig 1b).

Fig 2 : Clinical situation in a smoker at baseline (Fig 2a) and after 10 days of plaque accumulation (Fig 2b).

Fig 3 : Total amounts /20sec sample (±SD) of GCF IL-1β at day –2, day 0 and day 10, in smokers and non-smokers.

Fig 4 : IL-4 total amounts (pg/20sec sample) at day –2, day 0 and day 10, in smokers and non-smokers.

Fig 5 : IL-8 total amounts (pg/20sec sample) at day –2, day 0 and day 10, in smokers and non-smokers.

***Table 1.*** **Demographic and behavioural variables**

| | Non-smokers | Smokers |
|---|---|---|
| **N** | 12 | 10 |
| **Men/ Women** | 7 / 5 | 7 / 3 |
| **Mean age** | 27.5 ± 3.1 | 24.9 ± 4.5 |
| **Cigarettes/day** | 0 | 15 ± 4 |
| **N smoking years** | 0 | 7 ±3 |

***Table 2.*** **Clinical and biochemical parameters with the number of observations, mean of first values (day –2), and means of the differences (day –2 vs day 0). The standard deviation of the mean of the differences and the corresponding coefficient of variation are also shown.**

| | N | Mean values of day –2 | Means of differences (day –2, day 0) | standard deviation of the means of differences | Coefficient of variation |
|---|---|---|---|---|---|
| *Clinical data* | | | | | |
| PII | 22 | 0.09 | -0.06 | 0.22 | 0.05 |
| GI | 22 | 0.21 | 0.03 | 0.11 | 0.01 |
| PPD | 22 | 2.41 | 0.01 | 0.22 | 0.05 |
| BOP | 22 | 0.09 | -0.03 | 0.12 | 0.01 |
| *Biochemical* data | | | | | |
| IL-1β | 22 | 4.96 | -0.05 | 1.22 | 1.50 |
| IL-4 | 22 | 7.54 | 0.90 | 2.90 | 8.42 |
| IL-8 | 22 | 17.8 | 0.75 | 1.63 | 2.65 |

*Table 3.* Clinical parameters at the beginning (day –2) and the end of the experimental gingivitis trial (day 10),

**in smokers and non-smokers**

| **Data** | **Baseline** | | **Day 10** | |
|---|---|---|---|---|
| | Smokers | Non-smokers | Smokers | Non-smokers |
| **PII** | 0.200[a]±0.402 | 0.049[a]±0.216 | 1.758±0.534 | 1.569±0.563 |
| **GI** | 0.242[a]±0.430 | 0.188[a]±0.392 | 1.292±0.456 | 1.500[b]±0.502 |
| **PPD(mm)** | 2.333±0.491 | 2.479±0.515 | 2.375±0.486 | 2.382±0.502 |
| **BOP** | 0.058[a]±0.235 | 0.125[a]±0.332 | 0.367±0.484 | 0.576[b]±0.496 |

Means and SE are shown
PII : Plaque Index scores ; GI : Gingival Inflammation ; PPD : Periodontal probing depth ; BOP : Bleeding on Probing
a : significant differences between baseline and day 10
b : significant differences between groups (smokers and non-smokers)

***Table 4.*** **Total amounts of IL-1β, IL-4 and IL-8 in the GCF of smokers and non-smokers during the 10-days experimental gingivitis study**

| **Mediator** | **Baseline** | | **Day 10** | |
|---|---|---|---|---|
| | Smokers | Non-smokers | Smokers | Non-smokers |
| **IL-1β** | 6.2[a]± 4.60 | 3.99[a]± 2.7 | 13.6±5.6 | 9.8±4.5 |
| **IL-4** | 4.4[b]±2.2 | 10.2[a]±7.0 | 4.1[b]±2.2 | 5.5±2.6 |
| **IL-8** | 21.1[a,b]±4.8 | 15.1[a]±4.2 | 56.1[b]±12.4 | 36.5±13.3 |

Means and SE are shown
a : significant differences between baseline and day 10
b : significant differences between groups (smokers and non-smokers)