

# Outcomes of Periodontal Therapy in Smokers and Non-smokers with Chronic Periodontitis

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## **DEDICATION**

**This thesis is lovingly dedicated to Øystein, Selma, Mari, and Aksel**

*“Wisdom is not a product of schooling but of the lifelong attempt to acquire it”*

*Albert Einstein (1879-1955)*



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## SCIENTIFIC ENVIRONMENT

The studies comprising the thesis were conducted during the years 2011-2016 under the supervision of Professor **Knut N. Leknes** as main supervisor and Professor **Kamal Mustafa** and Professor **Anne N. Åstrøm** as co-supervisors. The scientific activities took place at the Department of Clinical Dentistry at the University of Bergen.

## ABSTRACT

Chronic periodontitis is a prevalent inflammatory disorder initiated by dental microbial plaque. Smoking is considered a major risk factor for chronic periodontitis and smokers are known to exhibit impaired treatment outcomes.

The overall aim of this work was to study clinical outcomes of active and supportive periodontal therapy in smokers and non-smokers with chronic periodontitis at patient, tooth, and site level. Moreover, to compare the periopathogenic microflora and inflammatory and bone remodeling markers in gingival crevicular fluid in smokers and non-smokers following therapy.

Eighty patients, 40 smokers and 40 non-smokers, with moderate to severe chronic periodontitis were included in this prospective cohort study and treated non-surgically and surgically, and then followed-up in a supportive periodontal therapy program for 12 months. Smoking status was validated measuring serum cotinine levels at pre-treatment and 12 months following supportive periodontal therapy. Clinical measurements included full mouth recordings of clinical attachment level, probing depth, bleeding on probing, and plaque index at pre-treatment and following active and supportive periodontal therapy. At the same timepoints, subgingival plaque samples of 20 subgingival periopathogenic bacterial species were analysed using checkerboard DNA–DNA hybridization. From a subsample including 25 smokers and 25 non-smokers, 27 inflammatory and two bone gingival crevicular fluid markers were analysed using bead-based multiplex assays. In all multilevel analyses probing depth  $\geq 5$  mm with bleeding on probing was used as the primary outcome variable.

In smokers and non-smokers all patient level clinical parameters improved following non-surgical and surgical periodontal therapy. Only non-smokers showed a significant reduction in red complex species. At site-level, impaired outcome was observed in smokers and particularly at dental plaque positive sites (Study I).

Following 12 months of supportive therapy bleeding on probing, dental plaque positive sites, and probing depths increased slightly for both groups. Nevertheless, a negative effect of smoking was observed, in particular at maxillary single-rooted teeth.



At patient level, the multilevel analysis showed a suppressed variation in treatment outcome following supportive periodontal therapy in smokers (Study II).

Smokers demonstrated suppressed gingival crevicular fluid levels of several inflammatory markers and only non-smokers responded to periodontal therapy by altered marker profiles. An overall negative association was revealed between smoking and subgroups of markers at sites presenting  $\geq 10^5$  red complex periodontal microbial species (Study III).

In summary, smokers demonstrated unfavourable site-specific treatment outcomes compared with non-smokers, especially at plaque positive sites and at maxillary single-rooted teeth. Further, there seemed to be an immunosuppressive effect of smoking regulating the local inflammatory and bone remodeling response following periodontal therapy. Collectively, the results indicate a site-specific tissue response in smokers superimposed on the patient-specific systemic effect of smoking.

## LIST OF PUBLICATIONS

The thesis is based on the following studies, which will be referred to by their Roman numbers:

- I. **Bunæs Dagmar F**, Lie SA, Enersen M, Åstrøm AN, Mustafa K, Leknes KN. Site-specific treatment outcome in smokers following non-surgical and surgical periodontal therapy. **J Clin Periodontol. 2015;42:993-42.**
  
- II. **Bunæs Dagmar F**, Lie SA, Åstrøm AN, Mustafa K, Leknes KN. Site-specific treatment outcome in smokers following 12 months of supportive periodontal therapy. **J Clin Periodontol. 2016;43:1086-1093.**
  
- III. **Bunæs Dagmar F**, Mustafa M, Mohamed HG, Lie SA, Leknes KN. The Effect of Smoking on Inflammatory and Bone Remodeling Markers in Gingival Crevicular Fluid and Subgingival Microbiota Following Periodontal Therapy. **Manuscript accepted 21.11.2016, J Periodontal Res.**

## **ABBREVIATIONS**

AAP	American Academy of Periodontology
BI	Full mouth gingival bleeding
BoP	Bleeding on probing
CAL	Clinical attachment level
DFB	Operator and Examiner
EFP	European Federation of Periodontology
ELISA	Enzyme linked immunosorbent assay
FGF	Basic Fibroblast Growth Factor
GCF	Gingival crevicular fluid
G-CSF	Granulocyte Colony Stimulating Factor
GM-CSF	Granulocyte-Monocyte Colony Stimulating Factor
ICC	Intra-class correlation coefficients
IFN	Interferon
IL	Interleukin
IP	Interferon Inducible Protein
ISO	International Organization for Standardization
OPG	Osteoprotegerin
OR	Odds ratio
MCP	Monocyte Chemo-attractive Protein
MIP	Macrophage Inflammatory Protein
MMP	Matrix metalloproteinase
NHANES	National Health and Nutrition Examination Survey
PCR	Polymerase chain reaction
PD	Probing depth
PDGF	Platelet-Derived Growth Factor

PI	Full mouth dental plaque
SPT	Supportive periodontal therapy
RANKL	Receptor activator for nuclear factor kappa-B ligand
RANTES	Regulated upon activation, normally T-Expressed, and Presumably Secreted
TNF	Tumor necrosis factor
T0	Baseline pre-treatment
T1	3 months following active periodontal therapy
T2	Following 12 months of supportive periodontal therapy
VEGF	Vascular Endothelial Growth Factor

# 1. INTRODUCTION

## 1.1 Periodontitis

The periodontium consisting of gingiva, periodontal ligament, cementum, and alveolar bone supports and protects the teeth in function. Gingivitis is a bacterial dental plaque induced minor inflammatory disorder of the gingiva and the prerequisite first stage of periodontal disease. When a gingivitis lesion advances to cause irreversible loss of the periodontal ligament and supporting bone, it has transformed into periodontal disease. Periodontal diseases, highly common and a major cause of tooth loss <sup>[1]</sup>, have also been linked to conditions and systemic diseases including adverse pregnancy outcomes, diabetes mellitus, and cardiovascular, atherosclerotic and pulmonary diseases <sup>[2]</sup>.

### 1.1.1 Definition and classification of chronic periodontitis

Periodontitis cases are characterized using clinical parameters including clinical attachment level (CAL), probing depth (PD), and bleeding on probing (BoP) and radiographic bone loss. Periodontal health is characterized by absence of these signs and symptoms <sup>[3]</sup>. However, the classification system of periodontal diseases is essentialistic, based on the aetiology of the disease <sup>[4]</sup>. Out of six destructive periodontal diseases, chronic periodontitis is the most prevalent and a major cause of tooth loss <sup>[5]</sup>. Assessment of the severity of periodontitis is based on the extent of attachment loss, 1-2 mm considered mild, 3-4 mm moderate, and  $\geq 5$  mm severe periodontitis. The cut-off level between aggressive and chronic periodontitis is not distinct and based on clinical features. Unlike aggressive periodontitis, chronic periodontitis lesions are generally observed in adults with no specific familial aggregation and characterized by a continuous loss of periodontal tissues associated with subgingival plaque, calculus, and individual risk factors <sup>[6, 7]</sup>. The progression of chronic periodontitis seems to be continuous with slow to moderate bursts of tissue destruction <sup>[8]</sup> slowing off later in life <sup>[9]</sup>.

### 1.1.2 Epidemiology

Periodontal diseases are characterized using surrogate parameters including CAL, PD, and BoP on a site and tooth level <sup>[3]</sup>. Thresholds defining periodontitis cases are critical to assess and compare epidemiological and patient related data. Case definitions presented by the American Academy of Periodontology (AAP) <sup>[10]</sup> and the European Federation of Periodontology (EFP) <sup>[11]</sup> represent present standard in clinical and epidemiological research.

Prevalence of periodontitis varies among populations <sup>[7]</sup>. Severe forms, however, seem to affect around 10% of diverse populations <sup>[12-14]</sup> with a global age standardized prevalence of 11% <sup>[15]</sup>. Comprehensive epidemiological data has been gathered from the National Health and Nutrition Examination Survey (NHANES) in the United States. In 2009-2012 and based on EFP definition, an estimated prevalence of severe periodontitis in the US adults older than 30 years approximates 12% for severe and 66% for incipient periodontitis <sup>[14]</sup>. Estimates based on the AAP definition approximate 9% for severe and 37% for mild and moderate periodontitis. Periodontitis is cumulative and the incidence increases gradually from age 30 to 80 years to peak at age 38 years <sup>[15]</sup>. Increasing life expectancy may increase the burden of periodontitis and the prevalence of non-severe periodontitis in US adults 65 years and older to 64% using the AAP case definition <sup>[16]</sup>. Risk factors including smoking, educational and socioeconomic status, diabetes mellitus, health care availability, and oral hygiene habits <sup>[17]</sup> may also contribute to an increase in the burden of periodontitis <sup>[3]</sup>. In particular, smoking appears strongly associated with increased prevalence and severity of periodontal disease in a dose dependent order <sup>[18, 19]</sup>.

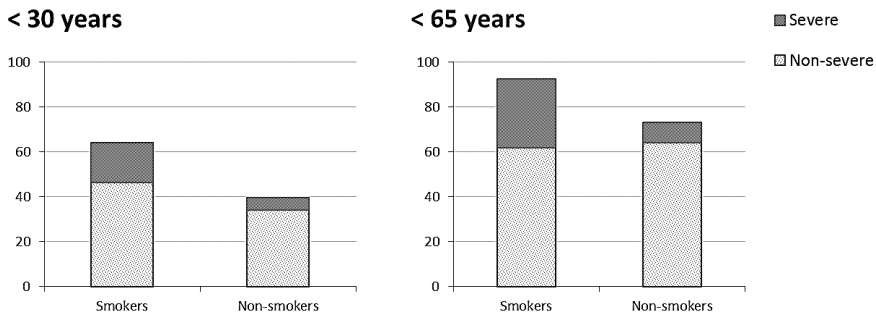


Figure 1. Prevalence of severe and non-severe periodontitis in smokers and non-smokers 2016, based on data from NHANES.

### 1.1.3 Aetiology

As bacterial dental plaque accumulates onto the tooth surface forming structured communities defined as dental biofilm [20], intractable biofilm formations provoke inflammatory processes in the subjacent gingiva to induce gingivitis [21]. A further increase in biomass and microbial complexity may disturb established tissue homeostasis and induce a destructive inflammatory process in the susceptible host [22]. Failing resolution or imbalance may further advance the inflammatory process into periodontitis – irreversible destruction of the periodontal attachment accompanied by migration and proliferation of the gingival sulcular epithelium onto the root surface [23]. Resulting deepening of the gingival sulcus establishes a periodontal pocket that provides protection and an anaerobic environment for bacterial species within the biofilm and growth of putative pathogenic species. The gingival tissues facing the biofilm provide a steady influx of molecules and immune cells within an inflammatory exudate known as gingival crevicular fluid (GCF). This new niche including GCF as principle nutritional source permits additional quantitative and qualitative variations within the biofilm [24]. Gram-negative bacteria associated with periodontitis include *Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema Denticola*, and *Aggregatibacter actinomycetemcomitans* [25, 26]. These bacterial species comprise a fraction of the total biomass [22]. Recent concept suggests, however, that only few bacterial species play a role as “keystone” periopathogenic bacteria [27]. “Keystone”

pathogens indirectly provoke periodontitis by transforming the normally symbiotic microbiota into a dysbiotic one which in turn activates the host immune response responsible for associated irreversible tissue damage <sup>[28]</sup>. *P. gingivalis* has in low abundance a potential to transform biofilms into a dysbiotic state and is hence recognized as a “keystone” pathogen <sup>[29]</sup>.

Dental biofilm activates the innate and adaptive immunity in highly complex events, involving recruitment of neutrophils, activation of lymphocytes, and activation of the complement system <sup>[30]</sup>. Cytokines, soluble small molecules responsible for crosstalk between immune cells and guidance of inflammatory responses, adjust the host responses to periodontal pathogens with up- and downregulation of genes <sup>[31]</sup>. The immune-inflammatory response intends to protect the host from infection and return the tissues to homeostasis. However, chronic stress may influence the inflammatory response and result in non-resolution of inflammation and periodontal tissue destruction. Re-establishing tissue homeostasis following infection appears to be an active coordinated process involving several biochemical pathways. Host factors, including immune components and resident cells, contribute by producing markers to the resolution of the inflammatory processes <sup>[30]</sup>.

The magnitude of the host response depends on genetics and environmental risk factors. Principally, periodontal tissue destruction is based on altered inflammatory response to subgingival biofilm <sup>[32]</sup>. The genetic component for a predisposition to chronic periodontitis is considered polygenetic and estimated to constitute 50% of the total risk for chronic periodontitis <sup>[33, 34]</sup>. Other risk factors known to increase susceptibility to periodontal disease may be modifiable such as cigarette smoking, stress, obesity, and diabetes mellitus <sup>[35]</sup>. Being male is also considered a modifiable risk factor as gender differences in lifestyle rather than genetic factors appear to predispose to periodontitis <sup>[35]</sup>. Cigarette smoking is also considered a causal factor for chronic periodontitis <sup>[36]</sup> and evaluation of clinical consequences for treatment outcomes in smokers is within the scope of this thesis.



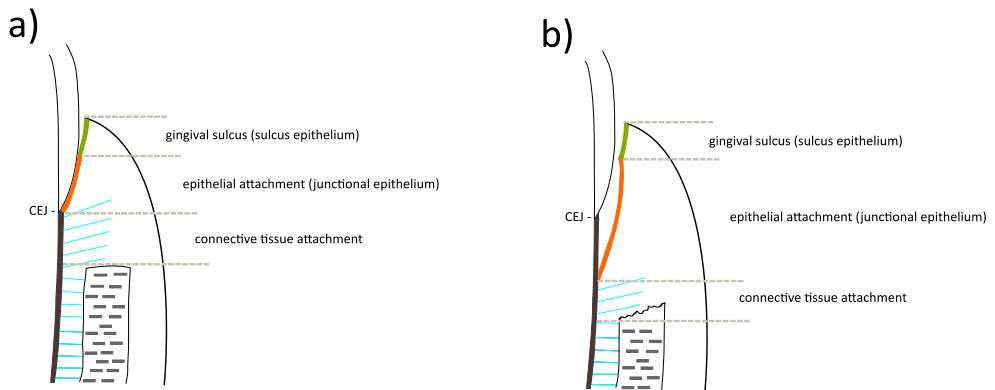


Figure 2. Anatomy of the periodontium in a) periodontal health and b) periodontal disease

## 1.2 Periodontal therapy

The ultimate goal in periodontal therapy is to prevent loss of periodontal support and tooth loss by self-performed plaque control creating equilibrium between plaque and host inflammatory responses. Lost periodontal tissues have a potential for regeneration. At present, however, a realistic goal for periodontal therapy is to control and eliminate periodontal disease by tissue repair.

### 1.2.1 Historical view

Oral hygiene and removal of calculus have been advocated in ancient civilizations and surgical removal of diseased periodontal tissues was described already in the 18<sup>th</sup> century [37]. Later, development of microscope, local anesthesia, and radiography contribute to a scientific understanding of a microbial aetiology of periodontal diseases [38]. Evidence based periodontal therapy developed in the second half of the 20<sup>th</sup> century following demonstration of bacterial dental plaque as an etiological factor in periodontal disease [39]. The principle of removing dental biofilm by self-performed oral hygiene and mechanical instrumentation developed non-surgical and surgical techniques to access subgingival plaque and not primarily surgical removal of diseased tissues. Longitudinal studies demonstrated the critical importance of high standard self-performed oral hygiene following periodontal therapy [40, 41] and minimally resective surgical approaches became the gold standard [42]. Therapeutic techniques were developed to regenerate lost periodontal tissues [43, 44] and paved the way for new

regenerative therapeutically approaches <sup>[45]</sup>. Documentation of pathogenic bacterial species in subgingival dental plaque led up to the specific plaque hypothesis suggesting a limited number of microbial species linked to the pathogenesis of periodontitis and a subsequent growth of local and systemic antimicrobial therapies <sup>[46]</sup>. However, as clinical trials failed to identify bacterial dental plaque as a prognostic factor for periodontal attachment loss <sup>[47, 48]</sup> and patient related risk factors were associated with periodontal disease <sup>[49]</sup>, the ecological plaque hypothesis emerged. This hypothesis introduced an effect of ecologic stress on enrichment of bacterial species <sup>[50]</sup>. Persistent inflammation was associated with disease progression <sup>[51, 52]</sup> and anti-inflammatory and host modulation approaches addressed to resolve inflammation were introduced as an adjunct to the mechanical plaque control <sup>[53]</sup>. The “keystone” pathogen hypothesis introduced bacterial species within subgingival biofilm to cause a dysbiotic biofilm triggering periodontal tissue destruction <sup>[27]</sup>. The simple concept that periodontal disease is caused solely by bacterial dental plaque appears no longer valid and a multifactorial causation has been documented and targeted <sup>[35, 54]</sup>. Therefore, supplement of mediators downregulating the inflammatory processes might be promising in the treatment of chronic periodontitis.

### **1.2.2 Treatment of chronic periodontitis**

Periodontal therapy constitutes a number of interventions considering the multifactorial aetiology of chronic periodontitis. Initially, patient related risk factors related to systemic health should be identified <sup>[55]</sup>. Risk of infections, endocarditis, bleeding disorders, and therapeutic efforts to modulate host-responses must be considered prior to active therapy <sup>[56, 57]</sup>. Counselling for smoking cessation and regulation of blood glucose, stress, diet, and weight should be integrated parts of active periodontal therapy <sup>[58-60]</sup>. Moreover, systemic medications and resolution-phase lipid mediators have the potential to downregulate and modulate inflammatory host responses <sup>[61]</sup>. However, this new treatment concept is not yet integrated in current systematic periodontal therapy <sup>[60]</sup>.

### ***1.2.2.1 Non-surgical therapy***

The hygiene phase of non-surgical periodontal therapy intends to establish a balance between bacterial insult and the host response through reducing the presence of bacterial dental plaque or biofilm. High standard plaque control appears pivotal to prevent gingivitis and maintain a healthy dentition <sup>[62, 63]</sup>. Prevention of gingivitis also prevents periodontitis <sup>[23]</sup>. Self-performed oral hygiene is the mainstay to prevent chronic periodontitis; a single manual tooth brushing exercise may reduce mean plaque scores up to 42% <sup>[64]</sup>. However, professional plaque control without instruction in self-performed oral hygiene appears of limited value <sup>[65]</sup> while repeated oral hygiene instruction may improve self-performed plaque control <sup>[64]</sup>. The duration of tooth-brushing correlates inversely with the presence of residual plaque and effective oral hygiene routines require careful selection of personal oral hygiene aides <sup>[66]</sup>. In perspective, power toothbrushes appear to increase the efficacy of plaque removal by 7-17% over manual toothbrushes <sup>[67]</sup>, whereas interproximal tooth surfaces are more efficiently cleaned using interdental brushes <sup>[68]</sup>.

As chronic periodontitis advances, the relative efficacy of high standard oral hygiene becomes reduced <sup>[69]</sup>. Mechanical instrumentation of the periodontal pocket then becomes a prerequisite to arrest the periodontal infection by removal of subgingival plaque and calculus from the root surfaces and the subgingival eoniche <sup>[70]</sup>. Nevertheless, removal of plaque and calculus remains demanding and subgingival debridement may result in varying presence of residual calculus <sup>[71, 72]</sup> depending PD, subgingival access, tooth type and surface, furcation involvement, local retentive factors, and operator skills <sup>[73-77]</sup>. Hand instruments, sonic or ultrasonic scalers, and Er:Yag lasers are all used for supra- and subgingival periodontal instrumentation. There seems to be no superior effectiveness between any of these approaches in treatment of chronic periodontitis <sup>[78]</sup>.

Part of an ongoing debate concerns preferred staging of non-surgical periodontal therapy whether to use a quadrant or sextant sequenced approach at 1-2 week intervals or a full-mouth approach. The principle of a full-mouth approach is to perform comprehensive periodontal instrumentation and elimination of

periopathogenic bacteria from other oral niches within 24 hours [79]. A meta-analysis shows modest clinical benefits of the full-mouth approach over sequenced quadrant-wise instrumentation [80]. Nevertheless, these various approaches are considered comparable options in the non-surgical therapy of chronic periodontitis [81]. The choice of protocol should rather be based on practical considerations related to time, patient preference, and clinical work load [82]. Healing response is usually assessed within 1-2 months following non-surgical therapy [83] and for patients with mild to moderate periodontitis, non-surgical treatment appears sufficient to maintain a stable attachment [84].

### ***1.2.2.2 Surgical therapy***

Chronic periodontitis patients enter a surgical phase of periodontal therapy principally to facilitate removal of subgingival plaque and calculus under visual inspection in sites that do not respond to non-surgical therapy and to prepare access for effective oral hygiene measures [84]. Open debridement surgery may reduce PDs promoting long-term preservation of the periodontium and thereby improving the prognosis of the tooth [85, 86]. Various access flap techniques have been developed. A systematic review indicated a cut-off PD value of 6 mm for surgical debridement aiming to reduce PD and gain clinical attachment. Open debridement in sites with PDs <4 mm may result in attachment loss [84]. The gingivectomy technique intends to remove the soft-tissue wall of the periodontal pocket in an apical direction creating access to the root surface for instrumentation and postsurgery oral hygiene [87]. The technique might not be performed at teeth in absence of attached keratinized gingiva and in intraosseous defects and thus appears more appropriate in cases with gingival enlargement [88].

Access flaps in combination with regenerative procedures aim to restore lost periodontal tissues [45]. Though current regenerative techniques are operator sensitive and predictability is limited to certain low-risk profile patients, promising advances may shift the paradigm of periodontal therapy from repair to regeneration. Guided tissue regeneration procedures using barrier membranes intend to preclude epithelial and connective tissue from occupying the wound area thus allowing periodontal ligament cells and alveolar bone to restore and regenerate new supporting tissues.

Other regenerative procedures include application of signalling molecules such as enamel matrix derivatives, platelet-derived growth factors, and bone morphogenetic proteins [89]. Further, several types of mesenchyme-derived cells with a potential to differentiate into periodontal tissue forming phenotypes have been investigated at a preclinical level [90]. Contextualized within periodontal regeneration, tissue engineering involves implantation of a scaffold incorporated with progenitor cells directly into a periodontal defect [91]. However, to develop the regenerative potential of the periodontal ligament is demanding and challenges remain before incorporated in a clinical setting [92].

### ***1.2.2.3 Supportive therapy***

Supportive periodontal therapy (SPT) is designed to maintain a healthy dentition to prevent or minimize disease recurrence or tooth loss over the lifespan of the patient [93-96]. A well-organized supportive periodontal therapy program may maintain periodontal health even in severe chronic periodontitis patients [94]. Absence of supportive therapy may jeopardize the successful outcomes of the non-surgical and surgical treatment [40, 97, 98].

Supportive periodontal therapy should consist of regular recall appointments including an update of systemic and oral health, examination of soft tissues and teeth, evaluation of self-performed oral hygiene, re-motivation, and complete plaque removal [99]. High standard self-performed oral hygiene and attendance to scheduled supportive periodontal therapy are critical [100-103]. Longitudinal studies have shown that favourable outcomes following active periodontal therapy may be lost due to poor compliance [94, 104]. Risk factors for tooth loss and recurrence of chronic periodontitis during supportive periodontal therapy are patient-, tooth-, and site-related factors including smoking, systemic disorders, compliance, furcation involvements, residual PDs, and BoP [52, 101, 105-107]. These factors may categorize patients based on risk profiles for future disease progression [108] and create a rationale for individually tailored supportive periodontal therapy [109]. However, following completion of active therapy, only a few predictive factors including smoking, plaque, and compliance are left to be modulated throughout supportive periodontal therapy. If the standard of daily

oral hygiene is inadequate and smoking cessation has failed, a more frequent recall interval appears necessary [110]. Based on previous studies, 3-month recall intervals are recommended for patients at risk [75, 111-113]. Only 26-77% of periodontal patients seem to fully comply with prescribed recall protocols [110]. In particular patients at high risk seem to have inconsistent compliance [114].

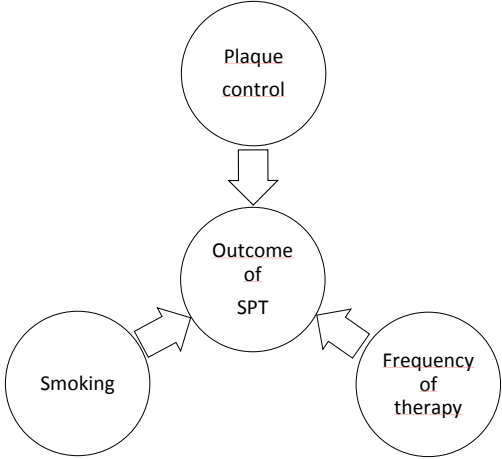


Figure 3. Modifiable predictive factors determining the outcome of supportive periodontal therapy

**1.2.2.4 Antimicrobial therapy**

The oral microflora is ecologically diverse as it includes at least 350 cultivable species. Culture-independent molecular approaches have identified about 1,200 different types of microbes that can inhabit the oral cavity [Human Oral Microbiome Database (<http://www.homd.org>)]. Development of biofilm may shelter bacteria from immune responses and antibiotic therapy [115]. Locally delivered antimicrobials are within minutes washed away from the periodontal pocket and thus may not reach relevant thresholds or substantivity [116]. Clinically relevant effects of the adjunctive use of locally delivered antibiotics in non-surgical therapy have been questioned as not being convincingly documented [117]. Nevertheless, a benefit of locally delivered devices has been estimated to 0.4 mm in PD reduction and 0.3 mm in CAL gain for deep periodontal sites [118]. A recent systematic review evaluating the use of local and systemic antimicrobials in smokers with chronic periodontitis as adjuncts to non-

surgical periodontal therapy, found an additional PD reduction of 0.81 mm and CAL gain of 0.91 mm for locally delivered antimicrobials at sites with deep baseline PD (PD  $\geq$  5mm). The review failed to detect similar adjunctive effects of systemic antimicrobial therapy<sup>[119]</sup>.

Some virulent bacterial strains are thought to penetrate the epithelial lining of the periodontal pocket<sup>[120]</sup>. As these pathogens appear inaccessible to instrumentation, a rationale for the use of systemic antibiotics emerges<sup>[116]</sup>. Positive effects of systemic antibiotics as adjunct to routine periodontal debridement have been shown for patients with inadequate response towards conventional non-surgical periodontal therapy, recurrent periodontitis, periodontal abscesses, and patients experiencing lymphadenopathy and fever post-therapy<sup>[118]</sup>. Preferred protocol appears to first mechanically disrupt any microbial reservoirs to make the residual contaminants more vulnerable to the antibiotic<sup>[121]</sup>. Nevertheless, it remains uncertain which patients should be targeted and what drugs and doses should be used for this approach.

Systemic antimicrobial therapy on periodontal indications should be contextualized in a growing concern of bacterial resistance to antibiotics and drug induced adverse effects<sup>[118]</sup>. Less stringent prescription routines and increasing non-supervised consumption of antibiotics seem to decrease bacterial susceptibility as well as increasing the possibility of drug resistant pathogens<sup>[122, 123]</sup>. In perspective, Scandinavian countries exercise restricted use of antibiotics in periodontal therapy compared with Southern Europe, US, and South America<sup>[124]</sup>. As periodontal pathogens differ in sensitivity towards antibiotics, microbiological testing may possibly increase the clinical efficacy by primarily targeting susceptible species<sup>[125]</sup>. However, the clinical relevance of microbiological testing has been questioned in studies obtaining excellent clinical outcomes following therapy without preceding microbiological testing<sup>[126]</sup>.

### **1.3 Periodontal wound healing**

Wound healing is the dynamic biological interplay between tissues, local and infiltrating cells, and signalling molecules released into the cellular environment. In

general, periodontal wound healing does not result in regeneration, complete functional and structural restoration of lost or injured tissues, but rather by scar formation <sup>[127]</sup>. Scar tissue represents a functional compromise which never exceeds 80% strength compared with the pristine tissue <sup>[128, 129]</sup>. Healing of periodontitis lesions occurs in complex settings with infected and inflamed periodontal tissues interacting with the tooth in a transgingival position displaying vascular soft tissue and non-vascular rigid wound margins. Plaque control is critical as infection may impair the healing process <sup>[130]</sup>. Despite the infectious environment, the oral mucosa tends to heal rapidly and with limited scar formation compared with cutaneous wounds <sup>[131]</sup>. Detailed mechanisms are not fully elucidated; however, components in saliva may be critical determinants of oral tissue homeostasis <sup>[132]</sup>.

### **1.3.1. Stages of wound healing/repair**

Periodontal wound healing may be conceptualized into integrated phases generally paralleling that in cutaneous wounds <sup>[133, 134]</sup>. Briefly, platelets exposed to extracellular matrix and collagen immediately form a fibrin clot. Activated platelets release cytokines, growth factors, and clotting factors initiating haemostasis, wound contraction, and formation of a fibrin-fibronectin mesh. As the fibrin clot in turn is infiltrated by neutrophils, monocytes/macrophages, fibroblasts, and endothelial cells, the haemostatic phase gradually progresses into an inflammatory phase <sup>[134]</sup>. Endothelial cells control the recruitment of cells in surrounding tissues, and within 1-24 hours neutrophils and monocytes appear. Neutrophils play a critical role cleaning the wound of tissue debris, effete blood cells, and destroying invading bacteria through phagocytosis, release of oxygen radicals, and activation of the complement system <sup>[135]</sup>. While the number of neutrophils peaks within 24-48 hours, the number of macrophages continues to increase <sup>[135]</sup>. Macrophages derived from monocytes supply the wound with a continuous flow of cytokines and growth factors transforming the fibrin clot into granulation tissue <sup>[136]</sup>, a highly vascularized immature tissue constituting extracellular matrix and fibroblasts.

In the proliferative phase, anabolic processes raise metabolic demands met by increased vascularity. Extracellular matrix factors and a variety of growth and matrix



factors stimulate migration and proliferation of endothelial cells from blood vessels and circulating endothelial progenitors <sup>[137]</sup> and thereby angiogenesis. One to 2 days post-injury, epithelial cells originating from the wound margins, separate from their basement membrane and migrate through degraded collagen and extracellular matrix by integrin receptors <sup>[136]</sup>. Following the migration and phenotypic transformation of the cells, attachment to the basement membrane is restored <sup>[138]</sup>. Keratinization of the epithelial cells is induced by tissue resources residing in the periodontal ligament and appears controlled by the sulcular environment <sup>[127]</sup>. Approximately 7 days post-injury, fibroblasts originating from the gingival connective tissue and the periodontal ligament populate the extracellular matrix to dominate the wound. Attached in a fibrin matrix, the fibroblasts mature into various phenotypes to produce diverse collagen species <sup>[139]</sup>. During the following remodeling phase, collagen is clustered into bundles by increasing cross-linking <sup>[140]</sup>. Maturation of the tissue continues slowly and may last for months, even years, regulated by factors released by macrophages, epidermal cells, endothelial cells, and fibroblasts <sup>[141]</sup>.

### **1.3.2 Wound healing following periodontal treatment**

Subgingival debridement inadvertently injures the sulcular and junctional epithelium. However, strict removal of these tissues is not advocated <sup>[142]</sup>. The resulting wound is left to heal by secondary intention and pending the magnitude of induced injury, healing by formation of a long junctional and sulcular epithelium is established within 1-2 weeks <sup>[143]</sup>. The junctional epithelium connects to the root surface through hemidesmosomes from the internal basal lamina.

Resective periodontal surgery including gingivectomy may generate larger connective tissue wounds readily exposed to oral bacteria and left to heal by secondary intention <sup>[87]</sup>. A fibrin clot will immediately cover the wound and within few days epithelial migration is initiated from the wound margins. The exposed wound may become epithelized within 1 week. Keratinization and reformation of rete pegs in attached gingiva will re-establish within 2 weeks, tissue maturation appreciable within 5-6 weeks <sup>[144]</sup>.

Using flap surgery techniques, ideally surgical wounds are left to heal by primary intention, the wound margins approximated and stabilized by sutures. A stabilized fibrin clot secures unimpeded absorption of plasma proteins onto the root surface and wound maturation into a connective attachment rather than epithelial down growth over exposed gingival tissues <sup>[133]</sup>. Careful tissue management and stable readaption of the wound margins appear critical to provide an epithelial attachment within 10-12 days and re-vascularization of the wound <sup>[145]</sup>. Thus, a new connective tissue attachment may be established following flap surgery.

Tissue resources sequestered in the periodontal ligament have the potential to support a connective tissue attachment with collagen fibers attaching to the root surface <sup>[127, 146]</sup>. Regenerative periodontal therapy may support formation of new alveolar bone, root cementum, and a functionally oriented periodontal ligament. It appears that regeneration can only be achieved if both the gingival epithelium and connective tissue are prevented from repopulating the surgically prepared wound, allowing cells from the periodontal ligament and alveolar base to recruit the defect <sup>[45]</sup>. Wound closure for healing by primary intention, space provision, and wound stability appear critical bioclinical factors to achieve these objectives <sup>[147]</sup>.

#### **1.4 Chronic periodontitis and smoking**

Smoking, the inhalation of smoke from burning tobacco, constitutes approximately 5000 different molecules inhaled through the oral and nasal cavity before the vaporized gases absorb in the lungs <sup>[148]</sup>. Worldwide, around 1.3 billion people smoke and the smoking epidemic is projected to increase linked to population growth in developing countries <sup>[149]</sup>. Smoking is considered a principal risk factor for a number of chronic diseases including cancer and pulmonary and cardiovascular diseases; in 2010 costing about 5 million lives globally with projected more than 10 million lives yearly a few decades ahead. Relatively few of the about 10% of adolescent women and 50% of adolescent men starting to smoke, will succeed to abstain due to physical addiction to smoking products and psychological addiction to smoking habits <sup>[150]</sup>. Extensive smoking cessation occurs in some high-income countries; in Norway for

example, the prevalence of smokers has been reduced by 30% from 43% to 13% within a decade <sup>[151]</sup>.

Smoking is recognized as a major patient related risk factor for chronic periodontitis. Evidence for the association between smoking and chronic periodontitis has been demonstrated in diverse populations <sup>[152]</sup>. Smokers tend to present with increased tooth loss, aggravated bone and attachment loss, and deeper periodontal pockets compared with non-smokers <sup>[18, 19, 153-155]</sup>. The most comprehensive analysis based on the NHANES III Study suggests approximately 50% of periodontitis cases being smokers <sup>[18]</sup>. Moreover, smokers have approximately four times greater risk of presenting with chronic periodontitis compared with non-smokers. The susceptibility to periodontitis progression appears dose-related with higher prevalence and increased loss of teeth and periodontal attachment in heavy smokers <sup>[156]</sup>. Based on multivariable logistic regression analysis in a South Brazilian population, attribution of smoking to attachment loss was estimated to 38% for heavy smokers and 16% for moderate smokers. Smoking impact was calculated by multiplying the number of days smoking with the number of cigarettes consumed per day divided by 20 (one pack of cigarettes) with a cut-off value >7300 packs for heavy smokers and 2735-7300 for moderate smokers. Compared with non-smokers, the odds ratio (OR) for heavy smokers to present with more sites with clinical attachment loss >5 mm was 3.6. The corresponding OR for moderate smokers was 2.0 <sup>[153]</sup>. In a prospective European study smoking in excess of 15 cigarettes per day was associated with >2 times higher risk of tooth loss in women and >3 times higher risk of tooth loss in men <sup>[155]</sup>. An Australian study defining heavy smoking as greater than 15 packyears (number of packs of cigarettes smoked per day multiplied with number of years smoked), showed that almost half the heavy smokers were periodontitis cases and less than one-fifth were never-smokers <sup>[154]</sup>. Compared with a smoking exposure of less than 20 packyears, heavy smoking in an adult US population revealed an OR for periodontitis of 2.1 according to the EFP and 2.4 based on the AAP definition <sup>[157]</sup>.

The association between chronic periodontitis and smoking seems not to be explained by differences in oral hygiene standards among smokers and non-smokers

[158]. Further, the association between chronic periodontitis and smoking is weakened over time following smoking cessation [18, 19, 159]. Longitudinal studies show comparable CAL in young smokers and non-smokers 6 years following smoking cessation. After 10-20 years cessation, former smokers tend to show less bone loss than smokers and approach the level of never smokers relative to tooth loss [155, 160, 161]. A causal association between smoking and tooth loss and smoking and chronic periodontitis is highly likely, with the strength of the association depending on chronicity and frequency of exposure [36, 162].



Figure 4. Clinical picture of a smoking severe chronic periodontitis case characterized by gingival recessions, minor clinical sign of gingival inflammation, and staining of the teeth.

### **1.5 Smokers responses to periodontal therapy**

Except for smoking cessation counselling, similar procedures are employed in treating chronic periodontitis in smokers and non-smokers. Non-surgical therapy by mechanical disruption of plaque is considered routine, even though less favourable therapeutic outcomes in smokers have repeatedly been documented [163-165]. A series of clinical studies in Sweden during the 1980s and 1990s initiated the systematic investigation of the effects of smoking on periodontal therapy [166-168], paving the way for prospective studies employing single-level statistics to compare means of PD, bleeding on probing, plaque, and periodontal pathogen levels in smokers and non-smokers following non-surgical periodontal therapy. PD considered the primary

outcome measure showed 0.2-0.9 mm less reduction in smokers with similar efficacy for one-stage full-mouth debridement and conventional quadrant-wide approaches [169-174]. Although these means appear small, PD reduction in the smoking populations is of clinical relevance. In favour of non-smokers, a meta-analysis of the influence of smoking on non-surgical therapy demonstrated a mean PD difference of 0.1 mm and for sites with initial probing depth >5 mm, 0.4 mm. No significant differences were documented for CAL and BI [164]. Prospective studies using multilevel statistics confirm the impaired outcomes in smokers following non-surgical therapy, especially for plaque positive sites, multi-rooted teeth, and deeper periodontal pockets [173, 175]. The probability of a 6-mm periodontal pocket to close ( $\leq 4$  mm) has been estimated to 31% at single-rooted and 51% at multi-rooted teeth in smokers compared with 43% and 64%, respectively, for non-smokers. Corresponding estimates for 7-mm pockets were 12% and 25% in smokers and 20% and 36 % in non-smokers [175].

Smokers affected by chronic periodontitis appear to harbour increased levels of putative periopathogenic bacteria [176, 177]. Investigations have reported a smaller reduction of periodontal pathogens in smokers following non-surgical therapy. Prospective studies suggest that *P. gingivalis*, *T. forsythia* and *T. denticola* may be more prevalent in smokers than in non-smokers following non-surgical therapy [62, 172, 178]. A reduced response to antimicrobial treatment in smokers is supported by cross-sectional and retrospective studies [176, 179]. In contrast, with an objective validation of smoking status, others have found similar reductions of periodontal pathogens in smokers and non-smokers following periodontal therapy [180]. Despite aggravated periodontal pathogens, adjunctive use of systemic and local antibiotics in conjunction with non-surgical periodontal therapy does not appear to advance PD reduction or CAL gain in smokers compared with non-smokers [181].

Non-surgical therapy in non-smoking chronic periodontitis patients is associated with reduced systemic inflammation, mirrored in reduction of systemic levels of c-reactive proteins and other inflammatory mediators [182, 183]. In contrast, smokers appear to have a suppressed antibody response to periodontal pathogens [180] with no significant reduction in c-reactive proteins (21 days) following non-surgical

therapy <sup>[184]</sup>. As GCF appears a relevant source of biomarkers of wound healing, prospective clinical studies have observed impaired outcomes of non-surgical therapy in smokers measured as local inflammatory responses in GCF. Following non-surgical periodontal therapy, altered treatment responses in smokers have been demonstrated for pro-inflammatory cytokines [interleukin (IL)-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$ , chemokines (IL-8), and matrix metalloproteinase (MMP)-8] <sup>[185-188]</sup>.

As smokers present with more severe chronic periodontitis and experience reduced response to non-surgical therapy, there may be an increased need for re-treatment. A retrospective study comparing sites with PD  $\geq$ 6 mm before and after non-surgical therapy found 30% remaining sites among smokers compared with 15% for non-smokers. At patient level, the possibility of requiring further treatment was 43% for smokers and 12% for non-smokers <sup>[189]</sup>. For both smokers and non-smokers, re-instrumentation following non-surgical therapy reduced pocket depth (PD  $\leq$ 4 mm) in 58% of sites presenting PD  $\geq$ 5 mm and 12% of PD  $>$ 6 mm <sup>[190]</sup>. For smokers, furcation involvement I and II appear particularly challenging by only 24% probability of improvement compared with non-smokers following re-instrumentation <sup>[191]</sup>.

In patients with advanced periodontal disease, periodontal surgery aims to achieve pocket closure and restore periodontal health. Current smokers may be candidates for periodontal surgery, though the benefit from PD reduction is estimated to only 50-75% of that accomplished in non-smokers <sup>[192]</sup>. The magnitude of differences between smokers and non-smokers correlates to responses to non-surgical therapy. A meta-analysis has quantified the outcomes in smokers following surgical therapy to be reduced 0.4 mm for PD and CAL compared with non-smokers <sup>[193]</sup>. Following flap surgery, furcation involvement I and II improved in 3% of degree I sites in smokers compared to 27% in non-smokers and smokers having 50% of the attachment gain of non-smokers <sup>[194]</sup>.

No evidence supports the use of systemic antibiotic as adjuncts to surgical periodontal therapy in smokers <sup>[119, 195]</sup>, though more favourable outcomes have been reported when using systemic antibiotic following regenerative treatment of furcation II defects <sup>[196]</sup>. Overall, smoking seems to impair periodontal wound healing in a dose-

dependent manner with negative effects on bone and attachment gain <sup>[197, 198]</sup>. A meta-analysis estimated the negative effect of smoking on bone gain in intrabony pockets to 2.1 mm<sup>[197]</sup>. Following guided tissue regeneration, a retrospective study found as much as 3.1 mm reduced attachment gain in intrabony pockets in smokers <sup>[199]</sup>. Regarding furcations, remaining defects were observed in 63% of smokers and 14% of non-smokers 24 months following surgery <sup>[200]</sup>.

Smokers enrolled in a maintenance program receiving regular supportive periodontal treatment show more tooth and bone loss and less PD reduction and CAL gain compared with non-smokers <sup>[86, 201-204]</sup>. As many as 90% of patients with recurrent chronic periodontitis are smokers <sup>[204]</sup> and heavy smoking is a risk factor for disease progression during supportive periodontal therapy <sup>[86]</sup>. The outcomes of periodontal therapy are associated with smoking consumption <sup>[201]</sup> and smoking cessation may improve the treatment response and reduce the risk of relapse of active disease during supportive periodontal therapy <sup>[19, 59, 204, 206, 207]</sup>.

## 2. RATIONALE FOR DESIGNING THE STUDY

Clinical parameters related to chronic periodontitis include PD, CAL, BoP, plaque accumulation, and GCF volume. By recording these parameters, previous periodontal destruction, ongoing disease and prediction of disease progression may be monitored [208]. Clinical parameters implemented in the diagnosis of chronic periodontitis are essential for treatment planning and to evaluate treatment outcomes. Smokers express clinical parameters differently than non-smokers with reduced BoP and GCF volume means [209, 210], and increased PD and CAL means [152]. Moreover, periodontal healing appears impaired in smokers following both active and supportive periodontal therapy. Smoking seems to affect active periodontal therapy through local and systemic pathways and impairs the inflammatory and the proliferative phase of periodontal wound healing [164, 178, 188]. Nevertheless, the aetiology behind the impaired healing response in smokers following active periodontal therapy is not clear.

When patients enter supportive periodontal therapy, acute periodontal wound healing has resolved and an overlapping phase of remodeling is initiated. Remodeling of the wound continues and the architecture gradually approaches normal [211]. The remodeling phase may continue for years and is responsible for wound contraction and scar formation. However, little is known about how smoking influences tissue maturation during supportive periodontal therapy.

Most studies have documented negative effects of smoking on periodontal therapies at a patient level [106]. To report patient-level treatment outcomes is appropriate as smoking represents a major patient-related risk factor for chronic periodontitis. Patient-level analysis provides limited evidence of associations between residual pockets and disease progression [86, 110]. Site or tooth as the unit of analysis is preferred in clinical studies. By averaging data, clinical relevant information is lost for both patient- or site-level analysis [212]. Teeth and sites share the same patient-related risk factors and are not independent units. Periodontal parameters are clustered in hierarchical structures within patients, in teeth within patients, and in sites within teeth. Furthermore, in follow-up studies, sites, teeth, and patients will be clustered



within repeated measures. Single level statistics will therefore not reflect the nature and dynamic diversity of site-specific responses to periodontal therapy<sup>[212]</sup>.

Multilevel statistics with the site as the unit of analysis may provide new insights into mechanisms of wound healing in smokers. Table 1 depicts the numbers of prospective studies over the last 30 years reporting the effect of smoking on treatment outcomes following active and supportive periodontal therapy. The literature screening shows that only two prospective studies have investigated the effect of smoking on non-surgical therapy using multilevel approaches but without objectively validating smoking status. As smoking modifies both local and systemic etiological risk factors, site-specific treatment responses in smokers may show altered site-specific wound healing over time following active and supportive periodontal therapy. Moreover, comparisons of microbiological and GCF inflammatory responses to periodontal therapy in smokers and non-smokers, contextualized with clinical treatment outcomes, may provide more in-depth understanding of the impact of smoking on active and supportive periodontal therapy. Summarizing, the research project on which this thesis is based was undertaken to analyse the impact of smoking on periodontal therapy, on the periodontal microflora, and on targeted inflammatory and bone remodeling markers in GCF at a patient, tooth, and site level.

Table 1. Numbers of prospective cohort studies of active and supportive periodontal therapy in smokers and non-smokers with chronic periodontitis.

Statistics	n	Months follow-up	Patients (n)	Smokers (n)	Reporting of smoking status			Case definition		Therapeutic intervention			Outcome variables						
					No	Yes		Yes	No	Non-surg.	Surg. Supp.	Local ab	Clinical Bacteria	Inflammatory markers					
						Subj.	Obj.							G	T	B	S		
<b>Single-level</b>	<b>51</b>	<b>15 (1.5-204)</b>	<b>52 (15-212)</b>	<b>24 (6-65)</b>	<b>9</b>	<b>39</b>	<b>3</b>	<b>34</b>	<b>17</b>	<b>33</b>	<b>10</b>	<b>10</b>	<b>4</b>	<b>45</b>	<b>9</b>	<b>6</b>	<b>2</b>	<b>5</b>	<b>3</b>
patient	49	15 (1.5-204)	52 (15-212)	25 (6-65)	8	38	3	34	15	31	9	10	4	43	9	5	2	5	3
-tooth	3	93 (9-204)	60 (50-74)	31 (10-46)	2	1	0	2	1	2	1	3	0	3	0	0	0	0	0
-site	2	9 (6-12)	44 (43-45)	16 (15-17)	1	1	0	0	2	2	1	0	0	2*	0	1	0	0	0
<b>Multi-level</b>	<b>2</b>	<b>9 (6-12)</b>	<b>67 (40-94)</b>	<b>20 (20-39)</b>	<b>1</b>	<b>1</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>2</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>

N; number, Subj.; subjectively validated, Obj.; objectively validated, Non-sur.; non-surgical, Surg.; surgical, G; gingival crevicular fluid, T; tissue, B; blood, S; saliva.

\* one study adjusted for clustering

### **3. AIMS**

The overall purpose of the present work was to evaluate the impact of smoking on the outcomes of periodontal therapy.

Specific aims were as follows:

- To study the site-specific effect of cigarette smoking on non-surgical and surgical periodontal therapy and to compare the composition of subgingival periopathogenic bacteria in smokers and non-smokers following non-surgical and surgical periodontal therapy (Study I).
- To study the site-specific effect of cigarette smoking on supportive periodontal therapy and to compare predictive values of clinical parameters on the outcome of supportive periodontal therapy in smokers and non-smokers (Study II).
- To determine the gingival crevicular fluid levels of inflammatory markers associated with periodontal inflammation and healing and the numbers of subgingival periopathogenic microflora following active and 12 months of supportive periodontal therapy in smokers and non-smokers.

## **4. MATERIAL AND METHODS**

### **4.1 Pre-study tests**

Intra-examiner (DFB) reproducibility was validated by registration of PD and CAL twice, one day apart, at six sites per tooth in a sample of 10 patients. Intra-class correlation coefficients (ICC) for the repeated measures for PD ranged between 0.92 and 0.96 and for CAL between 0.93 and 0.96. The sample size was estimated based on change in PD with a difference of 0.5 mm considered clinically relevant. The standard deviation of the differences between repeated PD measurements from the intra-calibration exercise was calculated to 0.5 mm. A power analysis based on 80 patients distributed into two groups and with the level of significance ( $\alpha$ ) set to 0.05, gave an 88% power to detect a true difference of 0.5 mm.

Prior to treatment, the hypothesis that it is not possible to mask an examiner to smoking status was tested in a pre-study sample of 30 chronic periodontitis patients, 16 smokers (>10 cigarettes/day for at least 5 years) and 14 non-smokers (never or not in the last 5 years) enrolled in a supportive periodontal therapy program at the University Dental Clinic, Department of Clinical Dentistry - Periodontics, University of Bergen. Following removal of calculus, plaque, and staining and after a mouth rinse with 0.2% chlorhexidine gluconate (Corsodyl, GlaxoSmithKline, London, UK) for 1 min, the examiner, wearing a face mask and masked to smoking status, scored smoking status as yes or no. Twenty-eight of 30 patients (93%) were correctly identified as either smoker or non-smoker. Thus, the pre-study hypothesis was accepted ( $p < 0.001$ ) indicating that the attempt to mask the examiner with regard to smoking status was unsuccessful.

### **4.2 Ethics**

The study protocol and informed consent were approved by the Institutional Medical Research Ethics Committee, University of Bergen, Norway. Prior to inclusion in the study, participating patients read and signed the official informed consent form.

### 4.3 Study sample and study period

Patients enrolled in the study were recruited from public and private dental clinics in three municipalities in Hordaland County, Norway, from December 2011 until June 2014. Patients fulfilling study inclusion criteria were consecutively included from March 2012 through September 2013. The thesis is based on a prospective cohort study with data collected from April 2012 until March 2015.

Study inclusion criteria accepted healthy subjects age 35-75 years diagnosed with chronic periodontitis and presenting with at least four non-adjacent teeth. These teeth should have proximal sites with a PD  $\geq$ 6 mm and clinical attachment loss  $\geq$ 5 mm with BoP and no radiographic signs of apical pathology. Subjects who presented with any current medical condition or used medications known to affect periodontal healing, or incorrectly reported smoking status, or had used antibiotics, or received subgingival scaling within 6 months of the study were excluded. The patients were either smokers ( $>$ 10 cigarettes per day for at least 5 years) or non-smokers (never smoked or not within the last 5 years). Each patient, based on subjectively reported smoking status, was allocated into smoking or non-smoking group.

Subjectively reported smoking consumption was calculated in packyears; the number of cigarettes smoked per day multiplied by the number of years divided by 20 (the number of cigarettes in a standard package) (Scott et al. 2001). Smoking status was objectively validated prior to treatment by measuring cotinine levels in serum. Peripheral venous blood was taken from each patient before and following supportive periodontal therapy and the concentration of cotinine in serum determined using enzyme linked immunosorbent assays (Cotinine ELISA Kit, MyBioSource, San Diego, USA).

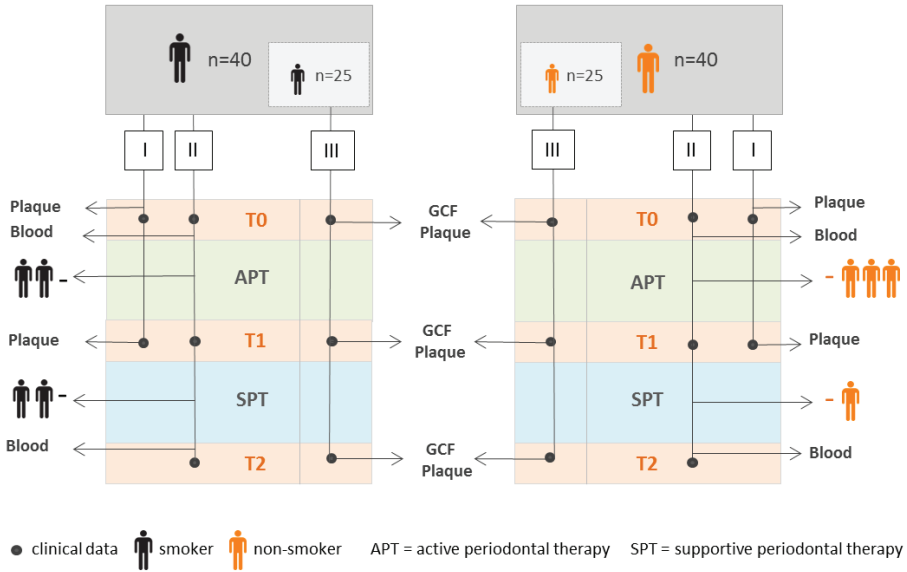


Figure 5. Study flow chart

#### 4.4 Clinical examination

Before clinical examination, a full mouth series of intraoral radiographs was taken. Each patient underwent clinical examination of teeth and oral soft tissues performed by the same examiner (DFB). Clinical data were collected at baseline pre-treatment (T0), 3 months following active periodontal therapy (T1), and following 12 months of supportive periodontal therapy (T2). Rounding up to the nearest mm, PD was recorded as the distance from the gingival margin to the probeable base of the pocket and CAL as the distance from the cemento-enamel junction or the margin of a dental restoration to the probeable base of the pocket at six sites per tooth using a manual periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA). Full mouth gingival bleeding (BI)<sup>[213]</sup> and full mouth dental plaque (PI) was recorded<sup>[214]</sup> at the patient level and BoP and plaque scored as present/absent at the site-level.

### **3.5 Collection and analysis of biological samples**

Subgingival plaque, GCF, and serum were sampled from 80 patients at T0, 75 patients at T1, and 72 patients at T2.

#### **4.5.1 Subgingival plaque**

##### ***4.5.1.1 Collection procedure***

Subgingival plaque was collected using two sterile paper points. Plaque samples were immediately labelled and immersed into a pre-reduced sterile transport medium (PRAS Dental Transport Medium, Morgan Hill, CA, USA). The samples were taken from the same site as the GCF samples.

##### ***4.5.1.2 DNA hybridization***

The subgingival plaque samples were analysed using DNA-DNA hybridization (checkerboard technique) <sup>[215]</sup> at Microbiological Diagnostic Service, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway. Each sample was qualitatively and quantitatively assessed for 20 periopathogenic bacteria. The analyses included red (*P. gingivalis*, *T. denticola*, and *T. forsythia*) and orange complex species (*Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum subsp polymorphum*, *Fusobacterium nucleatum subsp nucleatum*, *Fusobacterium nucleatum subsp vincentii*, *Parvimonas micra*), and *A. actinomycetemcomitans*.

#### **4.5.2 GCF**

##### ***4.5.2.1 Collection procedure***

GCF samples were collected using Periopaper strip (PERIOPAPER Gingival Fluid Collection Strips, Oraflow Inc., Smithtown, NY, USA). For each patient, one strip was inserted into the deepest periodontal pocket before treatment (T0) and resampled at the same site following active (T1) and supportive (T2) periodontal therapy. GCF volume was immediately estimated using the Periotron 8000 (Oraflow Inc., Smithtown, NY, USA), samples labelled, and stored at -80°C.

#### **4.5.2.2 Protein extraction and quantification**

GCF samples from 50 patients (25 smokers and 25 non-smokers) at T0, T1, and T2, were extracted using tris-HCl buffer and quantified using a commercially available kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). A plate reader (FLUOstar OPTIMA- BMG Labtech, Ortenberg, Germany) measured the absorbance at 570 nm, total protein per sample measured in µg.

#### **3.5.2.3 Multi- and singleplex assays**

Bead-based singleplex assay was employed to detect two bone-remodeling markers (Human Bone Magnetic Bead Panel, Multiplex MAP Kit, Billerica, MA, USA): *osteoprotegerin (OPG)* and *receptor activator for nuclear factor kappa-B ligand (RANKL)*. Bead-based multiplex assay was also employed to detect 27 inflammatory markers (Bio-Plex Human Cytokine Assay Bio-Rad Inc., Hercules, CA, USA): *Basic Fibroblast Growth Factor (FGF)*, *Eotaxin*, *Granulocyte Colony Stimulating Factor (G-CSF)*, *Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF)*, *IL-1β*, *IL-2*, *IL-1ra*, *IL-4*, *IL-5*, *IL-6*, *IL-7*, *IL-8*, *IL-9*, *IL-10*, *IL-12*, *IL-13*, *IL-15*, *IL-17*, *Interferon Inducible Protein-10 (IP-10)*, *Interferon-γ (IFN-γ)*, *Macrophage Inflammatory Protein-1α (MIP-1α)*, *Macrophage Inflammatory Protein-1β (MIP-1β)*, *Monocyte Chemo-attractive Protein-1 (MCP-1)*, *Platelet-Derived Growth Factor (PDGF)*, *Regulated Upon Activation, Normally T-Expressed and Presumably Secreted (RANTES)*, *(TNF-α)* and *Vascular Endothelial Growth Factor (VEGF)*.

### **3.6 Treatment**

Periodontal treatment was performed by one operator (DFB). Active periodontal therapy included non-surgical therapy, extraction of teeth with hopeless prognosis <sup>[216]</sup>, and periodontal surgery. In patients with PI less than 25% and sites with persistent PD >5 mm with BoP about 2 months following non-surgical therapy, surgical therapy was pursued. Individualized surgical protocols comprised gingivectomy, open flap surgery, or open flap surgery in combination with regenerative therapy (Emdogain®). Supportive periodontal therapy was scheduled at 3-month intervals beginning 3 months following active periodontal therapy. Smokers were regularly motivated to



reduce or stop smoking, and encouraged to participate in a public smoking cessation program (Røyketelefonen, Helsedirektoratet, Oslo, Norway).

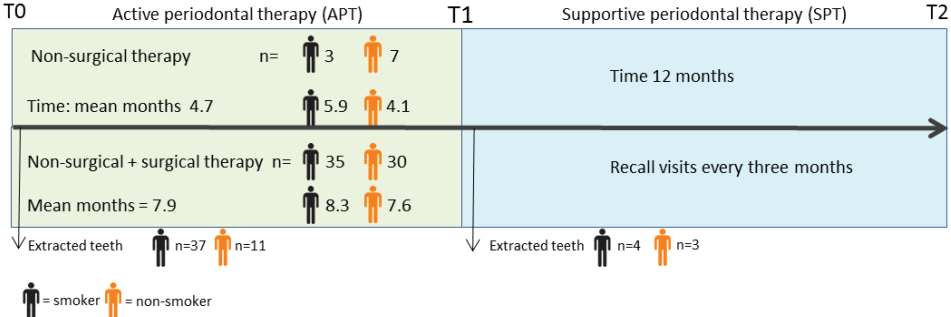


Figure 6. Study timeline.

### 3.7 Statistical analysis

The data were analyzed using the statistical software program Stata (Stata Corp., College Station, TX, USA), version 13 in Study I and II and version 14 in Study III. Distribution of continuous variables was tested in Study I with the skewness and kurtosis test and with the Shapiro-Wilk test in Study I and II. Numerical variables were expressed using means and standard deviations, means and standard errors of the mean, and means and range as appropriate. Differences between the continuous variables were assessed using a two-sample independent t-test and the Mann-Whitney test. Categorical variables were summarized as numbers and percentages and subsequently the chi-square test was used to identify differences. P-values <0.05 were considered statistically significant.

**Study I and II:** Logistic regression analysis was performed to study the associations of different predictive variables with the outcome of active and supportive periodontal therapy in smokers and non-smokers. The site, corrected for clustering within patients, teeth, and sites over time, was the unit of the analysis and PD  $\geq$ 5 mm with BoP was the outcome (dependent variable) dichotomized as present (1) or absent (0). Odds ratios (OR) with 95% confidence intervals (CI) were calculated. The OR expresses

differences in the risk to present the dependent variable between categories of independent variables. OR >1 imply an increased risk/chance, whereas OR <1 imply a decreased risk/chance. A non-significant outcome was understood if 1 was included in the 95 %. The sites presenting PD  $\geq$ 5 mm with BoP at teeth extracted between T0 and T1 were not included in the analysis.

**Study I:** Unadjusted analyses of relevant independent variables were performed. Variables with a  $p < 0.05$  in the unadjusted analysis plus gender, age, and baseline adjustments of four clinical parameters (PD, CAL, BI, and PI) were included in the unadjusted analysis to obtain adjusted ORs. Ordinary regression analysis, adjusted for clustering within patients over time, was undertaken to analyse the influence of active periodontal therapy on secondary outcome variables; clinical parameters and bacterial load.

**Study II:** Presented as adjusted ORs, logistic regression model adjusted for age, gender, marital status, and education was applied to test the associations between the outcome (dependent variable) at T2 and predictive variables at T0 and T1. For the smoking effect model, the adjustments were supplemented for smoke with two dummy variables: (T2 = 1 and Smoke = 0) as (1) and (T2 = 1 and Smoke = 1) as (0) and (T1 = 1 and Smoke = 0) as (1) and T1 = 1 and Smoke = 1) as (0). The model was constructed to study the effect of smoking at the differences between outcomes following active and supportive periodontal therapy. Multilevel analysis was used to calculate ICC within patients, teeth, and sites using linear mixed effects models. The multilevel model was set up as a random intercept model using melogit.

**Study III:** Based on a subsample of 25 heavy smokers and 25 non-smokers a post-hoc power analysis was performed. A level of significance ( $\alpha$ ) set to 0.05, gave a 71% power to detect a true difference of 0.5 mm. Due to skewed distribution of the data, square root transformation was employed. Ordinary regression analysis adjusted for clustering of time and for multiple comparisons (Sidak post-hoc test) was undertaken to analyse the influence of active and supportive periodontal therapy on the amounts and concentrations of markers in GCF. Red complex bacteria were categorized as > or

$<10^5$  number of species and plaque as present or absent. Stratified by plaque and red complex species, a regression model adjusted for age, gender, education, and clustering over time was applied to test the associations between smoking and amounts of markers. By calculation of the effect size, the impact of smoking on the amounts of markers before therapy and following active and supportive periodontal therapy was reported as Cohen's coefficient. Cohen's coefficient expresses the magnitude of smoking on a standardized scale; 0.2 imply small differences, 0.5 medium, and  $\geq 0.8$  large differences.

## 5. RESULTS

This section provides a brief summary of the results presented in the three reports (Study I, II, and III) that constitute the thesis.

### 5.1 Study I

The specific aim was to compare site-specific treatment outcomes in smokers and non-smokers following non-surgical and surgical periodontal therapy. Seventy-five of the 80 included patients, 40 smokers and 40 non-smokers, were available for examination following active periodontal therapy. Drop-outs included two smokers and three non-smokers. Mean age for smokers was 58 years (range 37-70) and for non-smokers 59 years (range 35-73); the percentage male smokers and non-smokers estimated to 38% and 58%, respectively. At baseline, mean reported smoking was 37 packyears (range 20-108) and mean cotinine level 471 ng/mL (range 168-861). None of the patients were excluded because of incorrect reporting of smoking status following cotinine validation and no patients reported starting or stopped smoking during Study I.

#### **Patient level clinical and microbiological outcomes following active periodontal therapy**

More teeth were extracted in smokers compared with non-smokers (37 vs 11) following active periodontal therapy. Both smokers and non-smokers demonstrated significant reductions in mean PD, CAL, BI, and PI ( $p < 0.001$ ). Total sites presenting PD  $\geq 5$  mm with BoP were 26.4% ( $n = 1471$ ) for smokers and 17.5% ( $n = 1049$ ) for non-smokers. Within arch and tooth type, the numbers of PD  $\geq 5$  mm with BoP were significantly higher at maxillary single rooted teeth in smokers compared with non-smokers ( $p = 0.030$ ). Both smokers and non-smokers responded to active periodontal therapy with a reduction of PD  $\geq 5$  mm with BoP to 132 (3%) and 52 (1%) sites, respectively. Following active periodontal therapy, five smokers (mean cotinine 727 ng/mL) presented more than 10 persistent sites with PD  $\geq 5$  mm with BoP, whereas no non-smokers presented more than seven sites. A significant reduction of red complex species was observed in non-smokers ( $p = 0.010$ ). For single species within the red

complex, *T. forsythia* was significantly reduced in smokers ( $p = 0.038$ ) and *T. forsythia* and *P. gingivalis* in non-smokers ( $p = 0.005$  and  $p = 0.013$ , respectively).

### **Site level clinical outcomes following active periodontal therapy**

PD  $\geq 5$  mm with BoP was more likely to occur in smokers than in non-smokers (unadjusted OR = 2.01,  $p = 0.004$ , adjusted OR = 1.90,  $p = 0.013$ ) following active periodontal therapy. Compared with a plaque free site in non-smokers, presence of plaque increased the risk for PD  $\geq 5$  mm with BoP in smokers and non-smokers (OR = 4.14,  $p < 0.001$  and OR = 3.09,  $p < 0.001$ , respectively). The risk was significantly greater in smokers within every tested location, except for maxillary buccal sites. Compared with plaque free sites in non-smokers, smokers were more likely to present with PD  $\geq 5$  mm with BoP at maxillary teeth (OR = 1.25,  $p = 0.029$ ) and at buccal sites in maxillary multi-rooted teeth (OR = 1.31,  $p = 0.045$ ).

## **5.2 Study II**

The specific aim was to compare site-specific treatment outcomes in smokers and non-smokers following supportive periodontal therapy. Seventy-two (36 smokers and 36 non-smokers) of the 80 included patients (40 smokers and 40 non-smokers) were available for examination following supportive periodontal therapy. In addition to the five patients lost during active periodontal therapy, two smokers and one non-smoker dropped out during supportive periodontal therapy. Assessments of socio-demographic status of the drop-out patients did not differ significantly from patients completing the study. Three smokers reported stopping smoking during supportive periodontal therapy, two of whom were confirmed by cotinine levels  $< 10$  ng/mL.

### **Patient level clinical outcomes following supportive periodontal therapy**

Mean PD, BI, and PI increased significantly in smokers and non-smokers and the number of sites presenting with PD  $\geq 5$  mm with BoP increased from 132 (3%) to 180 (3.8%) in smokers and from 52 (1%) to 79 (1.6%) in non-smokers. The greatest increase was observed in smokers at maxillary single-rooted teeth with an increase from 25 (2.6%) to 48 (5.4%) sites. At baseline, prior to active and supportive periodontal therapy, 39 smokers and 39 non-smokers presented nine or more sites with

PD  $\geq$ 5 mm with BoP. These smokers reported a smoking of 36.9 packyears (95% CI 36.4-37.4) and exhibiting a mean cotinine level of 474 ng/mL (95% CI 470-479). Following supportive periodontal therapy, five smokers and one non-smoker presented nine or more sites with PD  $\geq$ 5 mm with BoP and among the smokers, smoking estimated to 35.5 packyears (95% CI 35.0-36.0) with a cotinine level of 697 ng/mL (95% CI 682-719).

### **Site level clinical outcomes following supportive periodontal therapy**

Mean PD at baseline and following active periodontal therapy was slightly more predictive for PD  $\geq$ 5 mm with BoP in non-smokers than in smokers following supportive periodontal therapy. BoP was a significant predictor in both smokers and non-smokers. Following active periodontal therapy BoP was a stronger predictive factor in smokers than in non-smokers (OR = 13.26,  $p$  <0.001 and OR = 4.68,  $p$  <0.001, respectively). Plaque following active periodontal therapy was a more significant predictor in smokers (OR = 5.83,  $p$  <0.001) than in non-smokers (OR = 2.29,  $p$  = 0.041). Smoking had an overall negative effect on the outcomes of supportive periodontal therapy (OR = 2.78,  $p$  = 0.001), and the effect were more pronounced at maxillary single rooted teeth (OR = 5.08,  $p$  = 0.001). The random intercept models used to account for patient, tooth, and site over time, showed variations at site, tooth, and patient levels (ICC = 0.76, ICC = 0.59, and ICC = 0.11, respectively). The variance at patient level was less in smokers than in non-smokers (ICC = 0.137 and ICC = 0.051, respectively).

### **5.3 Study III**

The specific aim was to investigate the effect of smoking on the inflammatory response following active and supportive periodontal therapy as estimated in GCF. Moreover, the number of subgingival periopathogenic bacteria was estimated. A subsample of 25 heavy smokers and 25 non-smokers from the original 80 patients was selected based on baseline cotinine levels. Inclusion criteria for smokers and non-smokers were cotinine levels in serum  $\geq$ 300 ng/mL for smokers and <10 ng/mL for non-smokers. The following bone and inflammatory markers were reported: OPG, *G-*

*CSF, GM-CSF, IL-1 $\beta$ , IL-1ra, IL-4, IL-6, IL-7, IL-8, IL-9, IL-10, IL-17, IP-10, IFN- $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , PDGF, RANTES, TNF- $\alpha$ , and, VEGF.*

Measured by a negative effect size, smoking induced an overall reduced expression of bone and inflammatory markers in GCF at baseline and following supportive periodontal therapy (both,  $p < 0.001$ ). Subgroups of markers based on functionality, showed that smoking suppressed pro-inflammatory markers ( $p < 0.001$ ), chemokines ( $p = 0.007$ ), and growth-factors ( $p = 0.003$ ) at baseline, bone-remodeling markers ( $p = 0.003$ ) following active periodontal therapy, and pro-inflammatory markers ( $p = 0.019$ ) and chemokines ( $p = 0.005$ ) following supportive periodontal therapy. Only one specific marker, chemokine IL-8, was detected in significantly higher amounts in smokers compared with non-smokers following supportive periodontal therapy ( $p = 0.034$ ). Ten of the investigated markers in non-smokers were significantly upregulated following periodontal therapy whereas none in smokers. More specifically, for non-smokers a downregulation was observed for *IL-1 $\beta$ , TNF- $\alpha$ , IL-7, GM-CSF, IFN- $\gamma$ , IL-10, VEGF* and *IP-10* following active periodontal therapy and an upregulation for *TNF- $\alpha$ , IL-7, IL-9, IFN- $\gamma$ , IL-10, PDGF,* and *IP-10* following supportive periodontal therapy.

In the adjusted analysis, smoking was negatively associated with the amounts of pro-inflammatory ( $p = 0.042$ ), anti-inflammatory markers ( $p = 0.012$ ), and OPG ( $p = 0.001$ ) at sites presenting more than  $10^5$  red complex bacteria. Moreover, the amounts of markers for every investigated group (pro- and anti-inflammatory, chemokines, growth-factors and bone-remodeling markers) showed a significant negative association with smoking at plaque positive sites and a positive association at plaque negative sites ( $p < 0.05$ ).

## **6. GENERAL DISCUSSION**

### **6.1 Methods**

#### **6.1.1. Study design and patients**

Current smoking is considered an important modifiable risk factor for all severities of periodontitis <sup>[19]</sup>. The series of studies included in this thesis addresses the impact of smoking on periodontal therapy, on the subgingival microflora, and on inflammatory and bone remodeling GCF markers in chronic periodontitis patients. Generally, randomized controlled trials are considered the highest standard in clinical research providing the strongest level of evidence controlling for bias and confounding factors. However, a randomized controlled trial study design is not always possible or necessary particularly studying the effect of smoking exposure on periodontal treatment outcome. The present study can be defined as a prospective cohort study with one exposed and one non-exposed population. A prospective design may reduce selection bias by direct assessment of smoking exposure, controlling strict exclusion and inclusion criteria, and reducing recall bias by defining regular recall interval and time for data collection. On the other hand, lack of masking might introduce performance bias. However, masking the operator or examiner is not always possible and false masking could also introduce bias. The pre-study test demonstrated that a dentist cannot be masked relative to smoking status in current smokers. Thus, the critical issue of masking examiners or operators to smoking status is challenging and may introduce bias.

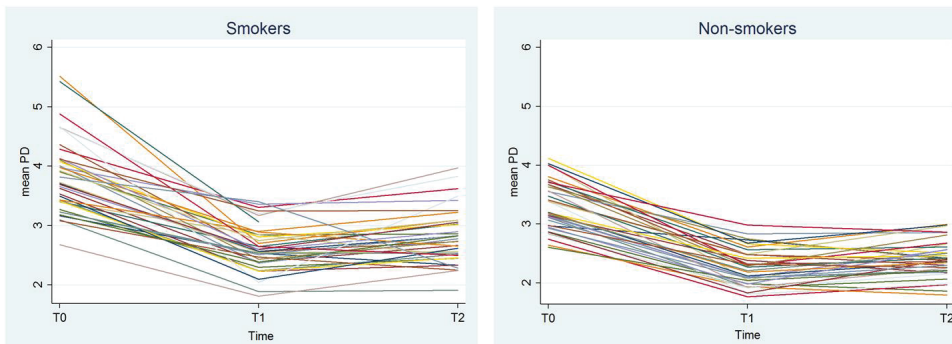
All patients in the present study were referred for periodontal therapy from general practitioners and represented adult motivated patients who previously had attended recall programs. The consecutively enrolled patients within the smoking and non-smoking cohort were not matched relative to sociodemographic or clinical characteristics. A higher female/male ratio in smokers compared with non-smokers occurred as more male than female smokers were excluded due to systemic health conditions affecting periodontal therapy. This could represent a confounder as females tend to exercise better compliance and oral hygiene regimens <sup>[217, 218]</sup> and exhibit a reduced pro-inflammatory response than males <sup>[219]</sup>. Marital status and education levels



were also unbalanced with smokers presenting lower education levels and living alone [220, 221]. Low education level is negatively associated with periodontal progression independent of smoking status [222, 223] and should be considered a confounder for impaired treatment outcomes in smokers.

The two cohorts were not balanced for disease severity and the study sample reflected an average severity of chronic periodontitis among adult smokers and non-smokers. Chronic periodontitis in smokers is generally manifested by deeper PDs and lesser BoP than in non-smokers [19]. Diagnosis and threshold levels for case definition did not secure an equal distribution of moderate and severe chronic periodontitis within the smoking and non-smoking cohort. As most smokers were exposed to smoking for decades prior to therapy, modulated inflammatory responses and aggravated periodontal pathogens have probably induced a more severe periodontal attachment loss, deeper PDs, and suppressed BoP compared with non-smokers [152]. Thus, severe periodontal disease in smokers may show less reduction of diseased sites following periodontal therapy [164, 224].

a)



b)

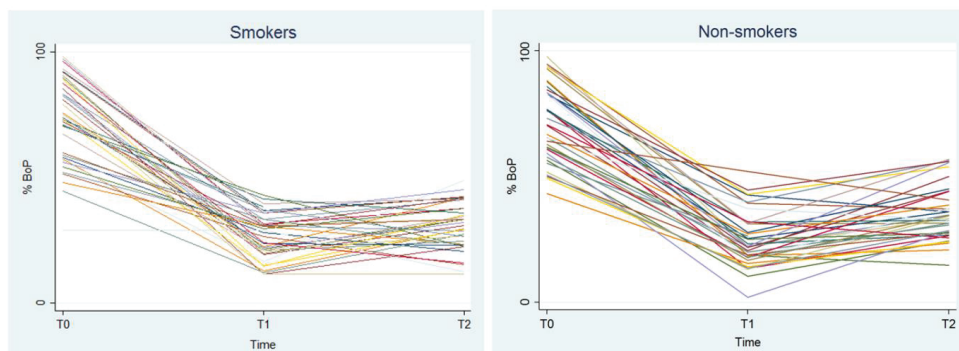


Figure 7. Mean PD (a) and BoP (b) in smokers and non-smokers at T0, T1, and T2.

In younger populations differences in clinical characteristics between smokers and non-smokers might have been more pronounced [225]. Since the two principle forms of periodontitis, chronic and aggressive, may not be discriminated in the setting of one clinical examination, the age group makes it more likely that enrolled patients represented true chronic periodontitis [226]. Treatment of severe chronic periodontitis is demanding as deeper PD and furcations generally predispose for more remaining plaque and calculus following non-surgical therapy [227]. Therefore, deeper PD and CAL presented in smokers at baseline could introduce a selection bias in the study sample by reduced treatment outcomes related to differences in subgingival plaque rather than smoking status. In total, periodontal surgery was performed in 35 smokers and 30 non-smokers and the greatest number of sites included in each surgery was in smokers. The surgical approach used depended on disease severity. As smokers presented with more advanced periodontal disease at baseline, more operator-sensitive surgical techniques were used. However, follow-up studies of periodontal therapy show that plaque control and adherence to a maintenance program may have a greater impact on treatment outcomes than the actual surgical intervention [40, 41, 228]. In perspective, smokers have demonstrated reduced compliance to supportive periodontal therapy [114]. A strict follow-up interval every 3 months was the setup during supportive periodontal therapy to standardize the therapy and to reduce potential compliance bias.

Sampling sites for GCF in smokers and non-smokers presented with no significant differences in PD, CAL, BI, PI, and GCF volume. However, regardless of smoking, the expression of markers in GCF is influenced by patient related factors including mean PD and CAL [229]. Therefore, local expression of GCF following periodontal therapy could be considered comparison of responses to subgingival plaque removal within specific host traits in smokers and non-smokers [32, 230].

A multicentre study design would have made it possible to increase the sample size. On the other hand, using one operator excluded inter-individual variability. Repeated measurements of PD performed by the examiner (DFB) were used to calculate the standard deviation and the sample size estimation by including 80 patients gave an 88% power to detect a true difference of 0.5 mm. In Study III, the sample size was reduced due to drop-outs, smoking cessation, and the objective of investigating effects of heavy smoking defined by cotinine levels in serum. Post-hoc power analysis was calculated to 71%. The eight (10%) drop-outs were balanced within the smoking (10%) and non-smoking (10%) cohorts and did not significantly vary by sociodemographic characteristics. Five (three smokers) of the excluded patients were removed from the study due to non-periodontal infections treated with systemic antibiotics. Systemic antibiotic could be considered an integrated part of performed periodontal treatment. However, antibiotic therapy was not an intervention and antibiotic used for systemic conditions could not be standardized nor controlled. Therefore, patients using systemic antibiotic were excluded throughout the study. One smoker not following the rigid supportive periodontal therapy interval was also excluded.

### **6.1.2 Choice of outcome variable**

Two levels of chronic periodontitis definitions were employed in Study I and II. At the patient level a periodontitis case was defined and diagnosed by periodontal parameters according to two consensuses for case definitions (AAP /EFP). At the site level a periodontal disease site was defined by  $PD \geq 5$  mm with BoP. At present, there is no generally accepted definition of a periodontal disease site and studies have generally reported mean number of sites with  $PD \leq 4$  mm and  $PD \geq 4$  mm, presenting periodontal

health and periodontal disease, respectively <sup>[231]</sup>. However, as a rule past experience of periodontitis is measured by attachment- and bone loss and present disease by BoP and/or PD <sup>[11]</sup>. Few studies have been conducted using site as unit of analysis. One multilevel study reporting 3-month outcomes following non-surgical periodontal therapy in non-smokers and smokers defined a periodontal healthy site as PD <4 mm without BoP <sup>[175]</sup>. In contrast, the present study defined a periodontal disease site as PD  $\geq$ 5 mm with BoP. One may argue that including BoP in the primary outcome variable might introduce a bias as smokers tend to show suppressed BoP and gingival bleeding compared with non-smokers <sup>[167, 232]</sup>. However, BoP in both smokers and non-smokers seems to indicate periodontal instability. A 5-year follow-up study reported increased mean BoP associated with periodontal instability and disease severity in smokers and non-smokers <sup>[107]</sup>. Another study applying site-specific data adjusted for clustering within patients and sites, reported 86% increased risk for transition of a non-bleeding to a bleeding site in smokers compared with non-smokers <sup>[233]</sup>. In contrast, excluding BoP as the primary outcome variable might introduce another bias by favouring PD reduction in non-smokers due to increased tissue probing penetration at baseline <sup>[234]</sup> and greater gingival shrinkage following resolution of inflammation <sup>[171]</sup>. As a site-level periodontal diagnosis including BoP appears associated with disease progression and periodontal instability irrespective of smoking status, both PD and BoP were included in the primary outcome variable.

### **6.1.3 Choice of biological sampling techniques and analysis**

#### ***6.1.3.1 Plaque***

The outcome of microbiological diagnostics may to some degree depend on the method of subgingival plaque sampling. Paper points and curette sampling are widely used. Compared with curettes, paper points may collect unattached bacteria and bacteria from the outer layer of plaque <sup>[235]</sup>. Conversely, in deep pockets, paper points are criticized to be saturated with moistness and plaque already in the shallower layer of the pockets <sup>[236]</sup>. Moreover, higher bacterial DNA counts have been detected from curette samples compared with paper points <sup>[26]</sup>. Nevertheless, the two sampling techniques have resulted in similar microbiological findings <sup>[237, 238]</sup>. In Study I and III

subgingival plaque samples were collected using sterile paper points (ISO size 40). As paper points absorb GCF, GCF sampling was performed in advance of subgingival plaque sampling [239]. GCF sampling prior to plaque sampling does not seem to interfere with detection of subgingival microbiota at periodontitis sites [240].

The plaque samples were analysed by using the semi-quantitative checkerboard DNA-DNA hybridization technique. The technique was developed for detection and quantification of periopathogenic bacteria and is useful in exploring presence of red complex species. Other techniques with a higher specificity and sensitivity could analyse the complete oral microbiome and reduce false negatives, especially following periodontal therapy [241]. Nevertheless, the DNA-DNA hybridization technique identifies associations between periopathogenic bacteria and periodontal health and has been shown to distinguish samples from different PD categories similarly as PCR, a high true put technique [242]. Furthermore, contextualized within the framework of the keystone pathogen hypothesis [27], limitations regarding quantification and specificity may not be of clinical relevance.

### **6.1.3.2 Gingival crevicular fluid**

GCF provides a protective role in host-parasite interactions by physically diluting bacteria and their metabolites and presenting antibacterial substances into the pocket. Inflammatory markers in GCF may be indicators of periodontal disease as well as periodontal wound healing. GCF can be collected by absorbent strips, capillary tubes, or gingival washing [243]. In Study III pre-sterilized periopaper filter strips were considered the preferred method compared with gingival washing [243, 244]. Detailed and calibrated collection methods that enable replication are recommended; standardized collection of GCF in broad terms performed using two approaches [244]. The first approach measures GCF volume and the second standardizes the collection time. The volume can be directly estimated using a Periotron reader that enables calculations of target cytokine markers at pg/ $\mu$ l-levels based on the sampled GCF volume. Weighing strips before and after sampling is not recommended due to minor changes in strip weight requiring very sensitive scales [245]. The alternative method is more prevalent and reports the total amount GCF collected in a standard period of time, frequently per

30 seconds. In Study III, collection time was standardized and the results reported as total amount per 30 seconds. Reduced periodontal inflammation following periodontal therapy resulted in decreased GCF volume for both smokers and non-smokers and decreased volume could have an excessive effect on concentration of markers and potentially elevate their concentrations in GCF. Therefore, reporting total amount per 30 seconds appears preferred compared with corrections for original GCF volume in prospective studies <sup>[244]</sup>.

Within the complex nature of chronic periodontitis, a single marker cannot reflect the overall inflammatory response to periodontal therapy and although ELISA is considered a preferred standard in the analysis of inflammatory markers, the multiplex technique offers the advantage of detecting up to 100 different markers in a relatively small sample. Moreover, multiplex is a high throughput technique offering similar sensitivity as ELISA <sup>[246]</sup>. Captured antibodies and antigens are freely suspended inside reaction wells in multiplex plates compared with antibodies attached at the bottom of the wells in ELISA plates <sup>[246]</sup>. Therefore, the multiplex technique used in Study III may provide an overall picture of the local inflammatory response to periodontal therapy in smokers and non-smokers.

#### **6.1.4 Statistical analysis**

The longitudinal study design was initiated to compare the changes in clinical parameters, subgingival plaque, and GCF markers following periodontal therapy in smokers and non-smokers. In Study I and II disease sites were the unit of the analysis. Reporting mean values of full-mouth measurements have tended to fail in investigating disease progression due to dilution by a major number of non-diseased sites <sup>[247]</sup>. Logistic regression analysis was a natural choice for statistical analysis with the binary outcome variable PD  $\geq$ 5 mm with BoP (present vs not present). Previous studies analysing periodontal therapy have mostly used single level statistical models assuming that site-level observations are independent. Patients are generally considered independent units and commonly site-specific measurements have been averaged to subject means. To take into account the dependency receding by the natural hierarchical structure of site-specific data within patients and teeth and by the

repeated measures in the prospective study design, all analysis was adjusted for dependencies.

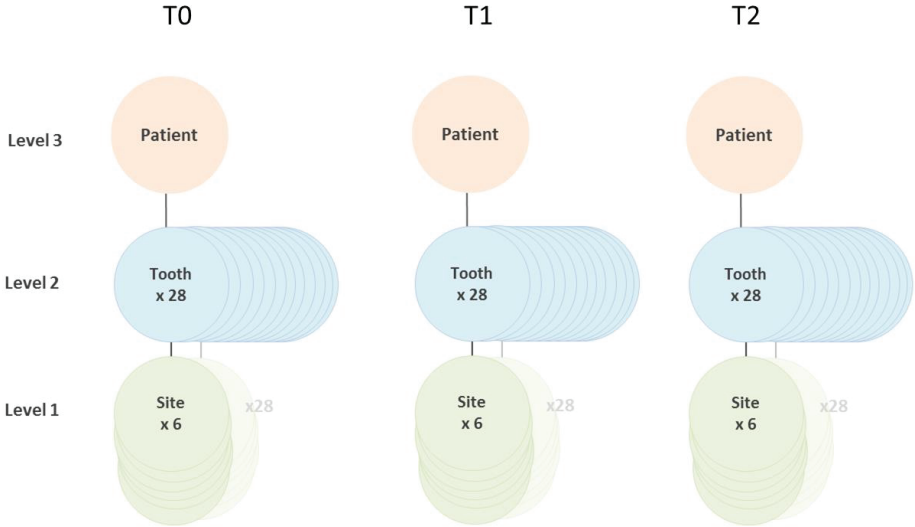


Figure 8. The hierarchical clustering of periodontal data, at T0, T1, and T2 and at six sites in maximum 28 teeth, in Study I and II.

The use of random effect models (logistic multilevel analysis) was in Study II an appropriate way to model and report the clustered data. Repeated measures for three time points were clustered within sites, teeth, and patients. At every level of hierarchical clustering of data the ICC indicates the proportion between cluster variances and total variance. The total variance regarding number of disease sites was related to within- and between-level differences. A high ICC implied hence that between-group variance dominated over within-group variance. In contrast, a small ICC indicated that more of the variance was related to differences within the groups, since the between observations within the group is smaller<sup>[248]</sup>.

In study III all analysis was performed at patient level and adjustments were done for clustering of repeated measures. By calculations of both p-values and effect sizes, the hypothesis could test if smoking affected expression of GCF markers

following therapy and moreover, how smoking affected the expression of GCF markers and the magnitude of the effect <sup>[249]</sup>.

## **6.2 Results**

### **6.2.1 Smoking and surgical and non-surgical periodontal therapy**

Non-surgical and surgical periodontal therapy efficiently reduced mean patient related variables as BI, PI, PD, and CAL and the number of diseased sites in smokers as well as in non-smokers. However, smokers were more likely than non-smokers to exhibit diseased sites (PD  $\geq$ 5 mm with BoP) following therapy (OR 2.01). The results are in accordance with previous findings documenting that non-surgical and surgical periodontal therapy has a therapeutic, but lower effect in smokers <sup>[163, 164, 193]</sup>. Responses to periodontal therapy are commonly compared with baseline recordings. As smokers tend to have deeper PD and CAL than non-smokers and when a baseline measure is part of the treatment outcome, the efficacy of therapy might be algebraically coupled to baseline measures <sup>[250]</sup>. Correction of differences in baseline variables in the adjusted analysis supported a suppression of site-specific treatment outcome following active periodontal therapy in smokers (OR 1.90). However, the risk of persisting diseased sites was more strongly associated with baseline PD in non-smokers, indicating that the outcome of active periodontal therapy is less dependent on initial PD.

Though the principle of active periodontal therapy is non-specific removal of plaque and calculus by various debridement techniques, PI was not a significant outcome factor in either smokers or non-smokers. Other studies as well have failed to demonstrate an association of PI and treatment outcomes <sup>[175, 251]</sup>. Few studies have considered plaque at a site-level as a determining outcome factor of periodontal therapy. In the present study, a plaque positive site increased the risk for persisting disease. This finding is in line with previous longitudinal studies reporting plaque at site-level as a critical outcome factor <sup>[173, 175]</sup>. Specifically, plaque positive lingual sites at multi-rooted mandibular teeth demonstrated the highest risk for persistent disease sites in smokers and non-smokers. Obtaining adequate plaque control at these sites



appears challenging <sup>[252]</sup>. Therefore, self-performed daily oral hygiene routines providing plaque free sites in those areas should be emphasized. Previous reports have shown reduced probability of pocket closure (PD  $\leq$ 4 mm without BoP) at plaque positive sites in smokers <sup>[175]</sup>. This finding is supported by the present study which indicated a four times higher risk of persistent disease at plaque positive sites. Smoking appears to reduce the site-specific tolerance to plaque thus effective self-performed oral hygiene routines in smokers appear critical. Site-specific variations were detected as plaque positive palatal sites at maxillary multi-rooted teeth in smokers presented a higher risk of disease sites while the palatal sites at single-rooted teeth in maxilla presented a lower risk. The explanation could be that smokers have more furcation involvements than non-smokers, especially in maxillary multi-rooted teeth <sup>[253, 254]</sup> with demanding access for instrumentation. Moreover, maxillary molar palatal sites appear particularly exposed to tar from tobacco smoke. Failure to discriminate attached tar from plaque may have led to misinterpretation of plaque positive sites in smokers.

### **6.2.2 Smoking and supportive periodontal therapy**

During supportive periodontal therapy a slight relapse of periodontal disease occurred as shown by increased patient level mean BI, PI, and PD and number of diseased sites in smokers and non-smokers. While active periodontal therapy reduced the number of diseased sites in all locations, the number of diseased sites increased followed supportive periodontal therapy. A higher risk for relapse was detected in smokers in all areas, demonstrating that the strict recall care every 3 months was not sufficient to outweigh the negative effect of cigarette smoking. Longitudinal studies have previously reported both reduced and similar responses in smokers compared with non-smokers over years with regular follow-up regimes <sup>[201, 255, 256]</sup>. However, as these studies compared changes in mean patient levels of both healthy and diseased sites, a direct comparison with the present study using site as the unit of the analysis is inappropriate.

Site-level differences are known to contribute most to the variance in outcomes following non-surgical periodontal therapy <sup>[173, 175, 251]</sup>. This also applies to the present

study. Moreover, stratified by smoking, ICC showed higher variation at patient level in non-smokers supporting smoking being a major patient related risk factor.

Following supportive periodontal therapy, the number of diseased sites at maxillary single-rooted teeth increased by 80.5 % in smokers (from 41% to 84%). Generally, in smokers, maxillary teeth present with the most advanced attachment loss and the deepest periodontal pockets <sup>[257]</sup>, also shown in the present study. Furthermore, the adjusted analysis revealed a stronger effect of cigarette smoke at maxillary single rooted teeth with less variation at patient level. A local effect of smoking on periodontal tissues has rarely been considered in previous studies, but an additive effect from direct exposure of smoking on the systemic effect has been suggested <sup>[258]</sup>. Maxillary single-rooted teeth are more directly exposed to smoking and may be more susceptible to impaired periodontal healing. The early phase of wound healing following active periodontal therapy might be less influenced by a direct exposure to cigarette smoke compared with the subsequent tissue remodeling during supportive periodontal therapy.

In the present study, BoP at site-level appeared to be a risk factor for disease progression and especially in smokers during supportive periodontal therapy. BoP from deep periodontal pockets has previously been associated with more prevalent and more intense BoP following non-surgical therapy <sup>[259]</sup>. Therefore, BoP at PD  $\geq$ 5 mm may be considered a predictive risk factor for inferior treatment outcome during supportive periodontal therapy in smokers. Generally, smokers appear with suppressed gingival bleeding and BoP <sup>[232]</sup>, most likely due to a different distribution of small and large capillaries in gingival tissues and vasoconstrictive adaption to periodontal disease <sup>[260]</sup>. The biological mechanisms, however, for suppressed gingival bleeding in smokers remain unclear.

### **6.2.3 Smoking exposure and response to periodontal therapy**

Smoking appears a major modifiable risk factor in periodontal diseases also affecting periodontal therapy, but unfortunately is not that easy to modify for patients and clinicians. Smoking cessation is a predictive factor for positive treatment responses

and already within days following smoking cessation periodontal parameters are positively affected <sup>[59, 261]</sup>. None of the patients reported to stop smoking during active periodontal therapy, but three out of 35 stopped smoking during supportive periodontal therapy. The reduced prevalence and the growing social disapproval of smoking, advocate more hardcore smokers, meaning smokers who have smoked for many years with no intention to stop <sup>[262]</sup>. Such hardening of smokers has been confirmed in Norway <sup>[263]</sup>, whereas in Netherlands the “hardening hypothesis” has been rejected, especially among highly educated persons <sup>[264]</sup>. Nevertheless, regulation and decline in smoking prevalence in Scandinavia have been successful, leading the way for smoking regulation in other countries <sup>[151]</sup>. A coincidental increased use of snuff in Scandinavia might partly explain the reduction in number of smokers <sup>[265]</sup>. There is an ongoing debate whether snuff actually prevents adolescents from starting smoking <sup>[266]</sup> and the magnitude of health profit by substituting smoke with non-combustible tobacco products <sup>[267]</sup>. A well-known tobacco researcher claimed that “*People smoke for the nicotine but they die from the tar*” <sup>[268]</sup>. By reducing or eliminating the content of nicotine from combustible tobacco products, the dependency and thereby the use of the products may decline. Therefore, in contrast to e-cigarettes and snuff, cigarettes with reduced nicotine might be a promising path to smoking cessation <sup>[269]</sup>.

Previous studies have demonstrated a dose-dependent detrimental effect of smoking on periodontal healing in which smoking exposure is subjectively reported in packyears or number of smoked cigarettes per day <sup>[86, 201]</sup>. However, lack of standardization of smoking status may to some extent explain an inconsistent dose-dependent effect of smoking on periodontal therapy <sup>[193]</sup>. A precise and objective measure of smoking exposure is essential to address the effect of smoking on periodontal healing. High cotinine levels in serum following supportive periodontal therapy were associated with nine or more remaining diseased sites within patients. Heavy smoking during therapy appeared to suppress the overall treatment responses resulting in more diseased sites, supporting a dose-response to periodontal therapy. A similar trend was not observed between subjectively reported number of cigarettes and treatment outcomes. Cotinine, a nicotine metabolite, is stable for up to 19 hours and considered an accurate measure of present smoking exposure <sup>[270]</sup>. In comparison,

packyear appears a less precise estimation of smoking exposure over several years. Underreporting smoking could mask an association between treatment outcome and exposure. Another confounding factor might be underreported use of other nicotine delivery products as moist snuff and electronic cigarettes [271], especially during smoking cessation. An effect of snuff and electronic cigarettes on chronic periodontitis has not been documented [272, 273].

#### **6.2.4 Periopathogenic bacteria and local inflammatory response to periodontal therapy**

Reduced numbers of anaerobic bacteria generally occur following periodontal therapy [274]. No significant reduction in the numbers of red and orange complex species was observed in smokers following active periodontal therapy, whereas a significant reduction in red complex species was observed in non-smokers. Smoking is considered to have a profound impact on the morphology, physiology, and formation of dental plaque [275] and on the distribution of periodontal pathogens [177]. Increased numbers of bacteria from the red and orange complex have been detected in smokers with chronic periodontitis before treatment and following periodontal therapy [62, 178]. Smoking may through changes in temperature, pH, nicotine, and release of chemicals disturb the periodontal ecology by modulating local and systemic host immune responses [275]. Furthermore, bacterial physiognomies may adapt to smoke. Especially the keystone pathogen *P. gingivalis* responds with enhanced ability to colonize and invade epithelial cells [276]. As smoking stimulates plaque formation in a dose dependent way and is reversed by removal of smoking exposure [277, 278], a local detrimental effect of smoking on periodontal disease and treatment outcome is endorsed. Nevertheless, *P.gingivalis* is not considered a primary inducer of inflammation causing periodontal attachment loss, but a manipulator of a dysbiotic periodontal microbiota giving rise to destructive host responses [28]. Smoking may exert a selective influence on dysbiotic development in the subgingival plaque persuading upgrowth of commensals or new subgingival bacteria, termed pathobionts, which may partly explain poor responses to periodontal therapy in smokers.

The periopathogenic species in subgingival plaque interact with release of biomarkers in GCF [279] and following active periodontal therapy in non-smokers, inflammatory markers in GCF were reduced. Compared with non-smokers, smokers demonstrated suppressed amounts of cytokines in GCF at baseline and following supportive periodontal therapy. Active periodontal therapy reduced the amount of inflammatory markers in non-smokers and thereby decreased differences in expression of markers between smokers and non-smokers. Variations in biomarkers in GCF generally mirror fluctuations of quiet periods and bursts of activity in chronic periodontitis [8]. However, smoking appears to outweigh expression related to activity by a downregulation of cytokines and, furthermore, by an overall suppression of pro- and anti-inflammatory markers at sites positive for plaque and for higher counts of red-complex species. A hypoexpression of cytokines in smokers with chronic periodontitis has previously been recognised [280-282]. In contrast, smoking has also been associated with increased pro-inflammatory responses to bacterial colonization [283] and not to be a major modifier of inflammatory markers in GCF in severe chronic periodontitis [32].

Only one investigated molecule, IL-8, was detected in significantly higher amounts in smokers following supportive periodontal therapy. This chemokine has been recognized as a marker with major pro-inflammatory properties attracting neutrophils and being upregulated by smoking in a dose-responding manner [284]. Levels of cotinine are known to vary considerably in different body fluids [285]. GCF may have 300-fold higher levels of nicotine than serum [286]. Therefore, local expression of IL-8 could be expected to vary from site to site in heavy smokers. Higher levels of IL-8 seem to increase survival of gingival fibroblasts [287] and alter chronic wound healing by inducing fibrosis of gingival tissues. Interestingly, increased IL-8 might impair periodontal healing, especially at maxillary single-rooted teeth where a local effect of heavy smoking appears more evident.

Bone formation and resorption is a dynamic process controlled by the bone-remodeling markers RANKL and OPG [288]. The remodeling process is regulated by the RANKL:OPG ratio as OPG binds to the cellular receptor of RANKL, blocking RANKL from activating osteoclasts. Increased GCF ratio has been detected in chronic

periodontitis patients <sup>[289]</sup> and is either caused by decreased OPG, increased RANKL, or both. Aggravated alveolar bone loss is a diagnostic feature of chronic periodontitis in smokers <sup>[160]</sup>. The concentration of RANKL was below detection level in the present study. However, OPG was significantly reduced in smokers compared with non-smokers following active periodontal therapy, indicating an increased RANKL:OPG ratio. Decreased OPG production has previously been revealed in osteoblasts following nicotine exposure in a dose dependent manner <sup>[290]</sup>. Using a threshold of smoking more than 20 packyears of cigarettes, a decrease in OPG and a higher RANKL:OPG ratio has been reported <sup>[291]</sup>. Findings in the present study support these findings and indicate that heavy smoking may increase tissue degradation by stimulating an imbalance in bone homeostasis.

## 7. CONCLUSIONS

Independently of disease severity and smoking status, overall non-surgical and surgical periodontal therapy resulted in favourable treatment outcomes. However, smokers exhibited an impaired site-specific response to active periodontal therapy documented by an increased risk to present with diseased sites in a dose-dependent manner. Only non-smokers demonstrated reduced numbers of red-complex microbiota. Plaque positive sites were at higher risk of limited treatment outcomes in smokers and reduced tolerance for plaque at site-level in smokers might be linked to aggravated subgingival dental plaque.

Following 12 months of supportive periodontal therapy, a higher incidence of diseased sites was detected in smokers than in non-smokers. A negative site-specific effect of smoking was observed, especially at maxillary single-rooted teeth. Elevated levels of cotinine were more related to numbers of disease sites than smoking estimated by packyears, indicating the relevance of reducing or terminating smoking during periodontal therapy. Following active periodontal therapy bleeding on probing was a strong predictor for disease site, especially in smokers.

No inflammatory and bone remodeling markers in gingival crevicular fluid responded to active and supportive periodontal therapy in smokers. In contrast, the local expression of inflammatory and bone remodeling markers in non-smokers was altered by active periodontal therapy. Only in non-smokers did inflammatory markers reflect the recurrence of chronic periodontitis during supportive periodontal therapy.

Within the limitations of the present study, it may be concluded that dampened local inflammation, especially at plaque positive sites and at sites harbouring high levels of red complex species, contribute to the impaired site-specific treatment outcomes in smokers.

## 8. FUTURE PERSPECTIVES

Globally, the number of smokers continues to increase. An obvious way to improve the outcome of periodontal therapy is tobacco regulations that prevent initiation of smoking, improve smoking cessation, and production of less addictive and toxic combustible tobacco products. Nevertheless, the future of periodontal therapy in smokers will be influenced by the growing populations of elderly with chronic periodontitis. As lifestyle and general health conditions might impair inflammatory host-responses, improved knowledge of smokers' inflammatory responses will provide pertinent information in individualizing targeted therapies.

Observed absence of local inflammatory responses to periodontal therapy in smokers may serve as a base for future research. It seems relevant to the ability of gingival fibroblasts to induce a prolonged and persistent local inflammatory cytokine production in smokers. Comparing inflammatory cytokines expressed in gingival fibroblasts derived from biopsies obtained from smokers and non-smokers, the impact of smoking on periodontal tissue response may be further elucidated. Identification of candidate genes or proteins *in vitro*, and determination of biological pathways that are affected by smoking, may provide important information of modulated local tissue responses in smokers. Moreover, fibroblasts derived from smokers and non-smokers may respond differently when exposed to periopathogenic bacterial species, to local expression of IL-8, and to high doses of cotinine.

Another interesting area of investigation would be to evaluate differences in local and systemic inflammatory responses to periodontal therapy in smokers and non-smokers. As smoking appears to stimulate an overall systemic hyper-inflammatory condition, comparisons of changes in systemic responses in smokers and non-smokers following periodontal therapy are warranted. Moreover, contextualized within suppressed local inflammatory responses, comparisons of local and systemic inflammatory responses to periodontal therapy appear critical in the search for biomarkers capable of resolving inflammatory processes in smokers and individualizing optimal therapy.



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## Study I

**Site-specific treatment outcome in smokers following non-surgical and surgical periodontal therapy**

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# Site-specific treatment outcome in smokers following non-surgical and surgical periodontal therapy

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## Abstract

**Aim:** To evaluate the effect of smoking at patient, tooth, and site level following non-surgical and surgical periodontal therapy.

**Material and Methods:** Eighty chronic periodontitis patients, 40 smokers and 40 non-smokers, were recruited to this single-arm clinical trial. Smoking status was validated by measuring serum cotinine levels. Periodontal examinations were performed at baseline (T0) and 3 months following non-surgical and surgical periodontal therapy (T1). At T0 and T1, subgingival plaque samples were collected from the deepest periodontal pocket in each patient and analysed using checkerboard DNA–DNA hybridization. Probing depth (PD)  $\geq$  5 mm with bleeding on probing (BoP) was defined as the primary outcome. Unadjusted and adjusted logistic regression analyses, corrected for clustered observations within patients and teeth, were conducted comparing smokers with non-smokers.

**Results:** Clinical parameters significantly improved in both groups ( $p < 0.001$ ). An association was revealed between smoking and PD  $\geq$  5 mm with BoP (OR = 1.90, CI: 1.14, 3.15,  $p = 0.013$ ), especially for plaque-positive sites (OR = 4.14, CI: 2.16, 7.96,  $p < 0.001$ ). A significant reduction of red complex microbiota was observed for non-smokers only ( $p = 0.010$ ).

**Conclusion:** Smokers respond less favourably to non-surgical and surgical periodontal therapy compared with non-smokers, in particular at plaque-positive sites.

Key words: chronic periodontitis; cluster analysis; microbiology; periodontal therapy; smoking

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Cigarette smoking appears a considerable behavioural risk factor for

periodontal diseases (Albandar 2002). Depending on the definition of the disease and exposure to smoking, a smoker has 3–25 times higher risk of developing chronic periodontitis compared with non-smokers (Bergström 2003, Hyman & Reid 2003). Nearly half of the cases diagnosed with chronic periodontitis are smokers (Tomar & Asma 2000,

Hyman & Reid 2003, Do et al. 2008). In perspective, there is a globally increasing prevalence of cigarette smokers (Samet & Wipfli 2010, Ng et al. 2014).

Chronic periodontitis patients generally respond favourably to conventional periodontal treatment. However, several studies indicate that smokers respond less favourably

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both to non-surgical (Preber & Bergström 1986, Apatzidou et al. 2005, Wan et al. 2009) and surgical approaches (Trombelli et al. 1997, 2003, Scabbia et al. 2001). In spite of this, smokers are in general treated following similar protocols as for non-smokers.

Cigarette smoking is considered an extrinsic modifying factor in the pathogenesis of periodontal diseases interacting with the host cells and affecting the inflammatory response to microbial challenge (Palmer et al. 2005). Plausible pathogenomic mechanisms include impaired neutrophil function, decreased lymphocyte proliferation and IgG production, altered release of cytokines (Al-Shammari et al. 2001, Orbak et al. 2003, Persson et al. 2003, Apatzidou et al. 2005), reduced revascularization, and decreased fibroblast proliferation, attachment and collagen synthesis (Gamal & Bayomy 2002, Mavropoulos et al. 2007, Semlali et al. 2011). Smokers also harbour increased levels of putative periodontal pathogens compared with non-smokers (Haffajee & Socransky 2001b, Van Winkelhoff et al. 2001, Guglielmetti et al. 2014, Joshi et al. 2014). Interestingly, the periodontal microbiota in smokers may return to normal within 6–12 months following smoking cessation (Fullmer et al. 2009).

The compromising effect of cigarette smoking on periodontal therapy appears dose-dependent (Kaldahl et al. 1996, Rieder et al. 2004). Thus, an objective estimation of smoking status emerges as an important prerequisite to identify and assess any harmful effects of smoking on the periodontium at a patient, tooth, and site level (Scott et al. 2001, Kotsakis et al. 2015). The use of self-reported smoking data is prone to bias in individuals who often are unwilling to disclose their smoking status. Therefore, self-reported smoking status needs to be objectively validated.

Besides the systemic effect, smoking may also exert local effects. Palatal sites and molar teeth seem to be more susceptible to advanced attachment loss throughout disease progression (Haffajee & Socransky 2001a). To predict the outcome of periodontal therapy in smokers, the effect of smoking needs to be explored at patient, tooth, and site

level. As variations in periodontal treatment outcomes to a great extent are explained by factors acting at a site level (D'Aiuto et al. 2005, Kim et al. 2007), the application of statistical models analysing sites, taking the clustering of data over teeth and patients into account, appears appealing. Focusing on the effects at particular sites may provide a more accurate explanation of the natural hierarchical structure of the treatment responses following periodontal therapy.

There seem to be no prospective studies evaluating the effect of cigarette smoking on the outcomes of non-surgical and surgical periodontal treatment at site level in chronic periodontitis patients, corrected for clustered observations. The overall purpose of this study was to compare the initial periodontal treatment outcome in smokers and non-smokers. More specifically, the aims were to evaluate the effect of smoking at patient, tooth, and site level following non-surgical and surgical periodontal therapy and to compare differences in the composition of the subgingival microflora during treatment at the patient level.

## Material and Methods

### Pre-study protocols and tests

The study protocol and informed consent approved by the Institutional Medical Research Ethics Committee (2011/151-6), University of Bergen, Norway followed the Helsinki Declaration of 1975, version 2008. Prior to inclusion, all patients read and signed a written consent form.

### Intra-examiner calibration

A calibration exercise was performed to obtain intra-examiner reproducibility for the clinical outcome variable probing depth (PD) and clinical attachment level (CAL). In a sample of 10 subjects, PD and CAL were measured twice, 1 day apart, at six sites per tooth and the intraclass correlation coefficients (ICC) were calculated separately for each site. The ICC ranged between 0.92 and 0.96 for PD and between 0.93 and 0.96 for CAL.

### Sample size

The sample size estimation was based on change in PD. A difference of 0.5 mm from T0 to T1 was considered clinically relevant. Standard deviation of the differences between repeated PD measurements from the intra-calibration exercise was 0.5 mm. A power analysis based on 40 subjects per group and with the level of significance ( $\alpha$ ) set to 0.05, gave an 88% power to detect a true difference of 0.5 mm.

### Blinding of the operator

The clinical examiner (DFB) was tested towards the smoking status of a sample of 30 chronic periodontitis patients, 16 smokers (>10 cigarettes/day for at least 5 years) and 14 non-smokers (never or not in the last 5 years). Calculus, plaque, and staining were removed and after rinsing with 0.2% chlorhexidine gluconate (Corsodyl, GlaxoSmithKline, London, UK) for 1 min, the examiner, wearing a face mask, scored the smoking status as yes or no. Twenty-eight patients (93%) were correctly identified as either non-smokers or smokers ( $p < 0.001$ ).

### Study group

Eighty patients, 40 smokers and 40 non-smokers, with moderate to severe chronic periodontitis (Armitage 1999) referred for periodontal treatment from general practitioners in a rural district of Norway, were enrolled in this single-arm clinical trial March 2012 through September 2013 (Table 1). A detailed medical, dental, periodontal, and smoking history for the patients was obtained from clinical examinations (including weight and height registrations), health forms, questionnaires, and by consulting their physicians. Furthermore, they were examined for eligibility and consecutively invited to participate.

The inclusion criteria were healthy subjects between 35 and 75 years, with no medication that could affect periodontal healing, having at least four non-adjacent teeth with inter-proximal PD  $\geq 6$  mm and clinical attachment loss  $\geq 5$  mm, bleeding on probing (BoP), and no signs of apical pathology

Table 1. Baseline (T0) patient characteristics by smoking status. Frequency/distribution of participants by sociodemographic and clinical characteristics, and smoking status.  $n = 80$

	Smokers, $n = 40$	Non-smokers, $n = 40$	$p$ -level
Age (n/%)			
<60 years	19/47.5	18/45	0.727
≥60 years	21/52.5	22/55	
Gender (n/%)			
Male	15/37.5	23/57.5	0.121
Female	25/62.5	17/41.5	
Marital status (n/%)			
Married or cohabitant	24/60.0	35/87.2	0.011
Single	16/40.0	5/12.8	
Income (n/%)			
Yes	18/45.0	28/70.0	0.069
No	22/55.0	12/30.0	
Education (n/%)			
≤9 years	30/75.0	20/50.0	0.025
>9 years	10/25.0	20/50.0	
Satisfaction with oral health (n/%)			
Content	10/25.6	14/35.9	0.368
Neither discontent or content, or discontent	28/74.4	25/64.1	
BMI (mean ± SD)	24.3 (4.1)	25.7 (2.9)	0.112
Number of teeth present (mean ± SD)	23.4 (5.2)	25.1 (2.9)	0.069
BI (mean ± SD)	66.7 (18.2)	67.3 (15.7)	0.865
PI (mean ± SD)	54.6 (21.9)	57.1 (20.3)	0.610
PD (mean ± SD)	3.8 (1.5)	3.4 (1.4)	0.028
CAL (mean ± SD)	4.6 (1.8)	4.0 (1.5)	<0.001

BMI, body mass index; BI, bleeding index; PI, plaque index; PD, probing depth; CAL, clinical attachment level.

(Tonetti & Claffey 2005, Page & Eke 2007). The subjects were either smokers (>10 cigarettes/day for at least 5 years) or non-smokers (never or not in the last 5 years). Exclusion criteria were any current medical condition affecting periodontal treatment and the use of systemic antibiotics or subgingival scaling in the 6 months before initiation of the study.

#### Smoking status

The subjectively reported smoking status was calculated in pack years; the number of cigarettes smoked daily multiplied by the number of years divided by 20 (a standard pack of cigarettes) (Scott et al. 2001). Before treatment, smoking status was objectively validated by measuring cotinine levels in serum. Peripheral venous blood was collected from each participant in a glass vacutainer. After coagulation, blood was centrifuged (1000 rpm/10 min) and the serum was stored in aliquots at  $-80^{\circ}\text{C}$ . Serum cotinine was assessed according to the instructions of the serum enzyme immunoassay kit (Cotinine ELISA Kit, MyBioSource,

San Diego, USA) by measuring the absorbance at 450 nm with a microplate reader (FluoStar Optima V1.32 R2, BMG Labtech, Offenburg, Germany).

#### Treatment

Non-surgical and surgical periodontal treatment was performed by one operator (DFB). All patients were subjected to non-surgical treatment consisting of motivation and instruction in oral hygiene and debridement using hand instrumentation (Hu-Friedy, Chicago, IL, USA; and American Eagle Instruments, Missoula, MT, USA) under local anaesthesia. Teeth with hopeless prognosis were extracted during the non-surgical treatment phase (McGuire 1991). Each treatment session lasted 60–90 min and mean number of treatment sessions was 5.5 for smokers and 5.0 for non-smokers. The smokers were motivated for smoking cessation and encouraged to participate in a public smoking cessation program (Røyketelefonen, Helsedirektoratet, Oslo, Norway). Sixteen patients (40%) accepted. After a healing period of 8 weeks, re-evaluation was per-

formed (Segelnick & Weinberg 2006). To further reduce PD and inflammation in patients presenting PD >5 mm with BoP and exhibiting adequate oral hygiene routines, periodontal surgery was implemented. Sixty-five patients, 35 smokers and 30 non-smokers, received periodontal surgery (Fig. 1). Mean number of surgeries per patient was 2.0 for smokers and 1.8 for non-smokers. Both periodontal flap and gingivectomy techniques were used following standard protocols. Sutures and periodontal dressings were removed at 7–10 days. A 0.2% chlorhexidine gluconate rinse (Corsodyl, GlaxoSmithKline, London, UK) was implemented for 4 weeks postsurgery. Postsurgical controls, including full mouth plaque removal and oral hygiene instruction, were conducted every second or third week until clinical evaluation at 12 weeks.

#### Clinical assessment

Before clinical examination, a full mouth series of intra-oral radiographs was taken. Clinical measurements were registered at baseline pre-treatment (T0) and at 3 months post-treatment (T1). PD was recorded as the distance in mm from the gingival margin to the probeable base of the pocket, and CAL as the distance in mm from the cemento-enamel junction or the margin of a dental restoration to the probeable base of the pocket. PD and CAL were measured with a manual periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA) at six sites per tooth rounding up to the nearest mm. Full mouth gingival bleeding was recorded as the percentage of sites showing bleeding after gentle probing (Ainamo & Bay 1975) and full mouth dental plaque as the percentage of tooth surfaces with visible plaque following staining with disclosing solution (O'Leary et al. 1972). As a supplement to staining, the periodontal probe was used to discriminate between plaque and pellicle.

#### Microbiological assessment

At T0, two sterile paper points were inserted into the deepest periodontal pocket in each patient, and the procedure was repeated at the same site at T1. Before sampling, the site was carefully cleaned of supragingival

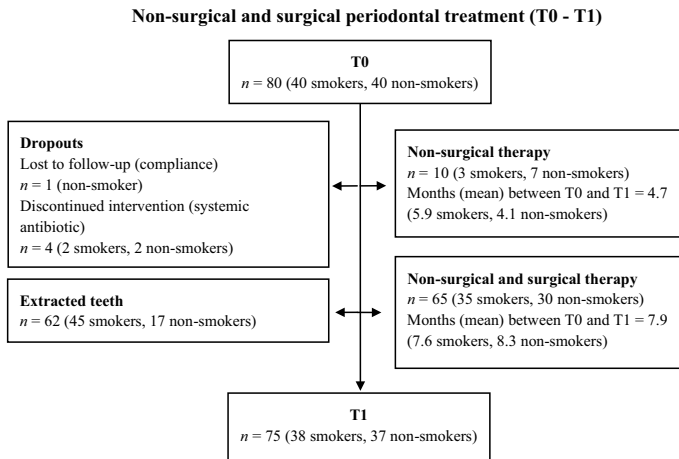


Fig. 1. Study flow chart.

plaque and kept dry. The paper points were gently inserted towards the apex of the pocket and kept in place for 20 sec (Renvert et al. 1992, Belibasakis et al. 2014) before removal and immersion into a pre-reduced sterile transport medium (PRAS Dental Transport Medium, Morgan Hill, CA, USA). The sample tubes were analysed at Microbiological Diagnostic Service, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway by DNA-DNA hybridization (checkerboard technique) (Socransky et al. 2004). The results were reported separately for each sample, showing both qualitative and quantitative results. Analysis included detection of red (*Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*) and orange (*Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum subsp polymorphum*, *Fusobacterium nucleatum subsp nucleatum*, *Fusobacterium nucleatum subsp vincentii*, and *Parvimonas micra*) complex species, and *Aggregatibacter actinomycetemcomitans* (Socransky et al. 1998).

#### Statistical analysis

Normality assumptions of the continuous variables were performed using the skewness and kurtosis test. Descriptive statistics were executed using frequencies and percentage for qualitative variables (chi-square test) and mean  $\pm$  standard deviation for

quantitative variables (ordinary two sample *t*-test and Mann-Whitney test).

PD  $\geq 5$  mm with BoP, defined as the primary outcome variable, was dichotomized as (1) present and (0) absent. In the logistic regressions each site, corrected for clustering of the data within teeth and patients, was the unit of the analysis. Patient-related explanatory variables were tested in unadjusted models and in a multiple model adjusted for covariates. In the analysis, time was categorized as T0 (0) and T1 (1), age as  $<60$  years (0) and  $\geq 60$  years (1), gender as male (0) and female (1), self-reported education as  $\geq 9$  years (0) and  $<9$  years (1), marital status as living alone (0) and married/cohabitant (1), and number of teeth at T0  $< 15$  teeth (0) and  $\geq 15$  teeth (1). For each patient an overall mean value for PD, CAL, BI, and PI was calculated at T0. This measure was applied to adjust for heterogeneity at T0. Sites presenting PD  $\geq 5$  mm with BoP at teeth extracted between T0 and T1 were not included in the analysis. Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated.

Secondary outcome variables, changes in plaque index (PI), bleeding index (BI), PD, and amount of bacteria, were analysed by conventional regression analysis, corrected for clustered observations. A *p*-value of less than 0.05 was considered statistically significant. All anal-

yses were conducted using Stata version 13 (Stata Corp., College Station, TX, USA).

#### Results

Patient characteristics are summarized in Table 1. Eighty patients were included; 40 smokers [mean age 57.6 years (range 37–70)] and 40 non-smokers [mean age 58.7 years (range 35–73)]. Seventy-five patients (94%) completed the study. During treatment, significantly more teeth with hopeless prognosis were extracted in the smoking group ( $p = 0.009$ ); 37 teeth in 16 smokers and 11 in nine non-smokers. In both groups mean PD, plaque, and bleeding index were significantly reduced ( $p < 0.001$ ) with no differences between the groups at T0 or T1. In smokers, mean PD was reduced from 3.8 to 2.6 mm (1.2 mm) and in non-smokers from 3.4 to 2.3 mm (1.1 mm). Figure 2 presents mean percentage of sites showing PD reduction of one mm or more between T0 and T1 for each PD category for smokers and non-smokers. Compared with non-smokers, smokers demonstrated between 5% and 8% less number of sites with mm reduction for PD categories between four and nine mm.

The distribution of PD  $\geq 5$  mm with BoP, at T0 and T1 is summarized in Table 2. At T0, smokers presented with 1471 (26%) and non-smokers with 1049 (18%) sites with PD  $\geq 5$  mm and BoP. The numbers decreased to 132 (3%) sites for smokers and 52 (1%) sites for non-smokers at T1. At the patient level, the mean number of sites with PD  $\geq 5$  mm and BoP per smoker was 36.8 (26%) and 26.3 (18%) per non-smoker at T0 (not tabulated). The corresponding estimates at T1 were 3.47 (3%) and 1.41 (1%). At T1 no patients in the non-smoking group presented with more than seven sites with PD  $\geq 5$  mm with BoP, whereas five smokers exhibited 10 or more sites. These five patients were all heavy smokers with a mean cotinine level of 725 ng/ml (range 501–861). Mean cigarette consumption in the smoking group was 37 pack years (20–108) and mean cotinine level 471 ng/ml (range 168–861). No patients reported starting or quitting smoking during the study.

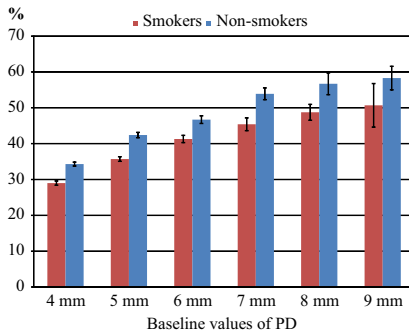


Fig. 2. Mean percentage of sites showing probing depth reduction of one mm or more between T0 and T1 for each probing depth category.

Between T0 and T1 a higher number of sites with PD  $\geq 5$  mm with BoP was removed by tooth extraction in the smoking compared with the non-smoking group ( $p = 0.002$ ); 177 and 46 sites, respectively. These sites were excluded from the analysis. Compared with non-smokers, an overall significantly higher risk was found in smokers to present with PD  $\geq 5$  mm with BoP at T1 [OR = 2.01, CI: 1.24, 3.23,  $p = 0.004$  (not tabulated)]. The adjusted analysis withstands the significant association with smoking and PD  $\geq 5$  mm with BoP at T1

[OR = 1.90, CI: 1.14, 3.15,  $p = 0.013$  (not tabulated)]. Results of unadjusted and adjusted logistic regression analysis of PD  $\geq 5$  mm with BoP are presented in Table 3. For both smokers and non-smokers the unadjusted analysis showed significant associations between PD  $\geq 5$  mm with BoP and mean T0 values of PD, CAL, and BI. For smokers, a significant association was shown for mean number of teeth at T0. For both groups the adjusted analysis revealed significant associations between PD  $\geq 5$  mm with BoP and mean baseline values

of PD, BI, and number of teeth. For smokers only, CAL at T0 and not living alone showed significant associations with the primary outcome.

Plaque-positive and plaque-negative sites were analysed for the association of PD  $\geq 5$  mm with BoP in the adjusted model, with plaque-negative sites in non-smokers as reference category. Plaque-positive sites in smokers had an overall higher risk to present with PD  $\geq 5$  mm with BoP at T1 compared with plaque-positive sites in non-smokers [4.14, CI:2.16, 7.96,  $p < 0.001$  and OR:3.09, CI:1.65, 5.79,  $p < 0.001$ , respectively (not tabulated)]. Further, within arch, teeth, and site, a highly significant effect of plaque was found with an interaction between plaque and smoking (Table 4). Presence of plaque more than doubled the risk of having PD  $\geq 5$  mm with BoP in smokers compared with non-smokers (OR= 4.98, CI: 2.50, 9.93,  $p < 0.001$  and OR = 2.40, CI: 1.09, 5.30,  $p = 0.030$ , respectively) at maxillary molar palatal sites.

Figure 3 shows number of patients harbouring target microbial species at T0 and T1. Mean sample site PDs did not differ between smokers and non-smokers at T0 (7.4 mm) and T1 (3.7 mm). No significant

Table 2. Numbers of PD  $\geq 5$  mm with BoP by smoking status before (T0) and following periodontal treatment (T1) at arch and tooth level

Localization within arch, tooth and site	T0			T1		
	Smokers n (%)	Non-smokers n (%)	<i>p</i>	Smokers n (%)	Non-smokers n (%)	<i>p</i>
Overall	1471 (26.4)	1049 (17.5)	<0.001	132 (2.6)	52 (1.0)	<0.001
Maxilla	894 (32.9)	638 (20.9)	<0.001	78 (3.2)	32 (1.2)	<0.001
Buccal	345 (26.4)	260 (17.1)	0.001	26 (2.1)	9 (0.7)	0.002
Palatal	549 (40.4)	378 (24.8)	<0.001	52 (4.3)	23 (1.7)	0.001
Multi-rooted	306 (46.4)	334 (40.1)	0.159	37 (7.1)	22 (3.1)	0.017
Buccal	131 (39.7)	136 (32.6)	0.105	10 (3.8)	7 (2.0)	0.209
Palatal	175 (53.0)	198 (47.5)	0.309	27 (10.2)	15 (4.2)	0.009
Single-rooted	588 (28.5)	304 (13.7)	0.110	41 (2.1)	10 (0.8)	0.045
Buccal	214 (20.8)	124 (11.2)	0.680	16 (1.7)	2 (0.2)	0.121
Palatal	374 (46.4)	180 (16.2)	0.030	25 (2.6)	8 (0.8)	0.159
Mandibula	577 (20.3)	411 (13.9)	0.009	54 (2.1)	20 (0.7)	0.013
Buccal	253 (17.8)	181 (12.3)	0.027	23 (1.7)	9 (0.7)	0.029
Palatal	324 (22.7)	230 (15.6)	0.009	31 (2.4)	11 (0.8)	0.028
Multi-rooted	236 (35.8)	211 (30.6)	0.236	21 (3.5)	16 (2.6)	0.538
Buccal	99 (30.0)	86 (24.9)	0.269	9 (3.0)	7 (2.3)	0.617
Lingual	137 (41.5)	125 (36.2)	0.326	12 (4.0)	9 (2.9)	0.561
Single-rooted	341 (15.6)	200 (8.9)	0.013	33 (1.6)	4 (0.4)	0.191
Buccal	154 (14.1)	95 (8.4)	0.148	14 (1.4)	2 (0.2)	0.628
Lingual	187 (17.1)	105 (9.3)	0.011	19 (1.9)	2 (0.2)	0.190

BoP; bleeding on probing; multi-rooted, molars; single-rooted, premolars and incisors; buccal, two proximal-buccal and one mid-buccal; palatal, two proximal-palatal and one mid-palatal; lingual, two proximal-lingual and one mid-lingual.

Table 3. Unadjusted and adjusted OR for PD  $\geq$  5 mm with BoP, stratified by smoking status

Characteristics	Smokers						Non-smokers					
	Unadjusted*			Adjusted†			Unadjusted*			Adjusted†		
	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>	OR	95% CI	<i>p</i>
Time												
T0	1			1			1			1		
T1	0.17	0.13, 0.22	<0.001	0.086	0.06, 0.12	<0.001	0.085	0.06, 0.13	<0.001	0.041	0.03, 0.06	<0.001
Age												
<60 years	1			1			1			1		
$\geq$ 60 years	0.93	0.62, 1.39	0.710	1.00	0.99, 1.01	0.350	0.75	0.48, 1.16	0.196	0.98	0.98, 0.99	<0.001
Gender												
Male	1			1			1			1		
Female	0.97	0.63, 1.50	0.904	0.88	0.74, 1.05	0.154	1.33	0.90, 1.98	0.152	1.03	0.84, 1.27	0.754
Education												
$\geq$ 9 years	1			1			1			1		
<9 years	1.30	0.86, 2.00	0.208	1.07	0.89, 1.29	0.488	1.35	0.94, 1.95	0.106	0.87	0.71, 1.05	0.150
Marital status												
Single	1			1			1			1		
Co-habitant	1.04	0.70, 1.55	0.855	0.84	0.71, 0.98	0.027	1.40	0.88, 2.22	0.151	0.90	0.63, 1.28	0.545
Teeth at T0												
<15 teeth	1			1			1			1		
$\geq$ 15 teeth	0.95	0.92, 0.99	0.015	1.07	1.03, 1.10	<0.001	0.99	0.94, 1.05	0.806	0.96	0.94, 0.98	<0.001
PD, per mm increase	3.10	2.70, 3.56	<0.001	3.54	2.95, 4.24	<0.001	3.42	2.98, 3.92	<0.001	4.36	3.74, 5.09	<0.001
CAL, per mm increase	1.78	1.60, 1.99	<0.001	1.14	1.04, 1.26	0.007	2.17	1.93, 2.42	<0.001	1.02	0.87, 1.20	0.761
BI, per 10% increase	1.15	1.03, 1.27	0.009	1.09	1.05, 1.13	<0.001	1.29	1.16, 1.44	<0.001	1.14	1.07, 1.22	<0.001
PI, per 10% increase	1.07	0.96, 1.19	0.225	1.05	0.99, 1.10	0.083	1.10	1.00, 1.21	0.062	0.97	0.94, 1.01	0.115

BoP, bleeding on probing; CI, Confidence Interval; OR, Odds Ratio; PD, probing depth; CAL, clinical attachment level; BI, bleeding index; PI, plaque index.

\*Unadjusted logistic regression for PD  $\geq$  5 mm with BoP.

†Adjusted logistic regression for PD  $\geq$  5 mm with BoP, and time, age, gender, education, marital status, teeth at T0, and mean values of PD, CAL, BI, and PI at T0. Sites with PD  $\geq$  5 mm with BoP extracted between T0 and T1 were not included in the analysis.

quantitative reduction was observed for the red ( $p = 0.35$ ) and orange ( $p = 0.16$ ) complex species in the smoking group (not tabulated). Nevertheless, a significant reduction of *T. forsythia* ( $p = 0.038$ ), *P. nigrescens* ( $p = 0.035$ ), and *F. nucleatum subsp vincentii* ( $p < 0.001$ ) was detected. Among non-smokers a significant reduction was observed for the red complex species ( $p = 0.010$ ), specifically for *P. gingivalis* ( $p = 0.013$ ) and *T. forsythia* ( $p = 0.005$ ). The orange complex species showed a borderline significant reduction ( $p = 0.060$ ) with a significant reduction of *P. intermedia* ( $p = 0.008$ ), *P. nigrescens* ( $p = 0.004$ ), and *F. nucleatum subsp polymorphum* ( $p = 0.035$ ). However, differences detected comparing single species were considered inconclusive due to multiple testing.

## Discussion

This prospective study appears to be the first to compare the effect of non-surgical and surgical periodontal therapy in smokers and non-smokers using clinical and microbiological

parameters and an objective validation of self-reported smoking habits. In both the smoking and non-smoking groups, PD, BI, and PI improved significantly following treatment. PD categories between 4 and 8 mm showed a less reduction in smokers compared with non-smokers. In perspective, Tomasi reported a 30% reduction in pocket closure in smokers following non-surgical periodontal therapy with a more limited effect in initially deep pockets (Tomasi et al. 2007). The present study using the same PD categories confirms a less reduction in initially deep pockets in smokers following non-surgical and surgical treatment. The relatively high number of patients receiving periodontal surgery was due to initially deep PDs in patients exhibiting adequate oral hygiene standards and good general health. Cigarette smoking is not a contraindication to periodontal surgery, but high cigarette consumption is considered a risk factor generating less favourable clinical outcomes (Matuliene et al. 2008). The negative effects of cigarette smoking increase

in a non-linear consumption model and patients consuming  $\geq 20$  cigarettes per day are considered at high risk for treatment relapse (Lang & Tonetti 2003).

In the present study, the five patients presenting with 10 or more sites with PD  $\geq 5$  mm with BoP following treatment were all heavy smokers with a mean serum cotinine level of 725 ng/ml. The discrepancy between measured cotinine level and subjectively reported cigarette consumption was more pronounced for heavy smokers, indicating a higher underreporting. A socioeconomic stigma of smokers and pressure towards smoking cessation, likely make smokers susceptible to underreporting their smoking habits (Scott et al. 2001, Stuber & Galea 2009). This should be a concern for clinicians in the everyday treatment planning and in projecting prognosis for teeth at risk in smokers.

The causality of cigarette smoking on the outcomes of periodontal treatment must be interpreted with caution as smokers tend to present with more advanced periodontitis



Table 4. Relative risks for PD  $\geq$  5 mm with BoP at arch, tooth, and site levels, with and without cigarette smoking and presence of plaque following periodontal treatment. Reference category: plaque-negative sites in non-smokers

Localization	Smokers plaque-negative sites			Non-smokers plaque-positive sites			Smokers plaque-positive sites		
	OR	CI	<i>p</i>	OR	CI	<i>p</i>	OR	CI	<i>p</i>
Maxilla	1.10	0.94, 1.29	0.224	2.21	1.22, 4.00	0.009	3.16	1.65, 5.90	<0.001
Mandibula	1.03	0.82, 1.30	0.783	7.12	2.47, 20, 52	<0.001	8.75	3.02, 25.33	<0.001
Multi-rooted	1.15	0.98, 1.37	0.097	2.92	1.51, 5.67	0.002	4.13	2.17, 7.87	<0.001
Single-rooted	1.01	0.81, 1.26	0.946	2.59	0.91, 7.38	0.075	3.96	1.51, 10.33	0.005
Buccal	1.04	0.85, 1.26	0.730	2.09	1.05, 4.18	0.037	3.07	1.47, 6.40	0.003
Palatal/lingual	1.08	0.91, 1.29	0.371	4.27	2.13, 8.56	<0.001	5.24	2.67, 10.30	<0.001
Teeth within arch									
Maxilla									
Multi-rooted	1.25	1.02, 1.53	0.029	1.64	0.84, 3.19	0.148	3.15	1.53, 6.49	0.002
Single-rooted	1.01	0.79, 1.29	0.921	2.87	0.99, 1.29	0.053	3.27	1.25, 8.51	<0.001
Mandibula									
Multi-rooted	1.05	0.83, 1.32	0.712	12.64	4.12, 38.26	<0.001	10.87	4.18, 28.26	<0.001
Single-rooted	1.00	0.71, 1.39	0.996	2.04	0.44, 9.41	0.361	5.50	1.37, 22.00	0.016
Sites within arch									
Maxilla									
Buccal	0.99	0.80, 1.22	0.920	1.17	0.58, 2.36	0.665	2.00	0.89, 4.49	0.095
Palatal	1.21	0.99, 1.47	0.066	3.59	1.80, 7.14	<0.001	4.45	2.38, 8.33	<0.001
Mandibula									
Buccal	1.15	0.82, 1.59	0.420	6.46	2.26, 18.53	0.001	7.79	2.54, 23.85	<0.001
Lingual	0.97	0.71, 1.33	0.844	7.66	2.19, 26.83	0.001	9.37	2.84, 30.97	<0.001
Sites within multi-rooted teeth									
Maxilla									
Buccal	1.31	1.01, 1.72	0.045	0.98	0.45, 2.13	0.964	1.54	0.50, 4.79	0.456
Maxilla									
Palatal	1.21	0.92, 1.58	0.180	2.40	1.09, 5.30	0.030	4.98	2.50, 9.93	<0.001
Mandibula									
Buccal	1.16	0.76, 1.77	0.503	11.41	3.04, 42.77	<0.001	9.88	3.01, 32.39	<0.001
Mandibula									
Lingual	0.96	0.70, 1.33	0.823	15.10	4.60, 49.61	<0.001	12.69	4.27, 37.73	<0.001
Sites within single-rooted teeth									
Maxilla									
Buccal	0.85	0.61, 1.19	0.337	1.17	0.26, 5.17	0.840	2.85	0.87, 9.32	0.083
Maxilla									
Palatal	1.13	0.86, 1.48	0.390	5.05	1.47, 17.34	0.010	3.56	1.31, 9.67	0.013
Mandibula									
Buccal	1.08	0.72, 1.61	0.709	2.44	0.48, 12.54	0.284	5.26	1.10, 25.23	0.038
Mandibula									
Lingual	0.96	0.61, 1.51	0.871	1.78	0.31, 10.42	0.521	5.17	1.23, 21.58	0.024

BoP, bleeding on probing; CI, confidence interval; OR, odds ratio; multi-rooted, molars; single-rooted, premolars and incisors; palatal, mesio-palatal and palatal and disto-palatal; lingual, mesio-ling and lingual and disto-lingual; buccal, mesio-buccal and buccal and disto-buccal. Logistic regression with outcome PD  $\geq$  5 mm with BoP adjusted for time, age, gender, education, marital status, mean PD, CAL, BI, BI, and number of teeth present at T0. Sites with PD  $\geq$  5 mm with BoP extracted between T0 and T1 were not included in the analysis.

(Hugoson & Rolandsson 2011). In this study, an association between smoking and PD  $\geq$  5 mm with BoP was shown both with and without baseline adjustments. This observation is consistent with a systematic review on the influence of cigarette smoking on the effect of non-surgical therapy (Labriola et al. 2005). The negative effect of cigarette smoking was shown by including BoP in the primary outcome, although smokers tend to have scarcer bleeding from deeper PDs than non-smokers (Preber & Bergström 1985). Excluding BoP might positively influence the primary outcome

in non-smokers by deeper probe penetration into the inflamed soft tissue at T0 and a more pronounced shrinkage of gingiva during resolution of the inflammation (Biddle et al. 2001). Further, BoP is considered an indicator for disease progression in high-risk patients at site level and absence of BoP indicates a lower risk for disease progression in both smokers and non-smokers (Lang et al. 1990, Claffey & Egelberg 1995).

A significant association was detected between PD  $\geq$  5 mm with BoP and presence of plaque, in smokers as well as non-smokers.

This association was highly significant for smokers and particularly pronounced for maxillary molar palatal sites. These sites are immediately exposed to cigarette smoke and thereby to nicotine and combustion products. Binding of nicotine and tar to root surfaces and a 300 times higher concentration of cotinine in GCF compared with plasma, are proposed causative factors for impaired treatment response (Cuff et al. 1989, Ryder et al. 1998, Wan et al. 2009). Further, plaque control being generally demanding in the posterior dentition may increase the probability of cigarette smoke aggra-

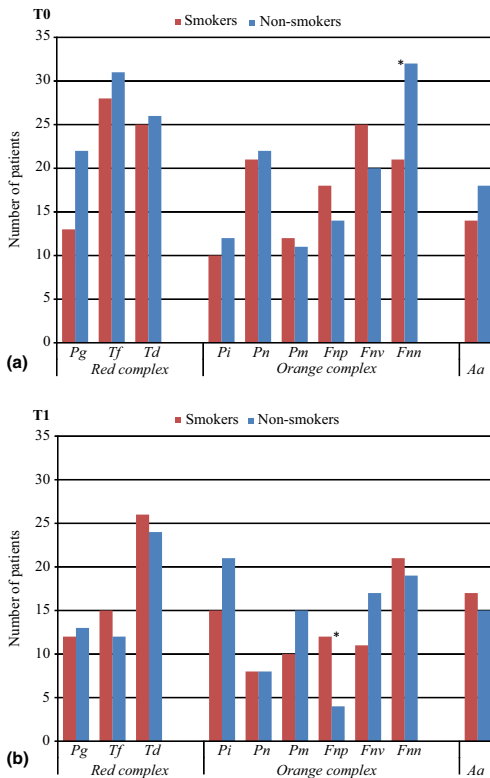


Fig. 3. (a) Number of patients harbouring different pathogens at baseline. (b) Number of patients harbouring different pathogens following non-surgical and surgical periodontal therapy. \*Statistical significance between the groups.

vating a plaque-induced inflammatory process.

An increased presence of red and orange complex species was found in smokers compared with non-smokers at T0 and T1. The non-significant reduced counts in the red and the orange complex species, especially *P. gingivalis* and *T. denticola* in smokers, are in agreement with previous reports (Grossi et al. 1997, Haffajee et al. 1997). In the present study, all smokers maintaining elevated red complex bacterial counts at T1 were heavy smokers. In perspective, early dysbiosis in subgingival plaque colonization is influenced by cigarette smoke and in a dose responding manner (Hutcherson et al. 2015). *P. gingivalis* has a potential to enhance early plaque

formation in smokers (Bagaitkar et al. 2011, Zeller et al. 2014). Early microbial colonization and poor correlation between the marginal and subgingival ecosystems in smokers might further impair resolution of inflammation during treatment (Joshi et al. 2014). As plaque formation adapts to cigarette smoke and the alterations are reversed when removing the cigarette stimulus, it is critical to avoid smoking exposure during periodontal therapy. The ability of the microbiota to adapt to tobacco exposure should encourage further multilevel investigation of clinical and microbiological effects of smoking cessation or reduction during periodontal treatment.

We acknowledge that the lack of blinding might be a limiting factor in

the present study. An attempt to blind the operator with regard to smoking status was unsuccessful. To reduce bias, all data plotting was performed by a person unaware of clinical registrations and smoking status. Moreover, personnel conducting the microbiological analysis were blinded to the smoking status of the patients. To reduce the risk of treatment variation, all patients were treated by one operator. The high-level oral hygiene standards achieved might be influenced by the Hawthorne effect, as participants were aware of being part of the study.

In conclusion, within limitations of the study, smokers show less favourable treatment response to non-surgical and surgical periodontal therapy in terms of residual PD  $\geq 5$  mm with BoP, reduced counts of red and orange complex bacterial species, and PD reduction. Elevated smoking exposure negatively influenced the number of PD  $\geq 5$  mm with BoP and the microbial counts. Correcting for clustered observations within patients and teeth revealed an increased risk for PD  $\geq 5$  mm with BoP at plaque-positive sites in smokers. Collectively, the results demonstrate a site-specific tissue response in smokers following initial periodontal therapy superimposed on a systemic effect.

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### Clinical Relevance

*Scientific rationale for the study:* Generally, smokers respond less favourable compared with non-smokers to non-surgical and surgical periodontal therapy. To predict the outcome of periodontal therapy in smokers, the effect of smoking needs to be critically

evaluated at patient, tooth, and site level.

*Principal findings:* Smokers showed impaired clinical and microbiological responses to non-surgical and surgical periodontal therapy. At tooth and site level, odds ratios for having probing depth  $\geq 5$  mm with bleeding on probing following treatment were

higher for smokers in all locations, especially for plaque-positive sites. *Practical implications:* Clinicians should consider including smoking cessation as a vital component of treatment to optimize the effect of non-surgical and surgical periodontal therapy.

## Study II

### **Site-specific treatment outcome in smokers following 12 months of supportive periodontal therapy**

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# Site-specific treatment outcome in smokers following 12 months of supportive periodontal therapy

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Bunæs DF, Lie SA, Åstrøm AN, Mustafa K, Leknes KN. Site-specific treatment outcome in smokers following 12 months of supportive periodontal therapy. *J Clin Periodontol* 2016; 43: 1086–1093. doi: 10.1111/jcpe.12619.

## Abstract

**Aim:** To evaluate the effect of cigarette smoking on periodontal health at patient, tooth, and site levels following supportive therapy.

**Materials and Methods:** Eighty chronic periodontitis patients, 40 smokers and 40 non-smokers, were recruited to a single-arm clinical trial. Periodontal examinations were performed at baseline (T0), 3 months following active periodontal therapy (T1), and 12 months following supportive periodontal therapy (T2). Smoking status was validated measuring serum cotinine levels. Probing depth (PD)  $\geq 5$  mm with bleeding on probing (BoP) was defined as the primary outcome. Logistic regression analyses adjusted for clustered observations of patients, teeth, and sites and mixed effects models were employed to analyse the data.

**Results:** All clinical parameters improved from T0 to T2 ( $p < 0.001$ ), whereas PD, bleeding index (BI), and plaque index (PI) increased from T1 to T2 in smokers and non-smokers ( $p < 0.001$ ). An overall negative effect of smoking was revealed at T2 (OR = 2.78, CI: 1.49, 5.18,  $p < 0.001$ ), with the most pronounced effect at maxillary single-rooted teeth (OR = 5.08, CI: 2.01, 12.78,  $p < 0.001$ ). At the patient level, less variation in treatment outcome was detected within smokers (ICC = 0.137) compared with non-smokers (ICC = 0.051).

**Conclusion:** Smoking has a negative effect on periodontal health following 12 months of supportive therapy, in particular at maxillary single-rooted teeth.

Key words: chronic periodontitis; multilevel analysis; periodontal therapy; smoking

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Active periodontal therapy (APT) followed by supportive periodontal therapy (SPT) has been demonstrated successful in a majority of patients (Axelsson & Lindhe 1981, Ramfjord 1987, Rosling et al. 2001, Axelsson et al. 2004). In perspective,

patients susceptible to recurrence of periodontal disease have been offered SPT at 3- to 4-month intervals with the intent to maintain treatment outcomes following APT, whereas less susceptible patients may be well served, using a less frequent SPT interval (Knowles et al. 1979, Lindhe & Nyman 1984). To facilitate identification of individuals at high risk for disease progression, a functional Periodontal Risk Assessment (PRA) diagram has been proposed (Lang & Tonetti 2003). In a longitudinal study validating the PRA

model, patients allocated to the high-risk category following APT showed a higher incidence of tooth loss compared with moderate- or low-risk patients (Eickholz et al. 2008).

In spite of clinical benefits, only a minority of patients appear to comply with recommended SPT regimens (Cecchi et al. 1994, Demetriou et al. 1995), and efforts to optimize compliance being only partly successful (Wilson et al. 1993). Further, it appears that SPT compliance decreases as the risk profile of the

## Conflict of interest and source of funding statement

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subject increases (Mendoza et al. 1991, Matuliene et al. 2010). Because of imperfect outcomes following APT (Bunæs et al. 2015) and inconsistent compliance (Matuliene et al. 2010, Ramseier et al. 2014), the selection of appropriate SPT intervals is of paramount importance for the maintenance of periodontal stability in cigarette smokers.

Smoking is a critical patient-related risk factor for chronic periodontitis and smokers exhibit fewer teeth and more advanced periodontal attachment loss compared with non-smokers (Kerdvongbudit & Wikesjo 2000, Calsina et al. 2002, Jansson & Lavstedt 2002). High cigarette consumption amplifies clinical manifestations of chronic periodontal disease and demands increased treatment needs (Dietrich et al. 2004, Susin et al. 2004, Do et al. 2008, Ramseier et al. 2015). Based on subjectively reported pack-year consumption, dose-dependent impaired clinical outcomes following SPT have been reported (Kaldahl et al. 1996a). It is unclear to what extent treatment response is influenced by a cumulative impact of smoking over years or by the consumption during SPT. However, a positive effect of smoking cessation on periodontal treatment outcomes may indicate the effect of present smoking exposure (Preshaw et al. 2005, Rosa et al. 2011).

Generally, optimal soft and hard tissue healing following APT is a critical point for successful treatment outcome. In a recently published study, Bunæs et al. (2015) reported impaired site-specific tissue responses to non-surgical and surgical APT in smokers compared with non-smokers. The multilevel approach using probing depth (PD) with bleeding on probing (BoP) as the primary outcome variable showed that plaque positive sites increased the risk for unfavourable treatment outcomes in smokers. A local additive detrimental effect of smoking is supported by studies reporting increased incidence of oral cancer and altered composition of the oral biofilm (Haffajee & Socransky 2001, Hashibe et al. 2007, Guglielmetti et al. 2014).

Longitudinal cohort studies have reported that smoking 20 or more cigarettes a day increased the risk of disease progression following APT

(Kaldahl et al. 1996a, Matuliene et al. 2008). In contrast, long-term follow-up studies have not found an association between smoking status and tooth loss (Fisher et al. 2008, Saminsky et al. 2015). These inconclusive findings indicate that the effect of subjectively reported smoking habits on the outcome of SPT needs to be addressed in a prospective study with an objective measure of smoking exposure.

To the best of our knowledge, there seems to be no prospective studies evaluating the patient, tooth, and site-related effects of cigarette smoking on the outcome of SPT in chronic periodontitis patients, using an objective measure of smoking status. Thus, the specific aims of this study were to determine the effect of smoking at patient, tooth, and site levels following 12 months of SPT and to compare the predictive value of clinical parameters for the outcome of SPT in smokers and non-smokers.

## Material and Methods

The study protocol and informed consent approved by the Institutional Medical Research Ethics Committee (2011/151-6), University of Bergen, Norway, followed the Helsinki Declaration of 1975, version 2008. Participating subjects read and signed the informed consent prior to inclusion in the study.

### Pre-study tests

Two pre-study exercises were performed. First, the intra-examiner (DFB) reproducibility was tested by measuring PD and clinical attachment levels (CAL) twice at six sites per tooth in 10 patients. Intra-class correlation coefficients (ICC) for repeated measures ranged between 0.92 and 0.96 for PD and between 0.93 and 0.96 for CAL. The sample size estimation was based on change in PD. A difference of 0.5 mm was considered clinically relevant. Standard deviation of the differences between repeated PD measurements from the intra-calibration amounted to 0.5 mm. A power analysis based on 40 subjects per group and with the level of significance ( $\alpha$ ) set to 0.05, gave an 88% power to detect a true difference of 0.5 mm. Second,

masking of the operator (DFB) towards smoking status was tested in 30 chronic periodontitis patients. Twenty-eight of 30 patients (93%) were correctly identified as smokers or non-smokers ( $p < 0.001$ ; for detail see Bunæs et al. 2015).

### Eligibility criteria, patient sample, and smoking status

Inclusion criteria were healthy subjects aged 35–75 years, none using medication that could affect periodontal healing, having at least four non-adjacent teeth with an interproximal PD  $\geq 6$  mm and clinical attachment loss  $\geq 5$  mm with BoP without signs of apical pathology (Tonetti & Claffey 2005, Page & Eke 2007). The patients were either smokers ( $>10$  cigarettes/day for at least 5 years) or non-smokers (never or not smoked within the last 5 years). Patients starting or discontinuing smoking during the study were not excluded. Exclusion criteria included any current medical condition affecting periodontal treatment, use of systemic antibiotics or subgingival scaling within 6 months prior initiation of the study, and delay of scheduled treatment visits by more than one month.

Eighty patients, 40 smokers and 40 non-smokers, with moderate to severe chronic periodontitis (Armitage 1999) referred for periodontal treatment from general practitioners in a rural district of Norway were consecutively enrolled in this single-arm clinical trial March 2012 through September 2013 (Table 1). Medical, periodontal, and smoking history of the patients was obtained from clinical examinations, health forms, questionnaires, and by consulting their physicians. All referred patients were examined for eligibility and consecutively invited to participate.

The subjectively reported smoking status was calculated in pack years; the number of cigarettes smoked daily multiplied by the number of years divided by 20 (a standard pack of cigarettes) (Scott et al. 2001). Before and at the end of the study, smoking status was objectively validated by measuring cotinine levels in serum. Peripheral venous blood was collected from each participant using a glass vacutainer. After coagulation, blood was centrifuged ( $700 \times g$  for 10 min.)



Table 1. Sociodemographic characteristics and health status in smokers and non-smokers at baseline (T0)

	Smokers n (%)	Non-smokers n (%)	<i>p</i>
≥60 years/<60 years	21/19 (52.5/47.5)	22/18 (55.0/45.0)	0.096
Male/female	15/25 (37.5/62.5)	23/17 (56.0/44.0)	0.121
Cohabitant/single	24/16 (60.0/40.0)	35/5 (87.2/12.8)	0.011
Elementary school/education beyond	30/10 (75.0/25.0)	20/20 (50.0/50.0)	0.025
Working/not working	18/22 (45.0/55.0)	28/12 (70.0/30.0)	0.069
Satisfaction/dissatisfaction with oral health	10/28 (26.3/73.7)	7/32 (18.0/82.1)	0.376
Alcohol consumption daily or weekly/ monthly or never	21/25 (58.3/41.7)	18/21 (46.2/53.9)	0.292
Dental visits regularly/irregularly	35/2 (94.6/5.4)	37/2 (94.9/5.1)	0.957

Students *t*-test and Chi-square: *p*-level <0.05.

and the serum was stored in aliquots at  $-80^{\circ}\text{C}$ . Serum cotinine was assessed according to the instructions of the serum enzyme immunoassay kit (Cotinine ELISA Kit; MyBioSource, San Diego, CA, USA) measuring the absorbance at 450 nm with a microplate reader (FluoStar Optima V1.32 R2; BMG Labtech, Offenburg, Germany).

#### Clinical assessments

A full-mouth intra-oral radiographs series was recorded before the clinical examination. Clinical recordings were collected at baseline pre-APT (T0), at 3 months post-APT (T1), and following 12 months of SPT (T2). PD was recorded as the distance from the gingival margin to the probeable base of the pocket, CAL as the distance from the cemento-enamel junction or the margin of a dental restoration to the probeable base of the pocket. PD and CAL were measured using a periodontal probe (PCPUNC 15; Hu-Friedy, Chicago, IL, USA) at six sites per tooth rounding up to the nearest mm. Full mouth gingival bleeding scores were recorded as the percentage of sites showing bleeding on gentle probing (Ainamo & Bay, 1975) and full mouth dental plaque scores as the percentage of tooth surfaces with visible plaque following staining with disclosing solution (O'Leary et al. 1972). As a supplement to staining, the periodontal probe was used to discriminate between plaque and pellicle.

#### Treatment

APT (T0-T1) and SPT (T1-T2) were performed by the same operator (DFB). APT included nonsurgical

and surgical periodontal therapy individualized to optimize treatment outcomes for each patient. Following ATP, a programme with regular appointments every three months was scheduled for SPT (Knowles et al. 1979, Lindhe et al. 1984). The 60-min appointments included re-motivation and re-instruction in oral hygiene, full mouth plaque removal, and supra- and subgingival debridement as needed. In addition, smokers were motivated to reduce or quit smoking, and encouraged to participate in a public smoking cessation programme (Røyketelefonen, Helsedirektoratet, Oslo, Norway). Mechanical debridement was carried out using conventional hand-instruments (Hu-Friedy, Chicago, IL, USA; and American Eagle Instruments, Missoula, MT, USA) and ultrasonic scalers (EMS, Nyon, Switzerland). For plaque removal, rotating rubber cups and glycine powder (EMS – Air Flow-Perio) in an air-polishing device (Dentsply Prophy-Jet<sup>®</sup>; Dentsply, York, PA, USA) were used.

#### Statistical analysis

The Shapiro–Wilk test was used to check for the assumption of normal distributed data. According to the test, the data were considered normally distributed. Means and standard deviations of secondary outcome variables (number of teeth, PD, CAL, BI, PI) were calculated and differences were tested, using the two sample *t*-test and Mann-Whitney test. Chi-square test was applied for testing of differences in frequencies and percentages between the categorical variables.

In an adjusted logistic regression model, gender was categorized as male (1) and female (0), age as ≥60 years (1) and <60 years (0), self-reported education as ≤9 years (1) and >9 years (0), and marital status as married/cohabitant (1) and living alone (0). The primary outcome variable PD ≥ 5 mm with BoP was dichotomized as (1) present and (0) absent. Each site, corrected for clustering of data within teeth and patients, was the unit of analysis. Sites presenting PD ≥ 5 mm with BoP at teeth extracted between T0 and T1 were not included in the analysis. Associations between PD ≥ 5 mm with BoP at T2 and clinical variables at T0 and T1 were tested using adjusted logistic regression analysis. Plaque positive sites categorized as (0) and plaque negative sites as (1), BoP positive sites as (0) and BoP negative sites as (1), and overall mean values calculated at T0 and T1 for PD and CAL were tested. For the smoking effect model following T1, specific teeth and sites were tested at T1 and T2. Two dummy variables were made for time and smoke and included in the adjusted model: (T2 = 1 and Smoke = 0) as (1) and (T2 = 1 and Smoke = 1) as (0) and (T1 = 1 and Smoke = 0) as (1) and T1 = 1 and Smoke = 1) as (0). Intra-class correlation coefficients (ICC) within patients, teeth, and sites were calculated using linear mixed effects models.

Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated. A *p*-value <0.05 was considered statistically significant. All analyses were conducted using Stata version 13 (Stata Corp., College Station, TX, USA).

#### Results

Eighty patients, 40 smokers (mean age 57.6 years, range 37–70 years) and 40 non-smokers (mean age 58.7 years, range 35–73 years), entered this study. Socio-demographic characteristics according to smoking status at baseline (T0) are summarized in Table 1. The experimental protocol started April 2012 to end March 2015. Thirty-six (90%) smokers and 36 (90%) non-smokers completed the study (Fig. 1). Drop-outs did not alter the socio-demographic characteristics at T1 and T2.

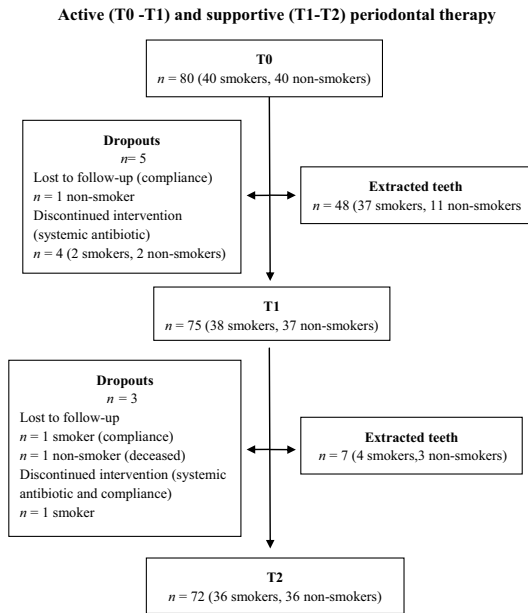


Fig. 1. Study flow chart.

Three (7.9%) smokers reported discontinuing smoking between T1 and T2, only one exhibited a cotinine level at T2 consistent with non-smokers (<10 ng/ml).

Compared with non-smokers, smokers presented significantly higher mean PD and CAL at all time-points (Table 2). Between T0 and T2 both groups responded favourable to periodontal therapy with significant reductions in mean PD, CAL, BI, and PI ( $p < 0.001$ ) (not tabulated). However, during SPT, from T1 to T2, mean PD, BI, and PI increased in both groups. In smokers mean PD increased from 2.63 to 2.80 mm ( $p = 0.007$ ) and in non-smokers from 2.27 to 2.42 mm

( $p = 0.002$ ), BI in smokers from 22.42 to 27.00 ( $p = 0.011$ ) and in non-smokers from 22.81 to 30.50 ( $p = 0.001$ ), and PI in smokers from 18.45 to 30.09 ( $p < 0.001$ ) and in non-smokers from 21.49 to 32.78 ( $p < 0.001$ ) (not tabulated). From T1 to T2, mean CAL did not change significantly in either smokers or non-smokers (not tabulated).

An overall distribution of PD  $\geq 5$  mm with BoP was 11.3% in smokers and 7.1% in non-smokers. In comparison, the corresponding percentages for PD  $\geq 5$  mm only were 14.8% in smokers and 8.3% in non-smokers. Compared with non-smokers, smokers had 4.2% more number of sites with PD  $\geq 5$  mm

with BoP compared with 6.5% more PD  $\geq 5$  mm. The number of sites with PD  $\geq 5$  mm with BoP at T0, T1, and T2 are summarized in Table 3. At T1, the total number in smokers were 132 (2.6%) and 52 (1.0%) in non-smokers ( $p < 0.001$ ), increasing at T2 to 180 (3.8%) in smokers and 79 (1.6%) in non-smokers ( $p < 0.001$ ). From T1 to T2, the increase was significant for all teeth and sites in smokers and non-smokers, except for multi-rooted buccal sites in non-smokers. At T2, a higher number of PD  $\geq 5$  mm with BoP was observed in smokers compared with non-smokers at maxillary molar palatal sites ( $p = 0.040$ ), at maxillary single-rooted palatal and buccal sites ( $p = 0.001$  and  $p = 0.002$ , respectively), and at mandibular single-rooted lingual sites ( $p = 0.032$ ).

Based on the number of PD  $\geq 5$  mm with BoP, patients were allocated into four different groups: (1) patients with 0 sites; (2) patients with 1–4 sites; (3) patients with 5–8 sites; and (4) patients with  $\geq 9$  sites. For both smokers and non-smokers at T0, 97.5% ( $n = 39$ ) had  $\geq 9$  sites and 2.5% ( $n = 1$ ) 5–8 sites. For smokers at T1, 13.2% ( $n = 5$ ) had  $\geq 9$  sites, 13.2% ( $n = 5$ ) 5–8 sites, 55.3% ( $n = 21$ ) 1–4 sites, and 18.4% ( $n = 7$ ) had 0 numbers of PD  $\geq 5$  mm with BoP (not tabulated). At T2, the corresponding percentages were 16.7% ( $n = 6$ ), 25.0% ( $n = 9$ ), 38.9% ( $n = 14$ ), and 19.4% ( $n = 7$ ). For non-smokers at T1, 0 patients had  $\geq 9$  sites (group 4) and 8.1% ( $n = 3$ ) had 5–8 sites (group 3) and at T2, the respective percentages were 2.8% ( $n = 1$ ) and 13.9% ( $n = 5$ ). The mean level of three different cigarette measures was recorded and presented for each patient group at T0 and T2 (Fig. 2). Compared with subjectively reported

Table 2. Patient-related clinical measures in smokers and non-smokers at T0, T1, and T2

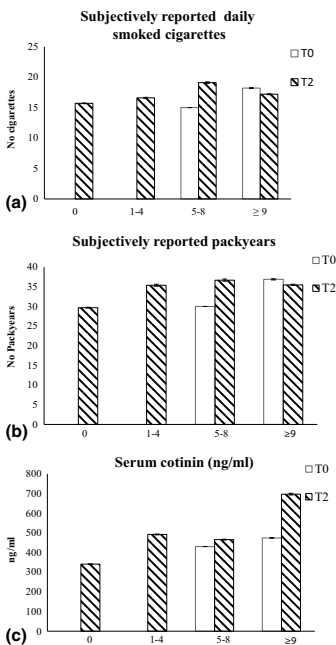
Clinical measures	T0 ( $n = 80$ )			T1 ( $n = 75$ )			T2 ( $n = 72$ )		
	Smokers mean ( $\pm$ SEM)	Non-smokers mean ( $\pm$ SEM)	$p$	Smokers mean ( $\pm$ SEM)	Non-smokers mean ( $\pm$ SEM)	$p$	Smokers mean ( $\pm$ SE)	Non-smokers mean ( $\pm$ SEM)	$p$
Number of teeth	23.35 (5.14)	25.08 (2.88)	0.069	22.53 (5.65)	24.81 (3.30)	0.036	22.11 (6.24)	24.61 (3.15)	0.058
PD	3.80 (1.63)	3.36 (1.52)	<0.001	2.63 (1.02)	2.27 (0.85)	<0.001	2.80 (1.11)	2.42 (0.88)	<0.001
CAL	4.55 (1.80)	3.97 (1.48)	0.001	3.57 (1.34)	3.06 (1.12)	<0.001	3.60 (1.52)	3.13 (1.11)	<0.001
BI	66.68 (17.93)	67.33 (15.57)	0.864	22.42 (8.41)	22.81 (10.97)	0.864	27.00 (8.02)	30.50 (9.78)	0.050
PI	54.62 (21.72)	54.63 (21.72)	0.607	18.45 (10.89)	21.49 (14.13)	0.304	30.09 (16.14)	32.78 (14.59)	0.352

BI, bleeding index; CAL, clinical attachment level; SEM, standard error of the mean, PD, probing depth; PI, plaque index.

**Table 3.** Numbers of sites with probing depth  $\geq 5$  mm with BoP before (T0), following active (T1), and supportive periodontal therapy (T2) at arch and tooth level

Localization	T0			T1			T2		
	Smokers n (%)	Non-smokers n (%)	<i>p</i>	Smokers n (%)	Non-smokers n (%)	<i>p</i>	Smokers n (%)	Non-smokers n (%)	<i>p</i>
Overall	1471 (26.4)	1049 (17.5)	<0.001	132 (2.6)	52 (1.0)	<0.001	180 (3.8)	79 (1.6)	0.001
Maxillary multi-rooted									
Buccal	131 (39.7)	136 (32.6)	0.105	10 (3.8)	7 (2.0)	0.209	11 (3.6)	12 (4.6)	0.731
Palatal	175 (53.0)	198 (47.5)	0.309	27 (10.2)	15 (4.2)	0.009	31 (12.0)	21 (6.4)	0.040
Maxillary single-rooted									
Buccal	214 (20.8)	124 (11.2)	0.680	16 (1.7)	2 (0.2)	0.121	26 (2.9)	6 (0.6)	0.002
Palatal	374 (46.4)	180 (16.2)	0.030	25 (2.6)	8 (0.8)	0.159	48 (5.4)	12 (1.3)	0.001
Mandibular multi-rooted									
Buccal	99 (33.0)	86 (24.9)	0.269	9 (3.0)	7 (2.3)	0.617	10 (3.4)	3 (1.1)	0.055
Lingual	137 (41.5)	125 (36.2)	0.326	12 (4.0)	9 (2.9)	0.561	17 (5.7)	12 (4.2)	0.473
Mandibular single-rooted									
Buccal	154 (14.1)	95 (8.4)	0.148	14 (1.4)	2 (0.2)	0.628	21 (2.2)	5 (0.5)	0.164
Lingual	187 (17.1)	105 (9.3)	0.011	19 (1.9)	2 (0.2)	0.190	16 (1.7)	8 (0.8)	0.032

BoP; bleeding on probing; multi-rooted, molars; single-rooted, premolars and incisors; buccal, two proximal-buccal and one mid-buccal; palatal, two proximal-palatal and one mid-palatal; lingual, two proximal-lingual and one mid-lingual.



**Fig. 2.** Means of smoking measures in patients with 0, 1-4, 5-8, and  $\geq 9$  sites of probing depth  $\geq 5$  mm and bleeding on probing at T0 and T2.

consumption, the serum cotinine levels were higher for smokers presenting  $\geq 9$  sites with PD  $\geq 5$  mm with BoP at T2. For this group, the objectively validated cigarette consumption showed 37.9% higher

mean serum cotinine level at T2 (697 ng/ml) compared with the mean serum cotinine level in the groups presenting a lower number of PD  $\geq 5$  mm with BoP (433 ng/ml).

At the site level, clinical parameters and numbers of teeth at T0 and T1 were tested in smokers and non-smokers as predictors for PD  $\geq 5$  mm with BoP at T2 (Table 4). All variables significantly increased the OR, except for number of teeth at T0 and T1 and for plaque positive sites at T0 in smokers. BoP at T0 was a strong predictor in smokers (OR: 8.93, CI: 3.28, 24.36,  $p < 0.001$ ) and non-smokers (OR: 10.99, CI: 3.33, 36.23,  $p < 0.001$ ). Compared with BoP at T0, BoP at T1 increased the OR in smokers (OR = 13.26, CI: 5.12, 34.38,  $p < 0.001$ ), but not in non-smokers (OR = 4.68, CI: 1.32, 16.61,  $p < 0.001$ ). Plaque positive sites at T0 predicted PD  $\geq 5$  mm with BoP only in non-smokers (OR = 3.05, CI: 1.19, 7.82,  $p = 0.020$ ), whereas an association was revealed between plaque positive sites at T1 in smokers (OR = 5.83, CI: 2.74, 12.42,  $p < 0.001$ ) and non-smokers (OR = 2.29, CI: 1.03, 5.07,  $p < 0.041$ ).

The overall effect of smoking at T2 on the number of sites with PD  $\geq 5$  mm and BoP was tested at different teeth and sites using adjusted logistic regression analysis (Table 5). An overall negative effect of smoking was demonstrated (OR = 2.78, CI: 1.49, 5.18,  $p = 0.001$ ) particularly at maxillary single-rooted buccal and

palatal sites (OR = 6.21, CI: 2.05, 18.88,  $p = 0.001$  and OR = 4.55, CI: 1.61, 12.85,  $p = 0.004$  respectively), mandibular single-rooted buccal sites (OR = 4.35, CI: 1.06, 17.82,  $p = 0.041$ ), and mandibular multi-rooted buccal sites (OR = 4.10, CI: 1.09, 15.38,  $p = 0.036$ ). The overall ICC were reported within patients (ICC = 0.114), teeth (ICC = 0.509), and sites (ICC = 0.761). The variation was highest at the patient level and least at the site level and was consistent within different teeth and sites (Table 5). At the patient level, the overall ICC for smokers (ICC = 0.137) was higher than for non-smokers (ICC = 0.051; not tabulated).

## Discussion

The present study evaluated the effect of cigarette smoking at patient, tooth, and site levels following 12 months of SPT. During SPT, smokers and non-smokers presented increased numbers of PD  $\geq 5$  mm with BoP with the greatest increase at maxillary single-rooted teeth in smokers; from 10 to 16 at buccal sites and from 25 to 48 at palatal sites. An overall negative effect of smoking was revealed at T2 with the strongest effect at maxillary single-rooted teeth. To a great extent, the site-specific effects explain the outcomes of periodontal therapy (D'Aiuto et al. 2005) and the patient-related effect of smoking seems to act as a modifier at the

Table 4. Clinical parameters and number of teeth as predictors for probing depth (PD)  $\geq$  5 mm with bleeding on probing (BoP) at T2 in smokers and non-smokers

	Smokers		Non-smokers	
	OR (95% CI) <sup>a</sup>	<i>p</i>	OR (95% CI) <sup>a</sup>	<i>p</i>
T0				
Teeth	1.02 (0.94, 1.10)	0.699	1.16 (1.01, 1.34)	0.035
CAL	1.48 (1.19, 1.83)	<0.001	1.63 (1.32, 2.02)	<0.001
PD	2.12 (1.74, 2.59)	<0.001	2.25 (1.68, 3.01)	<0.001
BoP	8.93 (3.28, 24.36)	<0.001	10.99 (3.33, 36.23)	<0.001
Plaque	2.21 (0.70, 6.36)	0.185	3.05 (1.19, 7.82)	0.020
T1				
Teeth	1.03 (0.94, 1.12)	0.527	1.16 (1.03, 1.31)	0.017
CAL	2.25 (1.80, 2.82)	<0.001	2.81 (2.14, 3.67)	<0.001
PD	5.63 (3.46, 9.16)	<0.001	7.39 (4.25, 12.85)	<0.001
BoP	13.26 (5.12, 34.38)	<0.001	4.68 (1.32, 16.61)	<0.001
Plaque	5.83 (2.74, 12.42)	<0.001	2.29 (1.03, 5.07)	0.041

T0-T2, baseline before active periodontal therapy (T0)-12 months of supportive periodontal therapy (T2), T1-T2; following completion of active periodontal therapy (T1)-12 months with supportive periodontal therapy (T2).

<sup>a</sup>Logistic regression showing effect of tooth and site-related conditions at T0 and at T1 adjusted for gender, age, marital status, and education.

Table 5. The effect of smoking on probing depth  $\geq$  5 mm with bleeding on probing at T2 presented with intra-class correlation coefficients (ICC) within patients, teeth, and sites

	OR (95% CI)	<i>p</i>	ICC <sup>a</sup>	ICC <sup>b</sup>	ICC <sup>c</sup>
Overall	2.78 (1.49, 5.18)	0.001	0.114	0.509	0.761
Maxillary multi-rooted	1.55 (0.74, 3.23)	0.238	0.165	0.472	0.758
Buccal sites	1.12 (0.46, 2.75)	0.802	0.161	0.286	0.686
Palatal sites	1.81 (0.84, 3.88)	0.129	0.182	0.371	0.746
Maxillary single-rooted	5.08 (2.01, 12.78)	0.001	0.184	0.476	0.752
Buccal sites	6.21 (2.05, 18.88)	0.001	0.156	0.351	0.754
Palatal sites	4.55 (1.61, 12.85)	0.004	0.210	0.406	0.733
Mandibular multi-rooted	2.51 (1.01, 6.23)	0.047	0.163	0.355	0.691
Buccal sites	4.10 (1.09, 15.38)	0.036	na	0.117	0.746
Lingual sites	2.12 (0.82, 5.49)	0.120	0.168	0.231	0.642
Mandibular single-rooted	3.09 (1.01, 9.43)	0.048	0.171	0.575	0.763
Buccal sites	2.34 (0.75, 7.26)	0.143	0.257	0.401	0.755
Lingual sites	4.35 (1.06, 17.82)	0.041	0.136	0.664	0.781

Logistic regression showing main effect of patient-related conditions at T2 adjusted for gender, age, marital status, and education.

<sup>a</sup>ICC, intra-class correlation coefficients within patients.

<sup>b</sup>ICC, intra-class correlation coefficients within teeth.

<sup>c</sup>ICC, intra-class correlation coefficients within sites na; not available.

site-specific level. As suggested, the magnitude of changes during SPT appears related to the initial defect size at site level and to heavy smoking at patient level (Matuliene et al. 2008). Moreover, a local effect of smoking appears to be superimposed on the systemic effect, particularly affecting maxillary single-rooted teeth. In smokers, the percentage of PD  $\geq$  5 mm with BoP at these teeth increased from 31% at T1 to 41% at T2, whereas the percentage for maxillary multi-rooted teeth declined from 28% to 23%, respectively. The percentage at T2 were comparable with baseline registration and in accordance with previous findings

demonstrating a high percentage of PD  $\geq$  5 mm in single-rooted teeth in smokers (van der Weijden et al. 2001). Interestingly, the results show slightly different site-specific treatment outcomes following APT and SPT, indicating altered local tissue responses to cigarette smoking during APT compared with SPT.

Including BoP in the primary outcome variable could introduce a bias due to less BoP in smokers compared with non-smokers (Preber & Bergström 1985, Bergström & Boström 2001). On the other hand, a site level periodontal diagnose including BoP seems to correlate with disease progression and

periodontal instability irrespective of smoking status (Ramseier et al. 2015). At a site level, absence of BoP is considered to predict long-term stability following treatment of chronic periodontitis patients (Lang et al. 1990), whereas presence of BoP predicts disease progression in both smokers and non-smokers (Ramseier et al. 2015). However, it is not clear whether BoP to the same extent is associated with disease progression at a site level in smokers and non-smokers. In this study, the association between BoP at T1 and PD  $\geq$  5 mm with BoP at T2 was stronger in smokers compared with non-smokers. More intense bleeding from deep pockets following non-surgical periodontal therapy in smokers (Ardais et al. 2014) can be explained by a hyper-inflammatory condition in gingival tissues, thus making BoP a strong predictor for disease progression during SPT.

A tendency towards recurrence of periodontitis during SPT was supported by a significant increase in PD, BI, and PI in both smokers and non-smokers. These findings are in agreement with previous studies showing a slight disease progression during the first years following ATP (Knowles et al. 1979, Preshaw & Heasman 2005). These longitudinal trends of treatment progression might reflect lack of compliance from highly susceptible patients during the first years of SPT. In this study, to compensate for variation in compliance among smokers

(Ramseier et al. 2014), a 3-month SPT frequency compatible with maintenance of highly susceptible patients, was offered. Preferably, the frequency of SPT should reflect the individual risk profile. However, in this prospective study, the SPT interval was standardized regardless of the susceptibility for recurrence of periodontitis, and patients exceeding a 4-month interval were excluded. The effort to adjust for compliance should be considered a merit in the analyses of evaluating the effect of smoking exposure on the efficacy of SPT.

The exposure of smoking was quantified and objectively validated by measuring serum cotinine concentration. At T2, an association was revealed between  $\geq 9$  sites of PD  $\geq 5$  mm with BoP per patient and high cotinine levels. Heavy smoking during periodontal treatment, quantified by high levels of cotinine, negatively influenced the outcome of SPT. This association was not detected at T0, indicating that doses of current smoking exposure do not to the same extent influence the level of periodontal disease. Consequently, when smoking cessation is not successful, reduced smoking exposure during therapy should be encouraged. A dose-related treatment response has been documented (Kaldahl et al. 1996a), however, not by objective measures of smoking exposure during therapy. In this study, 86% of the patients with  $\geq 9$  sites of PD  $\geq 5$  mm with BoP at T2 were heavy smokers. These findings are in agreement with a former study concluding that 90% of non-responders are smokers (Magnusson & Walker 1996). Non-responding periodontitis, characterized by multiple progressing sites following therapy, is considered a patient-specific more than site-specific entity. Smoking as a patient-related risk factor has previously been recognized (Kornman et al. 1997, Matuliene et al. 2010) and in this study, smoking outweighed other patient-related risk factors documented by a smaller variation in PD  $\geq 5$  mm with BoP at T2 within smokers compared with non-smokers.

A few limitations of this study, however, should be discussed. The lack of masking has been addressed previously (Bunæs et al. 2015).

Further, a follow-up period of 12 months is a relatively short time to study the effect of smoking on the outcome of SPT. An extension of the observation period might provide more substantiated information. On the other hand, during a longer follow-up period, more patients are prone to drop out and a higher number of smokers might quit smoking. Both factors could definitely have undermined the statistical analysis and the validity of the results. Three smokers reported smoking cessation between T1 and T2 and yet were not excluded from the study. Matching serum cotinine concentration confirmed smoking cessation for one, whereas the other two reported the use of snuff to substitute cigarette nicotine. In Scandinavia, the use of snuff has increased significantly during recent years, especially among adolescents (Hergens et al. 2014). Unregistered use of snuff may have disturbed the measured cotinine concentrations in serum and might be considered a confounder.

In summary, both smokers and non-smokers showed a slight recurrence of disease following 12 months of SPT. However, both smokers and non-smokers responded to periodontal therapy with significant reductions in mean PD, CAL, BI, and PI ( $p < 0.001$ ). An overall negative effect of smoking on PD  $\geq 5$  mm with BoP was demonstrated with a site-specific tissue response to smoking. Further, BoP at T1 in smokers was a strong site-specific predictor for PD  $\geq 5$  mm with BoP at T2. At the patient level, elevated cotinine measures at T2 were associated with  $\geq 9$  sites of PD  $\geq 5$  mm with BoP. The study reveals that cigarette smoking as a patient-related risk factor may modulate site-associated variables affecting outcomes of SPT. The magnitude of the effect of cigarette smoking on local tissue responses should be further explored in prospective studies with objective quantification of smoking exposure.

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## Clinical Relevance

**Scientific rationale for the study:** In general, smokers respond less favourably to periodontal therapy compared with non-smokers. To predict the long-term outcome of periodontal therapy in smokers, the effect of smoking needs to be evaluated at patient, tooth, and site level following active therapy.

**Principal findings:** An overall negative effect of smoking was demonstrated following 12 months of supportive periodontal therapy, especially at maxillary single-rooted teeth. At patient level, high serum cotinine levels were associated with  $\geq 9$  disease progressing sites. At site level, bleeding on probing following active periodontal therapy predicted

an increased risk of disease progression in smokers compared with non-smokers.

**Practical implications:** In perspective, smoking cessation or even smoking reduction may benefit treatment outcomes following supportive periodontal therapy.

Study III

**The Effect of Smoking on Inflammatory and Bone Remodeling Markers in  
Gingival Crevicular Fluid and Subgingival Microbiota Following Periodontal  
Therapy**

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# **The Effect of Smoking on Inflammatory and Bone Remodeling Markers in Gingival Crevicular Fluid and Subgingival Microbiota Following Periodontal Therapy**

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**Key words:** Chronic periodontitis, smoking, gingival crevice fluid, periodontal therapy, periodontal immunology, microbiology

**Running title:** Smoking affects GCF markers following periodontal treatment

## ABSTRACT

**Background and Objective:** Periodontal health is mediated by suppressing microorganisms inducing a local inflammatory host response. Smoking may impair this process. This study compares gingival crevicular fluid levels of inflammatory and bone remodelling markers in heavy smokers and non-smokers following active and supportive periodontal therapy in chronic periodontitis patients.

**Materials and Methods:** Gingival crevicular fluid (GCF) and subgingival plaque were collected from the deepest periodontal pocket in 50 patients, 25 smokers and 25 non-smokers, at baseline (T0), following active (T1) and 12 months of supportive periodontal therapy (T2). Smoking status was validated measuring serum cotinine levels. GCF levels of 27 inflammatory and two bone remodelling markers were analysed using multiplex and singleplex micro-bead immunoassays, and subgingival plaque samples using checkerboard DNA-DNA hybridization. Amounts of markers in smokers and non-smokers were compared calculating the effect size.

**Results:** Expression of inflammatory and bone-remodeling markers in smokers demonstrated an overall reduced effect size at T0 and T2 ( $p < 0.001$ ) More specifically, pro-inflammatory markers ( $p < 0.001$ ), chemokines ( $p = 0.007$ ) and growth-factors ( $p = 0.003$ ) at T0, osteoprotegerin ( $p = 0.003$ ) at T1, pro-inflammatory markers ( $p = 0.019$ ) and chemokines ( $p = 0.005$ ) at T2. At T2, IL-8 was detected in significantly higher levels in smokers. Ten different markers in non-smokers and none in smokers responded to periodontal therapy ( $p < 0.05$ ). An overall negative association was revealed between smoking and sub-groups of markers at sites presenting  $\geq 10^5$  red complex periodontal microbial species.

**Conclusion:** Except for an upregulation of IL-8, smokers exhibited reduced GCF levels of several inflammatory markers at baseline and following active and supportive periodontal therapy. Only inflammatory responses in non-smokers adapted to periodontal therapy. Apparently, there seems to be an immunosuppressant effect of smoking regulating the local inflammatory response and bone remodelling markers captured in GCF following periodontal therapy.

## INTRODUCTION

Cigarette smoking may affect periodontal tissues through modulating the immune response to periodontal pathogens. A dose-dependent hypo-immune inflammatory reaction has been suggested, mainly through systemic exposure following lung absorption (1-3). An additive local effect of smoking through direct exposure and absorption of toxic substances may adversely shift the periodontal equilibrium towards impaired repair and tissue breakdown (4, 5). Further, differences exist in composition of subgingival microbiota among smokers and non-smokers (6). Moreover, smoking appears to modulate composition, promote colonization of key periodontal pathogens, and influence bacterial aggregation (7, 8) rather than rate and amount of plaque accumulation (9, 10).

Periodontal pathogens induce release of inflammatory and bone remodelling markers orchestrating innate and adaptive immune responses. The interaction and balance of these markers determine whether the tissue response remains stable or leads to destruction and disease progression (11). Gingival crevicular fluid (GCF), an inflammatory exudate or transudate collected from the gingival crevice, contains components of host-derived inflammatory markers (12). Thus, GCF analysis appears a non-invasive approach to investigate site-specific inflammatory responses and assess presence of various inflammatory markers (13). In smokers, site-specific tissue responses are clinically expressed by a specific attachment loss profile (14). However, site-specific mapping of inflammatory and bone-remodelling markers in smokers with chronic periodontitis reports conflicting results (15). A majority of studies have reported decreased local expression of some pro-inflammatory cytokines and chemokines in smokers (16-18) indicating an immunosuppressant effect of smoking that may increase susceptibility to periodontitis. Conversely, elevated expressions of chemokines and pro-inflammatory cytokines have also been reported in smokers (19, 20). For bone remodelling, decreased GCF levels of osteoprotegerin (OPG) have been demonstrated following  $\geq 20$  years of smoking (21). As OPG blocks activation of receptor activator for nuclear factor kappa-B ligand (RANKL), the RANKL:OPG ratio, a surrogate marker for periodontal bone homeostasis, might increase in smokers and induce bone loss.

Compromised healing following periodontal therapy in smokers is well documented (22-24) and impaired site-specific treatment outcomes may to some extent be explained by altered inflammatory responses (25). A few prospective studies have investigated the relationship between smoking and levels of pro-inflammatory markers, chemokines, and bone markers in GCF following periodontal therapy. IL-1 $\beta$  and TNF- $\alpha$ , are mostly studied; IL-1 $\beta$  levels decreased in smokers and non-smokers following 6 weeks of non-surgical therapy (26), whereas at 6 months the levels were reduced in non-smokers only (27). TNF- $\alpha$  did not change in smokers and non-smokers following 1 month of non-surgical therapy (28). At 6 months, however, a decreased level was observed in smokers (29). Interestingly, smoking seemed to upregulate the chemokine IL-8 following therapy (30), whereas OPG decreased in both smokers and non-smokers (31).

Previous follow-up studies evaluating the local inflammatory status, have analysed a limited number of GCF markers. Multiplex immunoassay has the potential to simultaneously quantify multiple markers providing unique information necessary for a more complex understanding of the inflammatory response. By measuring several inflammatory markers over time, a site-specific tissue response to periodontal therapy can be monitored. Thus far, no prospective study has compared inflammatory responses in GCF following active and supportive periodontal therapy in smokers and non-smokers.

This prospective study was designed to test the hypothesis that smoking downregulates the expression of the inflammatory molecules in GCF during treatment of chronic periodontitis. The primary aim was to investigate the GCF levels of inflammatory markers involved in periodontal inflammation and healing following active and 12 months of supportive periodontal therapy in heavy smokers and non-smokers. The impact of smoking on the numbers of subgingival periopathogens following periodontal therapy was also studied.

## **MATERIAL AND METHODS**

### **Participants and study design**

From a sample of 80 patients, 40 smokers and 40 non-smokers, referred for periodontal treatment from general practitioners in a rural district of Norway, a subsample of 50 patients were enrolled in this single-arm clinical trial (for detail see Bunæs et al. 2015;(24). Briefly, criteria for inclusion were healthy subjects between 35-75 years with moderate to severe chronic periodontitis (32) having at least four non-adjacent teeth with interproximal probing depth (PD)  $\geq 6$  mm, clinical attachment loss  $\geq 5$  mm, and bleeding on probing (BoP) (33, 34). Exclusion criteria were any current medical condition or medication affecting periodontal treatment and the use of systemic antibiotic or subgingival scaling in the 6 months before initiation of the study. Demographic data were obtained from the study participants by means of health forms and questionnaires.

Based on predefined criteria, the 50 patients were allocated into two subgroups of 25 smokers reporting smoking  $>10$  cigarettes/day for at least 5 years and with baseline pre-treatment (T0) serum cotinine level  $\geq 300$  ng/mL, and 25 non-smokers reporting never or no smoking the last 5 years and with T0 serum cotinine level  $<15$  ng/ml. Whole blood sample obtained from each patient was coagulated and centrifuged ( $700 \times g$  for 10 min) and the serum was stored in aliquots at  $-80^{\circ}\text{C}$ . Serum cotinine was assessed according to the manufacturer's instructions of the serum enzyme immunoassay kit (Cotinine ELISA Kit, MyBioSource, San Diego, USA) measuring the absorbance at 450 nm using a microplate reader (FluoStar Optima V1.32 R2, BMG Labtech, Offenburg, Germany).

The study protocol and informed consent form was approved by the Institutional Medical Research Ethics Committee (2011/151-6), University of Bergen, Norway followed the Helsinki Declaration of 1975, version 2008. All patients had read and signed a written consent prior to enrolment in the study.

### **Clinical and microbiological examinations**

Clinical assessments, group allocations, and sampling selection were performed by a calibrated examiner (DFB). Clinical and microbiological sample collection were performed at baseline (T0), at 3 months following active periodontal therapy (APT)

(T1), and following 12 months of supportive periodontal therapy (SPT) (T2). Mean time between T0 and T1 was 7.9 months and comprised non-surgical and surgical treatment. Patients presenting persistent PD >5 mm with BoP and adequate oral hygiene following non-surgical treatment were subjected to periodontal surgery. SPT was conducted every 3 months. PDs were recorded as the distance in mm from the gingival margin to the probeable base of the periodontal pocket, and clinical attachment level (CAL) as the distance in mm from the cemento-enamel junction or the margin of a dental restoration to the probeable base of the periodontal pocket. PD and CAL were measured using a periodontal probe (PCPUNC 15, Hu-Friedy, Chicago, IL, USA) at six sites per tooth rounding up to the nearest mm. Full mouth gingival bleeding was recorded as the percentage of sites showing bleeding after gentle probing (35) and full mouth dental plaque as the percentage of tooth surfaces with visible plaque following staining with disclosing solution (36). As a supplement to staining, the periodontal probe was used to discriminate between plaque and pellicle. At T0 subgingival plaque were collected by inserting two sterile paper points into the deepest periodontal pocket in each patient, and the procedure was repeated at the same site at T1 and T2. Prior to sampling, the site was carefully cleaned of supragingival plaque and kept dry. The paper points were gently inserted towards the apex of the pocket and kept in place for 20 sec (37) before removal and immersion into a pre-reproduced sterile transport medium (PRAS Dental Transport Medium, Morgan Hill, CA, USA). The microbiological samples were analysed by DNA-DNA hybridization (checkerboard technique) at Microbiological Diagnostic Service, Department of Oral Biology, Faculty of Dentistry, University of Oslo, Oslo, Norway. The analysis included detection and quantification of red (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) and orange complex species (*Prevotella intermedia*, *Prevotella nigrescens*, *Fusobacterium nucleatum subsp polymorphum*, *Fusobacterium nucleatum subsp nucleatum*, *Fusobacterium nucleatum subsp vincentii*, and *Parvimonas micra*), and *Aggregatibacter actinomycetemcomitans (Aa)* (for details see Bunæs et al., 2015; (24).

Intra-examiner reliability of the examiner (DFB) was assessed using the intraclass correlation coefficients (ICC) for repeated measures. ICC ranged between

0.92 and 0.96 for PD and between 0.93 and 0.96 for CAL. A post.hoc power analysis based on 25 heavy smokers and 25 non-smokers and with the level of significance ( $\alpha$ ) set to 0.05, gave a 71% power to detect a true difference of 0.5 mm. Prior to treatment, the hypothesis that is not possible to blind an examiner towards smoking status was tested in a pre-study sample of 30 chronic periodontitis patients, 16 smokers (>10 cigarettes/day for at least 5 years) and 14 non-smokers (never or not in the last 5 years). Calculus, plaque, and staining were removed and after rinsing with 0.2% chlorhexidine gluconate (Corsodyl, GlaxoSmithKline, London, UK) for 1 min, the examiner wearing a face mask scored the smoking status as yes or no. Twenty-eight patients (93%) were correctly identified as either non-smokers or smokers. Thus, the pre-study hypothesis was accepted ( $p<0.001$ ) indicating that the attempt to blind the examiner with regard to smoking status was unsuccessful.

#### **GCF sampling and protein extraction**

GCF samples were collected using Periopaper strips, (PERIOPAPER Gingival Fluid Collection Strips, Oraflow Inc, Smithtown, NY, USA). Following removal of supragingival plaque with sterile curettes and cotton pellets, air dried, and isolated with cotton rolls, the deepest periodontal pocket in each participant was sampled. The paper strips were placed 1-2 mm into the entrance of the pocket and left in place for 30 sec. Strips visually contaminated with blood or saliva were discarded. Sampled strips were immediately evaluated for GCF volume using the Periotron 8000 (Oraflow Inc, Smithtown, NY, USA). Strips were then immediately inserted into separate and dry microtubes, labelled, and stored at  $-80^{\circ}\text{C}$  until further analysis.

Tris-HCl buffer (110  $\mu\text{L}$ ) with a final concentration of 12mM at pH 7.6 was added to each tube for protein extraction. The tubes were shaken in  $3\times 10$  min before centrifuged (1800 x g for 10 min at  $4^{\circ}\text{C}$ ) and subsequently the supernatant was pipetted to new tubes for protein quantification by a commercially available kit (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). A plate reader (FLUOstar OPTIMA- BMG Labtech, Ortenberg, Germany) measured the absorbance at 570 nm and total protein per strip was calculated in  $\mu\text{g}$ .

## **Analysed markers**

Based on inflammatory and bone remodeling molecules involved in the periodontal healing process, the following cytokines were determined (diluted 1:4) using the multiplex kit Bio-Plex Human Pro<sup>TM</sup> Assay (catalogue number LX10009222405) from Bio-Rad Inc., Hercules, CA, USA: *IL-1 $\beta$* , *IL-2*, *IL-1ra*, *IL-4*, *IL-5*, *IL-6*, *IL-7*, *IL-8*, *IL-9*, *IL-10*, *IL-12*, *IL-13*, *IL-15*, *IL-17*, *Interferon- $\gamma$*  (*IFN- $\gamma$* ), *Basic Fibroblast Growth Factor (FGF)*, *Granulocyte Colony Stimulating Factor (G-CSF)*, *Eotaxin*, *Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF)*, *Interferon Inducible Protein-10 (IP-10)*, *Monocyte Chemo-attractive Protein-1 (MCP-1)*, *Macrophage Inflammatory Protein-1 $\alpha$*  (*MIP-1 $\alpha$* ), *Macrophage Inflammatory Protein-1 $\beta$*  (*MIP-1 $\beta$* ), *Platelet-Derived Growth Factor (PDGF)*, *Regulated Upon Activation, Normally T-Expressed, and Presumably Secreted (RANTES)*, *Tumor Necrosis Factor- $\alpha$*  (*TNF- $\alpha$* ) and *Vascular Endothelial Growth Factor (VEGF)*. The standard curves for each marker present an overall range of 107489-0.064 pg/mL.

The level of *OPG* and *RANKL* were assessed (diluted 1:4) using Milliplex MAP Kit Human Bone Magnetic Bead Panel (catalogue number HRNKLMAG-51K-01) from EMD Millipore corp. (Billerica, MA, USA) and a range of 30367-7.28 pg/mL recombinant markers was used to establish the standard curves.

All measurements were performed using a Bio-plex 200<sup>®</sup> system (Bio-Plex Manager TM 6 software) based on the Luminex xMAP technology and the levels of all markers were reported in pg/30 sec and in pg/ml.

## **Statistical analysis**

The Shapiro-Wilk test was applied to test for distribution of continuous variables. Descriptive statistics of clinical and demographical data were analysed using a chi-square test for categorical variables (frequencies and percentages) and by a two-sample independent t-test for continuous data (mean  $\pm$  SD). As continuous data of the analysed mediators had a skewed distribution, natural logarithm transformation was employed to achieve normality prior to using the regression analysis to detect differences between smokers and non-smokers at T0, T1, and T2 and over time (T0 vs. T1, T0 vs. T2, and T1 vs. T2). Samples below detection of the standard curve were replaced by the lowest value of the standard curve (38). Regression analyses were



corrected for clustering of data within patients and the significance level of multiple comparisons were adjusted by the Sidak post-hoc test. A regression model, adjusted for age, gender, and education, and stratified by plaque (present/absent) or amount of red complex bacteria species ( $<10^5 / \geq 10^5$ ) was employed to test the overall association between the subgroups of quantified markers and smoking status.

Amounts markers (pg/30 sec) in smokers and non-smokers were compared by calculating the effect size. Effect size, reported as Cohens` s coefficient, was calculated as the difference between the means of each marker in smokers and non-smokers divided by the standard deviations. Cohen`s coefficient is generally classified into small (0.2), medium (0.5), and large differences ( $\geq 0.8$ ) and allows the size magnitude of the difference between smokers and non-smokers to be measured in a standardised scale. To present the results, forest-plots for the standardised effects sizes were used. An overall test of the difference between markers in smokers and non-smokers were analysed using fixed effects in the metan command (39).

A *p*-value  $\leq 0.05$  was considered statistically significant. All analyses were conducted using Stata 14 (Stata Statistical Software: Release 14. StataCorp LP, College Station, TX, USA).

## **RESULTS**

A total of 50 patients were evaluated, 25 smokers and 25 non-smokers. At T0, mean pack-year consumption in the smoking group was 37.0 (range 20-108) and mean cotinine level 478 mg/mL (range 340-861 mg/mL). For each patient, GCF samples were obtained from the same site at T0, T1, and T2. Data collection started April 2012 and ended December 2014. Baseline patient related clinical and demographic characteristics according to smoking status are shown in Table 1. Compared with non-smokers, significantly lower education level, higher mean PD and CAL were found for smokers. Mean patient related clinical measures of PD, CAL, BI, and PI, decreased significantly following ACT in smokers and non-smokers ( $p < 0.001$ ) and PD, BI, and PI increased significantly following SPT ( $p < 0.05$ ). Site-specific clinical and microbiological characteristics at T0, T1, and T2 are summarised in Table 2. No significant differences between smokers and non-smokers were detected for any of the

site-specific parameter at any time point, except for significantly higher numbers of *Aa* in non-smokers compared with smokers at T0 ( $p=0.041$ ).

GCF markers detected in less than 30% of the samples (IL-2, IL-5, IL-12, IL-13, IL-15, Eotaxin, FGF, MCP-1 and RANKL) were removed from the analysis. The surveyed markers at T0, T1, and T2 were stratified by smoking status and comparisons of unadjusted means of the quantities are presented as amounts per 30 sec (pg/30sec) (Table 3a) and as concentrations (pg/mL) (Table 3b). Further analyses were conducted on the amounts of markers per 30 sec (40). Compared with non-smokers, significant lower levels were detected in smokers for TNF- $\alpha$ , IL-9, G-CSF, GM-CSF, IFN- $\gamma$ , VEGF, MIP-1 $\alpha$  and RANTES at T0, for OPG at T1, and for IL-9, IFN- $\gamma$ , PDGF, MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES at T2. At T2, IL-8 was detected in significantly higher levels in smokers ( $p=0.034$ ). Only non-smokers responded to treatment with significant changes in surveyed markers over time. From T0 to T1, significant reductions were revealed for IL-1 $\beta$ , TNF- $\alpha$ , IL-7, GM-CSF, IFN- $\gamma$ , IL-10, VEGF and IP-10, and from T0 to T2 for IL-1 $\beta$  and GM-CSF. A significant upregulation of TNF- $\alpha$ , IL-7, IL-9, IFN- $\gamma$ , IL-10, PDGF, and IP-10 was observed from T1 to T2.

Based on the biological effects, the markers were distributed into subgroups of pro-inflammatory markers (IL-1 $\beta$ , IL-6, IL-9, IL-12 and TNF- $\alpha$ ), anti-inflammatory markers (IL-4 and IL-10), chemokines (IL-8, IP-10, MCP-1, MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES), growth factors (PDGF and VEGF), Th-1/Th-2 (INF- $\gamma$ / (IL-4, IL-6, IL-9, IL-10), and marker of bone remodelling (OPG). Since the markers were expressed in various amounts in smokers and non-smokers and measured in different scales (range 107489-0.064), the magnitude of the differences between smokers and non-smokers was calculated as effect size (Cohen's coefficient). Figure 1a, b and c illustrate the size of the effect of smoking on the expression of marker and subgroup at T0, T1, and T2, respectively. At T0 smoking significantly reduced effect size for pro-inflammatory markers ( $p=0.001$ ), chemokines ( $p=0.007$ ), and growth-factors ( $p=0.003$ ), at T1 for OPG ( $p=0.003$ ), and at T2 for pro-inflammatory markers ( $p=0.019$ ) and chemokines ( $p=0.005$ ).

The subgroups were tested for overall association with smoking status after stratification by dental plaque (present/absent) and red complex microbial species

(<10<sup>5</sup>/≥ 10<sup>5</sup>) (Table 4). The numbers of tested sites in the analysis are not tabulated: plaque positive sites [n=96 (45 smokers / 51 non-smokers)], plaque negative sites [n=54 (30 smokers / 24 non-smokers)], red complex positive sites [n=49 (23 smokers / 26 non-smokers)], and red complex negative sites [n=101 (52 smokers / 49 non-smokers)]. At plaque positive sites, a significant negative association with smoking status was revealed for pro-inflammatory markers, chemokines, and growth factors. For each group, adjusted analyses demonstrated an overall significant negative association with smoking status for plaque positive sites and an overall positive association for plaque negative sites. Further, in both unadjusted and adjusted analyses, negative associations were revealed between smoking status and groups of pro- and anti-inflammatory markers and OPG at sites presenting ≥10<sup>5</sup> red complex species. No significant associations were detected between smoking and groups of markers at sites presenting < 10<sup>5</sup> red complex species. The same tests were performed for orange complex species and *Aa* and a significant negative association between smoking and amounts of pro-inflammatory markers were only present for the adjusted analysis of sites < 10<sup>5</sup> orange complex species ( $p=0.033$ ) (not tabulated).

## **DISCUSSION**

Significantly smaller amounts of several inflammatory markers were detected in smokers compared with non-smokers at T0 and in the presence of increased clinical inflammation from T1 to T2. The expression of GCF markers at a site might be influenced by mean levels of PD and CAL (41). Sampling from the deepest PD in smokers and non-smokers rather from matched PD could have biased the analyses. However, the reliability of an overall reduced inflammatory response in smokers was substantiated by the fact that GCF samples were collected from sites exhibiting similar PD, CAL, BI, PI, and GCF volume in smokers and non-smokers. Another methodological concern when including smokers in clinical studies is the unsuccessful blinding of the examiner with regard to smoking status. This might in fact introduce a study bias. Further, strict sampling procedures and a trained operator intended to prevent a potential saliva contamination of the periopaper strips during the GCF collection.

An overall suppressed inflammatory response in smokers is supported by Tymkiw and suggests a local hypo-inflammatory state in smokers with chronic periodontitis (17). Reduction of important pro-inflammatory cytokines may initially alter local cytokine regulated inflammatory processes and persuade a dysfunctional response to stimuli such as bacterial lipopolysaccharides. In smokers at T0, significant reduced amounts of TNF- $\alpha$ , a multifunctional pro-inflammatory cytokine promoting cell migration and tissue destruction, might downregulate IL-1 $\beta$  and IL-6 and reduce production of chemokines (42). Though investigations of the impact of smoking on the expression of chemokines are limited, reduced amounts of chemokines in smokers have been confirmed by others (17, 43). In the present study, the slight rebound of periodontal disease during SPT coincided with increased amounts of MIP-1 $\alpha$ , MIP-1 $\beta$  and RANTES at T2 in non-smokers only. As these chemokines facilitate migration and activation of specific types of leukocytes in response to periodontal pathogens (44), a downregulation of chemokines in smokers might reduce recruitment of inflammatory cells into infection sites. Chemokines also stimulate bone remodelling driving osteoblast migration (45) and reduced expression might negatively influence bone metabolism. A negative impact of smoking on bone homeostasis might be further supported by lower levels of OPG in smokers compared with non-smokers (significant at T1). RANKL was not detectable and an influence of smoking on the RANKL:OPG ratio can only be speculated upon. Nevertheless, increased bone loss in smokers with chronic periodontitis could be attributed to decrease in OPG and a subsequent increase in the RANKL:OPG ratio with a potential stimulation of osteoclasts. Another study in periodontitis patients with varying smoking status has reported reduced GCF levels of OPG in high pack-years consumption groups compared with non-smokers (21).

IL-8 was the only inflammatory marker detected in significantly higher amounts in smokers compared with non-smokers. This is in agreement with previous studies (18, 20) indicating that smoking seems to upregulate the expression of IL-8 in a dose dependent manner (46). IL-8 is a chemokine associated with subclinical inflammation of initial periodontal lesions through migration of polymorphonuclear neutrophils (PMNs) to the infection sites (47, 48). Modulated inflammatory responses in smokers are strongly associated with impaired PMN functions (49) and higher amounts of IL-8

in smokers may increase chemotaxis and migration of dysfunctional PMN cells. Moreover, IL-8 has an important role in bone metabolism with direct actions on osteoclast activity and differentiation (50). Since IL-8 is suggested to play a crucial role in the pathogenesis of chronic periodontitis, an upregulation of IL-8 production in smokers might be a detrimental factor for impaired treatment outcome and recurrence of periodontal disease, especially in heavy smokers.

None of the analysed inflammatory and bone remodelling markers responded significantly to treatment in smokers. This may strengthen the perception that smoking has a capacity to overwhelm and suppress local inflammatory response to periodontal pathogens (17, 51). Non-smokers responded to treatment with changes in the amounts of inflammatory markers reflecting positive treatment responses and for several markers a significant reduction was observed from T0 to T1 followed by an increase from T1 to T2. The amounts of three principal pro-inflammatory markers, IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$ , reduced significantly as a response to therapy. IFN- $\gamma$  is related to Th-1 response and inhibition containment of periodontal infection by enhancing phagocytic activity of macrophages and neutrophils(52), whereas IL-1 $\beta$  and TNF- $\alpha$  are the first markers emerging during the periodontal inflammation processes. IL-1 $\beta$  is a major mediator for periodontal disease and involved in inflammatory cell migration and osteoclastogenesis (53). Another longitudinal study reported significant reduction of IL-1 $\beta$  at 4 months following non-surgical periodontal therapy, supporting present findings of a sustained decrease in IL-1 $\beta$  following treatment in non-smokers (47, 54). A persistent release of pro-inflammatory cytokines in smokers following active and supportive periodontal therapy may partially be explained by impaired resolution of inflammation and recurrence of periodontal disease following therapy.

As a response to active periodontal therapy, all clinical parameters improved significantly in smokers and non-smokers whereas the total numbers of red complex species, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, were significantly reduced in non-smokers only. The non-significant reduction of red complex species observed between T0 and T1 in smokers, could stimulate local inflammatory responses maintaining elevated amounts of GCF markers at T1. Otherwise, upregulated expression of inflammatory markers in GCF might favour

aggravation of the biofilm and delayed reduction of red complex species in smokers following ATP. Nevertheless, following SPT, a significant reduction of total red complex species took place in smokers without significant changes in the amounts of inflammatory markers. A dysregulated inflammatory response to periopathogenic bacteria in smokers was further supported by negative associations between smoking and groups of inflammatory markers at plaque positive sites and at sites harbouring  $\geq 10^5$  of red complex species. It would be of interest to investigate the plaque with high-throughput techniques, whereas DNA-DNA checkerboard hybridization technique has a rather crude accuracy. Keystone pathogens other than *P. Gingivalis* may be determined for host response in smokers and *Parvimonas Mirca* is considered a keystone pathogen associated with deep pockets in smokers (7, 55). Nevertheless, the majority of immune responses to periopathogens are known to occur locally within the periodontal tissues, in GCF, and between cells conjugated to another, rather than to systemic responses (56, 57). However, in smokers, the amounts of inflammatory markers appeared to be modulated not only by site-specific factors as presence of plaque and a high number of red-complex species, but also by the complex systemic influence of cigarette smoke.

In general, expressed markers in GCF using multiplex assays and ELISA's are detected with similar trends, but not directly comparable due to methodological variations (58), varying amounts of markers, and measurements at different scales. Two recent reviews support reporting of total marker content per 30 sec, especially for longitudinal studies, together with smoking status, and clinical parameters at collection sites (40, 53). They argue that low levels of gingival crevicular fluid volumes following periodontal therapy can negatively influence calculation of the concentration. Reduced GCF and BoP in smokers compared with non-smokers (59, 60), could be an explanation for the suppressed response and downregulation of markers collected per 30 sec in smokers. Related to progression of chronic periodontitis, it appears to be an association between BoP in both smokers and non-smokers (3, 61). Nevertheless, GCF volume did not vary significantly between smokers and non-smokers and analysis with concentrations of markers reported the same trends as for the total amounts. By calculating the effect size of each marker, the

differences in the amounts among markers are standardized and forest plots illustrate the magnitude of the difference between smokers and non-smokers. Further, non-pooled samples and an objective validation of smoking status, reduced the number of confounding factors in the comparisons of GCF markers following periodontal therapy in smokers and non-smokers and to some extent weight up for the relatively low sample size.

The results herein confirmed the study hypothesis by a suppressed inflammatory reaction presented as reduced expression of GCF markers in heavy smokers, especially for pro-inflammatory markers and chemokines. Furthermore, smokers did not respond to periodontal therapy with significant changes in the amounts of any marker in GCF and a negative association was detected between smoking and expression of markers at plaque positive sites. Future prospective studies should contextualize the local tissue responses in smokers within the influence of a larger systemic effect of smoking.

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## **Figure legends**

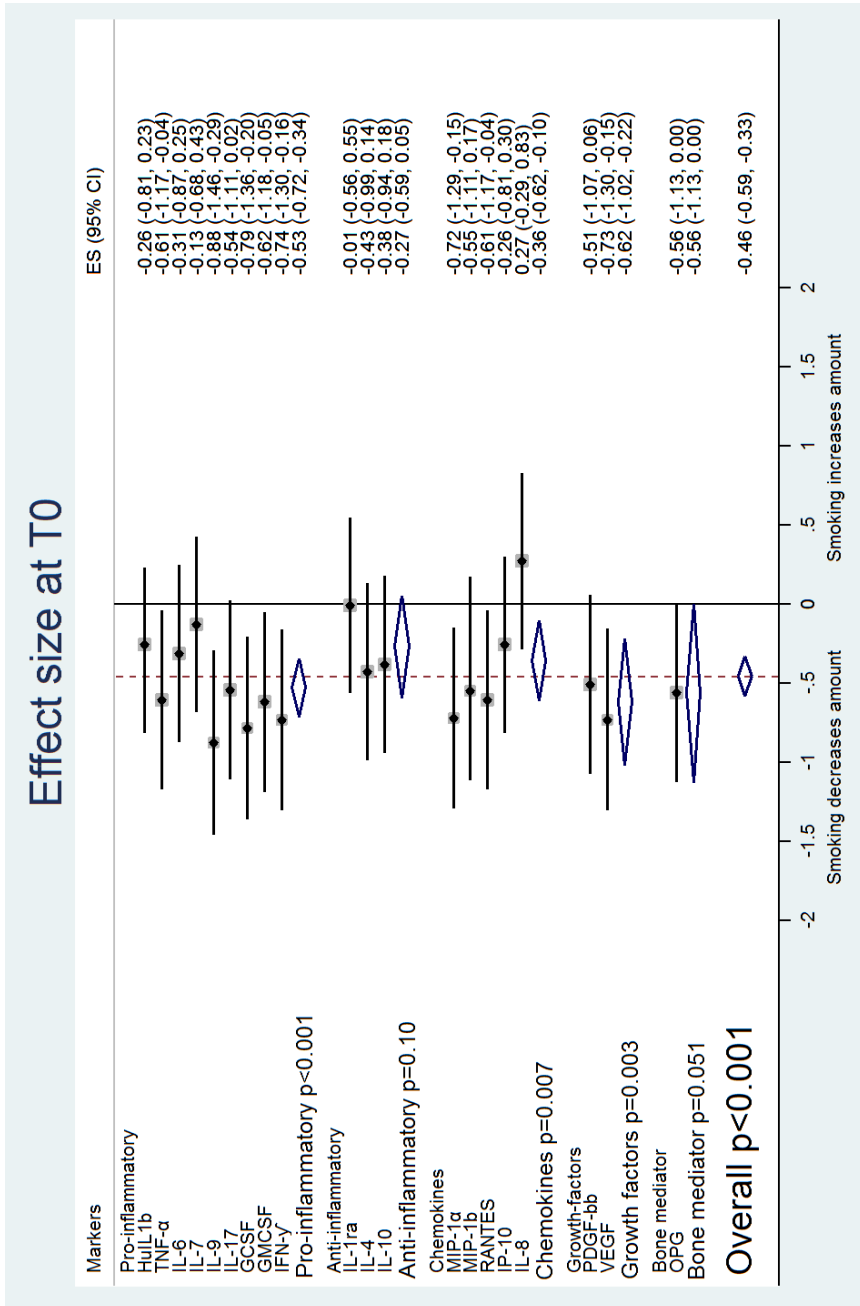
### **Figure 1**

- a. Forest plot for overall standardized effect of smoking at T0 for markers in GCF.
- b. Forest plot for overall standardized effect of smoking at T1 for markers in GCF.
- c. Forest plot for overall standardized effect of smoking at T2 for markers in GCF.

# Figures

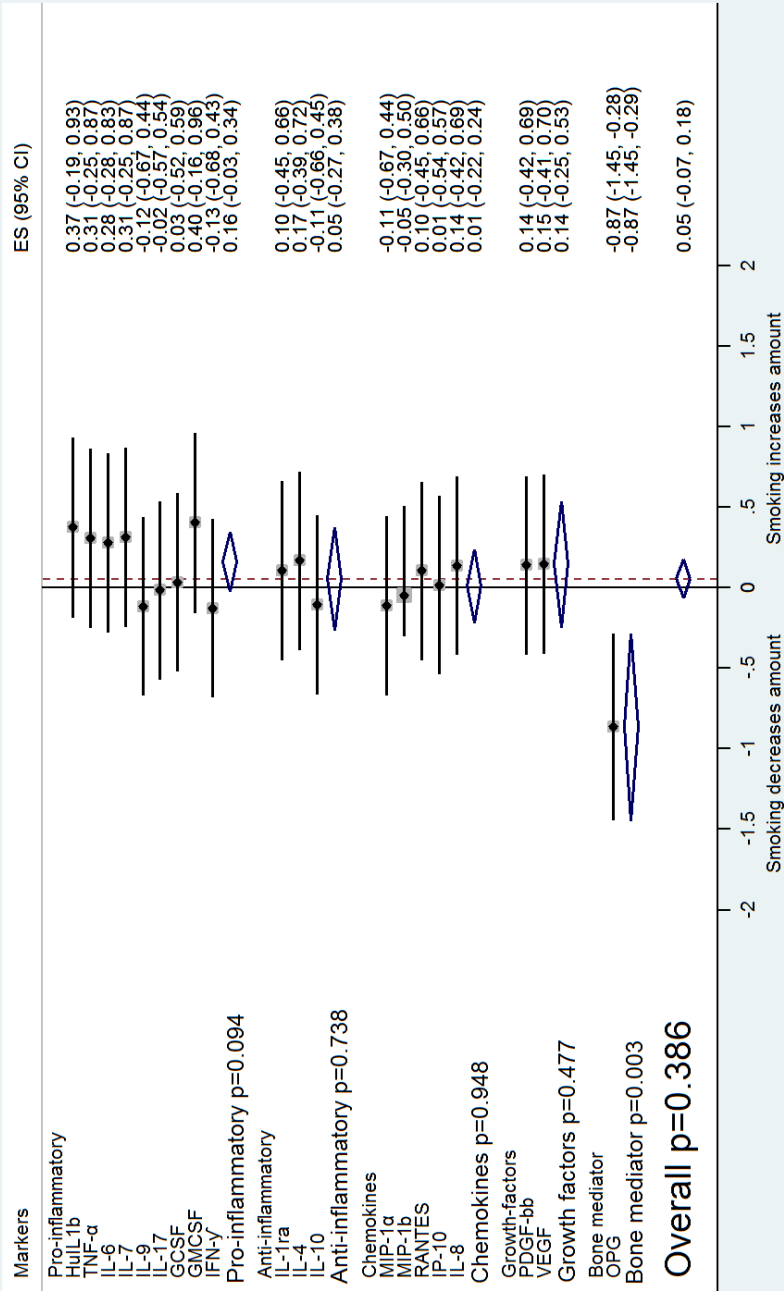
## Figure 1

a)



b)

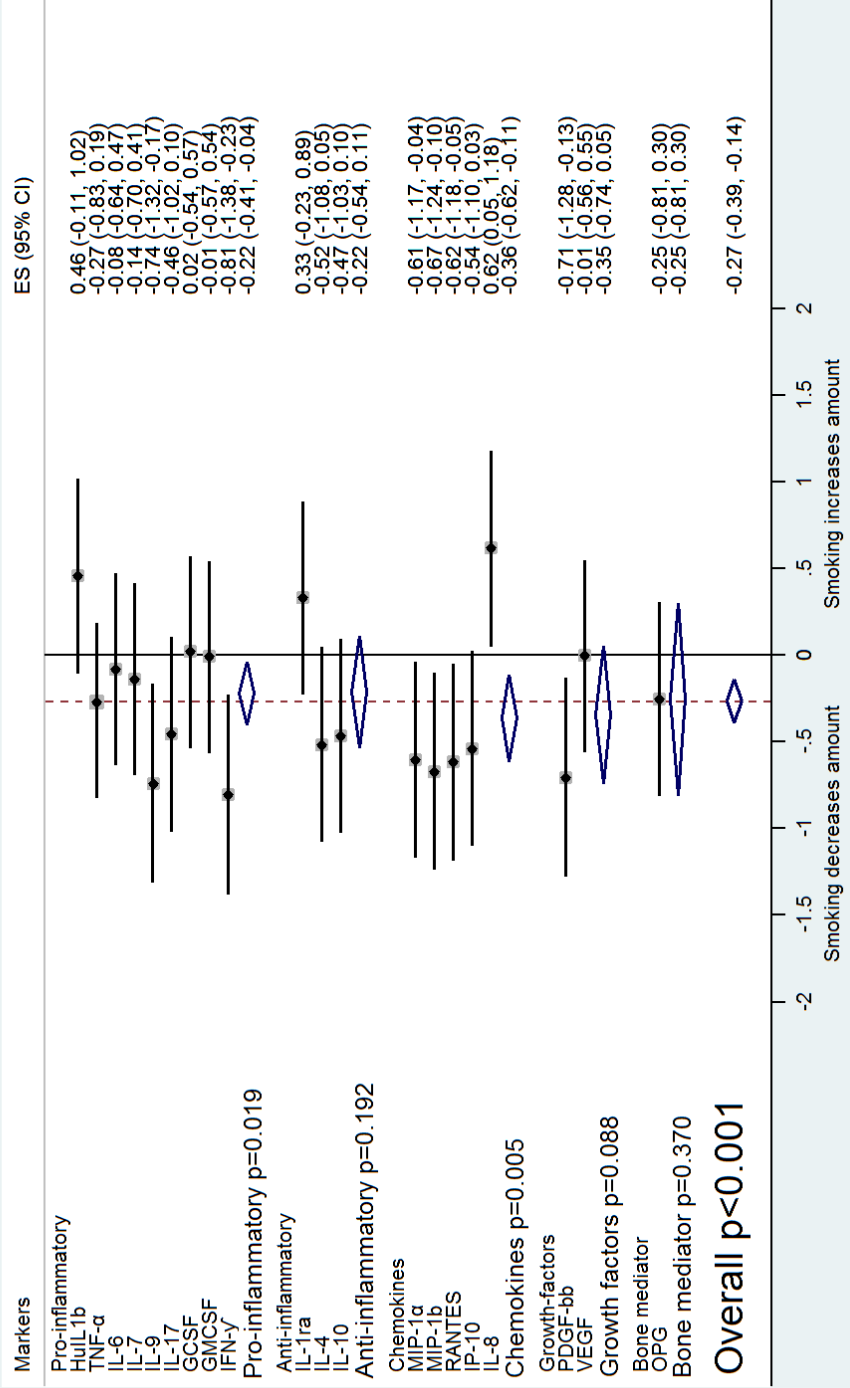
## Effect size at T1





c)

# Effect size at T2



**Overall p<0.001**

*Table 1.* Baseline patient related characteristics, presented as mean ( $\pm$  SD), or percentage (number), stratified by smoking status.

	Smokers (n=25)	Non-smokers (n=25)	<i>p</i>
Male <sup>1</sup>	32 % (8)	52 % (13)	0.158
Elementary school ( $\leq$ 9 years) <sup>1</sup>	76 % (19)	36 % (9)	0.004
Age <sup>2</sup>	56.6 (2.07)	57.9 (1.86)	0.658
Body mass index <sup>2</sup>	22.9 (1.32)	23.62 (1.69)	0.742
Number of teeth <sup>2</sup>	23.5 (1.19)	25.8 (0.48)	0.076
Probing depth <sup>2</sup>	3.9 (0.12)	3.3 (0.10)	0.001
Clinical attachment level <sup>2</sup>	4.6 (0.19)	4.0 (0.11)	0.008
Bleeding index <sup>2</sup>	69.7 (4.00)	66.5 (3.06)	0.532
Plaque index <sup>2</sup>	51.6 (3.96)	56.3 (3.78)	0.415

<sup>1</sup> chi-square test

<sup>2</sup> two-sample independent t-test

Table 2. Collection site\* characteristics at T0, T1, and T2, presented as mean ( $\pm$  SD) and percentage (number), stratified by smoking status.

	T0		T1		T2	
	Smokers	Non-smokers	Smokers	Non-smokers	Smokers	Non-smokers
Probing depth <sup>1</sup>	6.84 (0.27)	6.60 (0.22)	3.88 (0.28) <sup>A</sup>	3.76 (0.19) <sup>A</sup>	4.40 (0.27) <sup>C</sup>	3.72 (0.26) <sup>C</sup>
Clinical attachment level <sup>1</sup>	7.00 (0.30)	6.56 (0.27)	4.80 (0.36) <sup>A</sup>	5.08 (0.32) <sup>A</sup>	5.08 (0.32) <sup>C</sup>	4.92 (0.27) <sup>C</sup>
GCF volume ( $\mu$ l) <sup>1</sup>	0.46 (0.05)	0.58 (0.05)	0.37 (0.04)	0.34 (0.02) <sup>A</sup>	0.43 (0.07)	0.42 (0.04) <sup>C</sup>
Bleeding on probing, positive <sup>2</sup>	88% (22)	100% (25)	36% (9) <sup>A</sup>	48% (12) <sup>A</sup>	53% (13) <sup>C</sup>	76% (19) <sup>C</sup>
Plaque, positive <sup>2</sup>	84% (21)	96% (24)	36% (9) <sup>A</sup>	36% (9) <sup>A</sup>	60% (15)	72% (18) <sup>BC</sup>
$\geq 10^5$ red complex species <sup>2</sup>	40% (10)	68% (17)	40% (10)	36% (9) <sup>A</sup>	12% (3) <sup>B</sup>	0% (0) <sup>BC</sup>
$\geq 10^5$ orange complex species <sup>2</sup>	92% (23)	96% (24)	76% (19)	84% (21)	60% (15) <sup>C</sup>	60% (15) <sup>C</sup>
$\geq 10^5$ <i>Aa</i> species <sup>2</sup>	24% (6)	52% (13)	52% (13)	44% (11) <sup>A</sup>	24% (6) <sup>B</sup>	32% (8)

\*Collection site; the deepest periodontal pocket in each patient at T0

*Aa*: *Aggregatibacter actinomycetemcomitans*

Letters (A, B, and C) in superscript to the mean values denotes a significant difference ( $p < 0.05$ ) over time for that specific clinical and microbiological variable in smokers and in non-smokers. A: T0 vs T1; B: T1 vs T2; C: T0 vs T2.

<sup>1</sup> chi-square test

<sup>2</sup> two-sample independent t-test

Table 3a. Mean ( $\pm$  SEM) amount gingival crevicular fluid markers per site (pg/30 sec) in smokers and non-smokers at T0, T1, and T2.

	T0			T1			T2		
	Smokers	Non-smokers	p	Smokers	Non-smokers	p	Smokers	Non-smokers	p
IL-1 $\beta$	116.06 (21.79)	242.74 (63.44)	0.365	128.93 (47.85)	59.48 (59.48) <sup>A</sup>	0.194	104.28 (21.06)	67.8 (17.78) <sup>C</sup>	0.113
TNF- $\alpha$	12.38 (1.87)	19.39 (2.83)	0.037*	12.65 (1.95)	9.16 (0.94) <sup>A</sup>	0.281	10.62 (0.84)	16.60 (2.81) <sup>B</sup>	0.340
IL-6	5.34 (0.84)	10.28(2.37)	0.275	7.00(1.96)	5.42(1.09)	0.332	8.88(2.27)	9.41(1.89)	0.773
IL-7	1.49 (0.31)	1.70 (0.27)	0.653	1.12 (0.13)	0.96 (0.15) <sup>A</sup>	0.276	1.47 (0.22)	2.42 (0.77) <sup>B</sup>	0.619
IL-9	5.16 (1.11)	8.41 (1.02)	0.003*	4.05 (0.79)	3.88 (0.44)	0.678	3.59 (0.33)	7.62 (1.42) <sup>B</sup>	0.011*
IL-17	235.73 (83.88)	331.66 (78.69)	0.060	249.44 (14.91)	161.53 (36.04)	0.949	123.28 (31.28)	215.70 (52.52)	0.111
G-CSF	74.71 (20.26)	137.07 (23.84)	0.008*	68.66 (14.91)	77.20 (16.36)	0.910	156.91 (49.27)	102.61 (15.03)	0.951
GM-CSF	134.69 (14.75)	173.02 (14.38)	0.033*	108.98(11.89)	91.39 (7.31) <sup>A</sup>	0.161	124.95 (19.87)	119.75 (12.05) <sup>C</sup>	0.967
IFN- $\gamma$	36.87 (7.49)	56.64 (7.93)	0.012*	28.46 (4.39)	30.47 (3.69) <sup>A</sup>	0.652	24.22 (2.60)	62.60 (13.07) <sup>B</sup>	0.006*
IL-1ra	3239.77 (725.91)	3037.24 (515.87)	0.978	2301.01(441.55)	2185.45 (504.33)	0.712	4502.04 (812.65)	2720.69 (551.70)	0.249
IL-4	0.70 (0.17)	1.02 (0.19)	0.136	0.62(0.10)	0.61(0.10)	0.557	0.53(0.07)	1.14 (0.23)	0.073
IL-10	6.26 (1.20)	7.47 (1.03)	0.183	4.17 (0.58)	4.13 (0.43) <sup>A</sup>	0.705	6.46 (1.08)	8.27 (1.20) <sup>B</sup>	0.104
PDGF	6.44 (1.13)	10.87 (1.77)	0.077	4.99 (0.68)	5.41 (0.89)	0.625	4.84 (0.60)	11.97 (2.46) <sup>B</sup>	0.016*
VEGF	76.40 (11.41)	117.73 (14.55)	0.013*	74.05 (11.81)	59.74 (6.43) <sup>A</sup>	0.609	85.15 (13.64)	77.3 (9.14)	0.983
MIP-1 $\alpha$	3.42 (0.91)	5.53 (0.93)	0.014*	3.44 (0.72)	3.00 (0.41)	0.691	2.19 (0.33)	4.1 (0.77)	0.037*
MIP-1 $\beta$	7.18 (1.61)	10.00 (1.44)	0.057	7.87(1.14)	7.96 (1.07)	0.860	7.7 (1.46)	10.86 (1.25)	0.021*
RANTES	25.74 (6.81)	47.05 (8.55)	0.037*	25.36 (6.09)	23.54 (4.00)	0.716	18.16 (3.26)	36.41 (6.49)	0.033*
IP-10	23.16 (7.51)	17.97 (2.22)	0.368	13.67 (2.40)	12.27 (2.12) <sup>A</sup>	0.963	14.33 (2.67)	21.76 (3.61) <sup>B</sup>	0.062
IL-8	113.44 (18.74)	93.61 (13.90)	0.340	98.63 (21.18)	85.55 (19.01)	0.633	220.68 (47.41)	107.23 (26.00)	0.034*
OPG	3.29 (0.17)	3.84 (0.20)	0.052	3.08 (0.18)	3.89 (3.48)	0.003*	3.66 (0.22)	3.94 (0.22)	0.371

Letters A and C in superscript to the mean values denotes a significant decrease ( $p < 0.05$ ) over time between the log mean values for that specific marker in smokers and in non-smokers.

A: T0 vs T1; C: T0 vs T2. Letter B denotes a significant increase ( $p < 0.05$ ) over time. B: T1 vs T2.

Table 3b. Mean ( $\pm$  SEM) concentration gingival crevicular fluid markers per site (pg/mL) in smokers and non-smokers at T0, T1, and T2.

	T0			T1			T2			<i>p</i>
	Smokers	Non-smokers	<i>p</i>	Smokers	Non-smokers	<i>p</i>	Smokers	Non-smokers	<i>p</i>	
IL-1 $\beta$	248.96 (29.08)	435.53 (128.04)	0.898	373.01 (142.30)	165.72 (128.04)	0.211	294.50 (67.44)	157.76 (35.87)	0.092	
TNF- $\alpha$	28.11 (2.80)	32.69 (3.27)	0.350	34.92 (3.91)	27.36 (2.68)	0.288	29.80 (2.92)	41.45 (6.62)	0.278	
IL-6	12.87 (1.78)	16.88 (3.07)	0.937	19.81 (5.08)	15.97 (2.41)	0.368	23.09 (5.61)	23.52 (4.00)	0.738	
IL-7	3.67 (0.68)	2.96 (0.40)	0.385	3.42 (0.43)	3.03 (0.51)	0.339	4.08 (0.67)	6.31 (2.29)	0.580	
IL-9	10.69 (1.44)	14.50 (1.21)	0.019*	10.50 (1.15)	11.85 (1.35)	0.647	10.50 (1.15)	19.04 (3.71)	0.010*	
IL-17	496.52 (141.28)	559.14 (110.94)	0.185	575.99 (144.20)	503.27 (118.80)	0.932	393.19 (112.77)	543.48 (118.30)	0.108	
G-CSF	169.56 (42.01)	248.76 (51.57)	0.050	194.97 (27.28)	222.54 (35.75)	0.937	391.03 (104.83) <sup>C</sup>	260.32 (35.99)	0.957	
G-MCSF	300.50 (9.54)	300.04 (7.20)	0.909	296.02 (9.75)	263.82 (17.55)	0.132	293.21 (7.16)	298.04 (9.40)	0.841	
IFN- $\gamma$	75.05 (7.57)	95.12 (9.14)	0.078	79.41 (10.12)	92.77 (10.88)	0.612	66.34 (6.83)	154.61 (34.45)	0.002*	
IL-1ra	8203.28 (1659.82)	5615.66 (921.58)	0.310	7115.12 (1506.65)	6582.10 (1406.72)	0.751	12567.68 (2488.53)	7775.38 (1787.28)	0.258	
IL-4	1.45 (0.19)	1.70 (0.22)	0.625	1.75 (0.23)	1.82 (0.29)	0.571	1.51 (0.20)	2.84 (0.54)	0.057	
IL-10	15.09 (2.61)	13.38 (1.62)	0.739	12.71 (1.83)	12.21 (1.32)	0.682	17.23 (2.89)	21.35 (2.98) <sup>B</sup>	0.084	
PDGF	14.87 (2.57)	19.56 (3.55)	0.347	15.00 (2.13)	16.89 (2.98)	0.658	14.89 (2.21)	29.17 (5.88) <sup>B</sup>	0.018*	
VEGF	179.82 (18.35)	205.49 (19.60)	0.248	200.12 (20.52)	178.43 (18.18)	0.621	208.76 (21.31)	192.99 (12.89)	0.958	
MIP-1a	6.94 (1.19)	9.33 (1.16)	0.086	8.95 (1.31)	9.03 (1.20)	0.661	6.44 (1.11)	9.89 (1.24)	0.030*	
MIP-1b	16.24 (3.04)	17.74 (1.92)	0.387	22.40 (2.47)	23.55 (3.03)	0.832	19.40 (2.45)	27.45 (2.38) <sup>C</sup>	0.009*	
RANTES	53.46 (9.21)	80.21 (11.14)	0.147	63.02 (10.19)	71.54 (11.85)	0.735	51.48 (9.77)	87.90 (11.60)	0.022*	
IP-10	58.18 (21.23)	31.22 (2.84)	0.997	37.41 (4.42)	37.45 (7.56) <sup>B</sup>	0.986	35.54 (3.51)	55.14 (9.64) <sup>C</sup>	0.028*	
IL-8	272.68 (40.36)	180.11 (32.95)	0.057	285.25 (54.16)	224.93 (45.68)	0.650	517.44 (91.60)	254.58 (44.06)	0.017*	
OPG	2.38 (0.23)	3.21 (0.22)	0.013*	1.99 (0.17)	2.79 (0.18)	0.002*	2.65 (0.23)	2.97 (0.24)	0.354	

Letters A and C in superscript to the mean values denotes a significant decrease ( $p < 0.05$ ) over time between the log mean values for that specific marker in smokers and in non-smokers. A: T0 vs T1; C: T0 vs T2. Letter B denotes a significant increase ( $p < 0.05$ ) over time. B: T1 vs T2.

Table 4. Overall unadjusted and adjusted associations between gingival crevicular fluid markers (pg/30 sec) and cigarette smoking stratified by presence of plaque and numbers of red complex species.

	plaque negative sites		plaque positive sites		<10 <sup>5</sup> red complex species		≥10 <sup>5</sup> red complex species	
	Coef (95 % CI)	p	Coef (95 % CI)	p	Coef (95 % CI)	p	Coef (95 % CI)	p
<b>Unadjusted</b>								
Pro-inflammatory markers	2.11 (-1.61,5.82)	0.257	-3.23 (-5.57,-0.90)	0.008*	-0.74 (-3.2,1.7)	0.552	-3.36 (-6.60,-0.13)	0.042*
Anti-inflammatory markers	0.29 (-0.80,1.39)	0.590	-0.69 (-1.39,0.06)	0.052	-0.02 (-0.74,0.69)	0.954	-1.19 (-2.11,-0.26)	0.012*
Chemokines	0.94 (-1.19,3.06)	0.377	-2.05 (-3.56,-0.55)	0.009*	-0.74 (-2.17,0.69)	0.306	-1.81 (-3.81,0.18)	0.074
Growth factors	0.31 (-0.59,1.21)	0.487	-0.88 (-1.50,-0.26)	0.006*	-0.48 (-1.16,0.19)	0.158	-0.61 (-1.41,0.18)	0.125
Th1 / Th2 -ratio	-5.22 (-17.75,7.29)	0.402	-0.26(-0.93,0.42)	0.446	-3.79 (-10.81,3.22)	0.282	0.98 (-0.23,2.19)	0.108
OPG	-0.70 (-1.45,0.04)	0.062	-0.49 (-0.95,-0.03)	0.037*	-0.33 (-0.86,0.21)	0.227	-1.01 (-1.58,-0.45)	0.001*
<b>Adjusted<sup>§</sup></b>								
Pro-inflammatory markers	12.04 (10.46,13.62)	<0.001*	-5.90 (-9.85,-1.96)	0.004*	-3.52 (-9.51,2.47)	0.236	-6.77 (-12.99,-0.55)	0.034*
Anti-inflammatory markers	0.50 (0.41,0.59)	<0.001*	-1.50 (-5.50,-0.81)	0.016*	-0.70 (-2.79,1.40)	0.498	-1.68 (-3.02,-0.34)	0.016*
Chemokines	7.26 (4.78,9.75)	0.001*	-3.15 (-2.77,0.10)	0.010*	-1.94 (-5.21,1.33)	0.232	-3.00 (-6.98,0.99)	0.134
Growth factors	2.11 (1.63,2.58)	<0.001*	-1.32 (-2.26,-0.39)	0.007*	-0.98 (-2.32,0.34)	0.140	-1.41 (-2.97,0.14)	0.073
Th1 / Th2 -ratio	10.12 (-9.78,10.51)	<0.001*	-1.50 (-3.30,0.30)	0.101	-1.13 (-4.95,2.68)	0.544	0.71 (-2.69,1.28)	0.471
OPG	1.72 (1.05,2.38)	0.002*	-0.64 (-1.25,-0.03)	0.042*	0.01 (-0.92,0.94)	0.982	-1.10 (-1.90,-0.28)	<0.011*

\* significant p-value (<0.05); § regression analysis adjusted for age, gender, and education