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DISSERTATION

The influence of nicotine and smoking on the gene expression of human beta-defensins and proinflammatory cytokines in human keratinocytes (HaCaT) and in keratinized gingiva from smokers and non-smokers

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Dedicated to my parents “Giorgos, Vasileia” and
my brothers “Kyriakos, Manolis and Nikos”.

Contents

1 Introduction

Smoking is a significant risk factor in the development and progression of inflammatory periodontal diseases [Barbour *et al.* 1997; Bergstrom 2004]. Nicotine has a negative systemic and local effect on the host immune responses including vascular damage, impairment of neutrophil functions, decreased production of IgG, decreased proliferation of lymphocytes, altered functions of fibroblasts, altered production of cytokines and growth factors [Johnson & Hill 2004; Johnson & Slach 2001; Ryder 2007; Sopori 2002]. However, the impact of smoking and nicotine on the immune responses in the oral cavity and particularly on the expression and synthesis of antimicrobial peptides is not yet clearly understood.

Antimicrobial peptides (AMPs) such as human beta-defensins (hBDs) seem to play an important role in the oral cavity as a first-line defense against microorganisms [Dale 2002; Dale & Krisanaprakornkit 2001; Weinberg *et al.* 1998]. Cytokines are low molecular weight proteins regulating the immune host responses to infections, trauma and inflammations [Dinarello 2000].

Several studies have shown that smoking can modulate the host innate immunity producing a suppressive effect or impairment on various immune response cells such as monocytes, polymorphonuclear neutrophils (PMNs), lymphocytes and NK cells [Mehta *et al.* 2008]. Another study showed that cigarette smoke extract (CSE) can down-regulate the expression of hBD-2 in stimulated human gingival epithelial cells and up-regulate IL-8 [Mahanonda *et al.* 2009]. Furthermore, studies have shown that smoking, nicotine or cigarette smoke extract can modulate the cell functions and the expression of proinflammatory cytokines and defensins in various human tissues, cells and body secretions such as human periodontal ligament and fibroblast cells [Alpar *et al.* 1998], oral keratinocytes [Johnson *et al.* 2010], gingival crevicular fluid [Petropoulos *et al.* 2004; Tymkiw *et al.* 2011], human airway epithelial cells [Glader *et al.* 2006] and in human skin keratinocyte cell cultures [Radek *et al.* 2010].

There is growing evidence from various studies suggesting that smoking and nicotine affect the immune responses of the host and more specifically the expression of AMPs in various tissues and epithelia. AMPs are a part of the innate immune responses of the host and are the first line of defense against microbial infections. Impairment of this early defense mechanism, due to smoking and nicotine, may in part contribute to the susceptibility, initiation and progression of periodontal diseases. Further investigation is still required to elucidate the mechanisms of nicotine action

affecting the expression of antimicrobial peptides and especially human beta-defensins in oral epithelial keratinocytes.

To investigate the effect of nicotine and smoking on oral epithelial keratinocytes, a human cell line model has been used based on the keratinocyte cell line HaCaT, which were treated with nicotine, the proinflammatory cytokine TNF-α and their respective combinations. On the other hand, keratinized gingival tissue biopsies were collected during routine surgical treatment of patients, who were smokers or non-smokers. Total RNA from cell cultures and gingival biopsies was extracted, reverse transcribed into cDNA and analyzed with quantitative real-time polymerase chain reaction (QRT-PCR) for human beta-defensins-1, -2 (hBD-1, -2) and proinflammatory cytokines IL-1β and IL-6.

2 Literature Review

2.1 Periodontal Tissues in Health and Disease

2.1.1 Anatomy and Function of the Periodontium

The main functions of the periodontal tissues are to attach the tooth to the bone tissues of the jaws and to preserve the unity of the masticatory mucosa of the oral cavity. Periodontium is comprised of the gingiva, alveolar bone, periodontal ligament and root cementum [Nanci & Bosshardt 2006; Pollanen *et al.* 2003] (Fig. 1a). The oral mucosa consists of the masticatory, specialized and lining mucosa. One of the main functions of oral mucosa is to seal and protect the underlying tissues from oral environment, acting like a barrier to mechanical, chemical, microbial and other noxious agents [Pollanen *et al.* 2003].

The gingiva is that part of the masticatory mucosa, which covers the alveolar process and surrounds, like a collar, the cervical portion of the tooth, following the cementoenamel junction and creating a unique attachment called epithelial attachment apparatus. It consists of a stratified epithelial layer and an underlying connective tissue called the lamina propria, and can be differentiated into the free and the attached gingiva. The gingiva epithelia, depending on location demands, exhibit structural differences and is divided into oral, sulcular and junctional epithelium [Nanci & Bosshardt 2006] (Fig. 1b).

2.1.2 Periodontal Disease

The term “periodontal disease” refers to infectious diseases of the periodontium and includes both, gingivitis and periodontitis [Kinane 2001]. Although there are also non-plaque induced periodontal inflammations, the predominant etiology of inflammatory and immune reactions of gingivitis and periodontitis is the microbial plaque. Gingivitis is the inflammation of the soft tissues surrounding the teeth due to microbial plaque and is influenced by several factors such as hormonal changes, medication, genetic factors and smoking [Kinane & Mark Bartold 2007]. Periodontitis follows gingivitis in only a subset of the population, involving the inflammation and distraction of the supporting tissues of the teeth and the formation of pockets and/or gingival recession due to progressive attachment and bone loss [Hugoson & Norderyd 2008; Kinane & Mark Bartold 2007] (Fig. 1a).

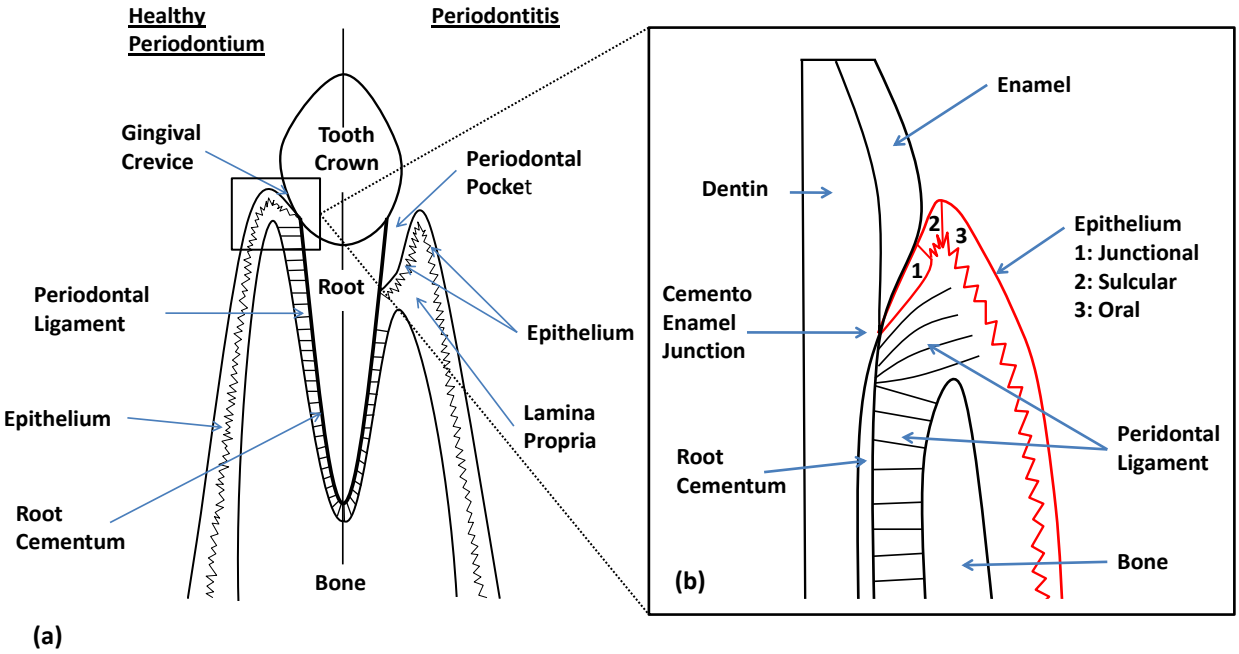


Fig. 1: (a) A schematic drawing of the anatomy and morphology of periodontal tissues in health and disease. (b) Magnification of the frame describing the composition of the gingiva and the contact preface between the gingiva and the enamel (modified and adopted from Pollanen MT 2003).

2.2 Etiology and Pathogenesis of Periodontal Diseases

The oral cavity has a very complex microflora with so far more than 700 identified bacteria species and furthermore it is the only part of the body where hard tissues penetrate epithelia surfaces, making oral cavity a very unique environment [He & Shi 2009]. So it is pertinent to suggest the importance of the oral epithelium and especially the periodontal epithelium as a barrier against a complex variety of noxious agents and microbes. Bacterial biofilm that is formed on the teeth and epithelia surfaces, commonly called microbial dental plaque, is in constant contact with the epithelial cells and is the main infectious factor for initiation and progression of periodontal disease [Dale & Fredericks 2005]. A wide range of products and substances are produced and released by the microorganisms of the dental plaque, affecting the epithelial cells and triggering inflammatory, innate and adaptive immune responses. The destruction of the integrity of the epithelial barrier is the result of the immune host responses, initiated by microbes but propagated by host cells [Kinane *et al.* 2007].

2.3 Aspects of Innate and Adaptive Immune Host Response in Periodontitis

2.3.1 Innate Immune Responses

Historically, the oral epithelium was considered as a passive covering of the underlying tissues, which can be damaged and ulcerated in the present of active disease and then healed with re-epithelialization. Nowadays, this perspective is being fundamentally revised, and the epithelium is considered not only a passive mechanical, chemical, water and microbial barrier to the oral cavity but thought to play an active role in innate host defense and signaling of the environment state to the underlying tissues [Dale 2003].

The first lines of defense that bacteria and their products have to overcome are the nonspecific innate defense epithelia mechanisms. The most significant are the mechanical displacement of microbial plaque and the continuous flushing of the oral epithelia by the secretion of gingival crevicular fluid and saliva. Gingival crevicular fluid and salivary components can prevent the microbial colonization of tissues and teeth or directly precipitate it [Kinane 2001; Schenkels *et al.* 1995]. The bacteria that successfully overcome these first defense obstacles now face other host defense mechanisms. Epithelia cell exfoliation removes bacteria that are attached to the surface, and production of active antimicrobial peptides such us defensins and lysosomal enzymes can directly destroy them [Pollanen *et al.* 2003].

In contrast, microbes produce a variety of enzymes and toxins that can damage the tissue, and also initiate inflammation. Their enzymes break down extracellular substances and intercellular connections between epithelial cells forming wider intercellular spaces [Darveau *et al.* 1997]. The formation of these spaces helps the penetration of other bacteria or its products through the epithelium deeper to the underlying connective tissue as well as the diffusion of various substances involved in the inflammatory response of the host. Via this mechanism the virulence factors initiate inflammation of the gingival vessels producing dilations of the microvascular bed and increasing of its permeability [Abe *et al.* 1991; Meikle *et al.* 1994; Zoellner *et al.* 2002]. Furthermore, mast cells are also activated at an early stage of inflammation producing vasoactive substances such as histamine which induce vascular permeability and vasodilatation [Payne *et al.* 1975; Zoellner *et al.* 2002].

Neutrophils or polymorphonuclear leukocytes (PMNs) are the characteristic dominant inflammatory cells of acute inflammation. They start to migrate from blood

vessels into the tissue towards chemotactic substances and heading to the site of the bacteria invasion [Anderson *et al.* 1985; Etzioni *et al.* 1992]. Clinically, the inflammatory changes of early gingivitis are visible and present as redness with edema and bleeding at probing, brushing or with mastication [Loe *et al.* 1965].

2.3.2 Adaptive Immune Responses

This initial tissue destruction caused by bacteria is continued by exacerbating the host's immune response [Kornman *et al.* 1997]. When bacteria stimuli persist, adaptive immune responses take over in order to enhance the innate immunity against the virulence factors. At the later stages of inflammation, the extravascular exudate is dominated by mononuclear cells. Migrated monocytes from the vessels into the tissue differentiate into macrophages, which exposed to lipopolyssacharides, show phagocytic activity and produce several cytokines and chemokines. Furthermore, after the acute phase of inflammatory response the tissue infiltrate is predominantly consist of lymphocytes, both B cells and T cells. These cells in the presence of antigens and various cytokines begin to differentiate into clones of $CD4^+$ and $CD8^+$ T cells and the B cells differentiate into antibody producing plasma cells [Kornman *et al.* 1997].

The first line of defense called innate immunity followed by the acquired immune responses associated with the activation of antigen-specific B and T lymphocytes [Kornman *et al.* 1997; Meikle *et al.* 1994; Teng 2003; Yamazaki *et al.* 2003]. Innate responses take place at the site of microbial irritation or penetration, as opposed to adaptive responses, which are generated at lymphoid tissues and then move to the site of the microbe invasion [Berglundh & Donati 2005].

2.4 Aspects of Innate Host Response and the Role of Epithelial Cells

There is ample evidence that the primary etiologic agent for the initiation of periodontal disease is the microbial plaque which is accumulate on oral epithelia and the gingival crevice. The initial rapid inflammatory processes against the virulence factors from the plaque are part of the innate immune response, so called because it is inherent in the host and does not require prior learning or experience. This innate host defense system limits the spread and penetration of the virulence factors by maintaining an intact epithelial barrier [Darveau *et al.* 1997].

Epithelial cells and more specific keratinocytes play an active role in the previous processes since they represent, with the contribution of Langerhans cells and melanocytes, the epithelial barrier between the underlying tissues and the environment

exposed to microbial irritation. Substantial evidence indicates that keratinocytes can respond to various microbial stimuli and produce a wide spread of proinflammatory mediators, chemokines and antimicrobial peptides [Jenssen *et al.* 2006; Kupper 1990; Stadnyk 1994]. Studies have shown that keratinocytes have the ability to "sense" the exoteric environment and analogously to respond with the production of autocrine and paracrine messages that are transmitted towards to the deeper underlying tissues and microvessels and thereby to induce an inflammatory response also by integrating innate and acquired immunity [Kornman *et al.* 1997].

2.4.1 Main Receptors and Signaling Pathways

There are a variety of host cell receptors, reflecting the inherent host resistance to microbial infections, which recognize bacterial components and initiate signaling pathways leading to inflammatory responses. These receptors are located at the interface between the body and the environment and include: Toll-like receptors (TLRs), CD14, nucleotide-binding oligomerization domain proteins (NOD), and G-protein-coupled receptors, including formyl-methionyl peptide receptors and protease-activated receptors [Akira 2001; Madianos *et al.* 2005; Medzhitov & Janeway 2000]. Toll-like receptors are the most studied and best characterized class of evolutionary ancient pattern-recognition receptors and are expressed predominantly in cells which are responsible for the first line of defense, such as neutrophils, monocytes/macrophages, dendritic cells and keratinocytes [Akira & Takeda 2004; Beutler 2004]. Epithelial cells express Toll-like receptors and thus have the ability to recognize and respond to microbe-associated molecular patterns (PAMPs) of microbes that are pathogens or commensals [Furrie *et al.* 2005; Kinane *et al.* 2006; Sugawara *et al.* 2006].

2.4.2 The Interleukin-1 Receptor/Toll-like Receptor

Agonist-induced activation of Toll-like receptor complex, provokes a variety of intracellular signaling pathways that can directly lead to both qualitative and quantitative expressions of host inflammatory response. Human Toll-like receptors are type I transmembrane proteins with an intracellular C-terminal domain called Toll/interleukin-1 receptor domain (TIR) and an extracellular leucine-rich repeat (LRR) domain. The intracellular domain is responsible for the interaction and recruitment of different adaptor molecules for the activation of a downstream signaling pathway [Kumar *et al.* 2009]. The best described Toll/interleukin-1 receptor-containing adapter molecules that impart specificity to a Toll-like reception signal transduction pathway include: myeloid

differentiation factor 88 (MyD88), Toll/interleukin-1 receptor-containing adaptor protein (TIRAP) for the MyD88-depended pathway and Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β (TRIF) and the Toll/interleukin-1 receptor domain-containing adaptor-inducing interferon-β-related adaptor molecule (TRAM) for the MyD88-independent pathway. These adaptor molecules provide the scheme required to recruit and activate downstream kinases and transcription factors such as NF-κB and IRFs [Akira *et al.* 2001; Fitzgerald *et al.* 2001; Hoebe *et al.* 2003; Kawai *et al.* 1999; Medzhitov *et al.* 1998; Yamamoto *et al.* 2003] (Fig. 2).

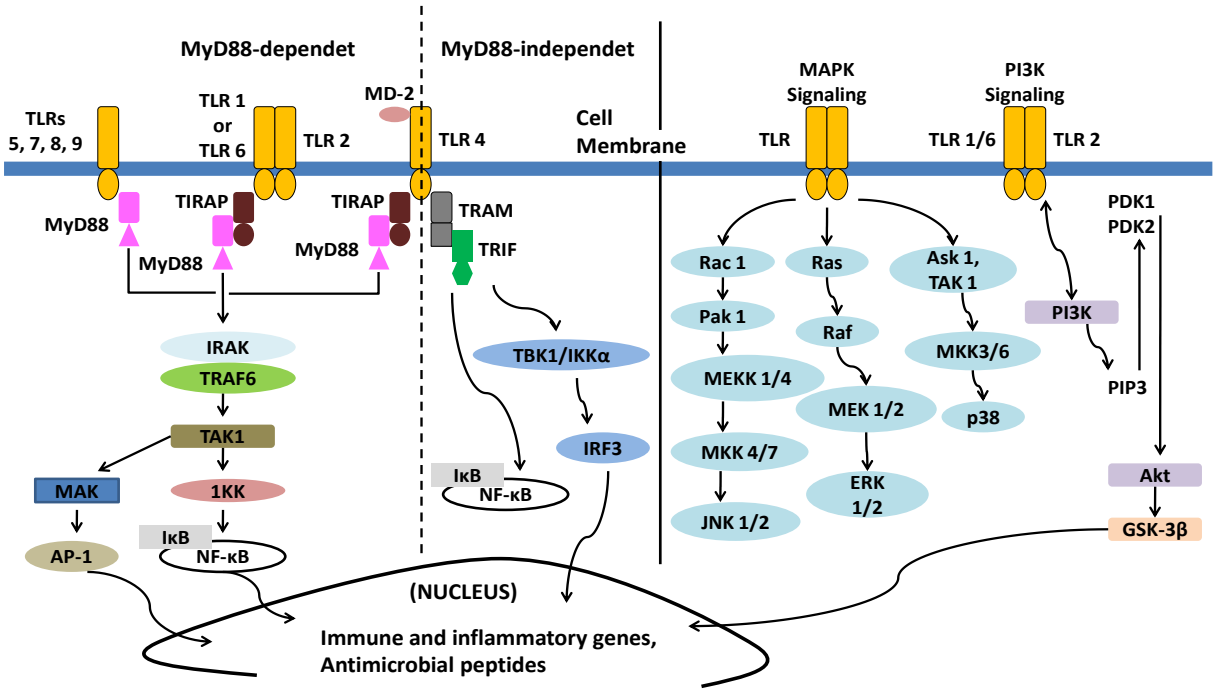


Fig. 2: The Toll-like receptors (TLRs) signal transduction pathways and their differences in terms of mitogen-activated protein kinase (MAPK), myeloid differentiation factor 88 (MyD88-dependet and MyD88-indipendet pathways) and phosphatidylinositol-3 kinase (PI3K) signaling. ERK1/2, extracellular signal-related kinase 1/2; GSK-3β, glycogen synthase kinase 3β; IRF, interferon regulatory factor; JNK, c-Jun N-terminal kinase; MEK, mitogen-activated protein kinase / extracellular signal-related kinase 1/2 kinase; MKK, mitogen-activated protein kinase kinase-4/7; NF-κB, nuclear factor- κB; PDK1, phosphoinositide-dependent kinase 1; PDK2, phosphoinositide-dependent kinase 2; PI3 K, phosphatidylinositol-3 kinase; PIP3, phosphatidy-linositol-3,-4,-5-trisphosphate; TAK1, transforming growth factor-β-activated kinase 1; TBK, tank-binding kinase 1; TIRAP, Toll / interleukin-1 receptor-containing adaptor protein; TRAM, Toll / interleukin-1 receptor domain-containing adaptor inducing interferon-β-related adaptor molecule; TRIF, Toll / interleukin-1 receptor domain-containing adaptor inducing interferon-β (modified and adapted from Mahanonda R. and Pichyangkul S. 2007, Kinane DF 2007).

2.4.3 The Mitogen-activated Protein Kinase (MAPK) Pathway

Furthermore, studies have shown that a major mechanism for the modulation and initiation of inflammatory responses, to a variety of Toll-like receptors agonists, involves the activation of specific kinase pathways [Takeda & Akira 2005]. These pathways activate signaling cascades in both innate and adaptive systems and include the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K). The best-described mitogen-activated protein kinases include the extracellular signal-related kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase and the c-Jun N-terminal kinase (JNK1/2) [Dong *et al.* 2002] (Fig. 2).

2.4.4 The Phosphatidylinositol-3 Kinase (PI3 K) Pathway

Activation of phosphatidylinositol-3 kinase occurs via its regulatory subunit (p85) binding to phosphotyrosine residues present in activated receptors located on the cell membrane. Once phsphatidylinositol-3 kinase is activated a downstream signaling pathway is initiated with the recruitment of various adaptor molecules such as phosphatidylinositol-3,4,5-trisphosphate (PIP3), phosphoinositide-dependent kinase 1 (PDK1), phosphoinositide-dependent kinase 2 (PDK2) serine-threonine kinase(Akt) and glycogen synthase kinase 3β (GSK-3β) [Arbibe *et al.* 2000; Cantley 2002] (Fig. 2).

2.4.5 The Equilibrium between the Host and the Environment

To maintain health, a certain degree of basal or constitutive activation of innate immune response is required from the host. This is achieved with the interaction between commensals and host Toll-like receptors that in normal situations leads to mild “physiological inflammation”, which helps to keep the commensals at the surface of the epithelium in a harmonious equilibrium of host-microbe coexistence [Dixon *et al.* 2004]. TLR signaling is responsible for the innate immune responses, involving the release of antimicrobial peptides such as beta-defensins, cathelicidin and calprotectin, and neutrophil-recruiting chemokine IL-8 [Mahanonda & Pichyangkul 2007]. Therefore, in part, the TLR signaling mechanism contributes to maintaining the microbial ecology of the healthy tissues and limiting the pathogenic infections, by keeping the microbial invaders under control [Dale 2002]. There is a vast variety of signal transduction pathways, cellular pattern-recognition receptors and interactions between them. This ancient form of host defense seems to maintain the epithelia tissue health, enhancing the epithelial barrier at the interface of host and environment. Analyzing and studying the causes of variability in innate immune responses between patients may elucidate

the differences in terms of susceptibility to gingivitis, periodontitis, and other chronic inflammatory diseases [Kinane *et al.* 2007].

2.5 Risk Factors and Susceptibility to Periodontal Disease

It is well documented that the primary etiological cause of the periodontal diseases is the microbial biofilm (plaque), and that a number of risk factors contribute to the susceptibility of individuals to the pathogenesis and severity of the disease. The term “risk factor” refers to “an aspect of personal behavior or lifestyle, an environmental exposure, or an inherited or inborn characteristic, which on the basis of epidemiologic evidence, is known to be associated with health-related conditions” [Loe *et al.* 1965; Papapanou *et al.* 1988]. The presence of a risk factor increases the probability of a disease to occur. Whereas specific microorganisms are considered as potential periodontal pathogens, it is well established that pathogens are necessary but not sufficient for the development of the disease [Lindhe *et al.* 1983]. The initiation and progression of periodontal disease is the result of interactions between the host and genetic, environmental and microbial factors [Kinane & Mark Bartold 2007; Page & Kornman 1997; Pollanen *et al.* 2003; Shiau & Reynolds 2010] (Fig. 3).

The crucial factor for the genesis of periodontal disease is the presence of microorganisms but the progression of the disease is related to host and environmental factors such as genetic, age, gender, smoking, socio-economic factors, specific systemic diseases and local risk factors. The interactions between these various risk factors may lead to influence the host responses generally and the immune responses specifically [Kinane 2001].

2.5.1 Microorganisms

Several studies have shown that specific microorganism such as *Prevotella intermedia, Porphyromonas gingivalis, Bacterioides forrsythus* are correlated with attachment loss, as a measure of periodontal disease, after adjustment for age, plaque, smoking and diabetes [Carlos *et al.* 1988; Grossi *et al.* 1995; Socransky & Haffajee 2005]. There are numerous additional examples such that the cumulative risk for periodontal disease for a given microflora can be estimated. It has been considered that Gram-negative anaerobes were the primary etiology for periodontal pockets, but for many years efforts to correlate specific microorganisms with the disease have been unsuccessful. More recently, studies have found that at diseased sites there are several putative pathogens that are consistently present. The predominant group comprises *Actinomyces*

actinomycetemcomitans (A.a.), *Bacterioides forsythus* (B.f.) (now *Tannerella foersythensis*), *Porphyromonas gingivalis* (P.g.), *Prevotella intermedia* (P.i.), *Fusobacterium nucleatum*, *Campylobacter rectus* and *Treponema denticola* [Albandar *et al.* 1997; Haffajee *et al.* 1998; Hamlet *et al.* 2001; Haraszthy *et al.* 2000; Machtei *et al.* 1997; Papapanou *et al.* 1997; Riggio *et al.* 1998; Ximenez-Fyvie *et al.* 2000].

2.5.2 Environment and Acquired Risk Factors

The prevalence of periodontal disease tends to increase in older age groups but it is not clear if ageing is related to an increased susceptibility to periodontal disease or if it is a cumulative effect of disease over a lifetime. This possibly explains the increased prevalence of disease among older people [Albandar *et al.* 1999]. Other studies suggest that the rate of periodontal destruction is the same throughout adulthood until the age of 70 [Genco *et al.* 1988; Holm-Pedersen *et al.* 1975; Machtei *et al.* 1994].

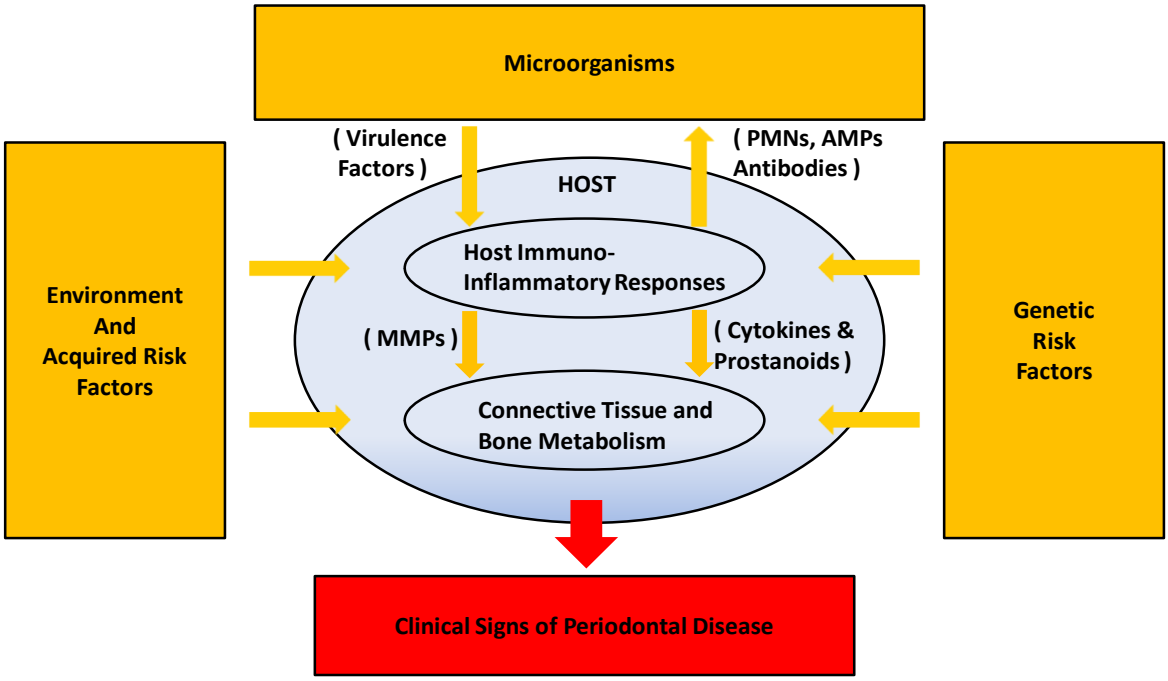


Fig. 3: Model demonstrating various factors and their interactions with the host contributing to the genesis and progression of periodontal diseases (modified and adapted from Page RC and Kornman KS 1997).

There are findings consistent with previous studies indicating that clinical attachment loss of all levels of severity is generally more prevalent in males than in females. The etiology of these gender differences has not been addressed in depth but is thought to be related to poorer oral hygiene, less compliance to suggested oral hygiene and less dental visits than to any genetic factors [Abdellatif & Burt 1987; Kelly &

Harvey 1979]. There are, of course, certain gender-related hormonal differences between males and females, contributing to periodontal diseases, such as pregnancy-associated gingivitis and puberty-associated gingivitis [Shiau & Reynolds 2010].

In general, those who are better educated, wealthier and live under desirable life circumstances have a better health status and are less prone to periodontal diseases [Astrom & Rise 2001; Thomson & Locker 2000]. Perhaps the relation between the socio-economic status and periodontal health is a result of better oral hygiene among the better educated, more positive attitude towards oral hygiene and frequent dental visits.

Systemic diseases can detrimentally affect host defense systems and thus could act as risk factors for periodontal diseases. Severe periodontitis has been strongly associated with depressed neutrophil numbers and functions such as in neutropenia, Chediak-Higashi syndrome, Down's syndrome and Papillon-Lefevre syndrome [Genco & Loe 1993]. There is also a significant evidence linking diabetes mellitus with a higher risk for the inflammatory periodontal disease [Katz *et al.* 1991; Oliver & Tervonen 1993]. Diabetic patients may be at higher risk for periodontal disease than the population in general but only a subgroup of those with poor oral hygiene and/or poor diabetic control and diabetic complications seem to be at particularly high risk [Holm-Pedersen *et al.* 1975]. In early reports, acquired immunodeficiency syndrome (AIDS) was associated with severe forms of gingivitis and periodontitis but recent cross-sectional studies have failed to detect differences between people with AIDS and healthy controls [Klein *et al.* 1991; Lamster *et al.* 1994; Masouredis *et al.* 1992; Swango *et al.* 1991].

It is important to consider, although general risk factors are currently under intensive investigation, that any microbial plaque retentive factor such as restorative deficiencies or overhangs may contribute to the local risk factors of periodontal disease [Kinane 2001].

2.5.3 Genetic Risk Factors

There is a growing amount of evidence indicating that genetic influences have a significant role in periodontal disease and our understanding has grown remarkably in recent years. In 1997, the first report describing an association between a specific genotype of the polymorphism IL-1 gene cluster and severe periodontitis was published [Kornman *et al.* 1997]. This gene polymorphism has received a lot of research attention since then [Cullinan *et al.* 2001; Diehl *et al.* 1999; Laine *et al.* 2001; Lang *et al.* 2000;

Mark *et al.* 2000; McDevitt *et al.* 2000]. However, not all studies found a link between genetic polymorphism of IL-1 and periodontal disease [Hodge *et al.* 2001; Kinane *et al.* 1999]. Furthermore, a gene mutation was identified as being responsible for prepubertal periodontitis. It overlaps the region of chromosome 11q14 containing the cathepsin C gene which is responsible for Papillon-Lefevre and Haim-Monk syndromes [Hart *et al.* 2000]. More research, especially epidemiological studies with subjects with or without disease, is essential before the genetic contribution to periodontal diseases can be specified [Burt 2005].

2.6 Smoking as a Risk Factor in Periodontal Disease

Numerous investigations and both cross-sectional and longitudinal studies have been performed over the past 15 years providing ample epidemiological evidence that smoking is clearly a risk factor for periodontal disease, with the odds ratio of having periodontitis attributed to tobacco use compared to non use of tobacco being 2.5 to 6.0 [Bergstrom & Preber 1994]. The evidence that smoking constitutes a considerably increased risk factor for periodontal disease has emerged from various studies [Amarasena *et al.* 2002; Bergstrom & Floderus-Myrhed 1983; Chen *et al.* 2001; Gonzalez *et al.* 1996; Grossi *et al.* 1995; Grossi *et al.* 1994; Haffajee & Socransky 2001; Ismail *et al.* 1990; Locker 1992]. Moreover, a 10-year longitudinal alveolar bone radiographic study showed that smoking is a significant predictor of future bone loss [Bolin *et al.* 1986], and a 5-year study found that smokers have an increased risk of attachment loss [Beck *et al.* 1997].

Furthermore, a great number of studies have investigated, in terms of potential mechanisms, the correlation of tobacco smoking and periodontal disease, and it appears that smoking may affect the vasculature, the humoral immune system, the cellular immune system and the inflammatory system [Kinane & Mark Bartold 2007]. It has been suggested that slower wound healing in smokers may be due to the inhibition of growth and attachment of fibroblasts in periodontal ligament [James *et al.* 1999] as well as the slower post-therapy decrease of neutrophils and white blood cells [Christan *et al.* 2002]. Evidence that tobacco smoking promotes the growth of specific periodontal pathogens is still under debate, some early investigations have found no difference in the prevalence of these bacteria subgingivally [Preber *et al.* 1992; Stoltenberg *et al.* 1993], but other recent studies have suggested the opposite [Haffajee & Socransky 2001; Zambon *et al.* 1996].

Smoking affects also other aspects of host responses by inhibiting granulocyte function [Soder *et al.* 2002] and by interacting with the IL-1 gene cluster. A study found no difference in mean clinical attachment loss between smokers and non-smokers, in those who were genotype-negative, but for those who were genotype-positive, smokers had greater clinical attachment loss than the non-smokers [Meisel *et al.* 2002]. Moreover, smoking stimulates the production of TNF-α and cytokines that may lead to tissue destruction [Fredriksson *et al.* 2002; Gustafsson *et al.* 2000]. Interestingly, smoking has been shown to be a greater risk factor than insulin-dependent diabetes [Moore *et al.* 1999].

There is also evidence suggesting that smoking suppresses the vascular reaction which follows gingivitis and depresses host response to infection in a variety of ways [Bergstrom *et al.* 1988]. Smokers with periodontal disease present less clinical inflammation [Feldman *et al.* 1983] and gingival bleeding [Bergstrom & Floderus-Myrhed 1983] compared with non-smokers. These findings support the hypothesis that smoking masks the signs of inflammation [Bergstrom & Preber 1994], and this may be explained by the fact that nicotine, one of a great number of tobacco smoke by-products, causes local vasoconstriction, reducing blood flow, edema, and clinical signs of inflammation. Another study has shown that cigarette smoke extract and nicotine downregulate the expression of human beta-defensin-2 in human gingival epithelial cells [Mahanonda *et al.* 2009].

2.7 Defensins and Cytokines (molecular biology of periodontitis)

2.7.1 Antimicrobial Peptides

Epithelial tissues are constantly exposed to the environment, and, analogously, have to serve as a barrier between the microorganisms that surround us and the underlying tissues. These microorganisms trigger the innate immune system of the host to produce cytokines and antimicrobial peptides, contributing to the maintenance of health of the upper respiratory tract and oral cavity, a major entry point for virulence factors [Campochiaro 1993; Durum *et al.* 1985; Ganz 2002; Hedges *et al.* 1994; Jung *et al.* 1995; Stadnyk 1994; Svanborg *et al.* 1994]. Anti-microbial proteins are early responses of the host to microbial invasions and can confer rapid protection from microorganisms, either before or parallel with the development of the acquired immune responses.

Studies have found that many known anti-microbial proteins are present in the oral cavity, such as neutrophil defensins, beta-defensins, lysozyme,

bactericidal/permeability-increasing proteins, bactericidal/permeability-increasing protein-like proteins, histatins, proline-rich proteins, salivary agglutinin (GP-340), cathelicidin LL-37, cystatins, lactoferrin, salivary peroxidase, mucins and secretory leukoproteinase inhibitor [Ghafouri *et al.* 2003; Guo *et al.* 2006; Hardt *et al.* 2005; Hu *et al.* 2005; Huang 2004; Vitorino *et al.* 2004; Walz *et al.* 2006; Wilmarth *et al.* 2004; Xie *et al.* 2005; Yao *et al.* 2003].

2.7.2 Human beta-defensins

Mammalian defensins are antimicrobial peptides consisting of 30-40 amino acid residues with a molecular weight of about 3-4 kD [Ganz & Lehrer 1994; Huttner & Bevins 1999]. All defensin molecules containing six cysteine residues that are paired with intramolecular disulfide bonds in various patterns, stabilizing a triple-stranded antiparallel β-sheet structure [Bauer *et al.* 2001; Ganz & Lehrer 1994; Kagan *et al.* 1994]. There are three main defensin subfamilies, α-, β-, and θ-defensins classified according to the length of the peptide segment between cysteine residues and the disulfide bond patterns [Bauer *et al.* 2001; Hill *et al.* 1991; Tang *et al.* 1999].

Human beta-defensins are expressed predominantly in all human epithelial tissues tested to date, providing the first line of defense of the host against the microbial pathogens of the environment [Huttner & Bevins 1999; Weinberg *et al.* 1998]. The first human beta-defensins, hBD-1 and hBD-2, were discovered in 1995 and 1997, respectively [Mathews *et al.* 1999]. Moreover, hBD-3 and hBD-4 have also been isolated [Garcia *et al.* 2001; Garcia *et al.* 2001; Harder *et al.* 2001]. Human beta-defensins constitutively or inducibly expressed, were found in the following tissues and organs: tracheal epithelia, kidney, urinary tract, human corneal epithelia cells, oral mucosa, tongue, gingiva and other epithelia [Diamond & Bevins 1994; Diamond *et al.* 1996; Dunsche *et al.* 2002; Dunsche *et al.* 2001; Krisanaprakornkit *et al.* 1998; Kumar *et al.* 2006; Schonwetter *et al.* 1995; Supp *et al.* 2004; Valore *et al.* 1998; Zhao *et al.* 1996].

Human beta-defensins are induced by inflammation, by proinflammatory cytokines and by bacterial lipopolysaccharide (LPS) [Diamond & Bevins 1994; Diamond *et al.* 1996; Russell *et al.* 1996; Singh *et al.* 1998]. More specific human beta-defensin-1 (hBD-1) is constitutively expressed, while hBD-2 and hBD-3 expression is up-regulated in inflamed skin and other epithelia [Harder *et al.* 1997; Harder *et al.* 2000]. Furthermore, gingival epithelia or cells derived from the tissue express hBD-1, hBD-2,

hBD-3 and hBD-4 [Bissell *et al.* 2004; Dommisch *et al.* 2005; Krisanaprakornkit *et al.* 2002; Krisanaprakornkit *et al.* 1998; Vankeerberghen *et al.* 2005].

Human beta-defensins show different patterns of expression depending on the layers of stratified epithelia that are produced. The expression of hBD-1 and hBD-2 is more potent in the upper portion of the tissue, especially at the gingival margin where the epithelia are in constant contact with microbial plaque [Dale & Fredericks 2005]. In the epithelial basal cells mRNA and proteins are absent, while mRNA expression in the first suprabasal layer and peptide accumulation in the mid-spinus region of the epithelia is observed [Dale & Krisanaprakornkit 2001].

2.7.3 Mechanisms of Action Reported for Antimicrobial Peptides

The antibiotic activity of defensins is thought to be depended on their cationic and amphipathic nature, which enables the peptides to bind to negatively charged membrane targets of bacteria [Tossi & Sandri 2002]. These targets include lipopolysaccharides in Gram-negative bacteria, polysaccharides and teichoic acids in Gram-positive bacteria, and phospholipids (phosphatidyl-glycerol) in the inner membrane of both Gram-negative and -positive bacteria [Ganz & Lehrer 1994; Quayle *et al.* 1998; Weinberg *et al.* 1998]. The eukaryotic cytoplasmic membrane is phosphatidylcholine-rich, which explains at least electrostatically the selectivity of these peptides for bacteria [Weinberg *et al.* 1998].

Although the exact mechanism of action of antimicrobial peptides remains under investigation and is a matter of controversy, some possible mechanisms are proposed. Through their affinity for lipopolysaccharides, cationic antimicrobial peptides displace cations such as Ca^{2+} and Mg^{2+} from the outer membrane of Gram-negative bacteria, leading to a disruption of the membrane by forming pores [Hancock 1997; Reddy *et al.* 2004; Tossi *et al.* 2000; Zimmermann *et al.* 1995]. This mechanism is called the “self-promoted uptake” pathway of antimicrobial peptides. Another mechanism suggested is that the antimicrobial peptides bind to the surface of bacterial phospholipid membranes as monomers, covering the membrane in a “carpet-like manner” and dissolve it like a detergent [Hancock & Diamond 2000; Reddy *et al.* 2004] (Fig. 4).

Human beta-defensin-1 (hBD-1) is produced constitutively in many epithelia, and, like other beta-defensins, hBD-1 is thought to control the microflora on epithelial surfaces [Huttner & Bevins 1999; Mathews *et al.* 1999]. Furthermore, hBD-1 kills

Escherichia coli at micromolar concentrations and other Gram-negative bacteria at concentrations ranging from 60 to 500 µg/ml [Schroder 1999].

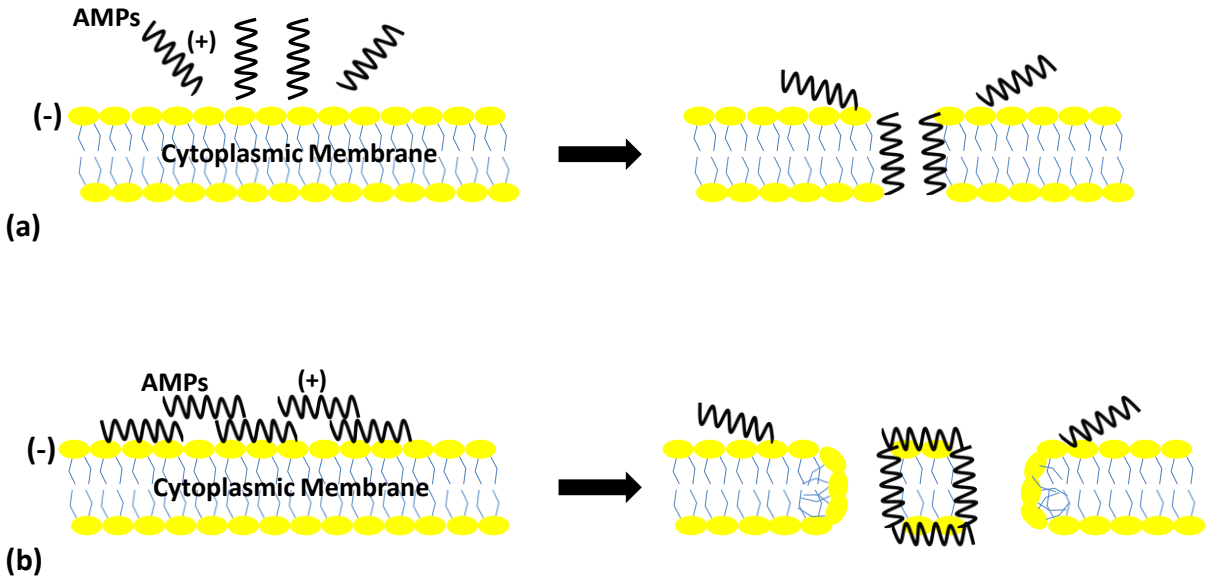


Fig. 4: Mode of action schemes of Antimicrobial Peptides (AMPs):
(a) Pore formation: The peptide monomers bind to the cell membrane followed by the aggregation of more peptide molecules, after which the peptide helices insert themselves into the hydrophobic cellular membrane forming pores, causing leakage of cytoplasmic material and hence death of the cell.
(b) Carpet formation model: The antimicrobial peptide monomers bind to the phospholipid head groups of the cellular membrane followed by the alignment of the peptides on the membrane such that the hydrophilic residues face the phospholipid head groups. Later, a reorientation of the peptides into the hydrophobic core of the membrane occurs, leading to the disintegration of the membrane due to disruption of the phospholipid bilayer (modified and adopted from Reddy KV 2004).

Human beta-defensin-2 is expressed in keratinocytes, the gingival mucosa and other tissues [Harder *et al.* 1997; Mathews *et al.* 1999]. The expression of hBD-2 can be induced by IL-1β, TNF-a, bacteria lipopolysaccharides, by contact with Gram-negative (*E. coli, Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus*), and the yeast *Candida albicans* [Chadebech *et al.* 2003; Harder *et al.* 1997; Huttner & Bevins 1999; Mathews *et al.* 1999; Zhao *et al.* 1996]. The hBD-2 expression is increased in inflammatory disorders [Hiratsuka *et al.* 1998; Schonwetter *et al.* 1995; Singh *et al.* 1998], whereas hBD-1 is constitutively expressed and may serve as a defense of epithelia in absence of inflammation [Singh *et al.* 1998]. HBD-2 is highly

effective in killing Gram-negative bacteria such as *E. coli* and *P. aeruginosa* with a LD_{90} (LD_{90} is the dose that achieves 90% reduction of colony-forming units) near 10 µg/ml [Harder *et al.* 1997]. HBD-2 also kills the yeast *C. albicans* with a LD_{90} of 25 µg/ml and has bacteriostatic effect on *S. aureus* at a concentration of 100 µg/ml. The previous data suggest that hBD-2 is predominantly active against Gram-negative bacteria and yeasts [Harder *et al.* 2001; Schroder & Harder 1999]. Furthermore, studies have shown that human beta-defensins can be effective not only against bacteria but also against viruses [Dale & Fredericks 2005; Zhang *et al.* 2002]. Especially hBD-2 and hBD-3 expression in oral epithelial cells is upregulated by human immunodeficiency virus-1 (HIV-1) and these defensins can also inhibit the replication of HIV *in vitro* [Quinones-Mateu *et al.* 2003].

The expression of hBD-2 is regulated by various intracellular signaling pathways such as the NF-κB pathway and the G-protein-coupled protease-activated receptors that mediate cellular responses to extracellular proteinases [Chung *et al.* 2004]. Moreover, it has been reported that expression of hBD-2 is also regulated by the mitogen-activated protein (MAP) kinase-signalling cascade that is associated with cytokine and stress responses [Krisanaprakornkit *et al.* 2002].

These results are in agreement with the hypothesis that stratified epithelia are capable of orchestrating a carefully staged and measured innate immune response in the presence of pathogen or commensal microorganisms, through the expression of antimicrobial peptides. It has also been suggested that commensal organisms aid the epithelial tissue in preparation for possible pathogen invasions and maintain the balance between health and disease without fully releasing other innate immune responses [Dale 2002].

2.8 Nicotine and Defensins

Tobacco smoke contains more than 3800 chemicals, including carbon monoxide, hydrogen cyanide, reactive oxidizing radicals, nicotine and others that are known or suspected to be carcinogens [Eriksen *et al.* 1988]. Nicotine is the addictive and the most extensively studied component of tobacco in terms of its effect on different types of cells and epithelia [Johnson & Guthmiller 2007]. The periodontal epithelium can be affected by nicotine, by its direct contact to the tobacco smoke with smoking or via the blood flow in the tissue and hence via the gingival crevice fluid whose nicotine concentration in

smokers is 300 times higher than that of plasma (20 ng/ml) [Benowitz & Jacob 1984; Ryder *et al.* 1998].

Although the epithelium of the oral cavity is the first tissue that comes into contact with cigarette smoke and that plays an important role to the innate immunity, there are not many studies about the effect of smoking on gingival epithelial cells. A study showed that cigarette smoke extract and nicotine suppress the expression of human beta-defensin-2 in human epithelial gingival cells [Mahanonda *et al.* 2009]. Another study found that acrolein, which is a component of cigarette smoke, inhibits the expression of mRNA and protein of human beta-defensine-2 *in-vitro* in the sinonasal epithelial cells [Lee *et al.* 2007]. Moreover another interesting study suggested that nicotine, via the activation of nicotinic achetylcholine receptors (nAChRs), suppresses the expression of antimicrobial peptides such as beta-defensins in the skin of mice and in normal human keratinocyte cell cultures and thus increases susceptibility to microbe infections [Radek *et al.* 2010].

Due to the fact that smoking and, more specifically, nicotine and its effects on oral epithelia cells (keratinocytes), have not been addressed in depth, the present *in-vitro* and *ex-vivo* study tries to elucidate some aspects of this interaction.

3 Aims and Questions of the Study

Aims of the study:

1. Determination of gene expression of the proinflammatory cytokines IL-1β and IL-6 and of human beta-defensins-1 and -2, in the immortalized human keratinocyte cell line HaCaT, after stimulation with nicotine, TNF-α and with their combination.

2. Determination of gene expression of the proinflammatory cytokines IL-1β and IL-6 and of human beta-defensins-1 and -2 in healthy gingiva of smokers and non-smokers.

Questions of the study:

1. Does gene expression of the proinflammatory cytokines IL-1β and IL-6 and of human beta-defensins-1 and -2 take place in the culture of human keratinocytes (HaCaT)?

2. Does alteration of the gene expression of proinflammatory cytokines and human beta-defensins take place after the stimulation of human keratinocytes (HaCaT) with TNF-α, nicotine, and their combination?

3. Does gene expression of proinflammatory cytokines and of human beta-defensins take place in the keratinized epithelium of healthy human gingiva of smokers and non-smokers?

4. What is the influence of smoking (use of 10 ≥ cigarettes per day) on the gene expression of proinflammatory cytokines and human beta-defensins in the keratinized epithelium of healthy human gingiva of smokers and non-smokers?

Control variables:

Gene expression (gene copies) of the cell-DNA

Null hypotheses:

The first null hypothesis ($H_{0\text{-}1}$) was: The gene expression of the proinflammatory cytokines IL-1β and IL-6 and of human beta-defensins-1 and -2, in non-stimulated ($\mu_{1\text{-}1}$) (HaCaT) keratinocytes does not differ from that of stimulated ($\mu_{2\text{-}1}$) with nicotine, TNF-α and their combination.

$$\mathbf{H_{0\text{-}1}: \mu_{1\text{-}1} = \mu_{2\text{-}1}}$$

The second null hypothesis ($H_{0\text{-}2}$) stated that gene expression of proinflammatory cytokines IL-1β and IL-6 and of human beta-defensins-1 and -2, in healthy human keratinized gingiva of non-smokers ($\mu_{1\text{-}2}$) does not differ from that of smokers ($\mu_{2\text{-}2}$).

$$\mathbf{H_{0\text{-}2}: \mu_{1\text{-}2} = \mu_{2\text{-}2}}$$

The alternative hypotheses ($H_{A\text{-}1}$ and $H_{A\text{-}2}$) are:

$$\mathbf{H_{A\text{-}1}: \mu_{1\text{-}1} <> \mu_{2\text{-}1}}$$

or

$$\mathbf{H_{A\text{-}2}: \mu_{1\text{-}2} <> \mu_{2\text{-}2}}$$

4 Materials and Methods

4.1 Overview

In the first part of the present study, the immortalized human keratinocyte cell line HaCaT was stimulated with the proinflammatory cytokine TNF-α, nicotine and a combination of the two. In the second part, keratinized gingival tissue biopsies were collected after routine surgical treatments of patients with clinically healthy periodontium who were smokers or non-smokers. Total RNA from HaCaT cells and gingival biopsies was extracted and analyzed by real-time RT-PCR for human beta-defensins-1, -2, and interleukins IL-1β and IL-6, as well as GAPDH-mRNA. Finally, the results were statistically evaluated (Fig. 5).

4.2 Cell Culture

The cell line HaCaT was used for the evaluation of the gene expression of proinflammatory cytokines and human beta-defensins. HaCaT cell line (human adult skin keratinocytes propagated under low Ca^{2+} conditions and elevated temperature) was developed by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg). This human cell line demonstrates similar characteristics with the primary oral keratinocytes and is easy to handle because of the steady rate of replication and proliferation [Boukamp *et al.* 1988].

4.2.1 Cultivation and Subcultivation (passaging)

The HaCaT cells were cultured in Dulbecco's Modified Eagles's Medium (DMEM, Biochrom, Berlin, Germany) containing 10% Fetal Bovine Serum (FBS, Biochrom, Berlin, Germany) with 100 U/ml Penicillin and 100 U/ml Streptomycin (Biochrom, Germany). Cells were grown in 75 cm^2 tissue culture flasks (TPP, Trasadingen, Switzerland), in an incubator (Heraus, Hanau, Germany), at 37 ˚C and in a humidified atmosphere of 5.5% CO_2. Every two days the cells were rinsed with 6 ml Dulbecco's phosphate buffered saline (PBS, Biochrom, Berlin, Germany) in order to remove catabolites and dead cells, and culture medium was replaced with fresh medium.

The passaging of the cells was performed when the cell confluence was ca. 60-70%. The cells were rinsed twice with PBS and then incubated in the incubator for ca. 3 minutes with 2 ml trypsin 0.05% - EDTA 0.02% (Biochrom, Germany) solution. With repeated tapping of the culture flask the cells were dissociated from the flask's ground and the enzymatic impairment of the cells was kept as small as possible.

Afterwards, the cells were resuspended with the same volume (2 ml) of trypsin inhibitor in order to stop the proteolytic activity of trypsin. The cells were then pelleted by centrifugation at 1,200 rpm for 5 minutes. The supernatant was aspirated with suction and the cells were resuspended in 5 ml of trypsin inhibitor. The cells were once more centrifuged at 1,200 rpm for 5 minutes and the supernatant was decanted. Afterwards the cell pellet was resuspended in DMEM and seeded in new culture flasks with a split ratio of 1:10. The first change of medium performed after 48 hours and the following passage of the cells was performed one week later with the same methods as described previously. In order to perform the treatments, the cells were equally seeded in six-well culture plates (TPP, Trasadingen, Switzerland) with the addition of DMEM, allowed to adhere overnight and grown until a confluence of 80-90%.

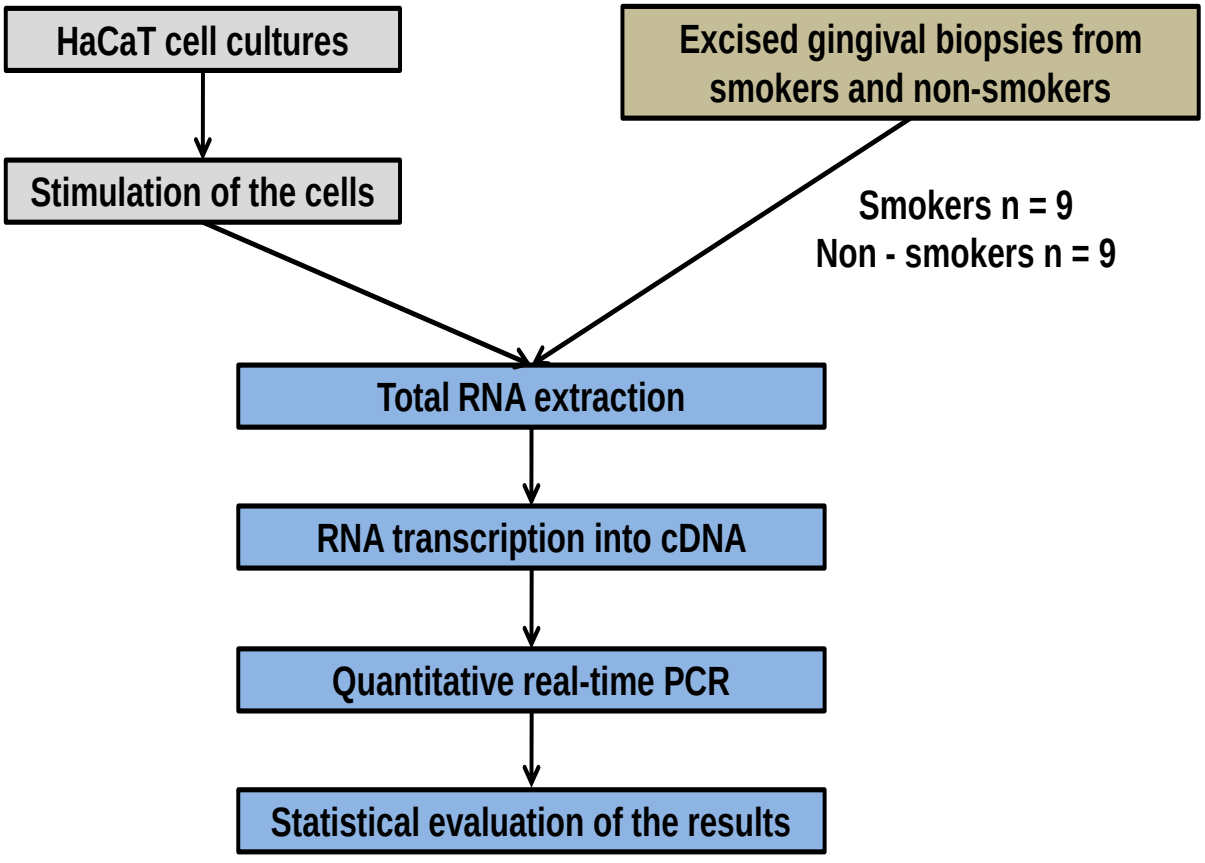


Fig. 5: Experimental set-up of the present study.

4.2.2 Stimulation of the Cell Culture

The adhered cells were incubated for 12 hours with starvation medium (modified DMEM with 0.5% serum) and then treated with the following alternatives: (1) 10 µg/ml nicotine (Sigma, St. Louis, MO, USA), (2) 50 ng/ml TNFα (PeproTech, Germany, Hamburg), (3) serum-free medium and (4) their combinations. All treatments were repeated at least three times, TNF-α served as a positive control and serum-free medium was used as negative control. The stimulation protocol is shown in figure 6. In two groups, cells were pretreated either with nicotine (well 5) or TNF-α (well 6) for 12 h each. The cells in the other wells (wells 1-4) were treated with serum-free medium also for 12 h. The next 12 h the treatments were as follows: negative control (serum free-medium; well 1), positive control (TNF-α; well 2), nicotine (well 3), simultaneously nicotine and TNF-α (well 4). The pretreated cells in wells 5 and 6 were now treated with TNF-α (well 5) and nicotine (well 6), respectively, for 12 h.

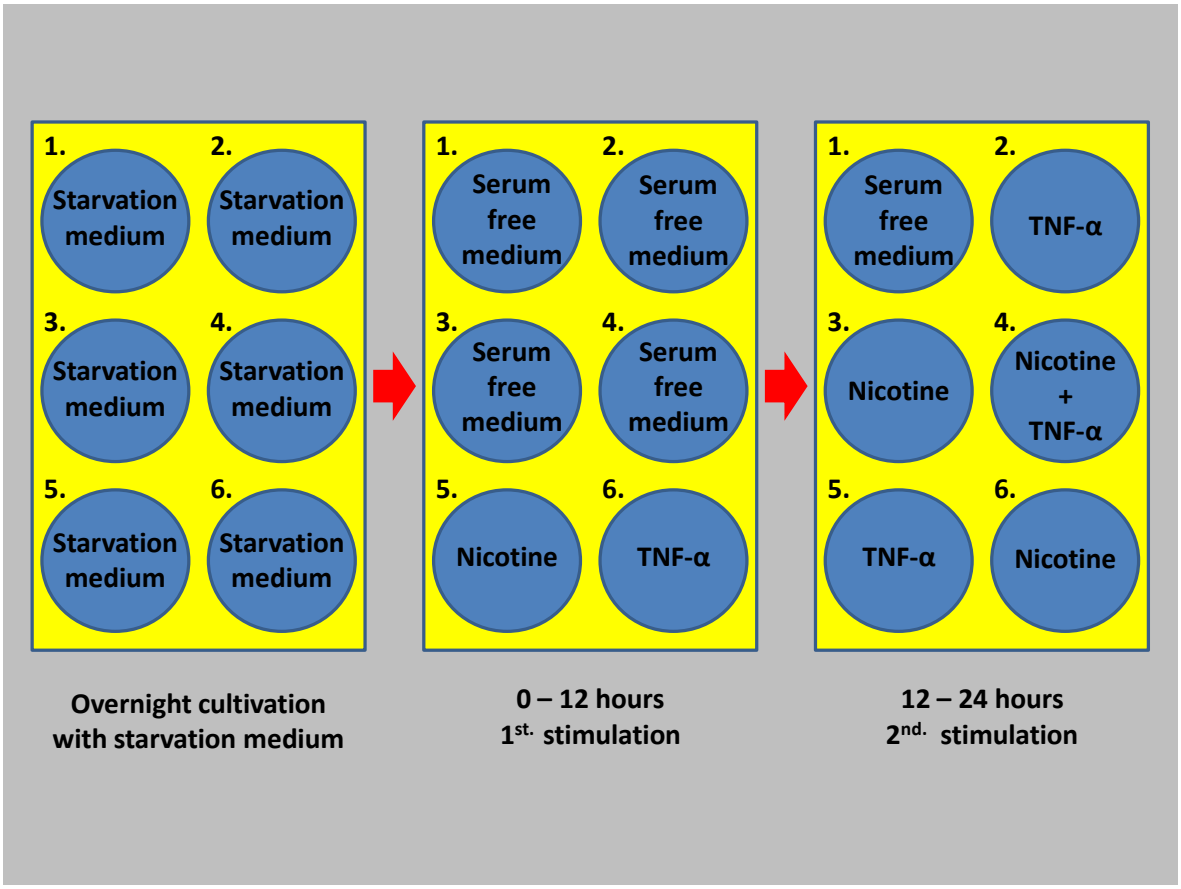


Fig. 6: 24 hours stimulation protocol for one six-well plate. All treatments were repeated in three independent experiments.

4.2.3 RNA-Extraction from the Cells

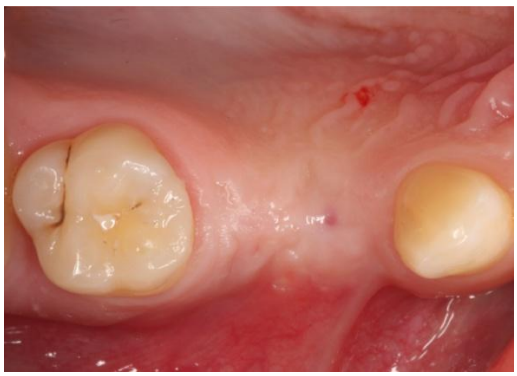
After the treatment, the HaCaT cells were washed twice with PBS and harvested with a cell scraper (TPP, Trasadingen, Switzerland). The cell lysates were passed through a 21-gauge needle several times, and total cellular RNA was extracted and cleaned using RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and stored at -80 ˚C. Afterwards, total RNA concentrations were quantified (see 4.3.2), and reverse-transcribed with reverse transcription-polymerase chain reaction (RT-PCR) (see 4.4.1).

4.3 Excision of Gingival Tissue Biopsies

The keratinized gingival tissue biopsies were excised from patients, during routine surgical treatment at ChariteCentrum3, Charité-Universitätsmedizin Berlin. All the patients had received printed information and signed a written consent according to the guidelines of the Central German Ethics Committee's referral, focusing on the use of body materials in medical research [Central-German-Ethics-Committee]. This pilot study population included 18 patients, 7 men and 11 women aged range 21-80 years, the median age being 59.3 years. The patients were interviewed about smoking habits and then divided into smokers n=9 and non-smokers n=9 groups (Tab. I). All smokers were cigarette smokers. Patients that reported the use of 10 ≥ cigarettes per day were classified as smokers [Erley *et al.* 2006] and non-smokers have never smoked tobacco. For ethical reasons patients were informed about the health effects of tobacco smoking and encouraged to quit smoking. Patients with systemic diseases, those who had taken analgesics in the previous 24 hours, were under medication or with untreated periodontal diseases were excluded from the study. The gingival biopsies were obtained either with gingival tissue punch technique from direct implantation (Fig. 7a, 7b), or from gingival excision performed to expose an inserted endosseous implant. The tissue biopsies were transferred immediately in cryotubes (Nunc, Roskilde, Denmark) containing a RNA stabilization reagent (RNAlater; Qiagen, Hilden, Germany) (Fig. 8a, 8b). Specimens were stored at -80 ˚C, and transported in a cryo carrier with liquid nitrogen.

Table I: Study population according to smoking status, gender and age (SD: standard deviation).

		Gender		Age (years)	
Smoking	***n***	**Men**	**Women**	**mean**	**SD**
Smokers	**9**	**3**	**6**	**45.3**	**14.3**
Non-smokers	**9**	**4**	**5**	**54.7**	**15.2**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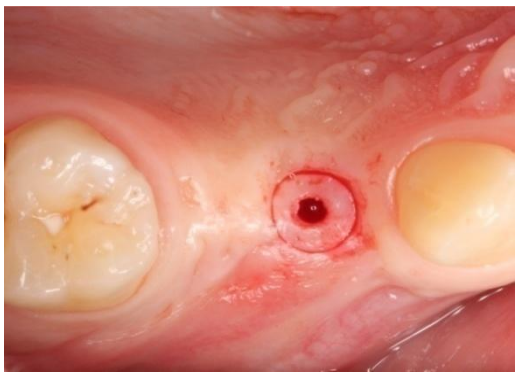
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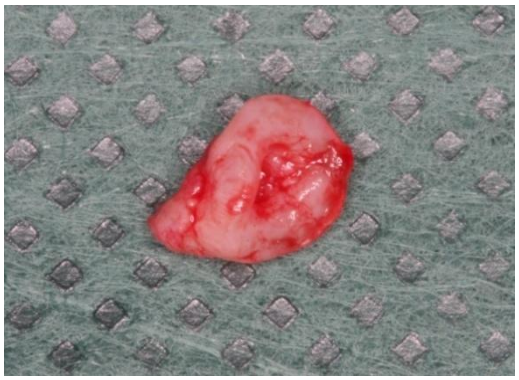
Fig. 7a, 7b: Excision of the gingival biopsy with the tissue punch technique.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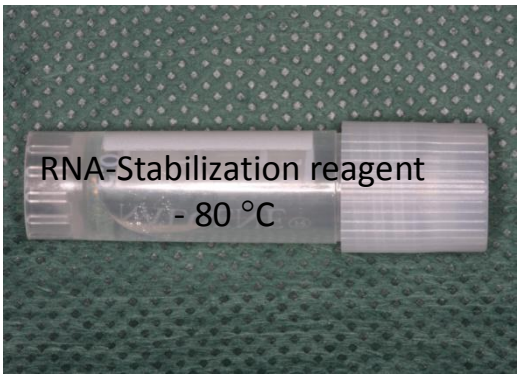

b

Fig. 8a, 8b: Excised gingival biopsy and a cryotube with the RNA Stabilization Reagent.

4.3.1 RNA-extraction from the Gingival Tissue Biopsies

Total RNA was extracted from gingival tissue biopsies using the QIAzol method (Qiagen, Hilden, Germany). The tissue was homogenized using 1000 µl QIAzol Lysis Reagent and a handheld rotor-stator homogenizer, TissueRuptor (Qiagen) according to the manufacturer's instructions, until the tissue lysate was uniformly homogenous. The

entire process was performed under constant cooling with ice, in order to inactivate the active RNases of the tissue.

The homogenate was transferred into a tube and incubated at room temperature for 5 minutes in order for the nucleoproteins complexes to be dissociated. Then 200 µl Chloroform was added, the tube was firmly closed and shaken with hand for 15 seconds. The mixture was again incubated at room temperature for 3 minutes and then centrifuged using a centrifuge machine (Eppendorf, Hamburg, Germany) at 10,000 rpm for 15 minutes at 4 ˚C. After the centrifugation the homogenous was separated into three phases. RNA partitions were at the upper phase (aqueous) while DNA partitions to the middle phase (interphase) and the proteins to the lower phase (organic phase). The aqueous phase was separated with suction, and, after the addition of 500 µl isopropanol, the mixture was incubated at room temperature for 10 minutes. Afterwards RNA precipitation was achieved by centrifugation at 10,000 rpm for 10 minutes at 4 ˚C.

The entire supernatant was decanted and the precipitated RNA (Pellet) was resuspended carefully with 1000 µl ethanol (75%). The mixture then was immediately centrifuged at 5,000 rpm for 5 minutes at 4 ˚C. The supernatant was decanted and the tube was left with its lid open until the residual ethanol evaporated. Subsequently, the pellet was redissolved with 50 µl RNase-free water (DEPC-treated water, diethyl pyrocarbonate with distilled aqua 1:1000 diluted) and incubated for 10 minutes at 55 ˚C in a hybridization oven (Hybaid, Shake ‘n’ Stack, Thermo Fisher Scientific, Waltham, USA).

Afterwards, the RNA was cleaned with RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions and stored at -80 ˚C ready for the later concentration quantification with photometry.

4.3.2 Quantification of Total RNA Concentrations (Photometry)

RNA concentrations were measured photometrically using a spectrophotometer (Biochrom, Cambridge, England), for the following values: absorbance ratio, RNA concentration, relevant purity, relative RNA concentration and protein concentration. The determination of RNA concentration and purity was performed by measuring the absorption at wavelengths of 260 nm (OD260) and 280 nm (OD280), when an optical density OD260 of 1 corresponds to a concentration of 40 µg/ml RNA [Sambrook *et al.* 1989]. The measurements were performed with the following settings: The ratio

OD260/OD280 was adjusted at 1.8 (this ratio gives an estimation of RNA purity) and the menu factor was 40 for the determination of the RNA concentration. The cuvette's width for the measurements was 5 mm and the RNA probes were diluted 1:5 (2 µl RNA probe + 8 µl DEPC water). The DEPC water was used also as a reference.

4.4 Real-Time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Real-time RT-PCR is a combination of the transcription of the expressed RNA (mRNA) into DNA (cDNA) followed by the amplification and quantification of the cDNA products under investigation using the quantitative real time-polymerase chain reaction (QRT-PCR). The isolated total RNA was reverse-transcribed into cDNA using the enzyme "reverse transcriptase". The Poly-$(A)^+$ tails of the mRNA are targets for the Oligo-(dt) primers used. Due to the presence of the reverse transcriptase, the synthesis of the complementary DNA-strand (cDNA) starts after the hybridization of these primers with the Poly-$(A)^+$ tails. Afterwards, the synthesized cDNA can be amplified with the PCR. The detection and quantification of the products can be achieved by various methods, and in this study the quantitative real-time, fluorescence-based polymerase chain reaction (QRT-PCR) was used.

4.4.1 Reverse Transcription (cDNA-Synthesis)

The reverse transcription (RT) of the expressed mRNA was performed with a RT-Kit (Promega, Wisconsin, USA), in accordance with the manufacturer's instructions. RT buffer-mixture was prepared in a PCR-tube using: 1 µl (200 U) reverse transcriptase, 1.25 µl (10 mM) dNTPs, 5 µl 5x buffer and 2.75 µl DEPC water. The total mixture volume per solution was approximately 10 µl. To avoid false measurements, a 10% pipette false was calculated (Tab. II). Afterwards, the RNA-mixtures were also prepared in PCR-tubes using: 2 µl oligo-(dt) primers, 2 µg/ml RNA that was estimated photometrically, and an analogous volume of DEPC water so that the total mixture volume per tube was 15 µl. All production procedures and preparation of reagents were performed on ice. Afterwards, the cDNA synthesis from the RNA was achieved in a PCR-cycler in accordance with the manufacture's instruction programs (UNO-Thermoblock, Biometra, Goettingen, Germany) (Tab. II).

After the incubation of the RNA-mix tubes in the PCR-cycler for 8 minutes at 70 ˚C, they were transferred instantly from the hot block into ice, preventing the backfolding of the RNA. Afterwards, in every RNA-mix tube was added 10 µl from the RT buffer-mix and the tubes were incubated in PCR-cycler for 60 minutes at 42 ˚C, followed by the

inactivation of reverse transcriptase at 70 ˚C for 10 minutes and finally cooled down at 4 ˚C (Tab. II). The cDNA was stored at -20 ˚C.

Table II: Reagents and their volumes that were used for the preparation of the RNA-mixture and RT buffer-mixture, and PCR cycler program.

Reagents RNA-mix	**volume**	**final concentration**	**Output concentration**
DEPC-water	Variable 1-13 µl		
Total RNA	Variable 1-13 µl	2 µg	variable
Oligo-dT	2 µl	1 µg	
Total Volume	**15 µl**		
Incubation in PCR-cycler for 8 min. at 70 ˚C. Afterwards immediately in ice			

Reagents RT buffer-Mix	**Volume**	**Final Concentration**
Reverse Transcriptase	1 µl	200 U
dNTP	1.25 µl	10 mM (1 µg)
5x Buffer	5 µl	5x
DEPC-water	2.25 µl	
Total volume	**10 µl**	

Total volume	**25 µl**	
PCR-cycler program	60 minutes at 42 ˚C	Reverse transcription
	10 minutes at 70 ˚C	Denaturation of the cDNA
	5 minutes at 4 ˚C	After storage at -20 ˚C

4.4.2 Quantitative Real-Time PCR (QRT-PCR)

The quantitative real-time polymerase chain reaction was performed using a real-time PCR cycler (Rotor-Gene 2000; Corbett Research, Sydney). PCR allows the exponential amplification of the double stranded segments of nucleic acids, which are marked with specific oligonoucleotides (primers). These primers function as a starting point for the synthesis of the complementary DNA. This DNA replication is catalyzed by a thermo-stable DNA polymerase. In the present study, the Taq polymerase that is originally isolated from the thermophilic bacterium, *Thermus aquaticus* was used. The thermo stability of the enzyme is essential for the PCR, because the reaction takes place at different temperatures up to 80-90 ˚C. An exponential amplification of the DNA was achieved in continuous cycle reactions with the following sequence: the denaturation of DNA (denaturation), the binding of the primers to the specific DNA segments (annealing) and the elongation of the complementary DNA (elongation). PCR is a very

sensitive method providing a large amount of DNA concentrations from the primary material.

There is a variation of different methods for the detection and quantification of real-time PCR products. In this study the fluorescence-based method was applied, using the fluorescent dye, QuantiTect® SYBR® Green (Qiagen, Hilden, Germany). This fluorescent dye binds to double stranded DNA and emits light with wavelength of 490 nm. The real-time PCR cycler measures this emission at the end of every elongation phase, in which the maximal amplified amount of DNA is presented. During the denaturation phase the solution contains only single-stranded DNA, consequently the dye is not bound with the DNA any more. The free dye molecules emit a low background fluorescence which is detected and subtracted from the signal by the RT-PCR software. Using the same program the increase in fluorescence is shown in graphic curves and subsequently can be evaluated directly.

4.5 Extraction of the Positive Control and the Standard Curves

The extraction of the positive controls and standard curves was essential for the later quantitative evaluation of the gene expression of human beta-defensins (hBDs), cytokines and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (see 4.6.1). Therefore, an external experiment with the culture and stimulation of human keratinocyte cell line (HaCaT) was used, in which the expression of the genes of interest was already demonstrated in past studies.

The cells were grown in 75 cm^2 culture flasks (TPP) with a modified medium DMEM (Biochrom) and incubated in an incubator (Heraus). Afterwards the cells were passaged several times with trypsin (see 4.2.1), seeded in a six-well plate (TPP) and stimulated with bacteria LPS from E. coli (Sigma) for four hours. The total cellular RNA extraction was performed using RNeasy minikit (Qiagen) in accordance with the manufacturer's instructions (4.2.3) and followed by reverse transcription (see 4.4.1). Afterwards, QRT-PCR was performed (see 4.6.2) using the appropriate primers (Tab. IV) for the genes of interest and every gene (GAPDH, hBD-1, -2, IL-1β and IL-6) was separately estimated.

The QRT-PCR products were separated by electrophoresis using a 3% agarose gel (Rotigarose, Carl Roth, Karlsruhe, Germany). Using UV-light, the QRT-PCR products in the agarose gel displayed fluorescence bands, whose fragment size was determined by a 20bp (base pairs) DNA marker (see 4.8 and Fig. 9).

Electrophoresis products were separated and purified using QIAquick PCR Purification Kit (Qiagen) in accordance with the manufacturer's instructions. Afterwards these products were used for plotting the standard curves (Fig. 10). In this study, a standard curve was plotted for every primer using a quantitative real-time nested PCR. The number of cDNA copies per µl was calculated using the following formula, before the performance of the nested polymerase chain reaction:

$$\text{Molecules/µl} = \text{DNA [µ/ml]/DNA-length [base pairs} \times 660 \times 10^{9}] \times 6.022 \times 10^{23}$$

Then every product was diluted in a series of $1x10^{2}$ to $1x10^{11}$ copies [Bongrazio *et al.* 2006]. At the first step of the nested PCR 'long primers' were used, and PCR was performed for a few cycles. At second step, PCR was performed with the products from the first step but using new primers 'short primers'. These short primers are complementary with a secondary target within the first PCR products, produced with the long primers, so in this second PCR were amplified only specific DNA segments from the first step. Nested polymerase chain reaction helps to reduce the contamination of the products due to the amplification of unexpected primer binding sites. 'Short primers' were used for plotting the standard curves and the performing of the PCR. The primers (MWG Biotech, Ebersberg, Germany) used for the RT-PCR are shown in table IV. The standard curves were used for the quantification of the other reactions.

4.6 Quantification of Expression

A real-time PCR cycler (Rotor-Gene 2000; Corbett Research, Sydney) was used for the quantification of the gene expression of hBD-1, -2, IL-1β, IL-6, and GAPDH. The fluorescence measurements were performed at the end of every elongation phase and were shown as an exponential graphic. After the end of the quantitative real-time PCR (QRT-PCR), the threshold and the base line were set. As Ct value (cycle threshold value) the number of cycles required for the fluorescent signal to cross the threshold was defined, and was used to the later estimation of the gene expression.

For every QRT-PCR a no template control (NTC) was performed with all the components of the reaction (primers, SYBR® Green and DEPC water) except the template. The NTC was considered as a reference evidence of the non contamination of the reagents. Furthermore, for every experiment also a no RT control was performed. For the no RT control was used the extracted RNA, primers, SYBR® Green and DEPC water except reverse transcriptase, and this served as an evidence of the non

contamination of the RNA with genomic DNA. These controls were used for the clean verification of the PCR reagents and the extracted RNA.

4.6.1 Quantification of the Housekeeping Genes

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as a useful and approved method for the normalization of the quantitative real-time PCR results. The GAPDH is a cell enzyme of the glucolysis and it catalyzes the transformation of Glyceraldehyde 3-phoaphate into 1,3-Bisphosphoglycerate. The GAPDH serves as a housekeeping gene, because it is produced constituently in every cell and is essential for the maintenance of basal cellular activity.

4.6.2 Quantification of the Gene Expression of Defensins and Cytokines

The contents of the PCR reagents mixes used in QRT-PCR for plotting the standard curves and the investigation of the probes are shown in the table III. The primer sequences that were used in this study for hBD-1, -2, IL-1β, IL-6 and GAPDH are shown in table IV. The desired products were produced with a Real-Time PCR cycler (Rotor-Gene 2000; Corbett Research, Sydney) under the following program:

• Initial denaturation	95,0 ˚C	300 s	
• Denaturation	95,0 ˚C	15 s	for 35 cycles
• Annealing	56,0 ˚C	30 s	
• Elongation	72,0 ˚C	30 s	
• Final elongation	72,0 ˚C	300 s	

4.7 Normalization of the QRT-PCR Results

All gene expression values were normalized using the gene expression value of the housekeeping gene GAPDH (see 4.6.1). The normalization was performed with the formation of a proportional relation between the value of genes of interest (hBD-1, -2, IL-1β, IL-6) and the value of the GAPDH using the software of the Real-Time PCR cycler.

4.8 Gel Electrophoresis

In this study a gel electrophoresis was performed to control the size and the specificity of the products. For the preparation of a 3% agarose gel, 3 g agarose (Rotigarose; Carl Roth) was dissolved in 97 ml 1 × TBE buffer (Tris-Borate-EDTA buffer) and boiled. After cooling down to about 50 ˚C, 0.5-1 µl ethidium bromide (10 mg/ml) (CarlRoth) was

added and the agarose solution was poured into a tray and allowed to set. The hardening of the 3% gel was followed by the addition of 10 µl of the PCR products mixed with 2 µl loading buffer (ABgene, Surrey, Great Britain). The electrophoresis of the products was performed in 500 ml 1x TBE buffer applying an electric field of 120 Volt for 60 minutes. The results were documented and recorded using a UV-light chamber (Vilber Lourmat, Marnela-Vallee, France) and the photo processing software Photo Finnish (GatorData, Inc. Lutz, USA) (Fig. 9).

Table III: PCR reagents volumes per tube for the quantitative real-time PCR. The concentration of the cDNA (template) in every tube was 160 ng and the final concentration of the used primers 0.6 mM

Components	Volume
Template	4 µl
Primer	4 µl
DEPC-water	2 µl
SYBR® Green	10 µl
Total	20 µl

Table IV: Primer sequences (long and short) for the genes of interest (hBD-1, -2, IL-1β, IL-6 and GAPDH)

AcNo	Gene	Forward	Reverse	Product length (kb)
NM 005218	HBD-1 (long)	tcattacaattgcagcag	ttgcagcacttggccttc	105
	HBD-1 (short)	ggagggcaatgtctctattctg	ctctgtaacaggtgccttgaa	64
AF 040153	HBD-2 (long)	ctgatgcctcttcaggtgt	tttgcagcattttgttccag	144
	HBD-2 (short)	tttggtggtataggcgatcc	gagaccacaggtgccaattt	102
NM 000576	IL-1β (long)	ctccagggacaggatatgga	tctttcaacacgcaggacag	134
	IL-1β (short)	caacaagtggtgttctccatgt	gcccaaggccacaggtat	72
NM 000600	IL-6 (long)	ctggtcttttggagtttgagg	atctgaggtgcccatgct	306
	IL-6 (short)	agagtagtgaggaacaagccaga	ttgccgaagagccctcag	233
NM 002046	GAPDH (long)	cctgacctgccgtctagaaa	tactccttggaggaggccatgtg	276
	GAPDH (short)	tcaagaaggtgtgaagcag	ccctgttgctgtagccaaat	198

4.9 Statistical Analysis

The statistical analysis was performed by means of Statistical Package for Social Science for Windows (SPSS Inc., Chicago, USA). Levels of mRNA expression for the genes hBD-1, -2, IL-1β, IL-6 were evaluated using quantitative real-time RT-PCR. The mean values of the mRNA expression levels from stimulated and non-stimulated HaCaT cells were statistically analyzed with one-way analysis of variance (ANOVA) and *post hoc* testing according to Tukey's B. The differences in the levels of mRNA expression in gingiva biopsies from smokers and non-smokers were assessed using the Mann-Whitney U Test. The accepted level of significance was $p \leq 0.05$ and line diagrams or box-and-whiskers-plots were used for the graphic presentation of the results.

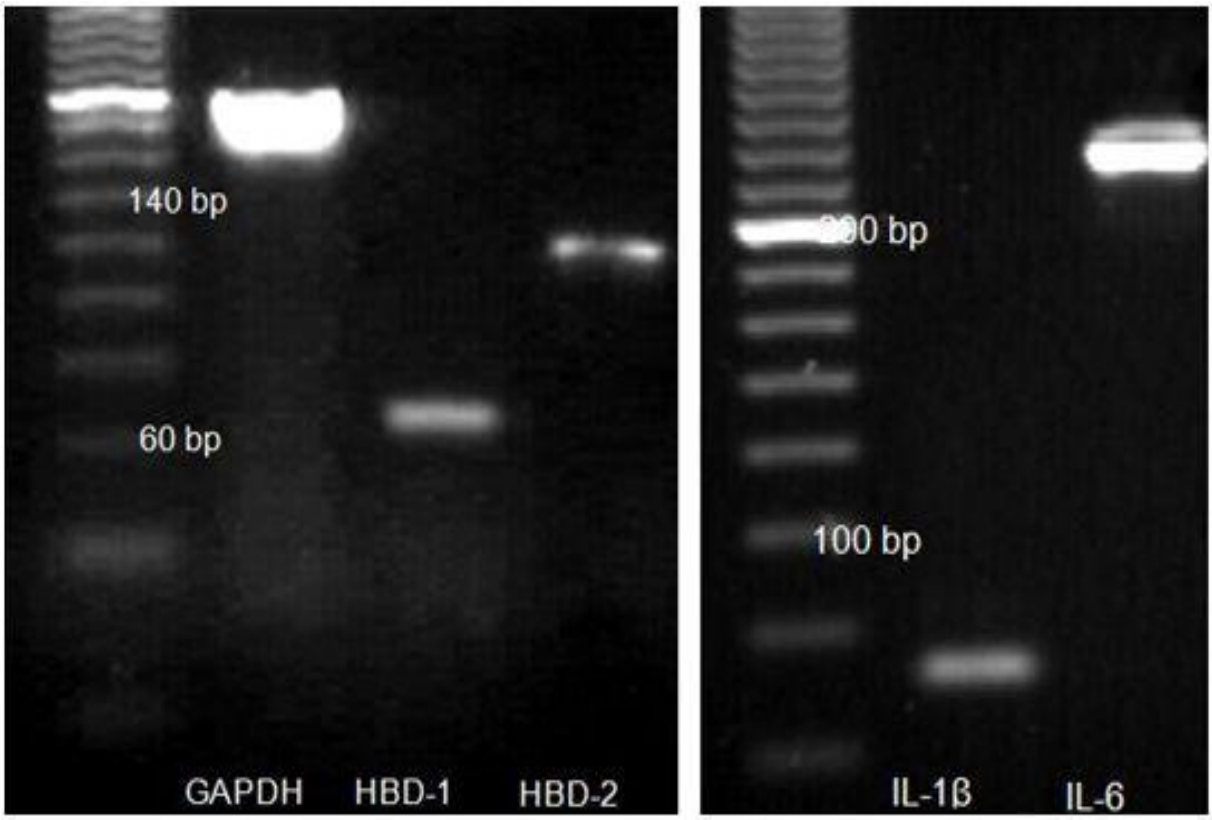

Fig. 9: Gel electrophoresis of the QRT-PCR products showing the fluorescence bands for the genes GAPDH, hBD-1, -2, IL-1β, IL-6 and the DNA marker. (Example)

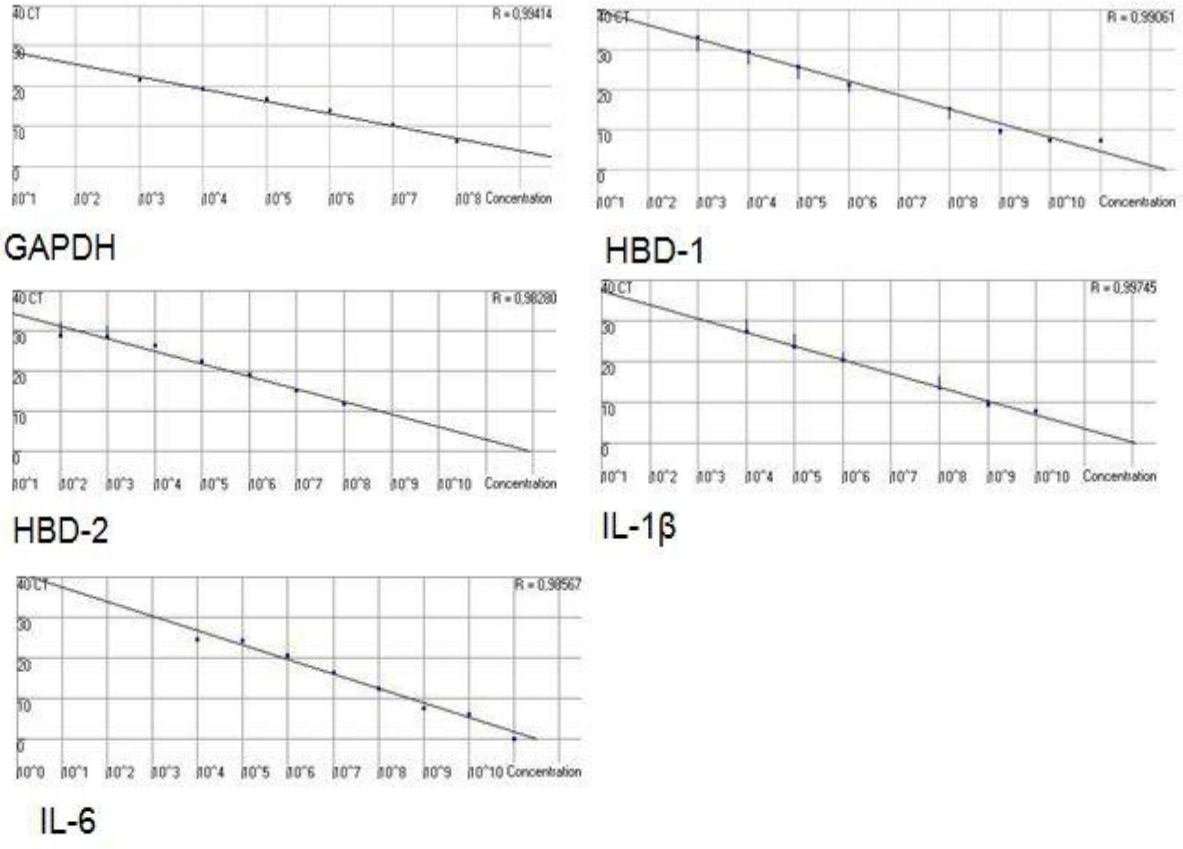

Fig. 10: Standard curves for every gene of interest (GAPDH, hBD-1, hBD-2, IL-1β and IL-6). Products were diluted in a series of $1x10^{2}$ to $1x10^{11}$ copy numbers. (Example)

5 Results

5.1 Gene Expression in HaCaT after Stimulation with Nicotine and TNF-α

Stimulation of HaCaT cells was performed in three independent experiments (n=3). The quantitative real-time RT-PCR results were normalized using the expression of GAPDH gene and then evaluated for statistical significance between treatment conditions using *post hoc* testing according to Tukey's B. TNF-α treatment was used as a positive control and was related for statistical significance with the other treatments, other correlations between treatments are reported. The accepted level of significance was $p \leq 0.05$, and line diagrams were used for the graphic representation of the results.

Nicotine-treated HaCaT, showed no statistically significant changes in the gene expression of IL-1β compared with the control (* p=0.260). Furthermore, there are no significant differences comparing simultaneous treatment with nicotine and TNF-α (** p=0.673), and the pretreatment with nicotine and then with TNF-α (*** p=0.723), with the positive control (Fig. 11).

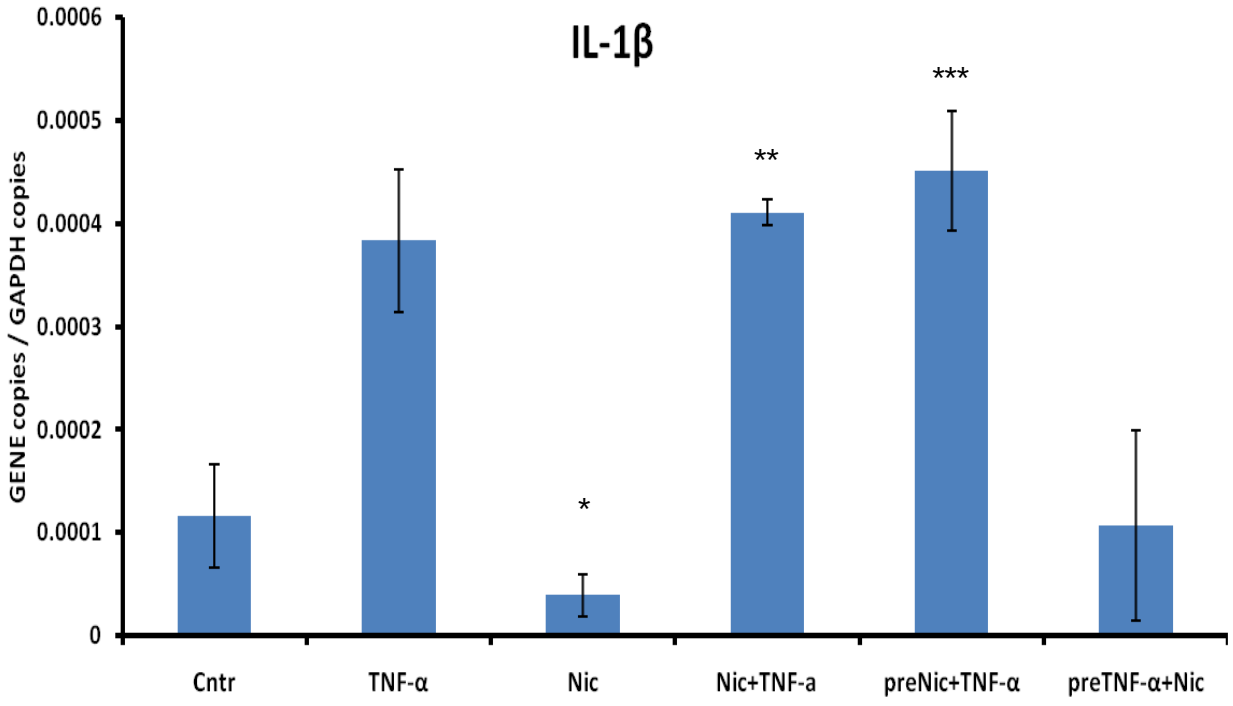

Fig. 11: Normalized expression of IL-1β (n=3). The results are represented as the mean values ± standard deviations of three separate experiments (Tukey's B). (Cntr: control, Nic: nicotine, Nic+TNF-α: simultaneous treatment with nicotine and TNF-α, preNic+TNF-α: pre-treatment with nicotine and then with TNF-α, preTNF-α+Nic: pre-treatment with TNF-α and then nicotine).

Likewise, nicotine caused no statistically significant changes in the gene expression of IL-6 compared with the control (* p=0.625). In addition, there were no significant differences comparing simultaneous treatment with nicotine and TNF-α (** p=0.843), or when comparing the pretreatment with nicotine and then with TNF-α (*** p=0.749) with the positive control (Fig 12).

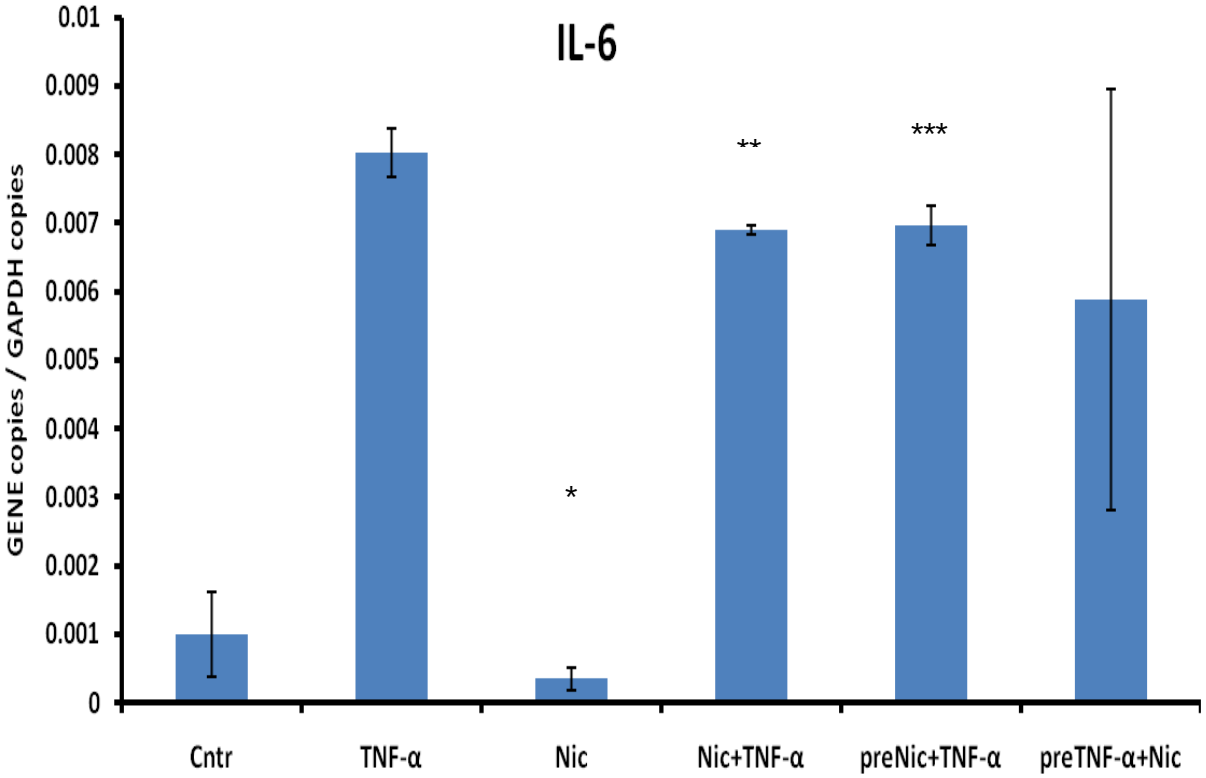

Fig. 12: Normalized expression of IL-6 (n=3). The results are represented as the mean values ± standard deviations of three separate experiments (Tukey's B).

Moreover, nicotine-treated HaCaT showed no statistically significant changes in the gene expression of hBD-1 compared with the control (* p=0.243), and there were no significant differences comparing simultaneous treatment with nicotine and TNF-α (** p=0.843), or when comparing the pretreatment with nicotine and then with TNF-α (*** p=0.749) with the positive control. Interestingly, TNF-α seems to downregulate significantly the expression of hBD-1, when comparing the control with the positive control (**** p<0.0005) (Fig. 13).

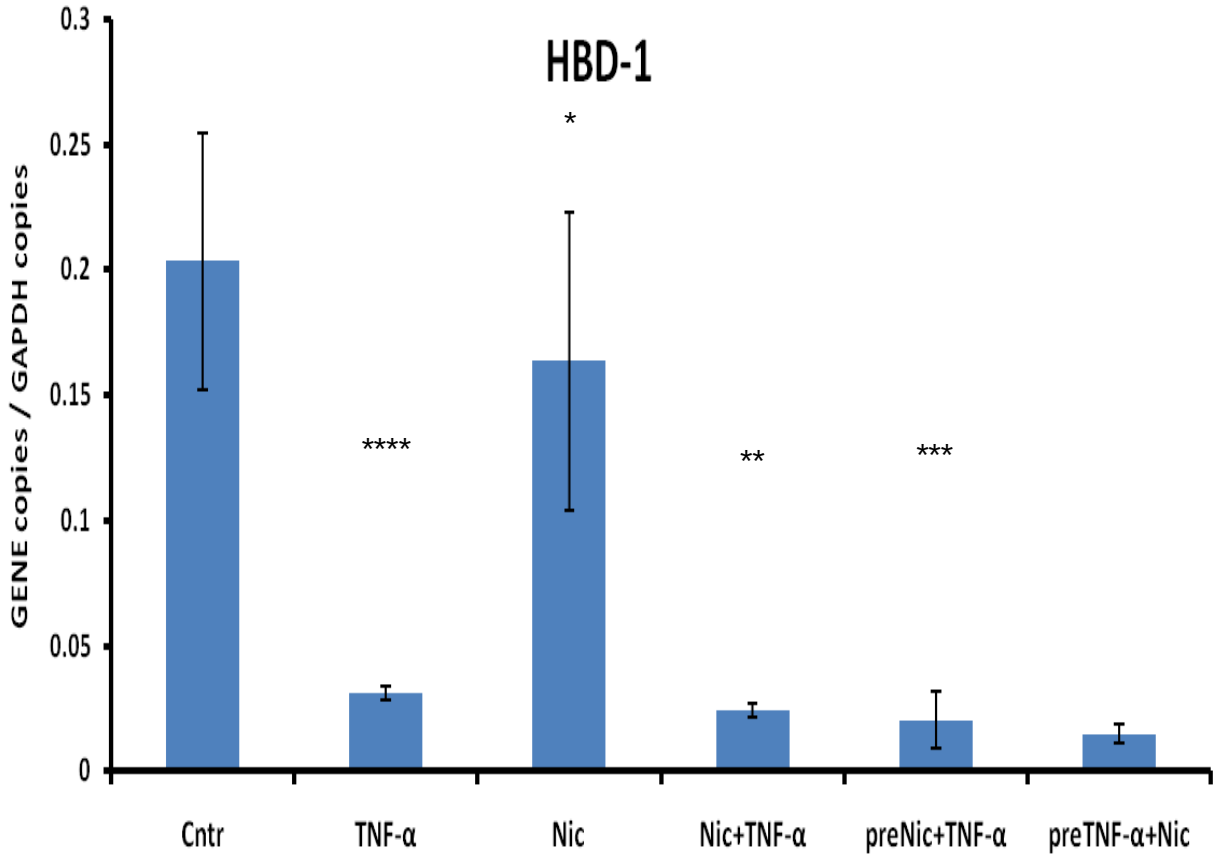

Fig. 13: Normalized expression of human beta-defensin-1 (n=3). The results are represented as the mean values ± standard deviations of three separate experiments (Tukey's B).

Furthermore, nicotine caused no statistically significant changes in the gene expression of hBD-2 compared with the control (* p=0.907). Interestingly, the simultaneous treatment with nicotine and TNF-α (** p=0.041), as well as the pretreatment with nicotine and then with TNF-α (*** p=0.004) caused statistically significant downregulation in the gene expression of hBD-2, compared with the positive control (Fig. 14).

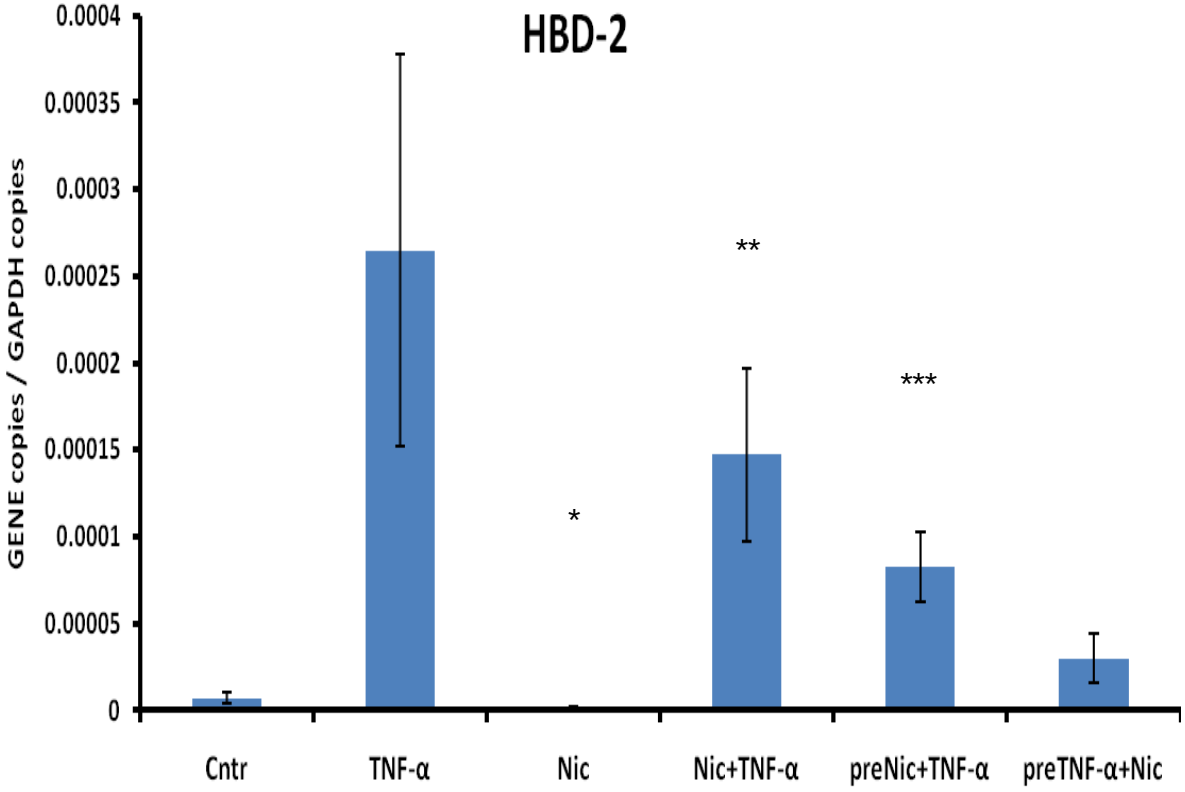

Fig. 14: Normalized expression of human beta-defensin-2 (n=3). The results are represented as the mean values ± standard deviations of three separate experiments (Tukey's B).

5.2 Gene Expression in Gingival Biopsies from Smokers and Non-Smokers

In total, 18 biopsies were collected from patients with healthy periodontium that were non-smokers (n=9) and smokers (n=9). The quantitative real-time RT-PCR results were normalized using the expression of GAPDH gene and evaluated for statistical significance using the Mann-Whitney U test. The accepted level of significance was $p \leq 0.05$ and box-and-whiskers-plots were used for the graphic representation of the results.

There were no statistically significant differences in the gene expression of the IL-1β and IL-6 between non-smokers and smokers (Figs. 15, 16). Interestingly, the expression of hBD-1 and hBD-2 was statistically significantly lower in smokers compared to non-smokers (Figs. 17, 18).

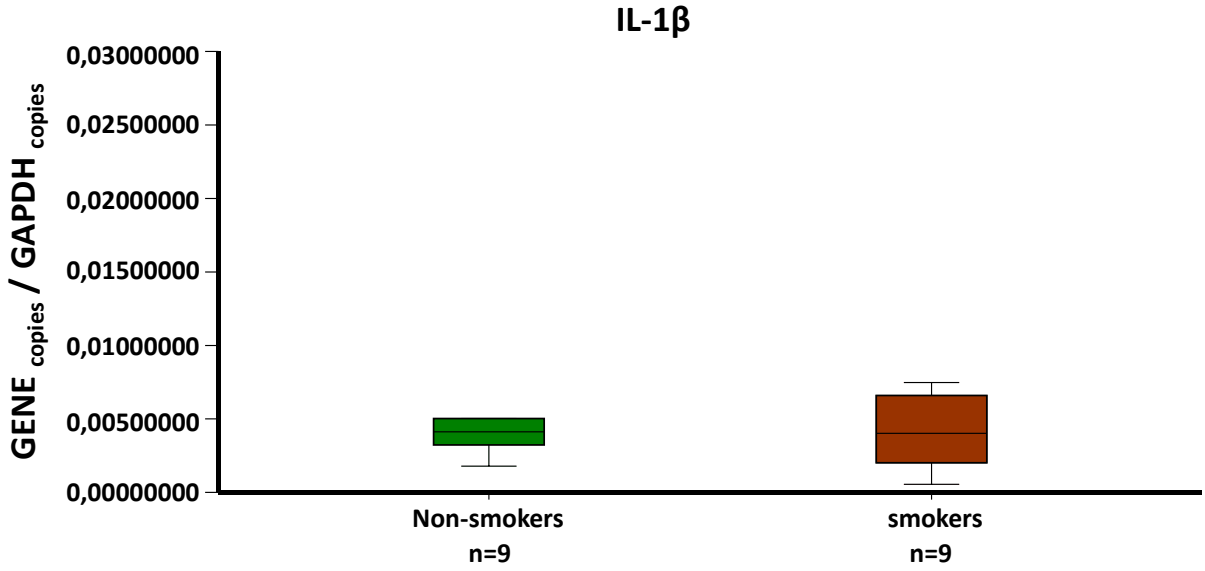

Fig. 15: Normalized expression of IL-1β. There is no significant difference in the expression of the IL-1β between non-smokers and smokers (p=0.508) (Mann-Whitney U test).

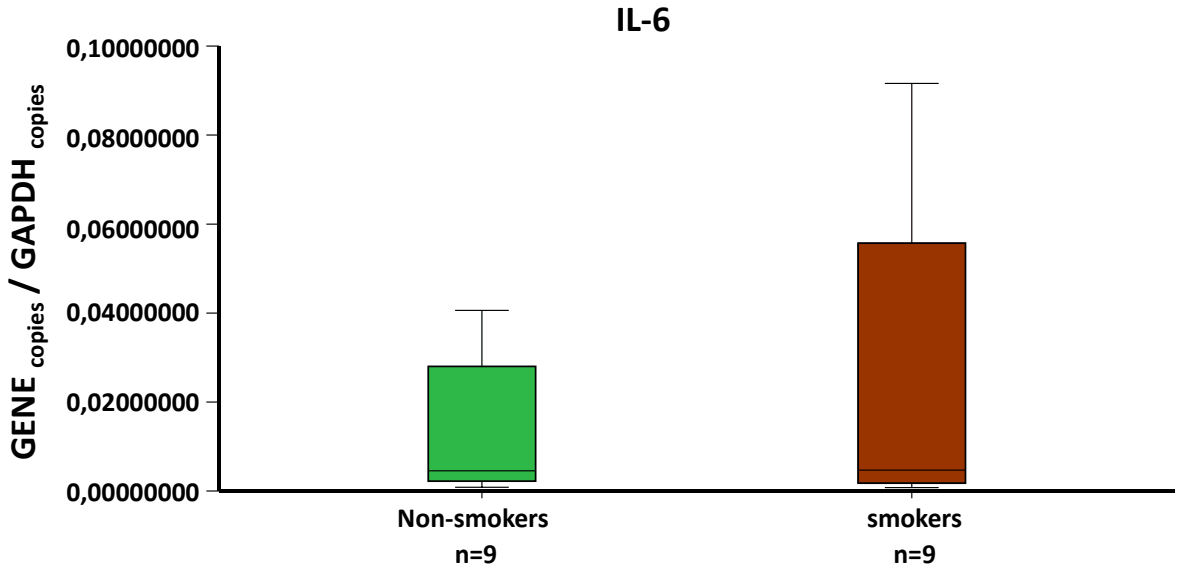

Fig. 16: Normalized expression of IL-6. There is no significant difference in the expression of the IL-1β between non-smokers and smokers (p=0.825) (Mann-Whitney U test).

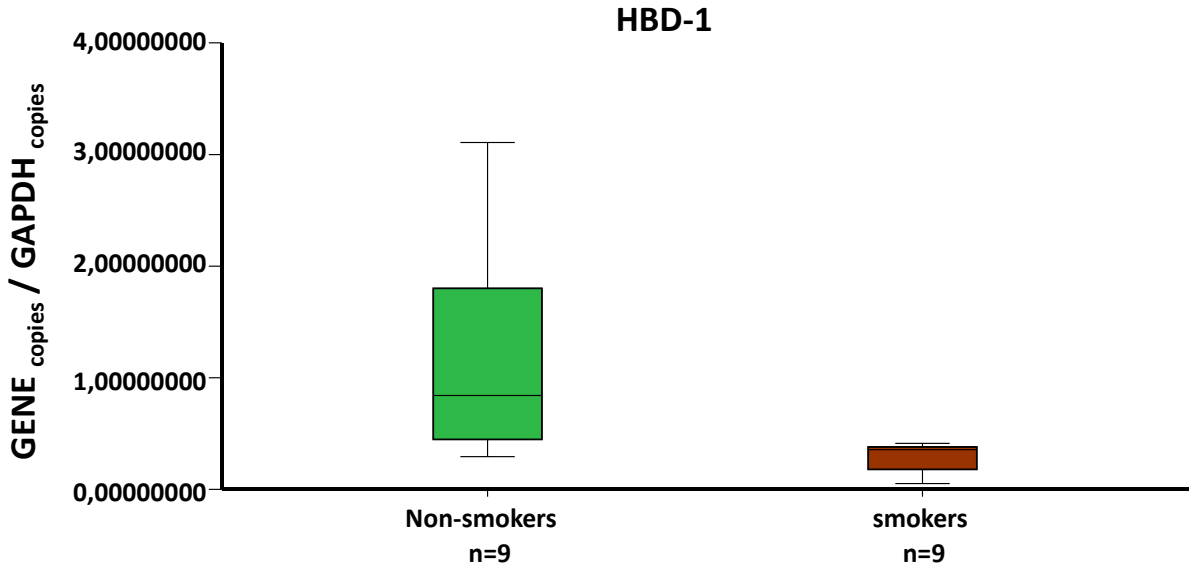

Fig. 17: Normalized expression of human beta-defensin-1. The expression of hBD-1 is statistically significantly lower in smokers compared to non-smokers (p=0.031) (Mann-Whitney U test).

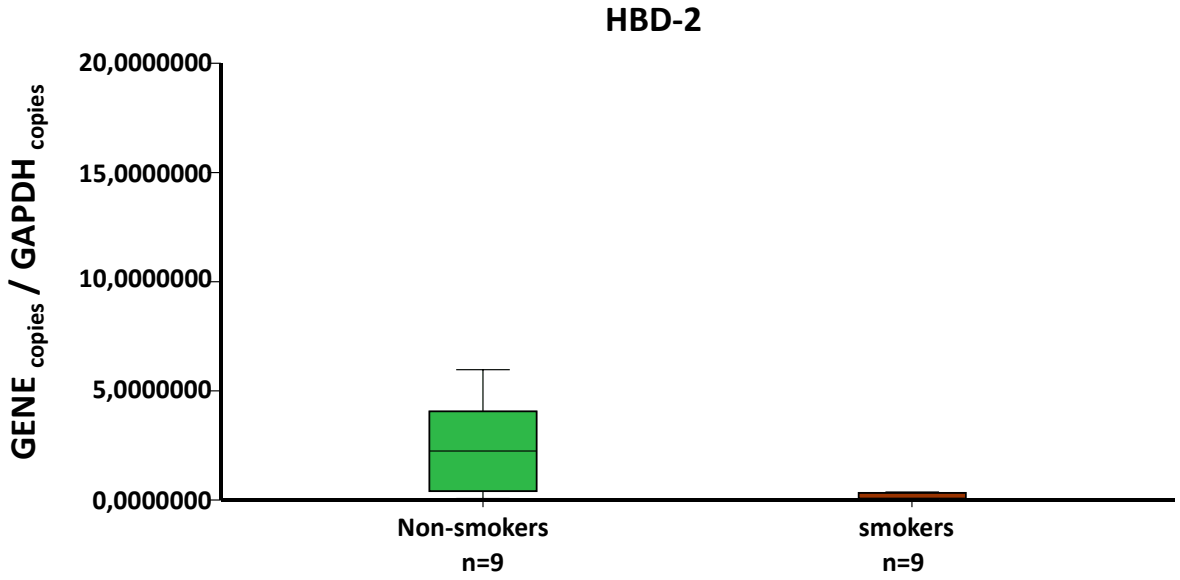

Fig. 18: Normalized expression of human beta-defensin-2. The expression of hBD-2 is statistically significantly lower in smokers compared to non-smokers (p=0.007) (Mann-Whitney U test).

6 Discussion

Numerous investigations and both cross-sectional and longitudinal studies have been performed over the past decades providing ample epidemiological evidence that smoking is clearly a risk factor for periodontal disease [Amarasena *et al.* 2002; Bergstrom & Floderus-Myrhed 1983; Chen *et al.* 2001; Gonzalez *et al.* 1996; Grossi *et al.* 1995; Grossi *et al.* 1994; Haffajee & Socransky 2001; Ismail *et al.* 1990; Locker 1992]. The effect of nicotine on the periodontal epithelium can be a result of its direct contact to the tobacco smoke with smoking or via the blood flow and gingival crevice fluid which in smokers the concentration of nicotine is 300 times greater that of in plasma (20 ng/ml) [Benowitz & Jacob 1984; Ryder *et al.* 1998].

Oral epithelial tissues as well as the periodontium are constantly exposed to the oral environment, and analogous to noxious reagents such as microbial flora and tobacco smoke. The microorganisms activate the innate immune system of the host to produce cytokines and antimicrobial peptides, to maintain the health of the oral epithelium as well as of the periodontium, which is considered a major entry point for virulence factors [Campochiaro 1993; Durum *et al.* 1985; Ganz 2002; Hedges *et al.* 1994; Jung *et al.* 1995; Stadnyk 1994; Svanborg *et al.* 1994]. Anti-microbial proteins are the first defense barrier of the host to microbial invasions and provide a rapid protection from microorganisms, either before or parallel with the establishment of the acquired immune responses. In the oral cavity, various anti-microbial proteins such as neutrophil defensins, beta-defensins, lysozyme, bactericidal/permeability-increasing protein, bactericidal/permeability-increasing protein-like proteins, histatins, proline-rich proteins, salivary agglutinin (GP-340), cathelicidin LL-37, cystatins, lactoferrin, salivary peroxidase, mucins and secretory leukoproteinase inhibitor have been described [Ghafouri *et al.* 2003; Guo *et al.* 2006; Hardt *et al.* 2005; Hu *et al.* 2005; Huang 2004; Vitorino *et al.* 2004; Walz *et al.* 2006; Wilmarth *et al.* 2004; Xie *et al.* 2005; Yao *et al.* 2003].

However, there are only few studies hitherto that have focused on the effect of nicotine and/or smoking on the oral keratinocytes, especially on the expression patterns of human beta-defensins. The present study addressed some parts of these effects, and tried to elucidate the interactions between smoking and the expression of antimicrobial peptides.

6.1 Discussion of Materials and Methods

The objective of the present study was to evaluate the effect of nicotine and of smoking on the expression patterns of the genes hBD-1, -2 and IL-1β, IL-6 in HaCaT cell culture treated with nicotine and in gingiva biopsies from healthy smokers and non-smokers.

The HaCaT cells were treated with nicotine and the proinflammatory cytokine TNF-α as a positive control for certain periods of time and in various combinations (see stimulation protocol figure 6). HaCaT cells are keratinocytes isolated from human adult skin and they were propagated under conditions of low Ca^{2+} and elevated temperature. The cells were spontaneously immortalized but are non-tumorigenic, and were developed by Dr. N. E. Fusenig (German Cancer Research Center, Heidelberg). This human cell line demonstrates characteristics similar to those of the primary oral keratinocytes, and is easy to handle because of the steady rate of replication and proliferation [Boukamp *et al.* 1988].

The keratinized gingiva biopsies were excised from healthy patients during routine surgical treatments such as exposing an inserted endosseous implant or obtained with gingival tissue punch technique from direct implantation.

In the present study, the method of choice for evaluating the expression level of mRNA derived from HaCaT cells and gingiva biopsies was the quantitative real-time RT-PCR. Real-time RT-PCR is an established and very sensitive method that traces a small number of transcripts and small changes in gene expression. The accuracy of the method is very high, reliable and the handling of the instruments and reactors is very easy. Real-time RT-PCR is a combination of the transcription of the expressed RNA (mRNA) into DNA (cDNA) followed by the amplification and quantification of the under investigation cDNA products with the quantitative real-time polymerase chain reaction (QRT-PCR). The fluorescence dye SYBR® Green was used for the detection and quantification of the target cDNA and the results were monitored in real time during every cycle of the PCR reaction [Bustin *et al.* 2005; Morrison *et al.* 1998; Pfaffl 2001].

This fluorescent dye binds to double stranded DNA and emits light with a wavelength of 490 nm that is measured by the real-time PCR cycler at the end of every elongation phase, in which the maximal amplified amount of DNA is presented. During the denaturation phase the solution contains only single-stranded DNA, consequently indicating that the dye is not bound to the DNA anymore. At every reaction cycle there is an exponential amplification of the DNA as well as an increased fluorescence emission. Using the real-time PCR cycler's software, the increased fluorescence is presented in

real time in graphic curves, and, subsequently, can be evaluated directly. The free dye molecules during the denaturation phase emit a low background fluorescence which is detected and subtracted from the signal by the same real-time RT-PCR cycler's software.

The extraction of the positive controls and standard curves was essential for the quantitative evaluation of the gene expression of human beta-defensins (hBDs), cytokines and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Therefore, an external experiment with the culture and stimulation of human keratinocyte cell line (HaCaT) was used, in which the expression of the genes of interest was already demonstrated in past studies. The extracted control-RNA from the HaCaT cells was analyzed using a quantitative real-time nested PCR and a standard curve for every primer was produced (see 4.5).

Finally, normalization for every target gene was performed using the expression of the housekeeping gene GAPDH. Housekeeping genes are considered to be expressed constituently in various tissues and cells, and are essential for the maintenance of basal cellular activity (see 4.6.1 and 4.7).

6.2 Discussion of Results

In the present study, HaCaT cells treated with nicotine showed no statistically significant changes in the gene expression of hBD-1 ($p=0.243$), hBD-2 ($p=0.907$), IL-1β ($p=0.260$), and IL-6 ($p=0.625$) compared with control (untreated cells). Interestingly, simultaneous treatment of HaCaT cells with nicotine and TNF-α caused a statistically significant down-regulation of hBD-2 ($p=0.041$) expression compared to positive control (TNF-α treated cells). Furthermore, pretreatment with nicotine and then with TNF-α also down-regulated significantly the expression of hBD-2 ($p=0.004$) compared to the positive control. In contrast, the differences between the same treatment protocols and the positive control, for the gene expression of hBD-1, IL-1β and IL-6 were not statistically significant.

Moreover, another interesting result was that the expression of hBD-2, IL-1β and IL-6 was up-regulated when cells were treated with TNF-α (positive control) compared with the control; however, in contrast, the expression of hBD-1 was down-regulated. Down-regulation of hBD-1 was observed also when cells were treated with TNF-α in combination with nicotine but not when treated with nicotine only.

On the other hand, after the analysis of the gingival biopsies from smokers and non-smokers, there were no significant differences in the gene expression of the IL-1β ($p=0.508$) or IL-6 ($p=0.825$). Interestingly, the expression of hBD-1 ($p=0.031$) and hBD-2 ($p=0.007$) was significantly lower in gingival biopsies from smokers than non-smokers.

Analyzing these findings, both null hypotheses $H_{0\text{-}1}$ and $H_{0\text{-}2}$ were partially rejected because nicotine and smoking seem to down-regulate the expression of hBD-2 in stimulated HaCaT cells and that of hBD-2 and hBD-1 in gingival epithelia from smokers, respectively. These results showed that nicotine and smoking can modify, especially down-regulate, the gene expression patterns of hBD-1 and -2 in HaCaT keratinocytes and in oral epithelium, and, thus, this can contribute to the initiation and progression of periodontal diseases, at least partially.

Previous studies have shown that the bacteria of the microbial plaque are the major noxious factors for the initiation of the inflammation processes and host immune responses that leading to periodontal diseases [Palmer *et al.* 2005]. There are highly controversial results as to whether smoking and especially nicotine, which is the component of the tobacco investigation is focused on, can modify the microflora of the subgingival microbial plaque to specific periopathogens. There are studies that support the two hypothesis, so further investigation is needed to confirm that microflora alteration is directly related to smoking and nicotine [Bostrom *et al.* 2001; Darby *et al.* 2000; Kamma *et al.* 1999; Preber *et al.* 1992; Van der Velden *et al.* 2003; Zambon *et al.* 1996].

In contrast, there is more evidence that smoking can modulate host innate immunity and have a suppressive or impairing effect on various immune response cells such as monocytes, polymorphonuclear neutrophils (PMNs), lymphocytes and NK cells [Mehta *et al.* 2008]. Furthermore, another study showed that cigarette smoke extract (CSE) can down-regulate the expression of hBD-2 in stimulated human gingival epithelial cells and up-regulate IL-8 [Mahanonda *et al.* 2009]. Moreover, studies have shown that smoking, nicotine or cigarette smoke extract can modulate the cell functions and the expression of proinflammatory cytokines and defensins in various human tissues, cells and body secretions such as human periodontal ligament and fibroblast cells [Alpar *et al.* 1998], oral keratinocytes [Johnson *et al.* 2010], gingival crevicular fluid [Petropoulos *et al.* 2004; Tymkiw *et al.* 2011], human airway epithelial cells [Glader *et al.* 2006] and in human skin keratinocyte cell cultures [Radek *et al.* 2010]. There is a growing body of evidence from various studies suggesting that smoking and nicotine affect the immune

responses, innate and acquired, of the host, causing a suppressive effect most of the time.

There are a variety of host cell receptors that are located at the membrane of the cells and that are responsible for the recognition of bacterial components and for initiating signaling pathways leading to inflammatory responses [Akira 2001; Madianos *et al.* 2005; Medzhitov & Janeway 2000]. The most studied pattern recognition receptors are the Toll-like receptors (TLRs) which are expressed predominantly in cells which are responsible for the first line of defense, such as neutrophils, monocytes/macrophages, dendritic cells and keratinocytes [Akira & Takeda 2004; Beutler 2004]. Keratinocytes, that are the predominant cells of epithelia and the first defense barrier of the host against the microbial noxious intrusions, express Toll-like receptors and thus have the ability to recognize and respond to microbe-associated molecular patterns (PAMPs) [Furrie *et al.* 2005; Kinane *et al.* 2006; Sugawara *et al.* 2006] producing various inflammation mediators and antimicrobial peptides such as human beta-defensins [Birchler *et al.* 2001; Dunsche *et al.* 2001; Krisanaprakornkit *et al.* 2000; Nagy *et al.* 2005].

The expression of hBD-2 is regulated by various intracellular signaling pathways such as the NF-κB pathway and the G-protein-coupled protease-activated receptors that mediate cellular responses to extracellular proteinases [Chung *et al.* 2004]. Furthermore, studies have shown that the induction of hBD-2 expression was due to activation of specific proinflammatory cytokine receptors involving the NF-κB downstream pathway [Diamond *et al.* 2000; O'Neil *et al.* 1999]. Moreover, it has been reported that expression of hBD-2 is also regulated by mitogen-activated protein (MAP) kinase-signalling pathway that is associated with cytokine and stress responses [Krisanaprakornkit *et al.* 2002]. Interestingly, it has been suggested that comensal or pathogenic bacteria utilizing the same TLRs can activate different downstream pathways for the induction of the hBD-2 but further investigation is needed to determine which pathway is used, from which bacteria component and why [Chung & Dale 2004].

Studies have shown that nicotine in various cell types seems to inhibit or prevent the phosphorylation (degradation) of the I-κB, an inhibitory protein that prevents NF-κB from activation and translocation into the nucleus initiating gene transcription, and thus NF-κB protein remains inactive [Borovikova *et al.* 2000; Saeed *et al.* 2005; Sugano *et al.* 1998; Wang *et al.* 2004]. On the other hand, studies have shown that oral keratinocytes express nicotinic achetylcholine receptors (nAChRs) and that agonists of these

receptors like nicotine or environmental tobacco smoke (ETS) can alter the functions of keratinocytes such as migration, intracellular downstream pathways, expression and activity of NF-kβ and finally gene expressions [Arredondo *et al.* 2006; Arredondo *et al.* 2005; Arredondo *et al.* 2005; Zia *et al.* 2000]. Another interesting study suggested that activation of nAChRs from endogenous (stress-induced nAChRs activation via the neuroendocrine system) and exogenous (nicotine) agonists suppressed the expression of antimicrobial peptides in the skin of mice and in normal human keratinocyte cell cultures and thus increased the susceptibility to microbe infections [Radek *et al.* 2010].

In the present study, the down-regulation of the hBD-2 in pre-treated HaCaT cells with nicotine or simultaneously treated with nicotine and TNF-α, as well as the down-regulation of the hBD-1 and hBD-2 in gingival epithelia of smokers were observed. The results from the present study (hBD-2 gene expression) agree with previous investigations that showed the same immune modulatory effect of nicotine on human epithelia cells [Mahanonda *et al.* 2009; Radek *et al.* 2010], and this might be caused by a modulating effect of nicotine on intracellular signalling pathways or by the activation of nAChRs of the keratinocytes as described. Further investigation is still required to determine which are the distinct mechanisms of nicotine action affecting the production of antimicrobial peptides and especially human beta-defensins in oral epithelial keratinocytes.

It is noteworthy that HaCaT cells pre-treated with nicotine for 12 hours and then with TNF-α showed a significantly enhanced down-regulation of hBD-2 (2.5 fold) compared to cells that were simultaneous treated with nicotine and TNF-α (2 fold). This might be due to an inhibiting effect of nicotine on the intracellular signaling pathway (I-κB degradation) or direct via the nAChRs activation, so the later stimulation with TNF-α is impaired. Interestingly, treatment with TNF-α for 12 hours in all cases caused up-regulation for the genes hBD-2, IL-1β and IL-6 (positive control) but down-regulation for the expression of hBD-1 compared to the control. This result might be interpreted from previous study data that would suggest that expression of hBD-1 is constitutive [Harder *et al.* 1997; Harder *et al.* 2000]. Furthermore, these results are consistent with a previous study that showed non-induced expression of hBD-1, when periodontal pocket epithelial cells were stimulated with TNF-α. The same study also found that hBD-1 can be induced with the proinflammatory stimuli phorbol 12-myristate 13-acetate (PMA) [Vankeerberghen *et al.* 2005].

Nicotine down-regulates the expression of hBD-2, and, thus, caused a modulating effect only when expression results were compared to cells stimulated with TNF-α (positive control) because expression of the human beta-defensins is very low in cultured HaCaT keratinocytes due to the lack of microbial noxious agents that flourish in normal human epithelia and induce antimicrobial peptide expression. Regarding the effect of nicotine on the expression of hBD-1, IL-1β and IL-6 in HaCaT cells stimulated using the same treatment protocol, no significant changes were observed compared with positive control. Moreover, in the literature there are controversial results about the effect of nicotine and the expression of IL-1β and IL-6. Stimulation of human tracheal smooth muscle cells with cigarette smoke extract (CSE) increased the release of IL-6 [Singh *et al.* 2009], while nicotine down-regulated the production of the IL-6 and IL-8 induced by LPS in human monocytes and coronary artery endothelial cells [Patton *et al.* 2006]. Other studies found an increased expression of IL-1, IL-8 and RANKL mRNA and protein in human osteosarcoma cells, whereas the LPS-induced IL-1, IL-8, and PGE_2 expression was reported to be inhibited in U937 cell lines by pretreatment with nicotine [Ho *et al.* 2009; Sugano *et al.* 1998]. Furthermore, the expression of IL-6 was slightly increased by nicotine in human gingival fibroblasts but further investigation is required [Almasri *et al.* 2007].

In the other part of the present study, the expression of hBD-1 and hBD-2 was significantly suppressed in oral epithelia keratinocytes from smokers compared to non-smokers. These findings agree with the results from the HaCaT cell culture regarding the expression of hBD-2 but not of hBD-1. This difference might be due to the origin and type of keratinocytes investigated. Moreover, the expression of IL-1β and IL-6 was not statistically different between smokers and non-smokers. This result is supports previous controversial studies about the modulating effect of nicotine on the expression of IL-1β and IL-6 in various tissues and cell types.

It would seem plausible that smoking and especially nicotine affect different cell and tissue types modulating their physiological functions in various and complex manners, and the understanding of these interactions calls for further studies. Furthermore, it is well established that smoking and nicotine can alter the activity of the cells responsible for the immune responses, producing most of the time a suppressive anti-inflammatory effect. In the literature hitherto there are still conflicts about the exact role and effect of nicotine in immune responses and this variability is concomitant with the multifactorial effect of smoking and nicotine on the immune responses. This can be varied from the

cell type and the endpoint tested in every study. Moreover, nicotine is not the only component of tobacco smoke but one of the 3800 chemicals, including carbon monoxide, hydrogen cyanide, reactive oxidizing radicals and others that are known or suspected to be carcinogens [Eriksen *et al.* 1988]. It is very likely that the other tobacco smoke products can cause the same or enhanced effects on the immune responses of the host and this area need further investigation. Summarizing, smoking and nicotine affect and modulate keratinocytes physiological functions but further studies need to determine the exact mechanisms of these actions.

7 Conclusions

In the present study using the quantitative real-time RT-PCR reaction the expression of hBD-1, -2, IL-1β and IL-6 was analyzed for HaCaT cells treated with nicotine, TNF-α and combinations of the two as well as for gingival biopsies from smokers and non-smokers. Both null hypotheses of the present study were partially rejected and the following can be concluded:

1. The proinflammatory cytokines IL-1β and IL-6 as well as human beta-defensins-1 and -2 are expressed in cultures of human keratinocytes (cell line HaCaT).

2. Pretreatment of human keratinocytes (HaCaT) with nicotine caused a significant down-regulation of hBD-2 compared to positive control.

3. Simultaneous treatment of human keratinocytes (HaCaT) with nicotine and TNF-α caused a significant down-regulation of hBD-2 compared to positive control.

4. Treatment of human keratinocytes (HaCaT) with nicotine caused no significant expression of IL-1β, IL-6 or of human beta-defensins-1 and -2 compared to negative control.

5. Treatment of human keratinocytes (HaCaT) with TNF-α caused a significant down-regulation of hBD-1 compared to negative control.

6. The proinflammatory cytokines IL-1β and IL-6 as well as human beta-defensins-1 and -2 are expressed in human keratinized oral epithelia.

7. The expression of hBD-1 and hBD-2 is significantly suppressed in keratinized gingival biopsies of smokers compared to non-smokers.

Understanding the effect of smoking and nicotine on oral epithelia, as well as the exact mechanisms of action in relation to antimicrobial peptides expression and initiation of periodontal diseases calls for further studies.

8 Abstract

Statement of problem: The effect of smoking and nicotine on the gene expression of human beta-defensins in oral epithelia and correlations to periodontal disease susceptibility is not well understood so far. **Objectives:** To evaluate the gene expression of human beta-defensins-1 and -2 and the proinflammatory cytokines IL-1β and IL-6 in the immortalized human keratinocyte cell line HaCaT, after stimulation with nicotine, TNF-α and their combination (TNF-α was used as positive control), and in healthy keratinized gingival biopsies from smokers and non-smokers. **Materials and methods:** HaCaTs cells were cultured in six-well plates in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% FBS at a density of $\times 10^6$. Cells were pretreated (during 12 h) with 10 μg/ml nicotine and then treated (during the following 12 h) with 50 ng/ml TNF-α or pretreated with 50 ng/ml TNF-α, and then treated with 10 μg/ml nicotine, or the cells were not pretreated but only stimulated with either nicotine or TNF-α or a combination of both. Furthermore, keratinized gingival tissue biopsies were excised, during routine surgical treatments, from healthy patients that were smokers (use of 10 ≥ cigarettes per day, n=9) and non-smokers (n=9). Total RNA from HaCaT cells and biopsies was extracted and analyzed by quantitative real-time RT-PCR for human beta-defensins-1, -2, and interleukins IL-1β and IL-6, as well as GAPDH-mRNA. Data were analyzed by Tukey's B multiple comparison test for post hoc analysis and Mann-Whitney U Test. **Results:** Pretreatment of HaCaT cells with nicotine caused a significant down-regulation of hBD-2 gene expression compared to positive control (TNF-α treatment). Moreover simultaneous treatment of HaCaT cells with nicotine and TNF-α also caused a significant down-regulation of hBD-2 gene expression compared to positive control. In contrast, there was no significant effect of nicotine on the expression of hBD-1, IL-1β or IL-6 compared to positive control. The expression of hBD-1 and hBD-2 was significantly suppressed in gingival biopsies from smokers compared to non-smokers as opposed to the expression of IL-1β, IL-6 that was not significantly different. The accepted level of significance was $p \leq 0.05$. **Conclusions:** Nicotine affects the expression of human beta-defensin-2 in human keratinocyte cell line HaCaT and smoking the expression of human beta-defensins-1 and -2 in keratinized gingival biopsies from smokers, producing a suppressive influence in both.

Clinical significance: Smoking and nicotine modulate the innate responses of the host and, more specifically down-regulate the expression of antimicrobial peptides and thus in part possibly increase the susceptibility to periodontal diseases. Smoking must be considered a significant risk factor for the initiation and progression of periodontal diseases and for the periodontal treatments outcome, so patients must be informed about the periodontal effects of tobacco smoking and the benefits of giving up smoking.

Keywords: beta-defensins; cytokines; nicotine; smoking; HaCaT; periodontitis

9 Zusammenfassung

Problemstellung: Die Effekte des Rauchens und des Nikotins auf die Genexpression von humanen beta-Defensinen in den oralen Epithelzellen sowie deren Bedeutung für die Entwicklung einer Parodontalerkrankung sind nicht ausreichend untersucht. **Zielsetzung:** Das Ziel der Studie war, die Genexpression von humanen beta-Defensinen-1 und -2, sowie von proinflammatorischen Zytokinen IL-1β und IL-6 in den mit Nikotin und TNF-α stimulierten, immortalisierten humanen Keratinozyten der Zellinie HaCaT, sowie in den gingivalen Biopsien von Rauchern und Nicht-Rauchern zu untersuchen. **Material und Methoden:** Die Zellen wurden in 6-Well-Platten in Dulbecco's Minimum Essential Medium (DMEM), angereichert mit 10 % FKS, kultiviert. Die Zellen wurden für 12 Stunden entweder mit Nikotin (10 µg/ml) oder mit TNF-α (50 ng/ml) prästimuliert um anschließend für weitere 12 Stunden korrespondierend mit beiden Stimulanzen behandelt zu werden. Die negative Kontrolle blieb unstimuliert; die positive Kontrolle erhielt eine Stimulation mit 50 ng/ml TNF-α. Des Weiteren wurde eine simultane Stimulation mit beiden Stoffen vorgenommen. Darüber hinaus wurden gingivale Biopsien von Rauchern und Nicht-Rauchern gewonnen. Die Proben stammten von gesunden Rauchern (Zigarettenkonsum ab 10 pro Tag, n=9) und Nicht-Rauchern (n=9), die sich einer routinemäßigen transgingivalen Zahnwurzelimplatation mit Gewebestanzung unterzogen hatten. Nach der RNA-Extraktion aus den Gingivaproben und Zellen wurde eine Reverse-Transkriptase-Reaktion durchgeführt, gefolgt von einer quantitativen Real-Time PCR für humane beta-Defensine-1 und -2 sowie für proinflammatorische Zytokine IL-1β und IL-6. Die Ergebnisse wurden mittels Tuckey's B sowie Mann-Whitney U Test statistisch ausgewertet. Das Signifikanzniveau wurde bei $p \leq 0{,}05$ festgelegt. **Ergebnisse:** Die Prästimulation der Zellen mit Nikotin verursachte, verglichen mit der positiven Kontrolle (TNF-α), eine signifikante Herabsetzung der Genexpression von hBD-2; darüber hinaus war die Genexpression von hBD-2 bei der simultanen Stimulation der Zellen mit Nikotin und TNF-α ebenfalls signifikante vermindert. Dagegen konnte kein Effekt der beiden Stimulanzen auf die Expression von hBD-1, IL-1β und IL-6 in den Zellen nachgewiesen werden. Die Genexpression von hBD-2 und -1, jedoch nicht für IL-1β und IL-6, war in den gingivalen Biopsien von Rauchern gegenüber Nicht-Rauchern signifikant vermindert. **Schlussfolgerung:** Nikotin scheint die Genexpression von hBD-2 in den humanen Keratinozyten zu beeinflussen. Das moderate bis starke Rauchen scheint die Expression von hBD-2 und hBD-1 in der menschlichen Gingiva inhibierend zu beeinflussen.

Klinische Bedeutung: Das Rauchen und das Nikotin modulierten die angeborene lokale Immunantwort, insbesondere durch Interaktionen mit dem Expressionsmechanismus von antimikrobiellen Peptiden. Dies legt die Vermutung nahe, dass das Rauchen den Verlauf und die Entwicklung der parodontalen Erkrankung negativ beeinflussen kann. Die Patienten sollten über negative Effekte des Zigarettenkonsums auf die orale Gesundheit informiert werden.

Schlagwörter: beta-Defensine; Zytokine; Nicotine; rauchen; HaCaT; Parodontitis

10 References

1. Abdellatif HM, Burt BA. An epidemiological investigation into the relative importance of age and oral hygiene status as determinants of periodontitis. J Dent Res 1987;66:13-8.
2. Abe T, Hara Y, Aono M. Penetration, clearance and retention of antigen en route from the gingival sulcus to the draining lymph node of rats. J Periodontal Res 1991;26:429-39.
3. Akira S. Toll-like receptors and innate immunity. Adv Immunol 2001;78:1-56.
4. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499-511.
5. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001;2:675-80.
6. Albandar JM, Brown LJ, Loe H. Putative periodontal pathogens in subgingival plaque of young adults with and without early-onset periodontitis. J Periodontol 1997;68:973-81.
7. Albandar JM, Brunelle JA, Kingman A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988-1994. J Periodontol 1999;70:13-29.
8. Almasri A, Wisithphrom K, Windsor LJ, Olson B. Nicotine and lipopolysaccharide affect cytokine expression from gingival fibroblasts. J Periodontol 2007;78:533-41.
9. Alpar B, Leyhausen G, Sapotnick A, Gunay H, Geurtsen W. Nicotine-induced alterations in human primary periodontal ligament and gingiva fibroblast cultures. Clin Oral Investig 1998;2:40-6.
10. Amarasena N, Ekanayaka AN, Herath L, Miyazaki H. Tobacco use and oral hygiene as risk indicators for periodontitis. Community Dent Oral Epidemiol 2002;30:115-23.
11. Anderson DC, Schmalsteig FC, Finegold MJ, Hughes BJ, Rothlein R, Miller LJ, Kohl S, Tosi MF, Jacobs RL, Waldrop TC, et al. The severe and moderate phenotypes of heritable Mac-1, LFA-1 deficiency: their quantitative definition and relation to leukocyte dysfunction and clinical features. J Infect Dis 1985;152:668-89.
12. Arbibe L, Mira JP, Teusch N, Kline L, Guha M, Mackman N, Godowski PJ, Ulevitch RJ, Knaus UG. Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. Nat Immunol 2000;1:533-40.
13. Arredondo J, Chernyavsky AI, Jolkovsky DL, Pinkerton KE, Grando SA. Receptor-mediated tobacco toxicity: cooperation of the Ras/Raf-1/MEK1/ERK and JAK-2/STAT-3 pathways downstream of alpha7 nicotinic receptor in oral keratinocytes. FASEB J 2006;20:2093-101.
14. Arredondo J, Chernyavsky AI, Marubio LM, Beaudet AL, Jolkovsky DL, Pinkerton KE, Grando SA. Receptor-mediated tobacco toxicity: regulation of gene expression through alpha3beta2 nicotinic receptor in oral epithelial cells. Am J Pathol 2005;166:597-613.
15. Arredondo J, Chernyavsky AI, Webber RJ, Grando SA. Biological effects of SLURP-1 on human keratinocytes. J Invest Dermatol 2005;125:1236-41.
16. Astrom AN, Rise J. Socio-economic differences in patterns of health and oral health behaviour in 25 year old Norwegians. Clin Oral Investig 2001;5:122-8.
17. Barbour SE, Nakashima K, Zhang JB, Tangada S, Hahn CL, Schenkein HA, Tew JG. Tobacco and smoking: environmental factors that modify the host response

(immune system) and have an impact on periodontal health. Crit Rev Oral Biol Med 1997;8:437-60.
18. Bauer F, Schweimer K, Kluver E, Conejo-Garcia JR, Forssmann WG, Rosch P, Adermann K, Sticht H. Structure determination of human and murine beta-defensins reveals structural conservation in the absence of significant sequence similarity. Protein Sci 2001;10:2470-9.
19. Beck JD, Cusmano L, Green-Helms W, Koch GG, Offenbacher S. A 5-year study of attachment loss in community-dwelling older adults: incidence density. J Periodontal Res 1997;32:506-15.
20. Benowitz NL, Jacob P, 3rd. Daily intake of nicotine during cigarette smoking. Clin Pharmacol Ther 1984;35:499-504.
21. Berglundh T, Donati M. Aspects of adaptive host response in periodontitis. J Clin Periodontol 2005;32 Suppl 6:87-107.
22. Bergstrom J. Tobacco smoking and chronic destructive periodontal disease. Odontology 2004;92:1-8.
23. Bergstrom J, Floderus-Myrhed B. Co-twin control study of the relationship between smoking and some periodontal disease factors. Community Dent Oral Epidemiol 1983;11:113-6.
24. Bergstrom J, Persson L, Preber H. Influence of cigarette smoking on vascular reaction during experimental gingivitis. Scand J Dent Res 1988;96:34-9.
25. Bergstrom J, Preber H. Tobacco use as a risk factor. J Periodontol 1994;65:545-50.
26. Beutler B. Inferences, questions and possibilities in Toll-like receptor signalling. Nature 2004;430:257-63.
27. Birchler T, Seibl R, Buchner K, Loeliger S, Seger R, Hossle JP, Aguzzi A, Lauener RP. Human Toll-like receptor 2 mediates induction of the antimicrobial peptide human beta-defensin 2 in response to bacterial lipoprotein. Eur J Immunol 2001;31:3131-7.
28. Bissell J, Joly S, Johnson GK, Organ CC, Dawson D, McCray PB, Jr., Guthmiller JM. Expression of beta-defensins in gingival health and in periodontal disease. J Oral Pathol Med 2004;33:278-85.
29. Bolin A, Lavstedt S, Frithiof L, Henrikson CO. Proximal alveolar bone loss in a longitudinal radiographic investigation. IV. Smoking and some other factors influencing the progress in individuals with at least 20 remaining teeth. Acta Odontol Scand 1986;44:263-9.
30. Bongrazio M, Da Silva-Azevedo L, Bergmann EC, Baum O, Hinz B, Pries AR, Zakrzewicz A. Shear stress modulates the expression of thrombospondin-1 and CD36 in endothelial cells in vitro and during shear stress-induced angiogenesis in vivo. Int J Immunopathol Pharmacol 2006;19:35-48.
31. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature 2000;405:458-62.
32. Bostrom L, Bergstrom J, Dahlen G, Linder LE. Smoking and subgingival microflora in periodontal disease. J Clin Periodontol 2001;28:212-9.
33. Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol 1988;106:761-71.
34. Burt B. Position paper: epidemiology of periodontal diseases. J Periodontol 2005;76:1406-19.

35. Bustin SA, Benes V, Nolan T, Pfaffl MW. Quantitative real-time RT-PCR--a perspective. J Mol Endocrinol 2005;34:597-601.
36. Campochiaro PA. Cytokine production by retinal pigmented epithelial cells. Int Rev Cytol 1993;146:75-82.
37. Cantley LC. The phosphoinositide 3-kinase pathway. Science 2002;296:1655-7.
38. Carlos JP, Wolfe MD, Zambon JJ, Kingman A. Periodontal disease in adolescents: some clinical and microbiologic correlates of attachment loss. J Dent Res 1988;67:1510-4.
39. Central-German-Ethics-Committee. Statement: The use of human body materials for purposes of medical research.2003.
40. Chadebech P, Goidin D, Jacquet C, Viac J, Schmitt D, Staquet MJ. Use of human reconstructed epidermis to analyze the regulation of beta-defensin hBD-1, hBD-2, and hBD-3 expression in response to LPS. Cell Biol Toxicol 2003;19:313-24.
41. Chen X, Wolff L, Aeppli D, Guo Z, Luan W, Baelum V, Fejeskov O. Cigarette smoking, salivary/gingival crevicular fluid cotinine and periodontal status. A 10-year longitudinal study. J Clin Periodontol 2001;28:331-9.
42. Christan C, Dietrich T, Hagewald S, Kage A, Bernimoulin JP. White blood cell count in generalized aggressive periodontitis after non-surgical therapy. J Clin Periodontol 2002;29:201-6.
43. Chung WO, Dale BA. Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. Infect Immun 2004;72:352-8.
44. Chung WO, Hansen SR, Rao D, Dale BA. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. J Immunol 2004;173:5165-70.
45. Cullinan MP, Westerman B, Hamlet SM, Palmer JE, Faddy MJ, Lang NP, Seymour GJ. A longitudinal study of interleukin-1 gene polymorphisms and periodontal disease in a general adult population. J Clin Periodontol 2001;28:1137-44.
46. Dale BA. Fascination with epithelia: architecture, proteins, and functions. J Dent Res 2003;82:866-9.
47. Dale BA. Periodontal epithelium: a newly recognized role in health and disease. Periodontol 2000 2002;30:70-8.
48. Dale BA, Fredericks LP. Antimicrobial peptides in the oral environment: expression and function in health and disease. Curr Issues Mol Biol 2005;7:119-33.
49. Dale BA, Krisanaprakornkit S. Defensin antimicrobial peptides in the oral cavity. J Oral Pathol Med 2001;30:321-7.
50. Darby IB, Hodge PJ, Riggio MP, Kinane DF. Microbial comparison of smoker and non-smoker adult and early-onset periodontitis patients by polymerase chain reaction. J Clin Periodontol 2000;27:417-24.
51. Darveau RP, Tanner A, Page RC. The microbial challenge in periodontitis. Periodontol 2000 1997;14:12-32.
52. Diamond G, Bevins CL. Endotoxin upregulates expression of an antimicrobial peptide gene in mammalian airway epithelial cells. Chest 1994;105:51S-52S.
53. Diamond G, Kaiser V, Rhodes J, Russell JP, Bevins CL. Transcriptional regulation of beta-defensin gene expression in tracheal epithelial cells. Infect Immun 2000;68:113-9.

54. Diamond G, Russell JP, Bevins CL. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. Proc Natl Acad Sci U S A 1996;93:5156-60.
55. Diehl SR, Wang Y, Brooks CN, Burmeister JA, Califano JV, Wang S, Schenkein HA. Linkage disequilibrium of interleukin-1 genetic polymorphisms with early-onset periodontitis. J Periodontol 1999;70:418-30.
56. Dinarello CA. Proinflammatory cytokines. Chest 2000;118:503-8.
57. Dixon DR, Bainbridge BW, Darveau RP. Modulation of the innate immune response within the periodontium. Periodontol 2000 2004;35:53-74.
58. Dommisch H, Acil Y, Dunsche A, Winter J, Jepsen S. Differential gene expression of human beta-defensins (hBD-1, -2, -3) in inflammatory gingival diseases. Oral Microbiol Immunol 2005;20:186-90.
59. Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. Annu Rev Immunol 2002;20:55-72.
60. Dunsche A, Acil Y, Dommisch H, Siebert R, Schroder JM, Jepsen S. The novel human beta-defensin-3 is widely expressed in oral tissues. Eur J Oral Sci 2002;110:121-4.
61. Dunsche A, Acil Y, Siebert R, Harder J, Schroder JM, Jepsen S. Expression profile of human defensins and antimicrobial proteins in oral tissues. J Oral Pathol Med 2001;30:154-8.
62. Durum SK, Schmidt JA, Oppenheim JJ. Interleukin 1: an immunological perspective. Annu Rev Immunol 1985;3:263-87.
63. Eriksen MP, LeMaistre CA, Newell GR. Health hazards of passive smoking. Annu Rev Public Health 1988;9:47-70.
64. Erley KJ, Swiec GD, Herold R, Bisch FC, Peacock ME. Gingival recession treatment with connective tissue grafts in smokers and non-smokers. J Periodontol 2006;77:1148-55.
65. Etzioni A, Frydman M, Pollack S, Avidor I, Phillips ML, Paulson JC, Gershoni-Baruch R. Brief report: recurrent severe infections caused by a novel leukocyte adhesion deficiency. N Engl J Med 1992;327:1789-92.
66. Feldman RS, Bravacos JS, Rose CL. Association between smoking different tobacco products and periodontal disease indexes. J Periodontol 1983;54:481-7.
67. Fitzgerald KA, Palsson-McDermott EM, Bowie AG, Jefferies CA, Mansell AS, Brady G, Brint E, Dunne A, Gray P, Harte MT, McMurray D, Smith DE, Sims JE, Bird TA, O'Neill LA. Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature 2001;413:78-83.
68. Fredriksson M, Bergstrom K, Asman B. IL-8 and TNF-alpha from peripheral neutrophils and acute-phase proteins in periodontitis. J Clin Periodontol 2002;29:123-8.
69. Furrie E, Macfarlane S, Thomson G, Macfarlane GT. Toll-like receptors-2, -3 and -4 expression patterns on human colon and their regulation by mucosal-associated bacteria. Immunology 2005;115:565-74.
70. Ganz T. Epithelia: not just physical barriers. Proc Natl Acad Sci U S A 2002;99:3357-8.
71. Ganz T, Lehrer RI. Defensins. Curr Opin Immunol 1994;6:584-9.
72. Garcia JR, Jaumann F, Schulz S, Krause A, Rodriguez-Jimenez J, Forssmann U, Adermann K, Kluver E, Vogelmeier C, Becker D, Hedrich R, Forssmann WG, Bals R. Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma

membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell Tissue Res 2001;306:257-64.
73. Garcia JR, Krause A, Schulz S, Rodriguez-Jimenez FJ, Kluver E, Adermann K, Forssmann U, Frimpong-Boateng A, Bals R, Forssmann WG. Human beta-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. FASEB J 2001;15:1819-21.
74. Genco RJ, Loe H. The role of systemic conditions and disorders in periodontal disease. Periodontol 2000 1993;2:98-116.
75. Genco RJ, Zambon JJ, Christersson LA. The origin of periodontal infections. Adv Dent Res 1988;2:245-59.
76. Ghafouri B, Kihlstrom E, Stahlbom B, Tagesson C, Lindahl M. PLUNC (palate, lung and nasal epithelial clone) proteins in human nasal lavage fluid. Biochem Soc Trans 2003;31:810-4.
77. Glader P, Moller S, Lilja J, Wieslander E, Lofdahl CG, von Wachenfeldt K. Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. Respir Med 2006;100:818-27.
78. Gonzalez YM, De Nardin A, Grossi SG, Machtei EE, Genco RJ, De Nardin E. Serum cotinine levels, smoking, and periodontal attachment loss. J Dent Res 1996;75:796-802.
79. Grossi SG, Genco RJ, Machtei EE, Ho AW, Koch G, Dunford R, Zambon JJ, Hausmann E. Assessment of risk for periodontal disease. II. Risk indicators for alveolar bone loss. J Periodontol 1995;66:23-9.
80. Grossi SG, Zambon JJ, Ho AW, Koch G, Dunford RG, Machtei EE, Norderyd OM, Genco RJ. Assessment of risk for periodontal disease. I. Risk indicators for attachment loss. J Periodontol 1994;65:260-7.
81. Guo T, Rudnick PA, Wang W, Lee CS, Devoe DL, Balgley BM. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. J Proteome Res 2006;5:1469-78.
82. Gustafsson A, Asman B, Bergstrom K. Cigarette smoking as an aggravating factor in inflammatory tissue-destructive diseases. Increase in tumor necrosis Factor-alpha priming of peripheral neutrophils measured as generation of oxygen radicals. Int J Clin Lab Res 2000;30:187-90.
83. Haffajee AD, Cugini MA, Tanner A, Pollack RP, Smith C, Kent RL, Jr., Socransky SS. Subgingival microbiota in healthy, well-maintained elder and periodontitis subjects. J Clin Periodontol 1998;25:346-53.
84. Haffajee AD, Socransky SS. Relationship of cigarette smoking to attachment level profiles. J Clin Periodontol 2001;28:283-95.
85. Haffajee AD, Socransky SS. Relationship of cigarette smoking to the subgingival microbiota. J Clin Periodontol 2001;28:377-88.
86. Hamlet SM, Cullinan MP, Westerman B, Lindeman M, Bird PS, Palmer J, Seymour GJ. Distribution of Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Prevotella intermedia in an Australian population. J Clin Periodontol 2001;28:1163-71.
87. Hancock RE. Peptide antibiotics. Lancet 1997;349:418-22.
88. Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. Trends Microbiol 2000;8:402-10.
89. Haraszthy VI, Hariharan G, Tinoco EM, Cortelli JR, Lally ET, Davis E, Zambon JJ. Evidence for the role of highly leukotoxic Actinobacillus

actinomycetemcomitans in the pathogenesis of localized juvenile and other forms of early-onset periodontitis. J Periodontol 2000;71:912-22.
90. Harder J, Bartels J, Christophers E, Schroder JM. Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 2001;276:5707-13.
91. Harder J, Bartels J, Christophers E, Schroder JM. A peptide antibiotic from human skin. Nature 1997;387:861.
92. Harder J, Meyer-Hoffert U, Teran LM, Schwichtenberg L, Bartels J, Maune S, Schroder JM. Mucoid Pseudomonas aeruginosa, TNF-alpha, and IL-1beta, but not IL-6, induce human beta-defensin-2 in respiratory epithelia. Am J Respir Cell Mol Biol 2000;22:714-21.
93. Hardt M, Thomas LR, Dixon SE, Newport G, Agabian N, Prakobphol A, Hall SC, Witkowska HE, Fisher SJ. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. Biochemistry 2005;44:2885-99.
94. Hart TC, Hart PS, Michalec MD, Zhang Y, Marazita ML, Cooper M, Yassin OM, Nusier M, Walker S. Localisation of a gene for prepubertal periodontitis to chromosome 11q14 and identification of a cathepsin C gene mutation. J Med Genet 2000;37:95-101.
95. He XS, Shi WY. Oral microbiology: past, present and future. Int J Oral Sci 2009;1:47-58.
96. Hedges S, Agace W, Svensson M, Sjogren AC, Ceska M, Svanborg C. Uroepithelial cells are part of a mucosal cytokine network. Infect Immun 1994;62:2315-21.
97. Hill CP, Yee J, Selsted ME, Eisenberg D. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. Science 1991;251:1481-5.
98. Hiratsuka T, Nakazato M, Date Y, Ashitani J, Minematsu T, Chino N, Matsukura S. Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. Biochem Biophys Res Commun 1998;249:943-7.
99. Ho YC, Yang SF, Huang FM, Chang YC. Up-regulation of osteolytic mediators in human osteosarcoma cells stimulated with nicotine. J Periodontal Res 2009;44:760-6.
100. Hodge PJ, Riggio MP, Kinane DF. Failure to detect an association with IL1 genotypes in European Caucasians with generalised early onset periodontitis. J Clin Periodontol 2001;28:430-6.
101. Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B. Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature 2003;424:743-8.
102. Holm-Pedersen P, Agerbaek N, Theilade E. Experimental gingivitis in young and elderly individuals. J Clin Periodontol 1975;2:14-24.
103. Hu S, Xie Y, Ramachandran P, Ogorzalek Loo RR, Li Y, Loo JA, Wong DT. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. Proteomics 2005;5:1714-28.
104. Huang CM. Comparative proteomic analysis of human whole saliva. Arch Oral Biol 2004;49:951-62.

105. Hugoson A, Norderyd O. Has the prevalence of periodontitis changed during the last 30 years? J Clin Periodontol 2008;35:338-45.
106. Huttner KM, Bevins CL. Antimicrobial peptides as mediators of epithelial host defense. Pediatr Res 1999;45:785-94.
107. Ismail AI, Morrison EC, Burt BA, Caffesse RG, Kavanagh MT. Natural history of periodontal disease in adults: findings from the Tecumseh Periodontal Disease Study, 1959-87. J Dent Res 1990;69:430-5.
108. James JA, Sayers NM, Drucker DB, Hull PS. Effects of tobacco products on the attachment and growth of periodontal ligament fibroblasts. J Periodontol 1999;70:518-25.
109. Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clin Microbiol Rev 2006;19:491-511.
110. Johnson GK, Guthmiller JM. The impact of cigarette smoking on periodontal disease and treatment. Periodontol 2000 2007;44:178-94.
111. Johnson GK, Guthmiller JM, Joly S, Organ CC, Dawson DV. Interleukin-1 and interleukin-8 in nicotine- and lipopolysaccharide-exposed gingival keratinocyte cultures. J Periodontal Res 2010;45:583-8.
112. Johnson GK, Hill M. Cigarette smoking and the periodontal patient. J Periodontol 2004;75:196-209.
113. Johnson GK, Slach NA. Impact of tobacco use on periodontal status. J Dent Educ 2001;65:313-21.
114. Jung HC, Eckmann L, Yang SK, Panja A, Fierer J, Morzycka-Wroblewska E, Kagnoff MF. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. J Clin Invest 1995;95:55-65.
115. Kagan BL, Ganz T, Lehrer RI. Defensins: a family of antimicrobial and cytotoxic peptides. Toxicology 1994;87:131-49.
116. Kamma JJ, Nakou M, Baehni PC. Clinical and microbiological characteristics of smokers with early onset periodontitis. J Periodontal Res 1999;34:25-33.
117. Katz PP, Wirthlin MR, Jr., Szpunar SM, Selby JV, Sepe SJ, Showstack JA. Epidemiology and prevention of periodontal disease in individuals with diabetes. Diabetes Care 1991;14:375-85.
118. Kawai T, Adachi O, Ogawa T, Takeda K, Akira S. Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 1999;11:115-22.
119. Kelly JE, Harvey CR. Basic data on dental examination findings of persons 1-74 years. United States, 1971-1974. Vital Health Stat 11 1979:1-33.
120. Kinane DF. Causation and pathogenesis of periodontal disease. Periodontol 2000 2001;25:8-20.
121. Kinane DF, Demuth DR, Gorr SU, Hajishengallis GN, Martin MH. Human variability in innate immunity. Periodontol 2000 2007;45:14-34.
122. Kinane DF, Hodge P, Eskdale J, Ellis R, Gallagher G. Analysis of genetic polymorphisms at the interleukin-10 and tumour necrosis factor loci in early-onset periodontitis. J Periodontal Res 1999;34:379-86.
123. Kinane DF, Mark Bartold P. Clinical relevance of the host responses of periodontitis. Periodontol 2000 2007;43:278-93.
124. Kinane DF, Shiba H, Stathopoulou PG, Zhao H, Lappin DF, Singh A, Eskan MA, Beckers S, Waigel S, Alpert B, Knudsen TB. Gingival epithelial cells heterozygous for Toll-like receptor 4 polymorphisms Asp299Gly and Thr399ile are hypo-responsive to Porphyromonas gingivalis. Genes Immun 2006;7:190-200.

125. Klein RS, Quart AM, Small CB. Periodontal disease in heterosexuals with acquired immunodeficiency syndrome. J Periodontol 1991;62:535-40.
126. Kornman KS, Crane A, Wang HY, di Giovine FS, Newman MG, Pirk FW, Wilson TG, Jr., Higginbottom FL, Duff GW. The interleukin-1 genotype as a severity factor in adult periodontal disease. J Clin Periodontol 1997;24:72-7.
127. Kornman KS, Page RC, Tonetti MS. The host response to the microbial challenge in periodontitis: assembling the players. Periodontol 2000 1997;14:33-53.
128. Krisanaprakornkit S, Kimball JR, Dale BA. Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappaB transcription factor family. J Immunol 2002;168:316-24.
129. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin 2 by Fusobacterium nucleatum in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. Infect Immun 2000;68:2907-15.
130. Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA. Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. Infect Immun 1998;66:4222-8.
131. Kumar A, Zhang J, Yu FS. Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. Microbes Infect 2006;8:380-9.
132. Kumar H, Kawai T, Akira S. Toll-like receptors and innate immunity. Biochem Biophys Res Commun 2009;388:621-5.
133. Kupper TS. The activated keratinocyte: a model for inducible cytokine production by non-bone marrow-derived cells in cutaneous inflammatory and immune responses. J Invest Dermatol 1990;94:146S-150S.
134. Laine ML, Farre MA, Gonzalez G, van Dijk LJ, Ham AJ, Winkel EG, Crusius JB, Vandenbroucke JP, van Winkelhoff AJ, Pena AS. Polymorphisms of the interleukin-1 gene family, oral microbial pathogens, and smoking in adult periodontitis. J Dent Res 2001;80:1695-9.
135. Lamster IB, Begg MD, Mitchell-Lewis D, Fine JB, Grbic JT, Todak GG, el-Sadr W, Gorman JM, Zambon JJ, Phelan JA. Oral manifestations of HIV infection in homosexual men and intravenous drug users. Study design and relationship of epidemiologic, clinical, and immunologic parameters to oral lesions. Oral Surg Oral Med Oral Pathol 1994;78:163-74.
136. Lang NP, Tonetti MS, Suter J, Sorrell J, Duff GW, Kornman KS. Effect of interleukin-1 gene polymorphisms on gingival inflammation assessed by bleeding on probing in a periodontal maintenance population. J Periodontal Res 2000;35:102-7.
137. Lee WK, Ramanathan M, Jr., Spannhake EW, Lane AP. The cigarette smoke component acrolein inhibits expression of the innate immune components IL-8 and human beta-defensin 2 by sinonasal epithelial cells. Am J Rhinol 2007;21:658-63.
138. Lindhe J, Haffajee AD, Socransky SS. Progression of periodontal disease in adult subjects in the absence of periodontal therapy. J Clin Periodontol 1983;10:433-42.
139. Locker D. Smoking and oral health in older adults. Can J Public Health 1992;83:429-32.

140. Loe H, Theilade E, Jensen SB. Experimental Gingivitis in Man. J Periodontol 1965;36:177-87.
141. Machtei EE, Dunford R, Grossi SG, Genco RJ. Cumulative nature of periodontal attachment loss. J Periodontal Res 1994;29:361-4.
142. Machtei EE, Dunford R, Hausmann E, Grossi SG, Powell J, Cummins D, Zambon JJ, Genco RJ. Longitudinal study of prognostic factors in established periodontitis patients. J Clin Periodontol 1997;24:102-9.
143. Madianos PN, Bobetsis YA, Kinane DF. Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva. J Clin Periodontol 2005;32 Suppl 6:57-71.
144. Mahanonda R, Pichyangkul S. Toll-like receptors and their role in periodontal health and disease. Periodontol 2000 2007;43:41-55.
145. Mahanonda R, Sa-Ard-Iam N, Eksomtramate M, Rerkyen P, Phairat B, Schaecher KE, Fukuda MM, Pichyangkul S. Cigarette smoke extract modulates human beta-defensin-2 and interleukin-8 expression in human gingival epithelial cells. J Periodontal Res 2009;44:557-64.
146. Mark LL, Haffajee AD, Socransky SS, Kent RL, Jr., Guerrero D, Kornman K, Newman M, Stashenko P. Effect of the interleukin-1 genotype on monocyte IL-1beta expression in subjects with adult periodontitis. J Periodontal Res 2000;35:172-7.
147. Masouredis CM, Katz MH, Greenspan D, Herrera C, Hollander H, Greenspan JS, Winkler JR. Prevalence of HIV-associated periodontitis and gingivitis in HIV-infected patients attending an AIDS clinic. J Acquir Immune Defic Syndr 1992;5:479-83.
148. Mathews M, Jia HP, Guthmiller JM, Losh G, Graham S, Johnson GK, Tack BF, McCray PB, Jr. Production of beta-defensin antimicrobial peptides by the oral mucosa and salivary glands. Infect Immun 1999;67:2740-5.
149. McDevitt MJ, Wang HY, Knobelman C, Newman MG, di Giovine FS, Timms J, Duff GW, Kornman KS. Interleukin-1 genetic association with periodontitis in clinical practice. J Periodontol 2000;71:156-63.
150. Medzhitov R, Janeway C, Jr. Innate immunity. N Engl J Med 2000;343:338-44.
151. Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S, Janeway CA, Jr. MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Mol Cell 1998;2:253-8.
152. Mehta H, Nazzal K, Sadikot RT. Cigarette smoking and innate immunity. Inflamm Res 2008;57:497-503.
153. Meikle MC, Hembry RM, Holley J, Horton C, McFarlane CG, Reynolds JJ. Immunolocalization of matrix metalloproteinases and TIMP-1 (tissue inhibitor of metalloproteinases) in human gingival tissues from periodontitis patients. J Periodontal Res 1994;29:118-26.
154. Meisel P, Siegemund A, Dombrowa S, Sawaf H, Fanghaenel J, Kocher T. Smoking and polymorphisms of the interleukin-1 gene cluster (IL-1alpha, IL-1beta, and IL-1RN) in patients with periodontal disease. J Periodontol 2002;73:27-32.
155. Moore PA, Weyant RJ, Mongelluzzo MB, Myers DE, Rossie K, Guggenheimer J, Block HM, Huber H, Orchard T. Type 1 diabetes mellitus and oral health: assessment of periodontal disease. J Periodontol 1999;70:409-17.
156. Morrison TB, Weis JJ, Wittwer CT. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 1998;24:954-8, 960, 962.

157. Nagy I, Pivarcsi A, Koreck A, Szell M, Urban E, Kemeny L. Distinct strains of Propionibacterium acnes induce selective human beta-defensin-2 and interleukin-8 expression in human keratinocytes through toll-like receptors. J Invest Dermatol 2005;124:931-8.
158. Nanci A, Bosshardt DD. Structure of periodontal tissues in health and disease. Periodontol 2000 2006;40:11-28.
159. O'Neil DA, Porter EM, Elewaut D, Anderson GM, Eckmann L, Ganz T, Kagnoff MF. Expression and regulation of the human beta-defensins hBD-1 and hBD-2 in intestinal epithelium. J Immunol 1999;163:6718-24.
160. Oliver RC, Tervonen T. Periodontitis and tooth loss: comparing diabetics with the general population. J Am Dent Assoc 1993;124:71-6.
161. Page RC, Kornman KS. The pathogenesis of human periodontitis: an introduction. Periodontol 2000 1997;14:9-11.
162. Palmer RM, Wilson RF, Hasan AS, Scott DA. Mechanisms of action of environmental factors--tobacco smoking. J Clin Periodontol 2005;32 Suppl 6:180-95.
163. Papapanou PN, Baelum V, Luan WM, Madianos PN, Chen X, Fejerskov O, Dahlen G. Subgingival microbiota in adult Chinese: prevalence and relation to periodontal disease progression. J Periodontol 1997;68:651-66.
164. Papapanou PN, Wennstrom JL, Grondahl K. Periodontal status in relation to age and tooth type. A cross-sectional radiographic study. J Clin Periodontol 1988;15:469-78.
165. Patton GW, Powell DA, Hakki A, Friedman H, Pross S. Nicotine modulation of cytokine induction by LPS-stimulated human monocytes and coronary artery endothelial cells. Int Immunopharmacol 2006;6:26-35.
166. Payne WA, Page RC, Ogilvie AL, Hall WB. Histopathologic features of the initial and early stages of experimental gingivitis in man. J Periodontal Res 1975;10:51-64.
167. Petropoulos G, McKay IJ, Hughes FJ. The association between neutrophil numbers and interleukin-1alpha concentrations in gingival crevicular fluid of smokers and non-smokers with periodontal disease. J Clin Periodontol 2004;31:390-5.
168. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
169. Pollanen MT, Salonen JI, Uitto VJ. Structure and function of the tooth-epithelial interface in health and disease. Periodontol 2000 2003;31:12-31.
170. Preber H, Bergstrom J, Linder LE. Occurrence of periopathogens in smoker and non-smoker patients. J Clin Periodontol 1992;19:667-71.
171. Quayle AJ, Porter EM, Nussbaum AA, Wang YM, Brabec C, Yip KP, Mok SC. Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. Am J Pathol 1998;152:1247-58.
172. Quinones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR, Marotta ML, Mirza M, Jiang B, Kiser P, Medvik K, Sieg SF, Weinberg A. Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. AIDS 2003;17:F39-48.
173. Radek KA, Elias PM, Taupenot L, Mahata SK, O'Connor DT, Gallo RL. Neuroendocrine nicotinic receptor activation increases susceptibility to bacterial infections by suppressing antimicrobial peptide production. Cell Host Microbe 2010;7:277-89.

174. Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 2004;24:536-47.
175. Riggio MP, Lennon A, Roy KM. Detection of Prevotella intermedia in subgingival plaque of adult periodontitis patients by polymerase chain reaction. J Periodontal Res 1998;33:369-76.
176. Russell JP, Diamond G, Tarver AP, Scanlin TF, Bevins CL. Coordinate induction of two antibiotic genes in tracheal epithelial cells exposed to the inflammatory mediators lipopolysaccharide and tumor necrosis factor alpha. Infect Immun 1996;64:1565-8.
177. Ryder MI. The influence of smoking on host responses in periodontal infections. Periodontol 2000 2007;43:267-77.
178. Ryder MI, Fujitaki R, Lebus S, Mahboub M, Faia B, Muhaimin D, Hamada M, Hyun W. Alterations of neutrophil L-selectin and CD18 expression by tobacco smoke: implications for periodontal diseases. J Periodontal Res 1998;33:359-68.
179. Saeed RW, Varma S, Peng-Nemeroff T, Sherry B, Balakhaneh D, Huston J, Tracey KJ, Al-Abed Y, Metz CN. Cholinergic stimulation blocks endothelial cell activation and leukocyte recruitment during inflammation. J Exp Med 2005;201:1113-23.
180. Sambrook J, Fritsch EF, Maniatis T. Extraction, purification, and analysis of messenger RNA from eukaryotic cells (chapter 7). In N Ford, C Nolan, M Ferguson (eds.). Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbour 1989:p.7.3–7.87.
181. Schenkels LC, Veerman EC, Nieuw Amerongen AV. Biochemical composition of human saliva in relation to other mucosal fluids. Crit Rev Oral Biol Med 1995;6:161-75.
182. Schonwetter BS, Stolzenberg ED, Zasloff MA. Epithelial antibiotics induced at sites of inflammation. Science 1995;267:1645-8.
183. Schroder JM. Epithelial antimicrobial peptides: innate local host response elements. Cell Mol Life Sci 1999;56:32-46.
184. Schroder JM, Harder J. Human beta-defensin-2. Int J Biochem Cell Biol 1999;31:645-51.
185. Shiau HJ, Reynolds MA. Sex differences in destructive periodontal disease: exploring the biologic basis. J Periodontol 2010;81:1505-17.
186. Singh PK, Jia HP, Wiles K, Hesselberth J, Liu L, Conway BA, Greenberg EP, Valore EV, Welsh MJ, Ganz T, Tack BF, McCray PB, Jr. Production of beta-defensins by human airway epithelia. Proc Natl Acad Sci U S A 1998;95:14961-6.
187. Singh SP, Mishra NC, Rir-Sima-Ah J, Campen M, Kurup V, Razani-Boroujerdi S, Sopori ML. Maternal exposure to secondhand cigarette smoke primes the lung for induction of phosphodiesterase-4D5 isozyme and exacerbated Th2 responses: rolipram attenuates the airway hyperreactivity and muscarinic receptor expression but not lung inflammation and atopy. J Immunol 2009;183:2115-21.
188. Socransky SS, Haffajee AD. Periodontal microbial ecology. Periodontol 2000 2005;38:135-87.
189. Soder B, Jin LJ, Wickholm S. Granulocyte elastase, matrix metalloproteinase-8 and prostaglandin E2 in gingival crevicular fluid in matched clinical sites in smokers and non-smokers with persistent periodontitis. J Clin Periodontol 2002;29:384-91.

190. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol 2002;2:372-7.
191. Stadnyk AW. Cytokine production by epithelial cells. FASEB J 1994;8:1041-7.
192. Stoltenberg JL, Osborn JB, Pihlstrom BL, Herzberg MC, Aeppli DM, Wolff LF, Fischer GE. Association between cigarette smoking, bacterial pathogens, and periodontal status. J Periodontol 1993;64:1225-30.
193. Sugano N, Shimada K, Ito K, Murai S. Nicotine inhibits the production of inflammatory mediators in U937 cells through modulation of nuclear factor-kappaB activation. Biochem Biophys Res Commun 1998;252:25-8.
194. Sugawara Y, Uehara A, Fujimoto Y, Kusumoto S, Fukase K, Shibata K, Sugawara S, Sasano T, Takada H. Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. J Dent Res 2006;85:524-9.
195. Supp DM, Karpinski AC, Boyce ST. Expression of human beta-defensins HBD-1, HBD-2, and HBD-3 in cultured keratinocytes and skin substitutes. Burns 2004;30:643-8.
196. Svanborg C, Agace W, Hedges S, Lindstedt R, Svensson ML. Bacterial adherence and mucosal cytokine production. Ann N Y Acad Sci 1994;730:162-81.
197. Swango PA, Kleinman DV, Konzelman JL. HIV and periodontal health. A study of military personnel with HIV. J Am Dent Assoc 1991;122:49-54.
198. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1-14.
199. Tang YQ, Yuan J, Osapay G, Osapay K, Tran D, Miller CJ, Ouellette AJ, Selsted ME. A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science 1999;286:498-502.
200. Teng YT. The role of acquired immunity and periodontal disease progression. Crit Rev Oral Biol Med 2003;14:237-52.
201. Thomson WM, Locker D. Dental neglect and dental health among 26-year-olds in the Dunedin Multidisciplinary Health and Development Study. Community Dent Oral Epidemiol 2000;28:414-8.
202. Tossi A, Sandri L. Molecular diversity in gene-encoded, cationic antimicrobial polypeptides. Curr Pharm Des 2002;8:743-61.
203. Tossi A, Sandri L, Giangaspero A. Amphipathic, alpha-helical antimicrobial peptides. Biopolymers 2000;55:4-30.
204. Tymkiw KD, Thunell DH, Johnson GK, Joly S, Burnell KK, Cavanaugh JE, Brogden KA, Guthmiller JM. Influence of smoking on gingival crevicular fluid cytokines in severe chronic periodontitis. J Clin Periodontol 2011;38:219-28.
205. Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB, Jr., Ganz T. Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J Clin Invest 1998;101:1633-42.
206. Van der Velden U, Varoufaki A, Hutter JW, Xu L, Timmerman MF, Van Winkelhoff AJ, Loos BG. Effect of smoking and periodontal treatment on the subgingival microflora. J Clin Periodontol 2003;30:603-10.
207. Vankeerberghen A, Nuytten H, Dierickx K, Quirynen M, Cassiman JJ, Cuppens H. Differential induction of human beta-defensin expression by periodontal commensals and pathogens in periodontal pocket epithelial cells. J Periodontol 2005;76:1293-303.
208. Vitorino R, Lobo MJ, Ferrer-Correira AJ, Dubin JR, Tomer KB, Domingues PM, Amado FM. Identification of human whole saliva protein components using proteomics. Proteomics 2004;4:1109-15.

209. Walz A, Stuhler K, Wattenberg A, Hawranke E, Meyer HE, Schmalz G, Bluggel M, Ruhl S. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. Proteomics 2006;6:1631-9.
210. Wang H, Liao H, Ochani M, Justiniani M, Lin X, Yang L, Al-Abed Y, Metz C, Miller EJ, Tracey KJ, Ulloa L. Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis. Nat Med 2004;10:1216-21.
211. Weinberg A, Krisanaprakornkit S, Dale BA. Epithelial antimicrobial peptides: review and significance for oral applications. Crit Rev Oral Biol Med 1998;9:399-414.
212. Wilmarth PA, Riviere MA, Rustvold DL, Lauten JD, Madden TE, David LL. Two-dimensional liquid chromatography study of the human whole saliva proteome. J Proteome Res 2004;3:1017-23.
213. Xie H, Rhodus NL, Griffin RJ, Carlis JV, Griffin TJ. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. Mol Cell Proteomics 2005;4:1826-30.
214. Ximenez-Fyvie LA, Haffajee AD, Socransky SS. Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis. J Clin Periodontol 2000;27:648-57.
215. Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301:640-3.
216. Yamazaki K, Yoshie H, Seymour GJ. T cell regulation of the immune response to infection in periodontal diseases. Histol Histopathol 2003;18:889-96.
217. Yao Y, Berg EA, Costello CE, Troxler RF, Oppenheim FG. Identification of protein components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. J Biol Chem 2003;278:5300-8.
218. Zambon JJ, Grossi SG, Machtei EE, Ho AW, Dunford R, Genco RJ. Cigarette smoking increases the risk for subgingival infection with periodontal pathogens. J Periodontol 1996;67:1050-4.
219. Zhang L, Yu W, He T, Yu J, Caffrey RE, Dalmasso EA, Fu S, Pham T, Mei J, Ho JJ, Zhang W, Lopez P, Ho DD. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. Science 2002;298:995-1000.
220. Zhao C, Wang I, Lehrer RI. Widespread expression of beta-defensin hBD-1 in human secretory glands and epithelial cells. FEBS Lett 1996;396:319-22.
221. Zia S, Ndoye A, Lee TX, Webber RJ, Grando SA. Receptor-mediated inhibition of keratinocyte migration by nicotine involves modulations of calcium influx and intracellular concentration. J Pharmacol Exp Ther 2000;293:973-81.
222. Zimmermann GR, Legault P, Selsted ME, Pardi A. Solution structure of bovine neutrophil beta-defensin-12: the peptide fold of the beta-defensins is identical to that of the classical defensins. Biochemistry 1995;34:13663-71.
223. Zoellner H, Chapple CC, Hunter N. Microvasculature in gingivitis and chronic periodontitis: disruption of vascular networks with protracted inflammation. Microsc Res Tech 2002;56:15-31.

11 Appendix

11.1 List of Materials and Equipments

1. 1x TBE-buffer (Rotiphorese, Carl Roth, Karlsruhe, Germany)
2. Agarose gel (Rotigarose, Carl Roth, Karlsruhe, Germany).
3. Biometra Uno-Thermoblock, PCR-cycler (Biometra, Goettingen, Germany)
4. Cell line HaCaT (German Cancer Research Center, Heidelberg)
5. Cell scraper (TPP, Trasadingen, Switzerland)
6. Centrifuge machine (Eppendorf, Hamburg, Germany)
7. Chloroform (Caelo, Hilden, Germany)
8. Cryo carrier (Taylor-Wharton, Husum, Germany)
9. Cryotubes (Nunc, Roskilde, Denmark)
10. Dulbecco's Modified Eagles's Medium (DMEM, Biochrom, Berlin, Germany)
11. Dulbecco's phosphate buffered saline (PBS, Biochrom, Berlin, Germany)
12. Ethidium bromide (10 mg/ml) (CarlRoth)
13. Fetal Bovine Serum (FBS, Biochrom, Berlin, Germany)
14. Fluorescent dye, QuantiTect® SYBR® Green (Qiagen, Hilden, Germany).
15. Hybridization oven (Hybaid, Shake 'n' Stack, Thermo Fisher Scientific, Waltham, USA).
16. Incubator (Heraus, Hanau, Germany),
17. Isopropanol (Carl Roth GmbH, Karlsruhe, Germany)
18. Laminar Flow Cabin (Heraeus, Hanau, Germany)
19. Loading buffer (ABgene, Surrey, Great Britain)
20. LPS (Sigma-Aldrich, München, Germany)
21. LPS from E. coli (Sigma)
22. Microscope (Carl Zeiss, New York, USA)
23. Nicotine (Sigma, St. Louis, MO, USA)
24. Nucleotides mix dNTPs (Carl Roth GmbH, Karlsruhe, Germany)
25. PCR Purification Kit QIAquick (Qiagen, Hilden, Germany)
26. Penicillin (Biochrom, Germany)
27. Pipettes (Nunc, Roskilde, Denmark)
28. Primer for the PCR (MWG Biothech, Ebersberg, Germany)
29. Reaction tubes 1,5 ml (Nunc, Roskilde, Denmark)
30. Real-Time PCR cycler (Rotor-Gene 2000; Corbett Research, Sydney)
31. Reverse Transcription-Kit (Promega, Wisconsin, USA)

32. RNA extraction kit QIAzol (Qiagen, Hilden, Germany)
33. RNA stabilization reagent (RNAlater; Qiagen, Hilden, Germany)
34. RNeasy Mini Kit (Qiagen, Hilden, Germany)
35. Six-well culture plates (TPP, Trasadingen, Switzerland)
36. Software Photo Finnish (GatorData, Inc. Lutz, USA).
37. Spectrophotometer (Biochrom, Cambridge, England),
38. SPSS-Software for Windows (SPSS Inc., Chicago, USA)
39. Streptomycin (Biochrom, Germany)
40. Tissue culture flasks (TPP, Trasadingen, Switzerland)
41. TissueRuptor (Qiagen, Hilden, Germany
42. TNFα (PeproTech, Germany, Hamburg)
43. Trypsin 0.05% - EDTA 0.02% (Biochrom, Germany)
44. UV-light chamber (Vilber Lourmat, Marnela-Vallee, France)

11.2 Abbreviations

1.	˚C	=	degree Celsius
2.	a	=	adenine
3.	AcNo	=	accession number
4.	AIDS	=	acquired immunodeficiency syndrome
5.	AMPs	=	antimicrobial peptides
6.	Borate	=	boric acid
7.	bp	=	base pairs
8.	c	=	cytosine
9.	ca.	=	circa
10.	CD	=	cluster of differentiation
11.	cig./day	=	cigarettes per day
12.	cm^2	=	square meter
13.	Cntr	=	control
14.	CO_2	=	carbon dioxide
15.	CSE	=	cigarette smoke extract
16.	Ct	=	Cycle threshold value
17.	DEPC	=	diethylpyrocarbonate
18.	Dept.	=	department
19.	DNA	=	deoxyribonucleic acid
20.	dNTPs	=	deoxyribonucleotides

21. EDTA = ethylenediaminetetraacetic acid
22. et al. = et alii, and others
23. Fig. = figure
24. g = guanine
25. GAPDH = glyceraldehyde 3-phosphate dehydrogenase
26. h = hours
27. HaCaT = human adult skin keratinocytes propagated under low Ca^{2+} conditions and elevated temperature
28. hBDs = human beta-defensins
29. HIV = Human immunodeficiency virus
30. IgG = immunoglobulin G
31. IL = interleukin
32. IRFs = interferon regulatory factors
33. kb = kilo base pairs
34. kD = kilodalton
35. LD = lethal dose
36. LPS = lipopolysaccharide
37. ml = milliliter
38. mM = millimolar
39. MMPs = matrix metalloproteinases
40. mRNA = messenger ribonucleic acid
41. n = variable quantity
42. nAChRs = nicotinic achetylcholine receptors
43. NF-κB = nuclear factor kappa
44. ng = nanogram
45. Nic = nicotine
46. NK cells = natural killer cells
47. NOD = nucleotide-binding oligomerization domain proteins
48. NTC = non template control
49. OD = optical density
50. Oligo-(dt) = a short sequence of deoxy-thymine nucleotides
51. p = statistically significant value
52. PAMPs = pathogen-associated molecular patterns
53. PCR = polymerase chain reaction

54.	PGE_2	=	prostaglandin E_2
55.	PMA	=	phorbol 12-myristate 13-acetate
56.	PMNs	=	polymorphonuclear neutrophils
57.	Poly-$(A)^+$ tail	=	a long sequence of adenine nucleotides
58.	preNic	=	pre-treatment with nicotine
59.	PreTNF-α	=	pre-treatment with TNF-α
60.	QRT-PCR	=	quantitative real time-polymerase chain reaction
61.	RANKL	=	receptor activator of nuclear factor kappa-B ligand
62.	rpm	=	revolutions per minute
63.	SD	=	standard deviation
64.	t	=	thymine
65.	Tab.	=	table
66.	Taq	=	thermus aquaticus
67.	TLRs	=	Toll-like receptors
68.	TNF-α	=	tumor necrosis factor-alpha
69.	Tris	=	tris(hydroxymethyl)aminomethane
70.	U	=	units
71.	U937	=	monocytes cell line
72.	UV-light	=	ultraviolet light
73.	µg	=	microgram
74.	µl	=	microliter

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13 Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

14 Declaration in lieu of an oath

Ich, Stylianos Liodakis, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: "*The influence of nicotine and smoking on the gene expression of human beta-defensins and proinflammatory cytokines in human keratinocytes (HaCaT) and in keratinized gingiva from smokers and non-smokers*", selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

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