Host-bacterial interaction in peri-implantitis

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Chapter 1:

General introduction

Lost natural teeth can be replaced with a variety of dental prosthesis, dental implants being one of the most successful methods. Contemporary dental implants are shaped similar to the root of a natural tooth and are almost exclusively made from commercially pure titanium or titanium alloys. A typical root-form endosseous implant consists of a titanium screw with a roughened or smooth surface. Endosseous dental implants are installed into the alveolar or basal bone by a single stage or two stage surgical procedures [1]. The healing process after dental implant placement results in osseointegration, which is the formation of a direct interface between an implant and bone without intervening soft tissue. Osseointegration was discovered and first described in 1983 by P.I. Brånemark [2].

Due to high success rates [3], the use of titanium dental implants has increased significantly during the past three decades. Success of an implant has been defined as implant and fixed prosthesis present in the mouth in the absence of biological and/or technical complications during the observation period [4]. However, failure of dental implants also occur and often leads to its removal. Failure of dental implant can occur due to the inability of tissues to establish osseointegration prior to the placement of dental prosthesis and is suggested to occur due to interference with the healing process [5]. Possible causes include lack of adequate bone volume, smoking, surgical trauma, lack of primary stability, intra-osseous infection and bacterial contamination of the recipient site [6, 7]. Failures of dental implants occurring after occlusal loading are suggested to occur due to the breakdown of the already established osseointegration [5, 8]. Breakdown of the established osseointegration may occur due to peri-implantitis and/or mechanical overload [6, 9, 10]. Depending on intraoral loading time, failure rates of 7.7 to 17 % have been reported for different implant systems [5, 11].

Chapter 1

Peri-implantitis

Definition, diagnosis and prevalence

The term peri-implantitis was introduced more than two decades ago to encompass infectious pathological conditions affecting dental implant [12-14]. According to the consensus report from the 7th European Workshop on Periodontology [15], in peri-implant mucositis, inflammatory response is limited to the soft tissues surrounding a functioning dental implant, whereas inflammatory response in peri-implantitis also results in loss of peri-implant marginal bone. Continued inflammation and bone loss around dental implant can lead to mobility and loss of the implant. Diagnosis of peri-implantitis is made similar to periodontitis and involves measuring clinical parameters such as peri-implant pocket depth (PPD), bleeding on probing (BoP), plaque index (PI), peri-implant loss of gingival attachment, suppuration and mobility [16]. Radiographic assessment of peri-implant bone loss also forms part of the current diagnostic tools for peri-implantitis. There is considerable variation in the reported prevalence of peri-implantitis in different studies. According to a review by Zitzmann and Berglundh [17], 28 to ≥ 56 % of the participants and 12 to 43% of the implants could be affected by peri-implantitis. Different disease definitions, follow-up periods or implant systems studied, could lead to such variations in the results of different studies. In a recent systematic review [18], peri-implantitis has been reported to occur in up to 18.8 % of patients and 9.6 % of implants. In terms of late implant failures, results of meta-analyses suggest that peri-implantitis accounts for 10-50% of failed implants after one year of loading [5, 19].

Etiology and microbiology

The etiology of peri-implantitis is multi-factorial but microorganisms play a central role in the development and progression of peri-implantitis [20, 21]. Implants surrounded by tissue free from clinical inflammation demonstrate microbiota associated with periodontal health which is dominated by Gram-positive facultative cocci and rods [22-24]. A complex subgingival microbiota is established during the tissue breakdown in peri-implantitis which is dominated by Gram-negative anaerobes and closely resembles the microbiota found in chronic adult periodontitis [25-27]. Oral bacteria that are strongly associated with peri-implantitis include *Porphyromonas gingivalis, Prevotella intermedia/Prevotella nigrescens, Tannerella forsythia, Treponema denticola* and *Aggregatibacter actinomycetemcomitans* [28]. However, several studies have found microorganisms not primarily associated with periodontitis such as *Staphylococcus* species, *Candida* species and *Enterics* in peri-implantitis lesions [22, 26, 29-33]. Interestingly, *Staphylococci* are also frequently isolated from infections of medically used metallic biomaterials other than dental implants [34, 35]. *In vitro* studies have shown an affinity of *S. aureus* to titanium surfaces in particular [36] and studies support association of *S. aureus* with therapy resistant cases of peri-implantitis [37-40]. However, importance of such reports for the pathogenesis of peri-implantitis needs further investigations. More recent studies, while maintaining the association of Gram-negative anaerobic microorganisms with peri-implantitis, reveal new microbial signatures that might be uniquely associated with peri-implantitis [41, 42]. Using 16S rRNA gene clone library technique for bacterial detection, it has been suggested that microbiota around implants in peri-implantitis is more complex when compared to periodontitis [41]. Another study has recently compared the microbiota around healthy implants, healthy teeth, periodontitis and peri-implantitis using 16S pyrosequencing [43]. This study suggested the

association of some previously unsuspected microbial species such as *Streptococcus mutans* and *Butryvibrio fibrisolvens* with peri-implantitis. However, in order to establish an association of these microorganisms with peri-implantitis, studies with a larger sample size are needed.

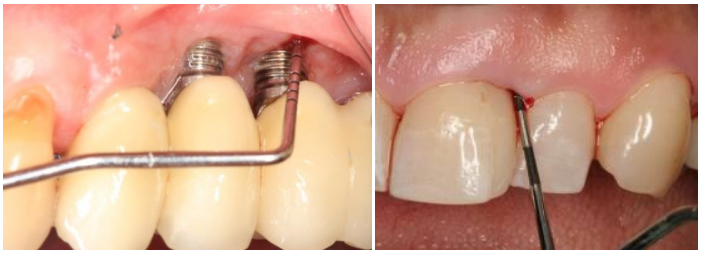
Besides bacteria, a history of periodontitis, smoking, an aberrant host response to microbial pathogens and poor oral hygiene are the most important known risk factors for developing peri-implantitis [44, 45]. Several systematic reviews have been published on the association of past history of periodontitis and development of peri-implantitis [46-50]. There is considerable heterogeneity in the study design, length of follow up, definition of patient population and outcome measures among the studies identified in these reviews. However, from these reviews, it can be concluded that past history of periodontitis is a risk factor for peri-implantitis [45]. Smoking is a known risk factor for several chronic conditions and peri-implantitis is no exception. Effects of smoking on implant have been systematically reviewed by Strietzel et al. [51]. Smoking has been associated with significantly increased inflammation related clinical parameters [52, 53], peri-implant bone loss [53, 54] and peri-implantitis [55]. In addition to the above mentioned established risk factors, preliminary data supports genetic susceptibility to increase the risk for developing peri-implantitis [56]. A recent review has concluded that *interleukin* (*IL)-1* gene polymorphisms (*IL1A -889* and *ILB +3954* genotype) in combination with smoking increases the risk for peri-implant bone loss in peri-implantitis [57]. Association of viruses such as human cytomegalovirus (HCMV) -2 and Epstein-Barr virus (EBV) -1 with peri-implantitis has also been reported [58].

Peri-implantitis lesions

Although peri-implantitis shares some characteristics with periodontitis, inflammatory lesions in peri-implantitis are different in certain aspects compared to periodontitis lesions [59]. Experimental studies on the tissue response to established biofilms have reported more pronounced inflammation around implants compared to natural teeth (figure 1, A&B)[60]. Peri-implantitis lesions tend to be more aggressive in nature and animal studies have revealed that peri-implantitis lesions extend more apically compared to periodontitis lesions [15]. Moreover, differences in the composition of inflammatory cell infiltrate have been found between peri-implantitis and periodontitis lesions [15]. In a study by Gualini & Berglundh [61], elastase-producing cells were found to be more common in peri-implantitis lesions, possibly indicating a more acute type of infection than periodontitis. Experimental animal models have demonstrated more pronounced and persistent bone loss around implants compared to natural teeth [62-64]. In addition, the circumferential pattern of bone loss seen in peri-implantitis is different from the often localized horizontal bone loss seen in periodontitis (figure 2, A&B). It has been suggested that the different surface properties of dental implants may favor colonization of specific microorganisms and thus may partly account for the differences seen between peri-implantitis and periodontitis lesions [65-67]. The absence of a periodontal ligament around implant, a low degree of vascularization and a higher collagen fiber to fibroblast ratio in comparison to the tooth, may all have an effect on the pattern and rate of bone loss in peri-implantitis [63, 68, 69]. Nevertheless, mechanisms behind the different tissue responses seen in peri-implantitis lesions are not known and needs further investigations.

Figure 1

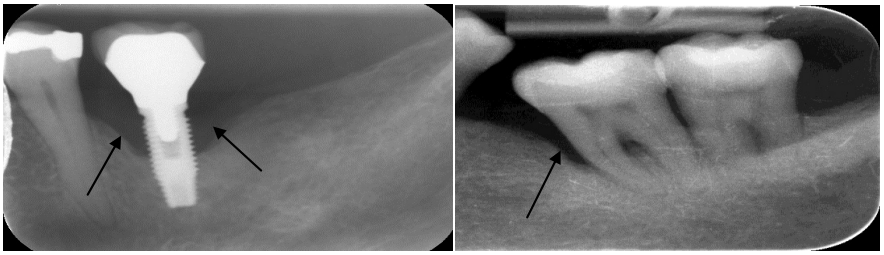
A. Peri-implantitis lesion B. Periodontitis lesion



Photos courtesy David Anssari Moin, department of oral function and restorative dentistry, ACTA.

Figure 2

A. Circumferential bone loss in peri-implantitis B. Localized bone loss seen in periodontitis



Photos courtesy David Anssari Moin, department of oral function and restorative dentistry/department of periodontology, ACTA.

Clinical management

Clinical management of peri-implantitis aims to eliminate plaque and calculus, decontaminate implant surface and regenerate lost tissue. Peri-implantitis is clinically managed in a similar way to periodontitis [70]. However, a systematic review by Esposito et al. [71] concluded that there is little evidence to support such an approach. For peri-implantitis, the effectiveness of a treatment protocol including surgical access, implant surface decontamination and systemic antimicrobials followed by a strict postoperative protocol has been shown recently [72]. Conservative non-surgical treatment for peri-implantitis includes mechanical debridement by scaling and root planning combined with local or systemic antibacterial agents. Use of lasers has also been reported for non-surgical treatment of peri-implantitis [73]. However, available evidence for the effectiveness of non-surgical treatment protocols for peri-implantitis is insufficient [74]. There is a lack of consensus on evidence based treatment modalities for peri-implantitis [75]. Therefore, more studies are needed to evaluate the clinical and microbiological outcomes of non-surgical treatment modalities for peri-implantitis. Moreover, interaction of oral bacteria associated with peri-implantitis, such as *P. gingivalis,* with host cells is of utmost importance to better understand the pathogenesis of peri-implantitis and devise better treatment strategies.

Chapter 1

Porphyromonas gingivalis

Porphyromonas gingivalis is strongly associated with disease initiation and progression in peri-implantitis [76]. It is a black-pigmented, Gram-negative, anaerobic, rod shaped bacterium. It is one of the most extensively studied oral bacterium. Therefore it is a valuable model organism to study the *in vitro* host-bacterial interaction in peri-implantitis. Through its varied virulence factors (figure 3), *P. gingivalis* can evade recognition by innate immune system, directly invade host cells, avoid killing by complement, corrupt the innate immunity in a way to maximize chances for its survival and may favorably alter the environment for other opportunistic pathogens in periodontal diseases [77]. Some of the most important virulence factors associated with *P. gingivalis* are described below;

P. gingivalis **proteinases**

P. gingivalis produces a variety of proteinases [78-80] which can degrade host proteins. The pathological role of proteinases produced by *P. gingivalis* has been recently reviewed [81]. These proteinases provide *P. gingivalis* with the necessary nutrition by breaking down host proteins [82]. The activity of trypsin-like proteinses (also called gingipains) produced by *P. gingivalis*, show a close relationship with *P. gingivalis* virulence [83]. *P. gingivalis* gingipains can also breakdown components of the host immune system and thus play an important role in modulation of host immune responses [84]. In addition, some *P. gingivalis* gingipains can play a role in its attachment to the host extracellular matrix such as collagen, thereby facilitating matrix breakdown [84]. *P. gingivalis* gingipains also play a role in biofilm formation with other bacterial species [85].

Fimbriae

P. gingivalis is also equipped with major and minor fimbriae which are thread-like proteins anchored in its outer membrane. Fimbriae have multiple functions and their importance has been shown for adherence and invasion of host cells [86, 87]. Specific *P. gingivalis* fimbriae can also play an immunomodulatory role by the release of cytokines from host cells via CD14 receptors and Toll-like receptors (TLR), like TLR-2 and -4 [88]. *P. gingivalis* fimbriae have been shown to play a role in periodontal bone resorption in animal models [89, 90].

Lipopolysaccharide

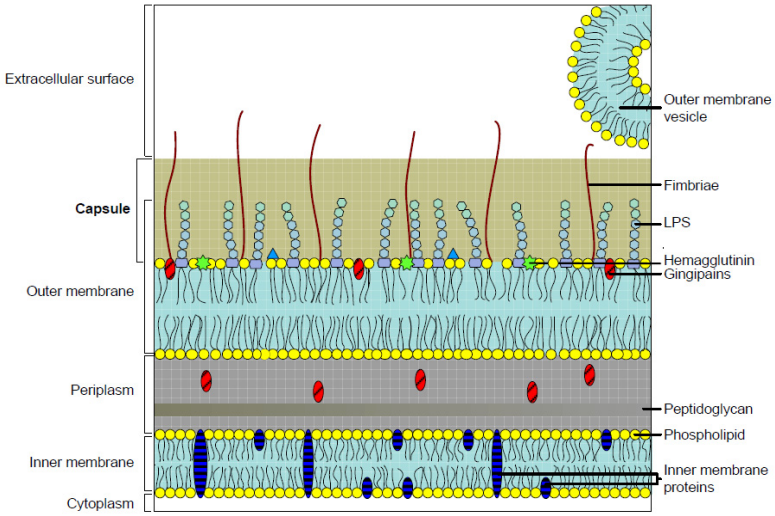
Another important virulence factor of *P. gingivalis* is its lipopolysaccharide (LPS), which is a major component of Gram-negative bacteria and is important for their structural integrity. LPS is a strong stimulator of cytokines in a variety of host cell types and has been reported to induce bone resorption [91, 92]. Upon exposure to *P. gingivalis* LPS, host cells such as gingival fibroblasts produce a range of pro-inflammatory cytokines including IL-1, IL-6, IL-8 and tumor necrosis factor (TNF)-α [93]. It has been suggested that binding of *P. gingivalis* LPS to TLRs rather than to CD14 on fibroblasts activates these cells to produce pro-inflammatory cytokines [93]. Interestingly, *P. gingivalis* LPS also suppresses the innate immune response via TLR-signaling [94]. It has been proposed that these opposing immune responses may be due to the heterogeneity in *P. gingivalis* LPS structure, resulting in the dysregulation of overall immune response commonly seen in periodontal diseases [95].

Capsular polysaccharide

Another major virulence factor of *P. gingivalis* is its polysaccharide capsule. Capsular polysaccharide (CPS) produced by *P. gingivalis* surrounds the bacterial cell and has antigenic

properties. On the basis of antigenic properties of its capsule, seven encapsulated (K1-K7) and one non-encapsulated (K-) serotypes of *P. gingivalis* have been described [96, 97]. *P. gingivalis* strains HG91 (originally strain 381) and ATCC 33277 are considered K-antigen negative as they lack a capsular-specific antibody response and strain HG91 has no capsular-polysaccharide layer as determined by electron microscopy and phase contrast analysis [97-99]. A number of studies have indicated that *P. gingivalis* CPS contributes significantly to its virulence [100-102]. Immunization with *P. gingivalis* CPS has been shown to protect mice from *P. gingivalis*-elicited bone loss [103]. CPS has been found to play a role in the evasion of host immune response by other microorganisms [104-106] and a similar function for *P. gingivalis* CPS has also been suggested [107]. Compared to non-encapsulated strains, encapsulated *P. gingivalis* strains are more virulent in an animal model (Laine et al. 1998), resistant to phagocytosis by polymorphonuclear cells [108] and their ability to activate the alternative complement pathway is reduced [100, 102, 108]. *P. gingivalis* CPS also decreases the ability of periodontal ligament fibroblasts to attach to the root surface of teeth [109]. Invasion of gingival fibroblast by *P. gingivalis* has been reported earlier [110] but it is not known whether *P. gingivalis* capsule affects its ability to invade host cells.

Figure 3



Schematic diagram of *P. gingivalis'* cell envelope with its major virulence factors [111]

Host-bacterial interaction in peri-implantitis

Adhesion and invasion

Oral bacteria associated with peri-implantitis colonize peri-implant sulcus/pocket by adhering to implant surface and various host cell-types. Adhesion to host cells is an important first step for the establishment of a successful infection. *P. gingivalis* can adhere to epithelial cells, endothelial cells and fibroblasts in the peri-implant connective tissues and the number of *P. gingivalis* adhering to periodontal epithelium has been shown to correlate strongly with the severity of inflammation in periodontal diseases [112]. Adhesion may proceed to invasion of the

host cells and thereby offer protection to *P. gingivalis* from external pressures such as host immune factors and antibiotics [110, 113-115]. Once inside the host cell, *P. gingivalis* can block apoptosis by up-regulating anti-apoptotic factors and down-regulating pro-apoptotic factors [116, 117]. As a result, *P. gingivalis* can persist and multiply within host cells and possibly play a role in recurrence of the infection in periodontal diseases [118].

Differences in the adhesion capacity of laboratory and clinical strains of *P. gingivalis* have been reported [119-123]. Host-cell adhesion and invasion of *P. gingivalis* depend on multiple factors. *P. gingivalis* fimbriae and gingipains are important for adhesion and invasion of host cells, and differences in the adherence and invasion capacity of different fimbrial strains of *P. gingivalis* have been shown [87, 124, 125]. In addition, surface hydrophobicity of the *P. gingivalis* strain is also an important determinant of its adherence to host cells [119]. CPS of *P. gingivalis* has been shown to decrease its ability to adhere to pocket epithelium *in vitro* [126]. However, it is not known whether the capsule also alters the ability of *P. gingivalis* to invade host cells. Furthermore, susceptibility of internalized *P. gingivalis* to antibiotics has not been studied.

Modulation of host immune response

The majority of implant complications including peri-implantitis tend to concentrate in a relatively smaller group of patients [11]. Therefore susceptibility of the host is an important determinant of the development of peri-implantitis. Bacterial interaction with host cells result in the release of certain inflammatory mediators and growth factors with the aim to eliminate the invading microorganisms and to repair the resulting tissue damage. To a larger extent, progression of tissue damage in peri-implantitis depends on the presence and activity of certain

bacteria and high local production of pro-inflammatory mediators, matrix metalloproteinases (MMPs) and prostanoids, accompanied by low local production of inhibitors of inflammation and growth factors [127-129]. These inflammatory mediators play an important role in peri-implant bone resorption by promoting osteoclastogenesis and subsequently activating the differentiated osteoclasts [130]. In addition, pro-inflammatory mediators such as IL-1β and TNF-α also induce the release of prostaglandin (PGE)-2 from monocytes and fibroblasts [131], thereby further enhancing tissue damage. An aberrant host response resulting in peri-implant tissue destruction has been reported for peri-implantitis [132-134].

As peri-implantitis is a site specific infection, local production of pro-inflammatory mediators in response to oral bacteria is important since these mediators increase the tissue perfusion and attract immune-competent cells such as monocytes/macrophages, lymphocytes and neutrophils. Immune cells from the circulation further enhance the inflammatory reaction and local tissue damage. Non-circulating cells such as gingival fibroblasts and gingival epithelial cells [113] are known to produce inflammatory mediators in response to microbial challenge. Due to their slower turnover rate compared to epithelial cells, fibroblasts can play an important role in the chronic inflammation associated with peri-implantitis. Studies reporting on the role of fibroblasts in the pathogenesis of peri-implantitis are limited [135, 136]. Interaction of oral fibroblasts with *P. gingivalis* has been shown to up-regulate production of inflammatory mediators including IL-1β, IL-6, IL-8 [137], monocyte chemotactic protein - (MCP) - 1[138] and matrix metalloproteinases (MMPs) [139]. In addition to extracellular matrix, MMPs can also cleave cytokines into smaller fragments and thus may activate or deactivate them [140]. Tissue inhibitors of metalloproteinases (TIMPs) tend to restrict matrix breakdown by MMPs [141] while members of transforming growth factor-beta (TGFβ) protein family regulate fibrogenesis

as well as vascular homeostasis [142]. MMPs are involved in physiological tissue remodeling in health but imbalances in the levels of MMPs and their inhibitors can result in breakdown of extracellular matrix.

Role of the implant material in peri-implantitis

Several types of synthetic biomaterials have been used for dental implants such as metals (titanium) and alloys (titanium-aluminum-vanadium alloy), ceramics (aluminum oxide, hydroxylapatite) and carbon, and polymers, mainly polymethylmethacrylate (PMMA) [143]. Dental implants used in current clinical practice are almost exclusively made from commercially pure titanium or its alloys. Furthermore, titanium dental implants can be coated with hydroxylapatite to produce a bio-active surface which promotes and induces a direct bond between the implant and bone tissue surrounding it [144].

Titanium (Ti) is considered the material of choice for dental implants due to its corrosion resistance and biocompatibility with and within living tissues [145]. However, corrosion resistance of Ti can decrease in oral environment [146] and wear debris in the form of titanium particles have been detected in peri-implant soft tissues [147, 148]. In a recent study [149], higher concentration of Ti particles were found in the peri-implant tissues of diseased implants when compared to healthy dental implants. There is an increasing interest in the interaction of Ti wear debris with host cells across different fields of study such as dental implantology and orthopedics. Ti is known to elicit inflammatory responses from human cells [150] and may also play a role in peri-implant bone resorption through facilitation of osteoclast differentiation [151]. In addition inhibition of bone formation by macrophage-like cells exposed to Ti particles has been reported [152]. In a clinical study, Nowzari et. al. [153] found elevated levels of pro-

inflammatory cytokines in the peri-implant sulcular fluid of clinically healthy dental implants. Furthermore, allergy to Ti has also been reported and may play a role in the pathogenesis of peri-implantitis [154].

A synergistic effect of Ti and bacterial-LPS on the pro-inflammatory responses of host cells has been described [155, 156]. However, since bacteria and implant material act in concert, affecting the host cells in the *in vivo* situation; it is important to study the interaction of host cells with implant-biomaterial and pathogenic microorganisms, together. Therefore, the current thesis also aims to study the effects of Ti and *P. gingivalis* on the inflammatory responses of peri-implant fibroblasts from peri-implantitis patients.

Chapter 1

Aims and thesis outline

Studies on the interaction between host cells and bacteria in the etiopathogenesis of peri-implantitis are limited. Therefore, the general aim of this thesis was to study interactions between *P. gingivalis* and fibroblasts in peri-implantitis. In addition, pro-inflammatory responses in fibroblasts to titanium particles and effects of non-surgical peri-implantitis treatment on the clinical and microbiological parameters were also studied.

In **Chapter 2** the ability of *P. gingivalis* to invade gingival fibroblasts and the role of *P. gingivalis* capsule in invasion of gingival fibroblasts was evaluated. Furthermore, survival of internalized *P. gingivalis* after *in vitro* antibiotic treatment was studied.

The host response is an important factor in peri-implantitis. Fibroblasts from peri-implant lesions may differ in their response to microbial challenge compared to fibroblasts from periodontitis lesions and healthy donors. Therefore, the aim of **Chapter 3** was to compare the pro-inflammatory and matrix-degrading responses of fibroblasts from periodontally healthy controls, peri-implantitis, and periodontitis lesions to an *in vitro* challenge with *Porphyromonas gingivalis*. In addition, the potential role of fibroblast from peri-implantitis lesions in sustaining inflammation in peri-implantitis was also studied.

Titanium particles can be detected in the peri-implant tissues of individuals with dental titanium implants and these released titanium particles are capable of inducing immune responses in human cells. In **Chapter 4** we aimed to determine influence of titanium micro-particles on the *in vitro* inflammatory responses of peri-implant granulation tissue fibroblasts to viable *P. gingivalis*.

Literature on the non-surgical treatment of peri-implantitis is scarce. Therefore, in **Chapter 5** the effects of non-surgical peri-implantitis treatment with or without antibiotics on clinical, microbiological and radiographic parameters of peri-implantitis were evaluated.

In **Chapter 6,** results of chapters 2-5 of this thesis are reviewed and discussed. Moreover, the potential clinical implications of our results and directions for relevant future research have been suggested.

Chapter 7 reiterates a short summary of the results and their clinical implications.

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Chapter 2:

In vitro invasion and survival of *Porphyromonas gingivalis* in gingival fibroblasts; role of the capsule

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Abstract

Background: *Porphyromonas gingivalis* is a Gram-negative, anaerobic bacterium involved in periodontitis and peri-implantitis that can invade and survive inside host cells *in vitro*. *P. gingivalis* can invade human gingival fibroblasts (GF) but no data is available about the role of *P. gingivalis'* capsule in GF invasion. In the current study we aimed to determine the ability of three strains of *P. gingivalis* (encapsulated wild type W83, non-encapsulated HG91 and the non-encapsulated insertional isogenic knockout mutant of W83, ΔEpsC) to invade GF and the ability of internalized *P. gingivalis* to survive *in vitro* antibiotic treatment.

Methods: The ability of *P. gingivalis* strains to invade GF was tested using an antibiotic protection assay at multiplicity of infection (MOI) 100 and 1000. The survival of internalized *P. gingivalis* cells was further analyzed by subsequent *in vitro* treatment with either metronidazole or amoxicillin alone or a combination of metronidazole and amoxicillin and anaerobic culture viability counts.

Results: All strains of *P. gingivalis* used in this study were able to invade GFs. The non-encapsulated mutant of W83 (ΔEpsC mutant) was significantly more invasive than the wild type W83 at MOI 100 (p-value = 0.025) and MOI 1000 (p-value = 0.038). Furthermore, internalized *P. gingivalis* was able to resist *in vitro* antibiotic treatment.

Conclusion: As demonstrated by the differences in invasion efficiencies of *P. gingivalis* strain W83 and its isogenic mutant ΔEpsC, the capsule of *P. gingivalis* makes it less efficient in invading gingival fibroblasts. Moreover, internalized *P. gingivalis* can survive antibiotic treatment *in vitro*.

Key words Periodontitis, Mechanism of antibiotic resistance, Internalization, Capsule,

Chapter 2

Introduction

Porphyromonas gingivalis is a Gram-negative, anaerobic, non-motile rod shaped bacterium found in sub-gingival plaque that has been associated with chronic adult periodontitis [1] and peri-implantitis [2]. *P. gingivalis* is equipped with a broad array of virulence factors [3] potentially involved in tissue colonization and destruction. *P. gingivalis* capsule is an important virulence factor and it has been shown that encapsulated *P. gingivalis* strains are more virulent than non-encapsulated strains in a mouse model [4], non-encapsulated strains adhere more to epithelial cells compared to encapsulated strains [5] and the capsule may be immunosuppressive for human gingival fibroblasts (GF) *in vitro* [6]. On the basis of capsular polysaccharides, seven capsular (K1-K7) and a non-capsular serotype of *P. gingivalis* have been described previously [7,8].

Invasion into host cells is one of the multiple ways in which pathogens interact with the host immunity [9]. *P. gingivalis* has been shown to invade into a variety of host cells in several *in vitro* studies [10-13], and to persist and multiply within the epithelial cells [14]. The capsule may impede the process of invading host cells by *P. gingivalis*, as partial dissolution of *P. gingivalis* capsule with amyloglucosidase has been shown to increase the invasion efficiency of *P. gingivalis* into endothelial cells [12].

Systemic metronidazole is most commonly used either alone or in combination with amoxicillin as an adjunct to periodontitis and peri-implantititis treatment [15]. Recurrence of periodontitis may still be a problem in some patients after the use of systemic antibiotics, probably due to a hampered eradication of periodontal pathogens such as *P. gingivalis* [16]. Intracellular presence of *P. gingivalis* in epithelial cells collected from patients after systemic

antibiotic treatment has been reported [17], and may reflect inadequacy of conventional antibiotics to eliminate internalized *P. gingivalis*.

Fibroblasts are the most abundant stromal cell type in the connective tissue of gingiva [18] and important cells in the pathogenesis of periodontitis [19]. *P. gingivalis* has been shown to penetrate multilayered epithelial cells and reach the underlying connective tissue in an *in vitro* model [20] which is also consistent with *in vivo* observations [21]. To the best of our knowledge, the role of *P. gingivalis* capsule in invading GF is not known and we hypothesize that the non-encapsulated *P. gingivalis* may be internalized more efficiently into GFs and survive antibiotic treatment inside GF. Therefore, the aim of our current study was to evaluate the effects of *P. gingivalis* capsule on its ability to invade GF and to investigate the *in vitro* effectiveness of metronidazole, amoxicillin and combination of metronidazole and amoxicillin, on intracellular *P. gingivalis*.

Material and methods

Bacterial strains

P. gingivalis strains W83 (K1 serotype), HG91 (K- serotype) and an isogenic non-encapsulated knockout mutant of W83 (ΔEpsC mutant, K- serotype), were grown anaerobically (80% N_2, 10% H_2, and at 10%CO_2 at 37˚C) until log growth phase in brain-heart infusion broth (BHI, Bacto™ Brain Heart Infusion, Becton, Dickinson and Company, USA) enriched with hemin (5 μg/ml) and menadione (1 μg/ml). Purity of the cultures was checked by Gram-staining. The ΔEpsC mutant is an insertional isogenic W83 knockout in the epimerase-coding gene EpsC that is located at the end of the capsular biosynthesis locus [6].

P. gingivalis were harvested by centrifugation at 5000 rpm, for 15 minutes. Bacterial pellets were washed twice in sterile phosphate-buffered saline (PBS) and resuspended in DMEM containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA) without antibiotics. The optical density (OD_{690}) of the fluid bacterial culture in BHI was measured at 690 nm wavelength. An OD_{690} = 0.8 corresponding to $2*10^9$ CFUs/ml on bacterial growth curves for different *P. gingivalis* strains (data not shown), was used to establish the number of colony-forming units (CFUs) and infect GFs.

Human gingival fibroblasts (GF)

Gingival fibroblasts isolated from an extracted third molar of a 62 years old, periodontally healthy, non-smoking female during a previous study [22] were used. The donor had given a written informed consent and the study was approved by the VUmc (Vrije Universiteit medical centre) medical ethical committee. Briefly, the tissue sample was taken by collecting the free gingiva around the tooth. The tissue sample was washed and maintained as explants in

Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose + L-glutamine + pyruvate; Invitrogen/Gibco Paisley, UK) supplemented with 10% fetal bovine serum (FBS; Invitrogen/Gibco, Paisley, Scotland, UK) and 2% antibiotics/antimycotics (PSF; 100 U/ml of penicillin, 100 μg/ml streptomycin and 250 ng/ml of amphotericin B; Sigma, St Louis, Mo, USA) in a humidified environment with 5% CO_2 at 37˚C until growth of fibroblasts was observed under the microscope. These fibroblasts were further expanded in DMEM containing 10% FBS and 1% PSF and stored frozen in liquid nitrogen at passage 4. All experiments were performed using fibroblasts between subsequent passages 4 and 10.

Minimum Inhibitory and Minimum Bactericidal Concentrations (MIC and MBC)

MIC and MBC values of the *P. gingivalis* strains W83, HG91 and ΔEpsC mutant were determined by a standard broth dilution method [23]. The MIC and MBC values were determined both in BHI and in DMEM enriched with 10% FBS. Briefly, *P. gingivalis* strains were grown anaerobically in BHI containing hemin and menadione for 18 hours at 37˚C, the OD_{690} was determined and based on the standard growth curves, the numbers of bacteria were calculated. Metronidazole (Sigma-Aldrich, the Netherlands) and amoxicillin (Beecham research laboratories, Beecham Pharma, Amstelveen, the Netherlands) solutions were prepared in sterile distilled water and filter sterilized with sterile polypropylene syringe filters (Whatman® FP 30/0.2, 0.2 µm pore size, GE Healthcare Europe GmbH, Belgium). Metronidazole and amoxicillin were serially diluted in a 24-wells-plate in BHI enriched with hemin and menadione and an inoculum containing 1×10^8 *P. gingivalis* was added to each well. *P. gingivalis* strains without antibiotics and antibiotics without *P. gingivalis* served as controls. Four different concentrations of metronidazole; 2 µg/ml, 1 µg/ml, 0.5 µg/ml and 0.25 µg/ml and amoxicillin; 0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml and 0.062 µg/ml were used for MIC and MBC determination. Each concentration of metronidazole (2 µg/ml, 1 µg/ml, 0.5 µg/ml and 0.25) was combined with respective concentration of amoxicillin (0.5 µg/ml, 0.25 µg/ml, 0.125 µg/ml and 0.062 µg/ml) for the *in vitro* combination antibiotic treatment.

Invasion efficiency

An antibiotic protection assay for *P. gingivalis* [12] was used to quantify the invasion efficiency of the three *P. gingivalis* strains. GFs were seeded into 24-wells-plates at a cell density of 1×10^4 cells/well, in antibiotic free DMEM containing 10% FBS and grown until sub-confluence. *P.*

gingivalis grown overnight in BHI broth with hemin and menadione, and harvested at the log phase (OD_{690} of 0.8) by centrifugation. These *P. gingivalis* were washed first with PBS (pH 7.4) and then with DMEM without antibiotics before suspending them in DMEM containing 10% FBS without antibiotics. The OD_{690} was again measured in DMEM to establish the CFUs. The numbers of *P. gingivalis* were adjusted to be used subsequently for infecting the fibroblasts with a multiplicity of infection (MOI) 100 (1×10^6/well) and 1000 (1×10^7/well) *P. gingivalis* per GF. Only DMEM containing 10% FBS was added to the control GFs. *P. gingivalis* without GFs in concentration of 1×10^8/ml were also included for each experiment as controls to evaluate efficiency of the antibiotic used for killing *P. gingivalis*. The bacterial suspensions in DMEM were added to the sub-confluent monolayers of GFs and co-incubated in humidified aerobic atmosphere for 90 minutes, in 5% CO_2 at 37˚C. After co-incubation, the unattached bacteria were removed by washing each well twice with PBS. Metronidazole (100 μg/ml) was added to the monolayers and incubated in DMEM for another 60 minutes to kill the external adherent bacteria. This concentration of metronidazole was shown in pilot experiments to kill 10^8 *P. gingivalis*/ml during 60 minutes exposure in BHI as well as DMEM and also reported elsewhere [5, 12]. Monolayers were washed twice with PBS and 1 ml of sterile distilled water was added to each well and further incubated for 30 minutes to lyse the fibroblasts. The cells were disrupted by vigorous and repeated pipetting. The lysate were plated in serial dilutions for CFUs count on horse blood agar plates (Oxoid no.2, Basingstoke, UK) containing hemin and menadione and incubated anaerobically at 37˚C. The remaining samples were stored at -80˚C. CFU counts were determined after 14 days of anaerobic incubation. Invasion efficiency was expressed as a percentage of the initial inoculum recovered after GF lysis [24].

Survival of internalized *P. gingivalis*

To study the survival of internalized *P. gingivalis*, the most invasive among the three *P. gingivalis* strains i.e. ΔEpsC mutant was chosen for further experiments. The highest invasion efficiency was seen with MOI 100, therefore only MOI 100 was used in further experiments.

This assay was performed in two steps. The first step was performed similar to the antibiotic protection assay except that the GFs were not lysed at the end of the experiment. In the second step GFs were washed twice with PBS and either metronidazole (100 μg/ml) alone, amoxicillin alone (12.5 μg/ml) or combination of metronidazole (100 μg/ml) and amoxicillin (12.5 μg/ml) were added to the wells containing GFs and incubated for 60 minutes under humidified aerobic conditions at 37˚C. Concentrations of antibiotics used in this step were equal to 100 times the MIC. Antibiotics free DMEM was added to the control GFs. After incubation the GFs were washed twice with sterile PBS and lysed as described previously.

***P. gingivalis* quantitation by real time Polymerase Chain Reaction (RT-PCR)**

P. gingivalis DNA was isolated from the lysate by MagNA Pure DNA Isolation kit III (Roche, Molecular Diagnostics, Almere, the Netherlands) as mentioned elsewhere [25]. The primer/probe sets and PCR conditions have been described earlier [26]. Briefly, RT-PCR amplification was performed in a total reaction mixture volume of 20 μL. The reaction mixtures contained 10 μL of LightCycler® 480 SYBR Green I Master mix, 1.8 picomole of *P. gingivalis* specific forward and reverse primers each, 0.4 picomole of LightCycler® 480 CYAN 500 Labeling Reagent and 4 μL of purified *P. gingivalis* DNA from the samples. The samples were subjected to an initial amplification cycle of 50°C for 2 minutes and 95°C for 10 minutes followed by 45 cycles at

95°C for 15 seconds and 60°Cfor 1 minute using the LightCycler® 480 Instrument. Data analysis was done with LightCycler® 480 software release 1.5.0 SP4.

Statistical analysis

Mean and standard error of the mean (SEM) were calculated from three separate experiments for antibiotic protection assays. Data from the culture and RT-PCR were compared and tested for significance either using a two tailed, non-parametric Mann-Whitney's test or a non-parametric Kruskal-Wallis and Dunn's multiple comparison post hoc tests. Data analysis was performed with GraphPad Prism software (version 5.00 for Windows, San Diego California, USA). Differences were considered statistically significant at a p value < 0.05.

Results

MIC and MBC

Table 1 presents the MIC and MBC values for different *P. gingivalis* strains as determined by broth dilution method. The MIC and MBC values of amoxicillin alone for the three *P. gingivalis* strains ranged between 0.125 μg/ml and 0.25 μg/ml. MIC and MBC values of metronidazole alone for the three *P. gingivalis* strains ranged between 1 μg/ml and 2 μg/ml. MIC and MBC values for the combination ranged between 1 μg/ml metronidazole plus 0.125 μg/ml amoxicillin and 2 μg/ml metronidazole plus 0.25 μg/ml amoxicillin. There were no significant differences in susceptibility to metronidazole and amoxicillin between the encapsulated and non-encapsulated *P. gingivalis* strains W83, HG91 and ΔEpsC mutant.

Table 1. MIC and MBC of antibiotics as determined by broth dilution method

P. gingivalis strain (serotype)	MIC/MBC (μg/ml)		
	Metronidazole	Amoxicillin	Metronidazole + Amoxicillin
W83 (K1)	1.0/2.0	0.25/0.25	1+0.125/ 2+0.25
HG91(K-)	2.0/2.0	0.25/ 0.25	1+0.125/1+0.125
ΔEpsC (K-)	1.0/2.0	0.125/0.25	1+0.125/1+0.25

MIC; minimum inhibitory concentration, MBC; minimum bactericidal concentration

Invasion of GF by *P. gingivalis*

P. gingivalis strains used in this study were able to invade and survive inside GF. Figure 1 shows the invasion efficiencies of the three *P. gingivalis* strains at MOI 100 and 1000, after 90 minutes of infection, as determined by anaerobic culture. Invasion efficiencies of W83 wild type, HG91 and ΔEpsC mutant at MOI 100 were 0,007%, 0.07% and 0.15% respectively. At MOI 1000, respective invasion efficiencies of W83 wild type, HG91 and ΔEpsC mutant were 0.016%, 0.04%, 0.09%. Invasion efficiencies of the non-encapsulated strains between MOI 100 and MOI 1000 were not statistically different.

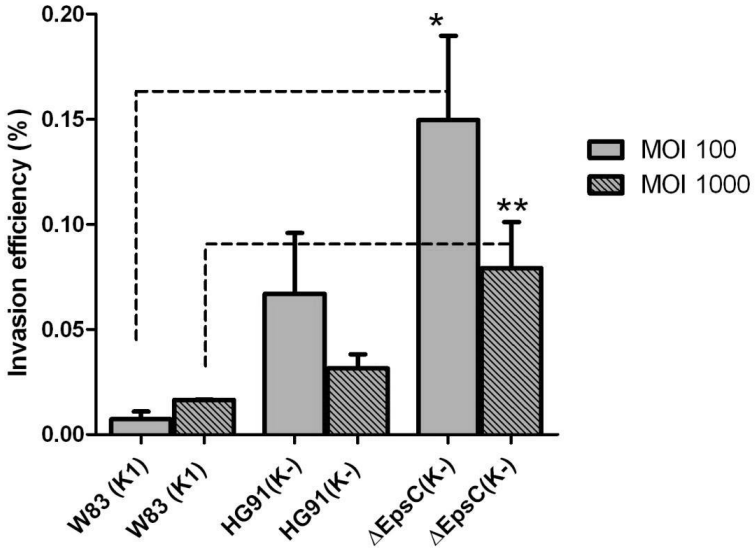
The non-encapsulated *P. gingivalis* ΔEpsC mutant was significantly more invasive than the encapsulated W83 parent strain at MOI 100 (p-value = 0.025) as well as MOI 1000 (p-value = 0.038) (Figure 1). Invasion efficiency of the non-encapsulated HG91 strain was also higher than the encapsulated W83 strain at MOI 100 and 1000, although the differences did not reach statistical significance.

Overall, the RT-PCR data show comparable results as seen for anaerobic culture (Figure 2). There were no statistically significant differences in the amounts of *P. gingivalis* detected by RT-PCR either between W83 and HG91 or HG91 and ΔEpsC mutant at MOI of 100 (Figure 2). However at MOI 1000, the detected amounts of ΔEpsC were significantly higher than the wild type W83 (p value = 0.027).

In vitro* survival of internalized *P. gingivalis

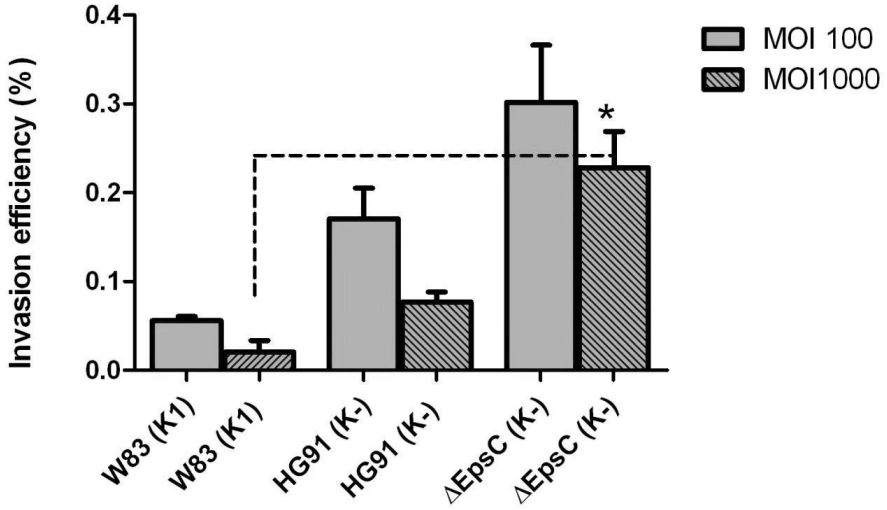
The internalized ΔEpsC mutant was further treated with different regimens of antibiotics. After the additional *in vitro* antibiotic treatment of internalized ΔEpsC mutant, viable *P. gingivalis* could still be recovered from the GFs regardless of the antibiotics used. When compared to the total amounts of internalized bacteria (i.e. bacteria left after the first antibiotic treatment), the mean percentages ranged from 13.5% to 17.8% depending on the antibiotic(s) used (Figure 3), differences were not significant. Survival of the internalized ΔEpsC mutant was significantly (p-value = 0.034) reduced compared to control, if a combination of metronidazole and amoxicillin was used (Figure 4). Either metronidazole alone or amoxicillin alone was not able to significantly reduce the internalized load of *P. gingivalis* (Figure 4).

Figure 1



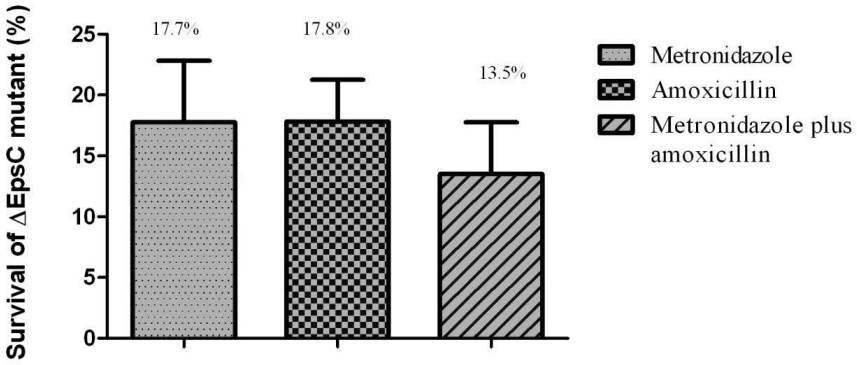
Invasion efficiencies of *P. gingivalis* strains into GF at MOI 100 and 1000, as determined by anaerobic culture. The non-encapsulated mutant of W83 (ΔEpsC) was significantly more efficient in invading GF compared to the encapsulated wild type (W83) both at MOI 100 (* p-value = 0.025) and MOI 1000 (** p-value = 0.038). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments.

Figure 2



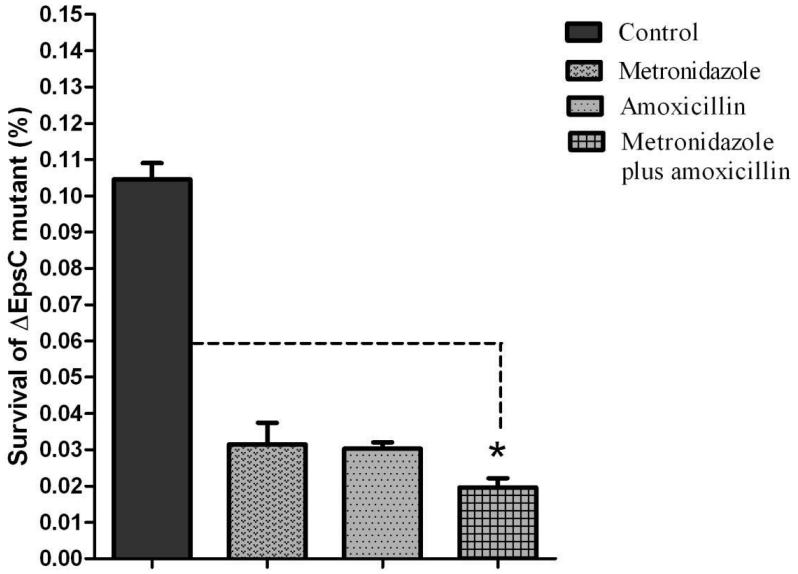
Invasion efficiencies of *P. gingivalis* at MOI 100 and 1000 as determined by RT-PCR. The invasion efficiency of ΔEpsC mutant into GFs was significantly higher than the wild type W83 at MOI 1000 (*p-value = 0.027). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments.

Figure 3



ΔEpsC *P. gingivalis* recovered by anaerobic culture after *in vitro* challenge of the internalized bacteria with metronidazole (100 μg/ml), amoxicillin (12.5 μg/ml) or combination o metronidazole (100μg/ml) and amoxicillin (12.5 μg/ml), shown here as mean percentages of the initially internalized bacteria at MOI 100. No viable *P. gingivalis* were recovered from the control.

Figure 4



Survival of *P. gingivalis* (ΔEpsC mutant) recovered by anaerobic culture after *in vitro* challenge of the internalized bacteria with metronidazole (100 μg/ml), amoxicillin (12.5 μg/ml) or combination of metronidazole (100 μg/ml) and amoxicillin (12.5 μg/ml). A significant reduction in the survival of internalized bacteria was observed after combination of metronidazole and amoxicillin treatment, compared to control (*p-value = 0.034). Results are presented as the mean and SEM of triplicate cultures and represent three independent experiments.

Discussion

Clinical studies on periodontitis and peri-implantitis patients treated with antibiotics have reported inability of antibiotics to completely eradicate *P. gingivalis* [27, 28]. This observation is in line with the finding that periodontal pathogens internalized into host cells might act as a potential reservoir for re-infection in periodontitis [29]. The re-emergence of *P. gingivalis* has particularly been attributed to failed eradication [30]. Invasion of host cells is one of the possible mechanisms that can offer protection to bacteria against antibiotic pressure [31]. GF appear to be important host cells in this regard because periodontal pathogens have been previously shown to invade into and survive inside GFs [13, 32]. Intracellular *P. gingivalis* undergoes morphologic changes and has been shown by scanning electron microscopy in the cytoplasm without membrane surrounding [13].

In the current study both encapsulated and non-encapsulated *P. gingivalis* strains were able to invade into GFs; however differences were found in their invasion efficiencies. The non-encapsulated ΔEpsC mutant was significantly more invasive than its encapsulated wild type strain W83. Invasion efficiency of the naturally non-encapsulated HG91 strain also tended to be higher than the encapsulated W83 but the difference did not reach statistical significance. This indicates that the capsule of W83 is mainly responsible for the differences in the invasion efficiencies between the parent W83 strain and its non-encapsulated isogenic ΔEpsC mutant. Factors other than the capsule may confound a comparison between a non-encapsulated *P. gingivalis* strain e.g. HG91, with an encapsulated one e.g. W83. Furthermore, in the current study we found the highest invasion efficiency with MOI 100, which is consistent with previous studies [13, 24]. There may be a threshold for a fibroblast to optimally internalize a certain number of *P. gingivalis* and increasing the number of bacteria beyond the threshold may not

further enhance invasion. The non-encapsulated ΔEpsC mutant, being the most invasive of the three *P. gingivalis* strains used, was chosen for further experiments to study the survival of internalized *P. gingivalis* to *in vitro* antibiotics treatment. The results show that *P. gingivalis* can resist antibiotic treatment inside fibroblasts. Persistence and/or multiplication of *P. gingivalis* after internalization and survival in GF, was not evaluated in the current study although other studies have shown that *P. gingivalis* can persist and multiply inside host cells after internalization [14]. In the current study, combination of metronidazole and amoxicillin was found to be significantly more effective in reducing the amounts of internalized *P. gingivalis* compared to control. Clinical isolates of *P. gingivalis* may be more difficult to eradicate compared to laboratory strains, with the antibiotic therapy for periodontitis and peri-implantitis because clinical isolates of *P. gingivalis* have been found to be considerably more resistant to antibiotic treatment inside host cells [33]. Since growth of fibroblasts can be affected by anaerobic conditions [34], antibiotic protection assays in the current study were carried out under aerobic conditions according to the standard protocol used in previous studies [35].

Metronidazole is used to treat periodontal and peri-implant infections either alone or in combination with amoxicillin [15]. Once taken up by the bacterial cell, metronidazole reacts with bacterial DNA, resulting in bacterial cell death [36]. Although we have not tested entry of metronidazole into gingival fibroblasts in the current study, metronidazole is known to cross cell membrane of gingival fibroblasts [37]. It has been shown in an *in vitro* study that *P. gingivalis* internalized into host cells can survive metronidazole concentrations of up to 100 times its MIC [33]. Amoxicillin is a β-lactam antibiotic which is bactericidal and act by inhibiting bacterial cell wall synthesis [38]. Penicillins do not readily cross plasma membrane which limits their ability

to kill intracellular bacteria [39]. The inability of metronidazole and amoxicillin to effectively eliminate internalized *P. gingivalis* is in line with clinical studies [16, 17].

Evasion of the host defence mechanisms and invasion of host tissues to maintain a successful infection are the prerequisites for a successful chronic infection. The capsule of *P. gingivalis* has been particularly implicated in the reduction of host immune response and increase in virulence [40]. Recent studies have confirmed the immunomodulatory role of *P. gingivalis* capsule in interaction with host cells [41]. Invasion of non-phagocytic host cells is yet another important mechanism of *P. gingivalis* to maintain a successful infection. The role of *P. gingivalis* capsule in its ability to invade GF has not been reported previously. Polysaccharide capsule of *P. gingivalis* may interfere with the initial step of bacterial binding to host cell membrane. For example, the polysaccharide capsule of *P. gingivalis* decreases their surface hydrophobicity [42], which may partly explain the higher affinity and increased invasion efficiency of non-encapsulated strains to host cells [10, 43]. The capsule has also been shown to impede host cell invasion in other pathogens such as *Klebsiella pneumoniae* [44] and *Haemophilus influenzae* [45]. In case of GF, the mechanism of invasion by *P. gingivalis* is not fully understood, although *P. gingivalis* has been reported to exploit host cell signaling pathways and cytoskeleton for its internalization into gingival epithelial cells [24, 46]. Other invasive bacterial species have also been shown to rely on the host cell actin cytoskeleton for a successful infection [47]. Once inside host cell, *P. gingivalis* can promote its survival by activating a variety of apoptotic pathways [48] and regulation of distinctive *P. gingivalis* proteins and genes [49]. These observations show the strategies that *P. gingivalis* has evolved to increase its chances of survival inside the host cell although their relevance for *P. gingivalis*-fibroblast interactions has yet to be determined. The *in vitro* inability of high concentrations of antibiotics to kill *P.*

gingivalis internalized into GF, shown in this study; provide further support to the idea that GF may play an important role in *P. gingivalis* resistance to antibiotics. Although the evidence that *P. gingivalis,* to a certain extent, can invade host cells and resist antibiotic treatment is in line with clinical studies, *in vitro* studies may not be directly extrapolated to *in vivo* situation.

Within the limitations of the study, we conclude that *P. gingivalis* can internalize human gingival fibroblasts *in vitro*, non-encapsulated *P. gingivalis* is more efficient in invading GFs than encapsulated *P. gingivalis* and once inside the host cell, *P. gingivalis* can survive antibiotic treatment.

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Chapter 3:

Cytokines and matrix metalloproteinase expression in fibroblasts from peri-implantitis lesions in response to viable *Porphyromonas gingivalis*

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Abstract

Background and Objective: To assess inflammatory reactions of fibroblasts in the pathophysiology of peri-implantitis, we compared the pro-inflammatory and matrix-degrading responses of gingival and granulation tissue fibroblasts from periodontally healthy controls, peri-implantitis, and periodontitis lesions to an *in vitro* challenge with *Porphyromonas gingivalis*. **Methods:** Fibroblasts from periodontally healthy, peri-implantitis and periodontitis donors were challenged with viable *P. gingivalis*. The inflammatory responses of fibroblasts were analyzed before and after 6h *P. gingivalis*-challenge, and 2.5h and 18h after removal of the challenge. Gene-expression and induction of pro-inflammatory mediators, and matrix-metalloproteinases (MMPs) were assessed by real-time-PCR. Protein expression was measured by ELISA. **Results:** Non-challenged fibroblasts from peri-implantitis and periodontitis lesions expressed higher levels of *interleukin (IL)-1β, IL-8* and *monocyte chemotactic protein (MCP) -1* than fibroblasts from periodontally healthy individuals. The *P. gingivalis*-challenge induced expression of *IL-1β, IL-8, IL-6, MCP -1* and *MMP-1* in periodontitis and peri-implantitis fibroblasts, but not in fibroblasts from periodontally healthy individuals. *MMP-8* expression was higher in the non-challenged peri-implantitis fibroblasts than in fibroblasts from periodontally healthy individuals. However, the *P. gingivalis*-challenge down-regulated *MMP-8* gene expression in peri-implantitis fibroblasts. After removal of the *P. gingivalis*-challenge, peri-implantitis fibroblasts had higher induction of *IL-1β, MCP-1* and *MMP-1* compared to periodontitis fibroblasts. **Conclusions:** Fibroblasts from peri-implantitis and periodontitis lesions gave a more pronounced inflammatory response to the *P. gingivalis*-challenge than fibroblasts from healthy donors. They may thereby be involved in the development of inflammation in peri-implantitis and periodontitis. Moreover, the sustained up-regulation of inflammatory mediators and *MMP-1* in peri-implantitis fibroblasts may play a role in the pathogenesis of peri-implantitis.

Chapter 3

Introduction

Peri-implantitis is an inflammatory disease affecting the alveolar bone and mucosal tissues surrounding the dental implant and is characterized by mucositis and loss of osseous integration [1]. Peri-implantitis shares important characteristics with chronic periodontitis, such as presence of chronic inflammation and bacterial species associated with the initiation and progression of the disease [2, 3]. Moreover, patients with a history of periodontitis may be more susceptible for developing peri-implantitis [4, 5]. Despite similarities, important differences exist between peri-implantitis and periodontitis. In contrast to the often localized horizontal bone loss around natural teeth in periodontitis, bone loss around implants shows a circumferential and angular pattern [6]. Inflammatory lesions in experimentally induced peri-implantitis have been shown to be more aggressive and extensive compared to similar lesions around natural teeth [7]. Additionally, it has been reported that inflammation around implants does not always resolve with the removal of infection [8].

Although the etiology of peri-implantitis is multifactorial, certain microorganisms are essential for its initiation and progression [2]. *Porphyromonas gingivalis* is a Gram-negative, anaerobic bacterium that is strongly associated with peri-implantitis [9]. It has a variety of ways to interact with host cells [10]. Tissue destruction in peri-implantitis in response to bacteria such as *P. gingivalis* is a result of the persistent and ineffective inflammatory immune responses from host cells. Host cells respond to bacterial challenge by releasing certain pro- and anti-inflammatory mediators which in turn mediate degradation of collagen and extracellular matrix, and bone resorption [11]. The role of these mediators in the pathogenesis of peri-implantitis has been reviewed in literature [12].

Despite the important role of bacteria in peri-implantitis, these bacteria can also be found in small numbers in apparently healthy oral cavities [13], which emphasises the fact that bacteria are not solely responsible for causing the disease. In addition, patients who have already lost an implant are at greater risk for subsequent implant losses [14]. Thus, host related factors are also important in the development of peri-implantitis.

Fibroblasts play an important immunomodulatory role in the disease progression and tissue destruction in peri-implantitis [15,16] for the following reasons: (i) fibroblasts respond to *P. gingivalis* infection by releasing inflammatory mediators such as interleukins (IL)-1β, IL-6, IL-8 [17] and monocyte chemotactic protein (MCP) -1 [18]. These inflammatory mediators increase tissue perfusion, recruit different immune cells, amplify the overall inflammatory process and thereby they can indirectly enhance tissue damage. [19] Fibroblasts challenged with *P. gingivalis* release matrix metalloproteinases (MMPs) [20], which, besides degrading extracellular matrix, can also break down some pro-inflammatory mediators into smaller fragments and thus may activate or deactivate these molecules [21]. (iii) Fibroblasts also produce tissue inhibitor of metalloproteinases (TIMPs), which tend to restrict matrix breakdown by MMPs [15, 22] and (iv) transforming growth factor-beta (TGFβ)-1 which regulates fibrogenesis as well as vascular homeostasis [23].

Even though specialized immune cells such as monocytes/macrophages, lymphocytes and neutrophils play important role in inflammation, these cells only transiently produce inflammatory mediators and they can develop tolerance to certain virulence factors of oral bacteria [24]. Fibroblasts on the other hand do not develop such tolerance and may play an important role in sustaining inflammation [25]. This is particularly relevant for peri-implantitis

lesions because in contrast to the effectiveness of non-surgical treatment for periodontitis [26], peri-implantitis lesions may not respond very well to non-surgical treatment [27].

Because of relative abundance in the peri-implant granulation tissue, it is important to study the role of fibroblasts in the inflammatory process and their responses to microbial challenge in peri-implantitis. It is not known how peri-implant granulation tissue fibroblasts (PIGFs) interact with bacteria associated with peri-implantitis such as *P. gingivalis*. Since fibroblasts from different anatomical sites or the same anatomical site but different diseases play distinct roles [28, 29], we hypothesized that fibroblasts from peri-implantitis lesions respond differently to *P. gingivalis* challenge compared to fibroblasts from periodontitis lesions and may play a role in the pathogenesis of peri-implantitis. Therefore, the aim of this study was to compare the pro-inflammatory and matrix-degrading responses of fibroblasts from periodontally healthy controls, peri-implantitis, and periodontitis lesions to an *in vitro Porphyromonas gingivalis*-challenge.

Material and methods

Tissue donors and fibroblast isolation

Peri-implant granulation tissue fibroblasts (PIGFs) were obtained from 7 peri-implantitis patients during flap surgery as part of peri-implantitis treatment. The surgical approach consisted of an incision extending mesially and distally to the implant after the removal of prosthetic suprastructure to facilitate access. A full thickness tissue flap was reflected and the inflamed granulation tissue was carefully removed with a curette. The tissue samples were further processed on the same day. The peri-implantitis patients were free from active periodontitis and had no known history of periodontitis. Gingival fibroblasts from 5 periodontally healthy controls (HGF) and 9 chronic periodontitis patients (PGF) were recovered by collecting gingiva-remains from extracted teeth using a scalpel as described before [18]. Periodontally healthy controls underwent tooth-extraction as part of treatment for other reasons than periodontitis and showed no signs of inflammation/periodontitis/loss of alveolar bone. From periodontitis patients, teeth displaying deepened pockets, bleeding on probing, and advanced loss of alveolar bone visible on radiographs were extracted as part of periodontitis treatment. None of the donors suffered from systemic diseases or were pregnant. None of the donors was a current smoker. All donors had given written informed consent, and the study was approved by the Medical Ethical committee of the VU University Medical Center.

Tissue samples were washed twice in Dulbecco's modified Eagle medium (DMEM; 4.5 g/L glucose + L-glutamine + pyruvate; Invitrogen/Gibco Paisley, UK) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and 2% antibiotics/antimycotics (PSF; 100 U/ml of penicillin, 100 μg/ml streptomycin and 250 ng/ml of amphotericin B; Sigma, St Louis, MO, USA) and cultured in DMEM containing 10% FBS and 2% PSF, in a humidified

atmosphere with 5% CO_2 at 37˚C until growth of fibroblasts was seen under the phase contrast microscope (Olympus CK2, Olympus, Japan). Cells were expanded in larger cell culture flasks and stored in liquid nitrogen at passage 4. Morphologically HGF, PIGF and PGF had similar appearance under the phase contrast microscope. Furthermore, the fibroblastic nature of HGF, PGF and PIGF was assessed by evaluating the expression of *FMOD* (*fibromodulin*) gene, a marker for HGF [30], which was expressed at similar levels in HGF, PGF and PIGF (data not shown). Experiments were performed with cells from passages 5-7.

Bacterial strain and culture

P. gingivalis HG91 (also designated as strain 381) was cultured anaerobically (80% N_2, 10%H_2, 10% CO_2) until log-growth phase in Brain-Heart Infusion (BHI)-broth supplemented with hemin (5mg/l) and menadione (1mg/l). Purity was checked with Gram-staining.

Viable *P. gingivalis* were harvested by centrifugation. Bacterial pellets were washed twice in sterile phosphate buffered salt solution (PBS, Gibco BRL, Paisley, Scotland) and re-suspended in antibiotic/antimycotic-free DMEM with 10% FBS. The optical density was measured at 690 nm to establish the number of colony forming units (CFUs). A suspension of 2 x10^8 CFU/ml was used to challenge the fibroblasts.

***P. gingivalis* challenge to fibroblasts**

Fibroblasts were challenged with viable *P. gingivalis* as reported previously [138]. In short, fibroblasts (10^4/well) were grown until sub-confluence in 24-well plates. Medium was removed and replaced with 0.5 ml of a *P. gingivalis* HG91 suspension of 2 x10^8 CFU/ml in antibiotic-free DMEM with 10% FBS. Only DMEM without PSF, supplemented with 10% FBS was added to control fibroblasts (non-challenged).

HGFs, PGFs and PIGFs were incubated with *P. gingivalis* for 6h. After the *P. gingivalis* challenge, fibroblast morphology was checked for abnormalities or cell-detachment by phase-contrast microscopy. Subsequently fibroblasts were washed with sterile PBS and lysed in lysis-buffer as supplied with RNeasy Mini Kit for RNA extraction (Buffer RLT, Qiagen, Hilden, Germany), supplemented with β-mercapto-ethanol. Experiments were performed in quadruplicate.

Priming of fibroblasts by *P. gingivalis* and subsequent removal of the challenge

PIGFs from 7 peri-implantitis donors and PGFs from 7 periodontitis donors were used. Fibroblasts (10^4/well) were grown until sub-confluence in 24-well plates. Medium was removed and replaced with 0.5 ml of a *P. gingivalis* suspension in antibiotic-free DMEM with 10% FBS and incubated for 6h as described above. At the end of the 6h challenge, *P. gingivalis* was removed by washing three times with sterile PBS. Cells were then incubated in DMEM supplemented with 10% FBS and 2% PSF for 30 minutes to kill the remaining *P. gingivalis*. Susceptibility of *P. gingivalis* to penicillin *in vitro* is known in the literature [31] and in our preliminary *in vitro* experiments (data not shown) this protocol proved to completely kill 2×10^8 CFU/ml of *P. gingivalis* HG91. After 30 minutes, cells were washed three times with sterile PBS and the medium was replaced with DMEM without antibiotics. Supernatants and cell lysates were collected at 6h after the *P. gingivalis* challenge (baseline), and 2.5 and 18h after removing *P. gingivalis*. At each time point the cells were washed three times with sterile PBS before lysing them. The non-challenged fibroblasts underwent the same treatments and acted as controls for different time points. All experiments were performed in quadruplicate.

mRNA expression

Fibroblast RNA was isolated using the Qiagen RNeasy Mini Kit according to manufacturers' protocol. The RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, Delaware, USA). mRNA was reverse-transcribed to cDNA using the MBI Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers according to manufacturers' protocol.

Real time PCR primers for *IL-1β*, *IL-6*, *IL-8*, *MCP-1*, *TIMP-1*, *MMP-1*, *MMP-2* and *MMP-8* were used as published previously [18, 32]. Primers for *TGFβ-1* were designed using PRIMER EXPRESS software, version 2.0 (Applied Biosystems, Foster City, CA, USA). All primers were ordered from SIGMA-ALDRICH (Sigma-Aldrich Co. LLC). Sequences of the relevant forward and reverse primers for the respective genes are given in Table 1. We used a serial dilution of human reference total RNA (Stratagene, La Jolla, CA, USA) to create a standard curve to check the PCR-efficiency. Real-time PCR was performed on Roche LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). Reactions were performed with 2 ng of cDNA in a total volume of 11 µl containing the LightCycler® 480 SYBR Green I Master Mix (F. Hoffmann-La Roche AG, Basel, Switzerland), consisting of DNA double-strand-specific SYBR Green I dye for product detection and characterization, FastStart Taq DNA Polymerase and 0.91 pM/μl of each primer. After an activation step with the FastStart Taq DNA Polymerase for 5 min at 95°C, 40 cycles were run of a two-step PCR consisting of a denaturation step at 95°C for 10 s, annealing and extension steps of 60°C for 5 s, 72°C for 10 s and 78°C for 5 s. Subsequently, the PCR products were subjected to melting curve analysis to test if any nonspecific PCR products were generated. Samples were normalized for the expression of the

housekeeping gene *β2-microglobulin*, which was not affected by the experimental conditions. Relative gene expression was calculated by the method proposed by Livak et al. [33], by calculating the ΔCt ($Ct_{\text{gene of interest}} - Ct_{\text{housekeeping gene}}$), and the expression of different genes was expressed as $2^{-(\Delta Ct)}$. Fold increase in gene expression (induction) was expressed as $2^{-(\Delta\Delta Ct)}$, wherein $\Delta\Delta Ct = \Delta Ct_{\text{challenged}} - \text{average } \Delta Ct\text{-value}_{\text{non-challenged}}$.

Table 1. Real-time PCR primer sequences.

Gene	Primer sequences	
	5'-3' Fw	**5'-3' Rev**
IL-1β	CTTTGAAGCTGATGGCCCTAAA	AGTGGTGGTCGGAGATTCGT
IL-6	GGCACTGGCAGAAAACAACC	GGCAAGTCTCCTCATTGAATCC
IL-8	GGCAGCCTTCCTGATTTCTG	CTGACATCTAAGTTCTTTAGCACTCCTT
MCP-1	CAGCCAGATGCAATCAATGC	TGCTGCTGGTGATTCTTCTATAGCT
TGFβ-1	CTCGGAGCTCTGATGTGTTGAA	CACCCGCGTGCTAATGGT
TIMP-1	CCTTCTGCAATTCCGACCTCGTC	CGGGCAGGATTCAGGCTATCTGG
MMP-1	GAAGTTGATGAAGCAGCCCAGATGT	CAGTTGTGGCCAGAAAACAGAAGTGAAA
MMP-2	ATCCGTGGTGAGATCTTCTTCTT	AGCCAGGATCCATTTTCTTCTT
MMP-8	GCTGCTTATGAAGATTTTGACAGAG	ACAGCCACATTTGATTTTGCTTCAG
β2-microglobulin	AAGATTCAGGTTTACTCACGTC	TGATGCTGCTTACATGTCTCG

Protein production

Cell culture supernatants from 5 peri-implantitis and 5 periodontitis patients challenged with *P. gingivalis* for 6h were used for determining the protein production of IL-8, MMP-8 and TIMP-1. The protein levels of IL-8 were determined by Enzyme-linked immunosorbant assays (ELISA) (PeliKine ELISA kits, Sanquin Blood Supply Foundation, Amsterdam, the Netherlands), according to the manufacturers' protocol. The sample quantities were normalized against a serial dilution of a known concentration of the relevant protein provided by the manufacturers. Sensitivity of the IL-8 assay was 1-3 pg/ml. MMP-8 and TIMP-1 concentrations in 10 × diluted

culture supernatants were measured by ELISA (Biotrak MMP-8 human ELISA system, Amersham Pharmacia Biotech, Buckinghamshire, UK; Quantikine human TIMP-1 immunoassay, R&D Systems, Minneapolis, MN, USA) according to the manufacturers' protocol. The average lower limits of detection were 0.032 ng/ml and 0.08 ng/ml respectively. All ELISA experiments were performed in duplicate.

Statistical analysis

Comparisons between the total mRNA expression, induction of gene expression and protein production between groups were tested with Student's t test when data were normally distributed. Wilcoxon matched pairs and Mann-Whitney tests were used to analyze the non-parametric data. If variances in groups were not equal, Welch's correction for unequal variances was applied. Differences were regarded statistically significant at a value of $p<0.05$. Tests were performed with GraphPad Prism software (version 5, by MacKiev Software ™).

Results

General and clinical characteristics of the donors included in this study are shown in Table 2. No significant differences were found between the ages of different groups of donors. Bleeding on probing (BOP) was more frequently detected around target implants in peri-implantitis patients compared to periodontally healthy controls ($p = 0.008$). Compared to target teeth in periodontally healthy controls, higher values for probing depths were found around target implants ($p = 0.005$) in peri-implantitis and teeth ($p = 0.01$) in periodontitis patients.

Table 2. Patient characteristics and clinical parameters of fibroblast donors and target teeth/implants.

	Periodontally healthy controls	Peri-implantitis	Periodontitis
N donors	5	7	9#
Gender (Male/Female)	1/4	6/ 1	3/ 6
Mean age (±SD) in years	54.4 (±18.7)	57.8 (±12.4)	54.4(±9.2)
BOP (N)	1	7 *	5
Mean PD (±SD)	2.7 (±0.8)	5.1 (±0.9) **	6.6 (±2.8) ***

BOP: number of target teeth/implants showing bleeding on probing. Mean PD: mean probing depth of pocket in millimeters around target teeth/implants; #: fibroblasts from 5 or 7 periodontitis patients were used in different experiment; * $p = 0.008$, ** $p = 0.005$, *** $p = 0.01$ (significant difference compared to periodontally healthy controls)

mRNA expression of *IL-1β*, *IL-6*, *IL-8* and *MCP-1* before and after the *P. gingivalis* challenge

mRNA expression of *IL-1β*, *IL-6*, *IL-8* and *MCP-1* was significantly up-regulated in response to the *P. gingivalis* challenge in PIGFs ($p = 0.031, 0.015, 0.0078, 0.023$ respectively) and PGFs ($p = 0.016, 0.015, 0.015, 0.03$ respectively), but not in HGFs (Figure 1A-D). mRNA expression levels of *IL-1β*, *IL-8* and *MCP-1* in PIGFs were higher compared to HGFs both in the non-challenged ($p = 0.030, 0.045, 0.0016$ respectively) as well as challenged cells ($p = 0.0025, 0.045, 0.029$ respectively) (Figure 1A, C, D). mRNA expression levels of *IL1-β*, *IL-6*, *IL-8* and *MCP-1*

were higher ($p = 0.048, 0.0025, 0.002, 0.002$ respectively) in PGFs than HGFs in the non-challenged but not in the challenged cells (Figure 1A-D). Differences in mRNA expression of *IL-1β*, *IL-6*, *IL-8* and *MCP-1* between PIGFs and PGFs did not reach statistical significance in the non-challenged as well as challenged cells.

Differential expression of MMPs in response to the *P. gingivalis* challenge

Compared to the non-challenged cells, the *P. gingivalis* challenge up-regulated expression of *MMP-1* in PIGFs ($p = 0.039$) and PGFs ($p = 0.047$), but not in HGFs (Figure 2A). In the non-challenged cells, mRNA expression levels of *MMP-8* in PIGFs were higher than in HGFs ($p = 0.029$, Figure 2B). Interestingly, mRNA expression of *MMP-8* was down-regulated in the challenged PIGFs ($p = 0.039$) compared to the non-challenged PIGFs (Figure 2B). A similar trend was observed in the mRNA expression of *MMP-8* in PGFs, but the decrease in mRNA expression was not significant. mRNA expression of *TIMP-1* was not significantly changed in response to *P. gingivalis* challenge in any group of fibroblasts (Figure 2C), although compared to HGFs the expression of *TIMP-1* was higher in PIGFs in the non-challenged ($p = 0.048$) as well as the challenged cells ($p = 0.0051$). *TIMP-1* mRNA expression was also significantly higher in PGFs challenged with *P. gingivalis* compared to the challenged HGFs ($p = 0.0025$, Figure 2C).

No significant differences were observed in mRNA expression levels of *TGFβ-1* and *MMP-2* either between the non-challenged versus the challenged fibroblasts of the same group or among different groups of fibroblasts (data not shown).

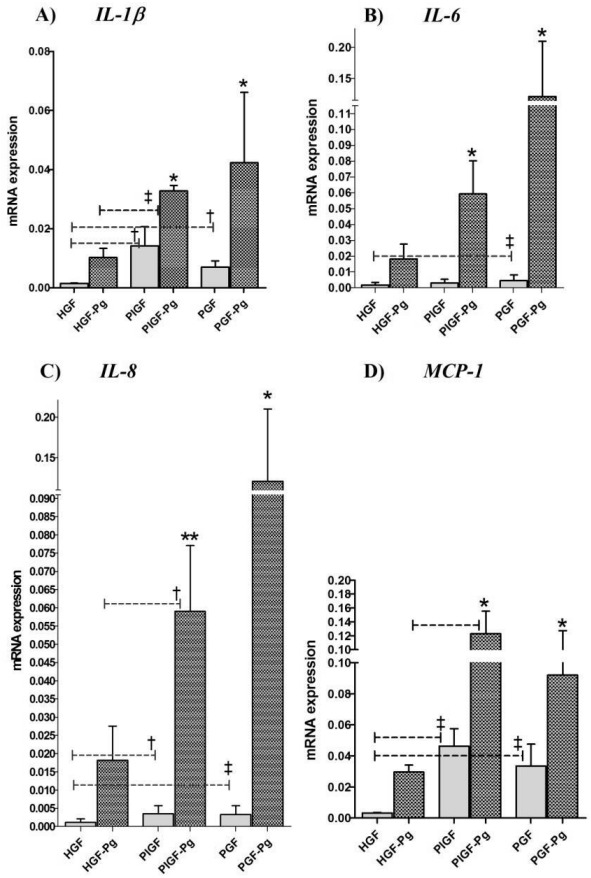

Figure 1

mRNA expression levels of pro-inflammatory mediators before and after a 6h *P. gingivalis* challenge.

mRNA expression levels of *IL-1β* (A), *IL-6* (B), *IL-8*(C) and *MCP-1* (D) relative to housekeeping gene (*β2- microglobulin*) in fibroblasts from periodontally healthy individuals (HGF, *N*=5), peri-implantitis patients (PIGF, *N=7*) and periodontitis patients (PGF, N=7) before and after a 6h challenge with viable *P. gingivalis (Pg)*. Bars represent the average ± SEM of mRNA expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. *$p < 0.05$, **$p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. † $p < 0.05$, ‡ $p < 0.01$ represent significant difference between different groups.

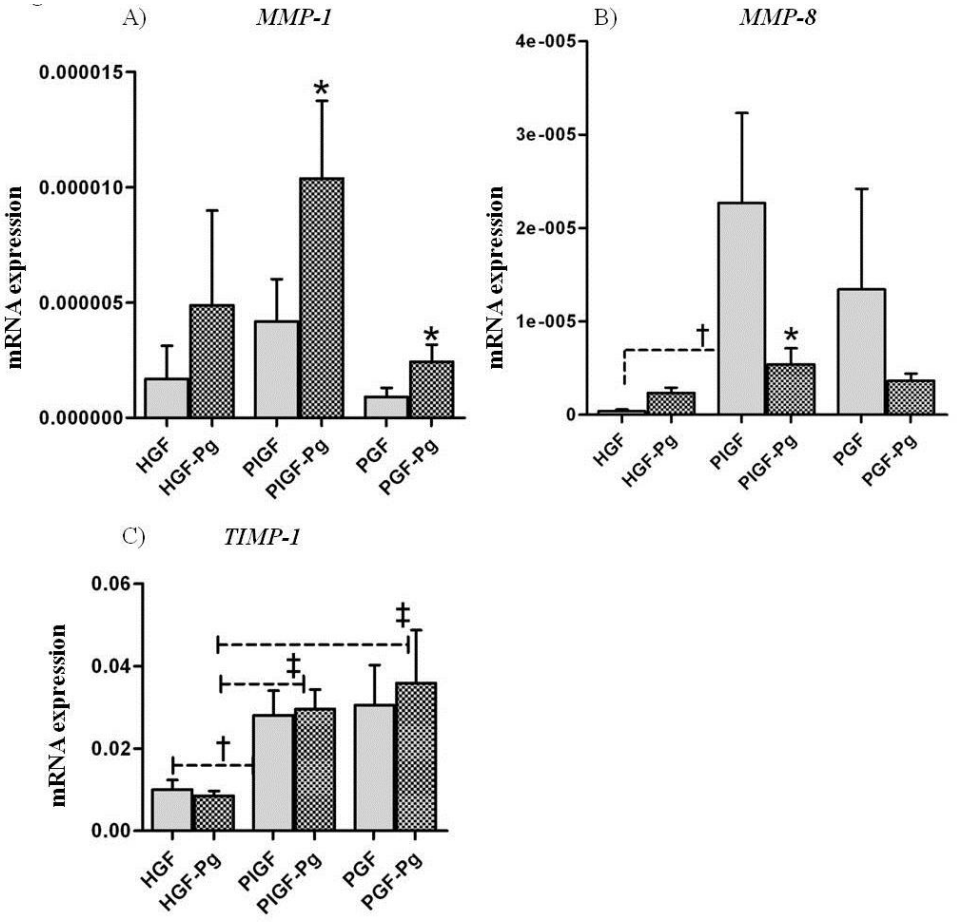

Figure 2

mRNA expression levels of *MMP-1*, *MMP-8* and *TIMP-1* before and after a 6h *P. gingivalis* challenge.

mRNA expression levels of *MMP-1* (A), *MMP-8* (B) and *TIMP-1* (C) relative to housekeeping gene (*β2-microglobulin*) in fibroblasts from periodontally healthy individuals (HGF, *N*=5), peri-implantitis patients (PIGF, *N*=7) and periodontitis patients (PGF, N=7) before and after a 6h challenge with viable *P. gingivalis (Pg)*. Bars represent the average ± SEM of mRNA expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. *$p < 0.05$, **$p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. † $p < 0.05$, ‡ $p < 0.01$ represent significant difference between different groups.

Protein production levels of IL-8, MMP-8 and TIMP-1 correspond with mRNA expression

To confirm whether the statistically significant changes observed in mRNA expression levels also lead to similar changes in protein production, we measured the protein production of IL-8, MMP-8 and TIMP-1 by ELISA. Since there were no significant differences in the mRNA expression of the studied genes (except for MMP-8) between challenged and non-challenged HGFs, protein productions were only determined for PIGFs and PGFs. Moreover, *P. gingivalis* proteases have been reported to cleave inflammatory mediators such as IL-1β and IL-6 [18, 34]; therefore we chose to measure protein production levels of IL-8, MMP-8 and TIMP-1. Consistent with the increased mRNA expression, IL-8 protein production was also significantly increased in PIGFs ($p = 0.002$) and PGFs ($p = 0.031$) challenged with *P. gingivalis*, compared to the non-challenged cells (Figure 3A). Protein production of MMP-8 and TIMP-1 from PIGFs and PGFs before and after the *P. gingivalis* challenge showed similar trends to the mRNA expression although the differences were not significant (Figure 3B and C). Protein production of TIMP-1 by PIGFs was significantly higher ($p = 0.008$) compared to PGFs in the non-challenged cells (Figure 3C).

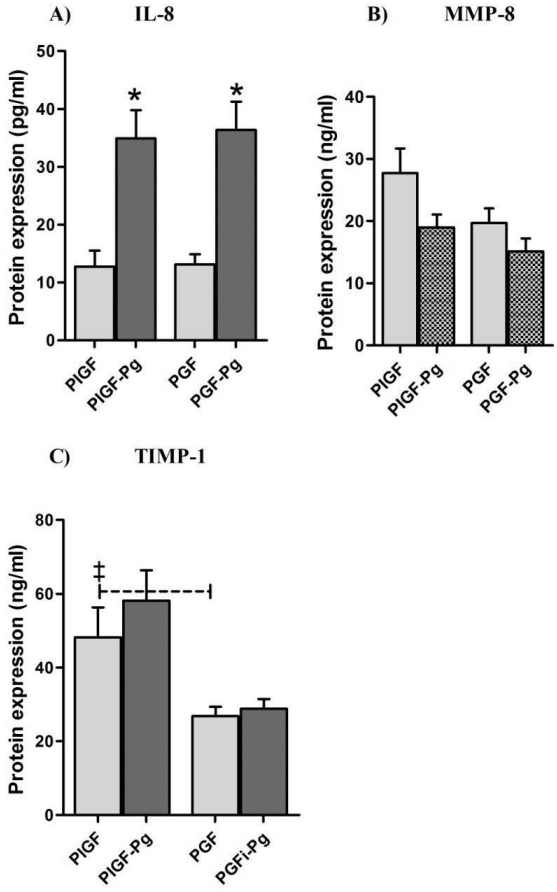

Figure 3

Protein expression of IL-8, MMP-8 and TIMP-1 at before and after a 6h *P. gingivalis* challenge.

Protein expression levels of IL-8 [pg/ml] (A), MMP-8 [ng/ml] (B) and TIMP-1 [ng/ml] (C) in cell culture supernatant from the non-challenged and challenged fibroblasts (ELISA) from peri-implantitis patients (PIGF, *N*=5) and periodontitis patients (PGF, N=5) before and after a 6h challenge with viable *P. gingivalis (Pg)*. Bars represent the average ± SEM of protein expression levels from the non-challenged and challenged fibroblasts from experiments performed in quadruplicate. *$p < 0.05$, **$p < 0.01$ represent significant difference between the non-challenged and challenged cells of the same group. † $p < 0.05$, ‡ $p < 0.01$ represent significant difference between different groups.

Gene induction of *IL-1β, MCP-1* and *MMP-1* differ between PIGFs and PGFs after removal of the *P. gingivalis* challenge

Since there was no significant up-regulation of pro-inflammatory mediators by HGFs in response to *P. gingivalis*, only PIGFs and PGFs were used in further experiments. To assess differences in the dynamics of pro-inflammatory and matrix degrading responses between PIGFs and PGFs, we removed the *P. gingivalis* from fibroblast cultures after the 6h challenge and measured mRNA expression at 2.5h and 18h after the removal. Induction of mRNA expression for *IL-1β*, *IL-6, IL-8, MCP-1* and *MMP-1* in PIGFs and PGFs were compared. Significantly higher gene induction of *IL-1β, MCP-1* and *MMP-1* was observed in PIGFs compared to PGFs after removal of *P. gingivalis*. No significant differences were found in the gene induction of any other mediators studied (data not shown). Gene induction of *IL-1β* was higher in PIGFs compared to PGFs 2.5h ($p = 0.0003$) as well as 18h after the *in vitro* removal of *P. gingivalis* ($p = 0.008$) (Figure 4A). Gene induction of *MCP-1* was higher in PIGFs ($p = 0.01$) compared to PGFs 2.5h, but not 18h after removal of *P. gingivalis* (Figure 4B). *MMP-1* gene induction was also higher in PIGFs 2.5h ($p = 0.02$) and 18h ($p = 0.016$) after removal of *P. gingivalis* (Figure 4C).

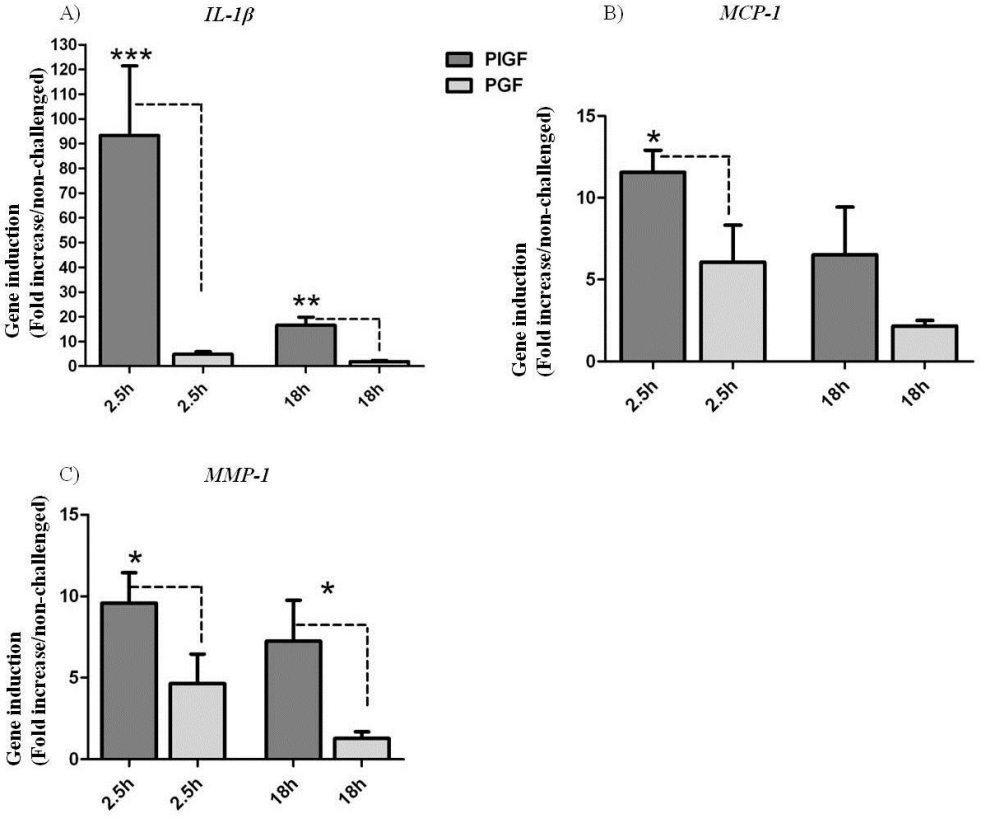

Figure 4

Gene induction of *IL-1β*, *MCP-1* and *MMP-1*, 2.5h and 18h after removal of the *P. gingivalis* challenge.

Gene induction (fold increase in mRNA expression in challenged compared with non-challenged cells) of *IL-1β* (A), *MCP-1* (B) and *MMP-1* (C) in fibroblasts from peri-implantitis patients (PIGF, N=7) and periodontitis patients (PGF, N=7). Bars represent the mean induction level ± SEM at 2.5h and 18h after the removal of *P. gingivalis* challenge from experiments performed in quadruplicate (Due to undetectable levels of mRNA in the non-challenged cells of some donors, the bars for *IL-1β* represent data from 5 PIGF donors at 18h; 6 and 4 PGF donors at 2.5 and 18h respectively. For *MMP-1* the bars represent data from 5 PIGF and 5 PGF donors at 18h). *$p < 0.05$, **$p < 0.01$, $p<0.001$ represent significant difference between the groups.

Discussion

In the current study, the role of fibroblasts in peri-implant inflammation and tissue breakdown in peri-implantitis was assessed. Studies focusing on the role of bacterial interaction with fibroblasts in the pathogenesis of peri-implantitis are scarce. Important differences between fibroblasts from peri-implantitis patients, periodontitis patients and healthy individuals have been reported [15, 16].

In the present study we found that PIGFs and PGFs were in a more pro-inflammatory state compared to fibroblasts from HGFs before a *P. gingivalis* challenge, which is consistent with an earlier report [16]. This implies that PIGFs and PGFs are in an activated state and maintain their activated state for several passages in culture. When challenged with viable *P. gingivalis*, in contrast to HGFs, PIGFs and PGFs up-regulated their *IL-1β*, *IL-6*, *IL-8* and *MCP-1* gene-expression. This more pronounced response from PIGFs and PGFs to *P. gingivalis* may reflect their hyper-reactivity to bacterial challenge. *MMP-1* expression was also up-regulated in response to *P. gingivalis* in PIGFs and PGFs, but not in HGFs. Interestingly, this up-regulation of *MMP-1* was not accompanied by a significant change in expression of *TIMP-1* and the fibrogenic factor *TGFβ-1,* which indicates that *P. gingivalis* plays a role in the matrix breakdown through fibroblasts. It has been reported earlier though that fibroblasts from peri-implantitis patients play a role in matrix breakdown by both an increased production of MMP-1 as well as decreased production of TIMP-1 and TGFβ-1 [15].

We also found that non-challenged PIGFs expressed more *MMP-8* compared to fibroblasts from HGFs, which is consistent with earlier reports that demonstrated increased MMP-8 levels in the peri-implant sulcular fluid of peri-implantitis patients [35]. MMP-8 is produced in relative large quantities by neutrophils [36] and production of MMP-8 by

neutrophils in peri-implant sulcus may also contribute towards the higher expression in peri-implant sulcular fluid. In the current study, PIGFs showed a down-regulation of *MMP-8* in response to the *P. gingivalis* challenge. This down-regulation of MMP-8 in response to the *P. gingivalis* challenge is interesting since a protective role for MMP-8 against site specific alveolar bone loss in response to *P. gingivalis* has been suggested [37]. This might be due to the potential of MMP-8 to breakdown and deactivate inflammatory mediators that are involved in periodontal bone resorption [38]. The down-regulation of MMP-8 in the current study may indicate that *P. gingivalis* decreases protection by MMP-8 against inflammation in peri-implantitis. However, fibroblasts are not the primary cells responsible for MMP-8 production. It should be noted that we measured total MMP-8, the expression of which could be different from active forms of MMP-8.

Interestingly, we observed that after removal of *P. gingivalis*, the gene induction of *IL-1β, MCP-1* and *MMP-1* remained significantly elevated in PIGFs. A recent study indicated that interaction of *P. gingivalis*-LPS with fibroblasts may play a role in the persistence of inflammation in periodontal diseases [25]. Such continuous production of inflammatory mediators by fibroblasts in response to *P. gingivalis* may play a significant role in sustaining the chronic inflammation and tissue damage by promoting migration of primary inflammatory leukocytes [39]. IL-1β is an important mediator in periodontal inflammatory diseases since it stimulates the production of other cytokines, chemokines, cyclooxygenase products, and MMPs which enhance the inflammatory process and tissue damage [40, 41]. Sustained induction of *IL-1β* and *MMP-1* expression by PIGFs after removal of the *P. gingivalis* suggests a more aggressive and persistent nature of inflammatory lesions around implants compared to similar lesions around natural teeth [7, 8]. Since we have used a non-capsular *P. gingivalis* strain in our

experiments, which can efficiently internalize into gingival fibroblasts [42], the internalized *P. gingivalis* may play a role in this persistent inflammatory response. Nevertheless, our results represent *in vitro* experiments and should be interpreted cautiously to explain the inflammatory response of PIGFs to *in vivo* infection in peri-implantitis. Moreover, in contrast to gingival or periodontal ligament fibroblasts, it is possible that PIGFs represent a collection of different fibroblast sub-populations in which case inflammatory responses from HGFs, PGFs and PIGFs may not be directly comparable.

P. gingivalis plays an important role in the pathogenesis of peri-implant and periodontal diseases by compromising the host immune system and interacting with host cells [9, 43]. Peri-implantitis is a multifactorial disease and besides bacteria, the inflammatory process around implants may also be affected by the altered anatomy resulting from the presence of a foreign body and absence of a physical barrier around implants in the form of periodontal ligament. Furthermore, the difficulty to decontaminate dental implant surface and the possible interaction between peri-implant tissues and implant material [44] may also play a role in the pathogenesis of peri-implantitis.

In conclusion, granulation tissue fibroblasts from peri-implantitis and gingival fibroblasts from periodontitis lesions have a higher pro-inflammatory and matrix degrading properties and give a more pronounced pro-inflammatory response to an *in vitro P. gingivalis*-challenge when compared to gingival fibroblasts from periodontally healthy individuals. In addition, fibroblasts from peri-implantitis lesions differ from fibroblasts from periodontitis lesions by the persistent up-regulation of *IL-1β* and *MMP-1* which may play a role in the more aggressive and persistent behavior of peri-implantitis inflammatory lesions.

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Chapter4:

Influence of titanium on *in vitro* fibroblast-*Porphyromonas gingivalis* interaction in peri-implantitis

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Chapter 4

Abstract

Aim: Titanium-wear-particles have been found in peri-implant tissues but their role in the pathogenesis of peri-implantitis remains unclear. We aimed to determine the *in vitro* inflammatory responses of peri-implant-granulation-tissue fibroblasts (PIGFs) to titanium-particles alone and in the presence of viable *Porphyromonas gingivalis*.

Materials and methods: PIGFs were challenged either with TiO_2-particles, *P. gingivalis*, or a combination of TiO_2-particles and *P. gingivalis*. Gene-expression and protein-production of pro-inflammatory mediators by PIGFs were measured with PCR and ELISA, respectively.

Results: Higher doses of TiO_2 were toxic to PIGFs and in sub-toxic doses, TiO_2 caused an increase of gene-expression of tumor-necrosis-factor *(TNF)-A* and increased protein-production of TNF-α, interleukin (IL)-6 and IL-8. A challenge with *P. gingivalis* alone induced gene-expression of *TNF-A, IL-1β, IL-6* and *IL-8*. A combined challenge with TiO_2 and *P. gingivalis* caused a stronger increase in gene-expression of *TNF-A* and protein-production of TNF-α and MCP-1 than *P. gingivalis* alone.

Conclusions: TiO_2-particles and *P. gingivalis,* individually, can induce pro-inflammatory responses in PIGFs. Furthermore, TiO_2-particles and viable *P. gingivalis* further enhance gene-expression and production of TNF-α by PIGFs. Therefore, Ti-wear-particles in the peri-implant tissues in combination with *P. gingivalis* infection, may contribute to the pathogenesis of peri-implantitis by enhancing the inflammation in peri-implant tissues.

Introduction

After the introduction of osseointegrated implants [1], the use of titanium (Ti) dental implants has become a successful and popular procedure in dentistry. Despite reported high success rates for dental implants [2], failures can occur due to the inability of tissue to establish osseointegration, or due to breakdown of established osseointegration. Breakdown of established osseointegration may occur due to peri-implantitis and/or mechanical overload [3]. Peri-implantitis is defined as an inflammatory lesion around the dental implant, accompanied by loss of supporting bone [4]. Peri-implantitis accounts for 10-50% of implant failures after at least one year of loading [5] and constitutes a major clinical problem.

Multiple factors contribute to the etiology of peri-implantitis, although the general consensus is that peri-implantitis is infectious in nature [4]. Gram-negative anaerobic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia/Prevotella nigrescens* and *Aggregatibacter actinomycetemcomitan*s dominate the sub-gingival biofilm associated with peri-implantitis [6, 7]. *Porphyromonas gingivalis* is strongly associated with peri-implantitis [8] and one of the most extensively studied oral microorganisms. It is a black-pigmented, Gram-negative, anaerobe with a variety of virulence factors [9] such as proteases, fimbriae, LPS, and capsular polysaccharides, which make it a potent pathogen for the initiation and progression of peri-implantitis. *P. gingivalis* can invade and survive inside host cells [10], and it interacts with different host cell types to induce a network of inflammatory responses that may lead to breakdown of extracellular matrix and bone resorption [11, 12]. Furthermore, *P. gingivalis* has been indicated as a "keystone pathogen" [13], meaning that it can orchestrate inflammatory disease by altering a normally benign microbiota.

Chapter 4

Due to its favorable biocompatibility [14], contemporary dental implants are almost exclusively made from commercially pure Ti or its alloys. Rutile oxidization of Ti implants is used to modify its surface [15], leading to increased wear debris production [16]. Furthermore, the corrosion resistance of Ti is decreased under low dissolved-oxygen conditions such as in the oral cavity, particularly in the presence of small amounts of fluoride [17]. Consequently, Ti particles have been detected in the peri-implant tissues of individuals with dental Ti implants [18, 19]. In a recent study [19], the size of Ti particles detected in peri-implant tissue ranged from $0.9\pm0.7\mu$m to $3\pm2\mu$m. Released Ti is capable of inducing immune responses in human cells [20, 21], and may play a pivotal role in bone resorption at the interface of bone and dental implants [22, 23]. An association between wear debris from Ti implants and peri-implantitis has also been suggested [24]. It was demonstrated that Ti and *Escherichia coli*-LPS together have a synergistic effect on the inflammatory responses of host cells [21]. Moreover, Ti particles have been detected by transmission electron microscopy inside fibroblasts and macrophages of patients treated with Ti-miniplates to stabilize fractured bones [25].

Fibroblasts play an important role in the pathogenesis of peri-implantitis and periodontal diseases [26]. Apart from their structural role, fibroblasts in the periodontal and peri-implant tissues also take part in host immune responses. Upon a *P. gingivalis*-challenge , fibroblasts can release a variety of inflammatory mediators including interleukins (IL)-1β, IL-6, IL-8, monocyte chemotactic protein (MCP)-1, prostaglandin E2 and matrix metalloproteinases (MMPs) [27-29].

To the best of our knowledge, the influence of Ti on fibroblast-bacterium interaction in peri-implantitis has not been studied previously. To mimic *in vivo* conditions, it is important to evaluate the inflammatory responses of fibroblasts to *P. gingivalis* in the presence of Ti. We hypothesized that Ti particles elicit inflammatory responses in peri-implant granulation tissue

fibroblasts (PIGFs) and alter inflammatory responses of PIGFs to a *P. gingivalis*-challenge. Therefore, the purpose of this study was to determine the influence of Ti on the *in vitro* inflammatory responses of peri-implant granulation tissue fibroblasts to viable *P. gingivalis*.

Materials and methods

Preparation of TiO_2 particles

Commercially pure rutile TiO_2 microparticles (Sigma-Aldrich, Buchs, Switzerland, size <5μm, average=2.3μm) were treated with nitric acid and sodium hydroxide to remove adherent endotoxins as described earlier [30]. After deactivation with 25% nitric acid at 70°C for 1h, the particles were washed three times with endotoxin free phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) and incubated in 70% alcohol at room temperature for 30min. Subsequently, the particles were incubated in five alternating cycles of 0.1N NaOH/95% ethanol (20h, 30°C) and 25% nitric acid (20h, room temperature) with three times sterile PBS wash between each cycle. Hereafter, particles were washed and resuspended in PBS. Particle numbers were determined with flow cytometry (BD Accuri^{TM}C6, BD Biosciences). The TiO_2 powder contained on average$\pm$ SD, 17.5 ($\pm$ 3.6) particles/ng. The particles were resuspended in Dulbecco's modified Eagle medium (DMEM; 4.5g/L glucose+L-glutamine+pyruvate; Invitrogen/Gibco Paisley, UK).

Tissue donors and fibroblast isolation

Peri-implant granulation tissue fibroblasts (PIGFs) from 10 peri-implantitis patients were obtained during open flap debridement as part of the peri-implantitis treatment. The surgical approach consisted of an incision extending mesially and distally to the implant after the removal of prosthetic suprastructure to facilitate access. A full-thickness tissue flap was reflected and the inflamed granulation tissue was carefully removed with a sterile curette. The tissue samples were processed further on the same day. All donors had given written informed consent, and the study was approved by the Medical Ethical committee of the VU University Medical Center.

Tissue samples were processed further as described before [27]. Cells were stored in liquid nitrogen at passage 4. The fibroblastic nature of PIGFs was assessed by the expression of *fibromodulin (FMOD*), a marker for gingival fibroblasts [31], which was expressed at similar levels in gingival fibroblasts from healthy individuals and PIGF. Experiments were performed with cells from passages 5-8.

Cell viability assay

To establish the TiO_2 particle concentration range not significantly reducing the viability of PIGFs, the proportions of surviving PIGFs were measured by the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST-1) assay (Roche Diagnostics Nederland BV, Almere, The Netherlands) according to the manufacturers' protocol. PIGFs from 6 peri-implantitis patients were seeded in 24-well plates at a concentration of 10^5cells/well in DMEM containing 1% PSF and 10% FBS. After 24h, TiO_2 particles suspended in DMEM at concentrations of 100, 50, 25 and 10 particles/PIGF were added to the wells containing PIGFs. PIGFs in DMEM served as positive controls while 5% Triton X-100 in DMEM was added to PIGFs to serve as negative controls. After incubation of the cells for 24h, WST-1 at a concentration of 100ul/ml of DMEM was added to each well. After incubating the cells with WST-1 reagent for 2h, the 24-well plates were shaken for 1min and 100 μl of cell culture supernatant was transferred to a 96-well plate which was analysed in a microplate reader (Synergy HT, Biotek Instruments). Absorbance was measured at 450nm (reference wavelength: 650nm). Taking metabolic activity of the untreated cells as a reference (100% viability), viability of the test samples was expressed in percent viable cells.

Bacterial culture and effect of TiO_2 on *P. gingivalis* growth

P. gingivalis HG91 (FDC381) was cultured anaerobically (80%N_2, 10%H_2, 10%CO_2) until log-growth phase in Brain-Heart Infusion (BHI)-broth supplemented with hemin (5mg/l) and menadione (1mg/l). Purity of bacterial cultures was checked with Gram-staining.

Viable *P. gingivalis* were harvested by centrifugation, pellets were washed twice in sterile PBS and re-suspended in antibiotic/antimycotic-free DMEM. The optical density was measured at 690nm to establish the number of colony forming units (CFUs). A suspension of $2x10^8$ *P. gingivalis* CFU/ml was used to challenge the PIGFs.

To study the effect of TiO_2 on *P. gingivalis* in different experiment conditions, pellets were re-suspended in; i) BHI with hemin and menadione, containing TiO_2 particles (285μg/ml, equal to 25 TiO_2 particles/PIGF) and grown anaerobically for 6h, ii) DMEM containing TiO_2 particles (285μg/ml) and grown for 6h or iii) DMEM without TiO_2 and grown aerobically for 6h. The suspensions were plated in serial dilutions for CFU count on horse blood agar plates (Oxoid no.2, Basingstoke, UK) supplemented with hemin (5mg/l) and menadione (1mg/l), before and after TiO_2 incubation. Three independent *P. gingivalis* HG91 cultures were used for different experiment conditions.

Pre-incubation of fibroblasts with TiO_2 followed by a *P. gingivalis*-challenge

PIGFs (10^5/well) were grown in DMEM containing 1%PSF and 10%FBS in 24-well plates. At sub-confluence, PIGFs were washed with DMEM without antibiotics and serum, and pre-incubated for 18h either in DMEM alone or DMEM containing 10 or 25TiO_2 particles/PIGF (Ti10 and Ti25, respectively) in a humidified atmosphere with 5%CO_2 at 37˚C. Medium was removed and replaced with either 0.5ml of DMEM alone (control) or 0.5ml of DMEM

containing *P. gingivalis* HG91 suspension of $2x10^8$CFU/ml and further incubated for 6h. Morphology of PIGFs was checked for abnormalities or cell-detachment by phase-contrast microscopy (Olympus CK2, Olympus, Japan). Subsequently PIGFs were washed with sterile PBS and lysed in lysis-buffer as supplied with GeneJET RNA Purification Kit (Fermentas, Vilnius, Lithuania), supplemented with β-mercapto-ethanol. Cell culture supernatants from the different experimental conditions were stored at -80ºC. Experiments were performed in quadruplicate.

mRNA expression

PIGF RNA was isolated using the GeneJET RNA Purification Kit according to manufacturers' protocol. The RNA concentration was measured using a Nanodrop spectrophotometer (NanoDrop Technologies; Thermo-Fischer Scientific, Wilmington, Delaware, USA). mRNA was reverse-transcribed using the MBI Fermentas RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers according to manufacturers' protocol.

Real-time PCR primers for *TNF-A*, *IL-1β*, *IL-6*, *IL-8* and *MCP-1* were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA, USA) and used as published previously [27]. To avoid amplification of genomic DNA, each amplicon spanned at least one intron. A serial dilution of human reference total RNA (Stratagene, La Jolla, CA, USA) was included as an external standard to check PCR efficiency. To test if nonspecific products were generated, PCR-products were subjected to melting curve analysis. PCR was performed on Roche LightCycler 480 (F. Hoffmann-La Roche AG, Basel, Switzerland). Reactions were performed on Roche LightCycler 480, with 2ng of cDNA in a total volume of 11µl containing the LightCycler® 480 SYBR Green I Master Mix (F. Hoffmann-La Roche AG, Basel,

Switzerland). Gene expression was normalized for the expression of housekeeping gene *β2-microglobulin*.

Protein production

Pelikine Enzyme-linked immunosorbant assays (ELISA) (Sanquin Blood Supply Foundation, Amsterdam, the Netherlands) were performed to determine protein levels of TNF-α, IL-1β, IL-6 and IL-8 in cell-culture supernatants according to manufacturers' protocol. Undiluted cell culture supernatants from PIGFs of 10 peri-implantitis patients were used. MCP-1 ELISA kits were purchased from Diaclone (Besancon, France) and 5x diluted samples were used to determine MCP-1 protein levels, according to manufacturers' protocol. The sample quantities for all assays were normalized against a standard dilution of the relevant protein as provided by the manufacturers. ELISAs were performed in duplicate

Statistical analysis

Comparisons between total mRNA expression, induction of gene expression, and protein production were tested with paired t-test when data were normally distributed. Wilcoxon-matched pairs test was used to analyze non-parametric data. Differences were regarded significant at $p<0.05$. Tests were performed with GraphPad Prism software (version5, by GraphPad Software Inc. San Diego, CA, USA).

Results

Patient characteristics

Table 1 presents clinical data of the PIGF donors. The average age ($\pm$SD) of 5 male and 5 female patients included in this study was 59.9 ($\pm$9.65) years. 3 out of the 10 patients were current smokers and 4 patients had a known history of periodontitis. The mean ($\pm$SD) pocket depth

around target implants was 6.1mm (±1.5mm) and soft tissues around all target implants showed bleeding on probing (BoP). Pus was present around target implants in 2 patients.

Table 1. Demographic and clinical data of patients included in the study.

Patient characteristics (N = 10)	
Gender (male/female)	5/5
Mean (± SD) age in years	59.9 (±9.65)
Current smokers (N)	3
History of periodontitis (N)	4
Mean (± SD) PD	6.1 (±1.5)
BoP	10
Pus	2

PD: probing depth of pocket in millimeters around target implants; BoP: number of target Implants showing bleeding on probing; Pus: number of implants showing pus on examination.

Effects of TiO_2 on cell viability

Figure 1 shows the viability of PIGFs after treatment with different concentrations of TiO_2 particles. The highest TiO_2-concentration (100 particles/PIGF) tested in our experiment resulted in only 13.5% survival of PIGFs. At concentrations of 100 and 50TiO_2 particles/PIGF, PIGF viability was reduced ($p=0.0001$ and 0.004, respectively) compared to non-challenged cells. 93.8% and 77.8% of the PIGFs survived a 24h incubation with concentration of 10 and 25TiO_2 particles/PIGF, respectively. At concentrations of 10 and 25TiO_2 particles/PIGF (Ti10 and Ti25, respectively), the viability of PIGFs was not different from non-challenged PIGFs. Concentrations of Ti10 and Ti25/PIGF were therefore chosen for further experiments.

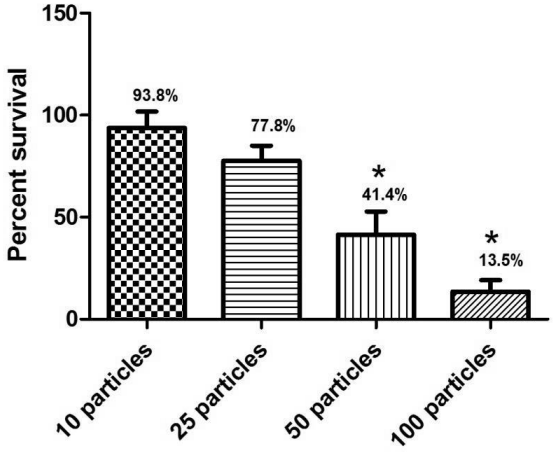

Figure 1. Viability of PIGFs cultured in the presence of TiO_2

Viability of PIGFs cultured 24h in the presence of increasing concentrations of TiO_2 particles (expressed as % of metabolic activity in comparison to the absorbance of fibroblasts incubated without TiO_2) . Bars represent the average $\pm$SEM of viable cells from experiments performed in triplicate. * $p<0.05$.

***P. gingivalis* growth in the presence of TiO_2**

Growth of *P. gingivalis* was not affected by the presence of TiO_2 in any of the tested experimental conditions. Before incubation with TiO_2 particles, $5.9x10^8(\pm 2.5\ x10^8)$ CFU/ml *P. gingivalis* were recovered on blood agar plates. After treatment with TiO_2 particles (285μg/ml equal to the concentration of TiO_2 for 25 particles/PIGF) suspended in BHI under anaerobic conditions, on average $2.4x10^8(\pm 6.0x10^7)$ CFU/ml was recovered.

Aerobic incubation of *P. gingivalis* in DMEM resulted in a reduction in the number of viable *P. gingivalis* ($2.2\ x10^7+1.0x10^7$CFU/ml). But the addition of TiO_2 particles to DMEM did not affect the number of viable *P. gingivalis* ($2.6x10^7+0.8x10^7$CFU/ml).

TiO_2 induces gene expression of pro-inflammatory mediators in PIGFs

To assess inflammatory responses of PIGFs to TiO_2 and *P. gingivalis*, mRNA expression of pro-inflammatory mediators was determined in challenged and non-challenged cells. Incubation with Ti10 alone resulted in a significant (p=0.002) up-regulation of *TNF-A* gene expression compared to control PIGFs, (Figure 2A), while no significant changes were detected in the expression of other genes studied (Figure 2B).

A challenge with *P. gingivalis* alone resulted in increased expression of *TNF-A* (Figure 2A, p=0.027), *IL1-β* (p=0.004), *IL-6* (p=0.037), *IL-8* (p=0.027) and *MCP-1* (Figure 2B), although for *MCP-1*, significance was not reached (p=0.06).

When PIGFs were pre-incubated with Ti10, followed by challenge with *P. gingivalis* (Ti10-Pg), gene expression of *TNF-A IL1-β, IL-8* and *MCP-1* was increased (p =0.002, 0.004, 0.002, 0.001, respectively) compared to non-challenged PIGFs (Figure 2A,B). Ti10-Pg increased *TNF-A* expression even more than *P. gingivalis* alone (p=0.002, Figure 2A). Compared to PIGFs challenged with Ti10 alone, *IL-1β* gene expression was higher with Ti10-Pg (p=0.008), and also *IL-8* gene expression was increased with Ti10-Pg (p=0.08).

Similar to Ti10, incubation with Ti25 caused up-regulation of *TNF-A*, and Ti25-Pg caused up-regulation of *INF-A*, *IL1-β, IL-8* and *MCP-1* (data not shown).

Protein production

Protein production of TNF-α, IL-1β, IL-6, IL-8 and MCP-1 by PIGFs was determined to substantiate the gene-expression data from our experiments. Very low levels of TNF-α protein (Figure 3) were detected in the control PIGFs and PIGFs challenged with *P. gingivalis*. PIGFs

incubated with Ti10 alone produced increased levels of TNF-α, IL-6 and IL-8 (Figure 3) compared to non-challenged PIGFs (p=0.002, 0.0001 and 0.002, respectively) as well as PIGFs challenged with *P. gingivalis* alone (p=0.0006, 0.0001 and 0.013, respectively).

Pre-incubation of PIGFs with Ti10 followed by a *P. gingivalis*-challenge resulted in higher concentrations of TNF-α and MCP-1 when compared to non-challenged PIGFs (p=0.0001 and 0.04, respectively) or, to PIGFs challenged with *P. gingivalis* alone (p=0.002 and 0.01, respectively). In contrast, lower levels of IL-6 (p=0.0001) and IL-8 (p=0.002) were detected in culture supernatant from PIGFs challenged with Ti10-Pg, compared to PIGFs incubated with Ti10 alone. IL-6 production was also lower in culture supernatant from PIGFs challenged with Ti10-Pg compared to non-challenged PIGFs (p=0.01).

Concentrations of IL-1β were low in all culture supernatants, and no differences existed between the experimental conditions tested.

Ti25 alone and Ti25-Pg gave protein production results comparable to Ti10 and Ti10-Pg results (data not shown).

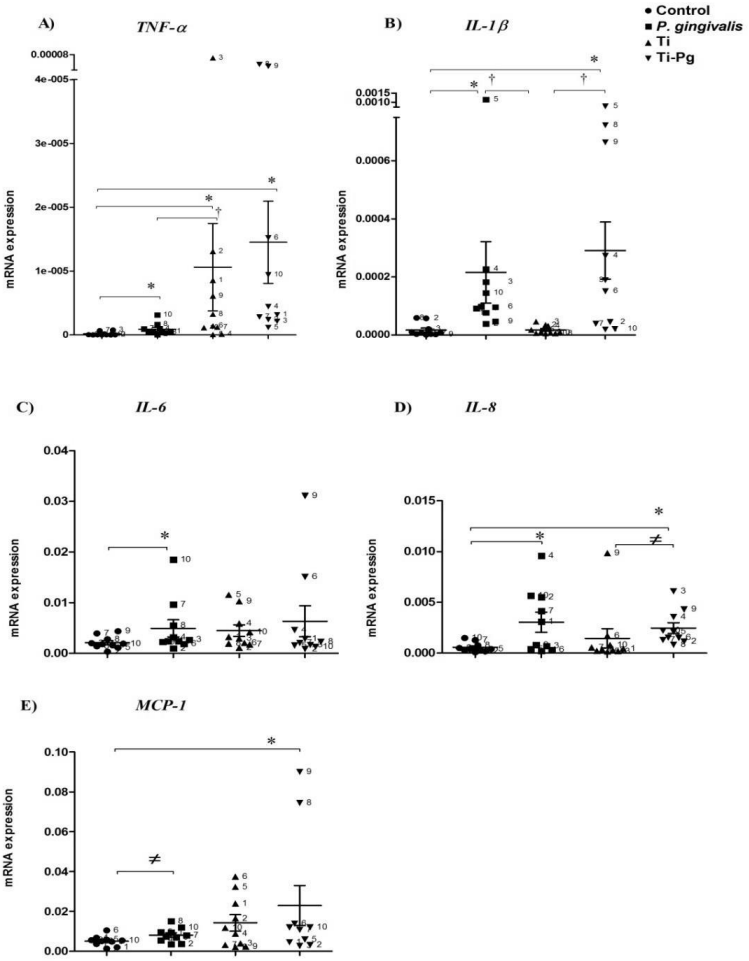

Figure 2. Gene expression of proinflammatory mediators in PIGFs

mRNA expression levels of (A) *TNF-A,* (B) *IL-1β,* (C)*IL-6,* (D) *IL-8* and (E) *MCP-1* in fibroblasts from 10 peri-implantitis patients (PIGF, N=10, mean±SD). Fibroblasts were either non-challenged (Control, circles) or challenged with *P. gingivalis* alone (*P. gingivalis*, squares), 10 TiO_2 particles/fibroblast alone (Ti, upward triangles) or pre-incubated with 10TiO_2 particles/PIGF followed by *P. gingivalis*-challenge (Ti-*P. gingivalis*, downward triangles). Symbols represent the mean mRNA expression levels of each individual patient from experiments performed in quadruplicate. *p<0.05 represents differences between control and challenged cells, † p<0.05 represents differences between cells challenged with different conditions, ≠ indicates a trend (p<0.1)

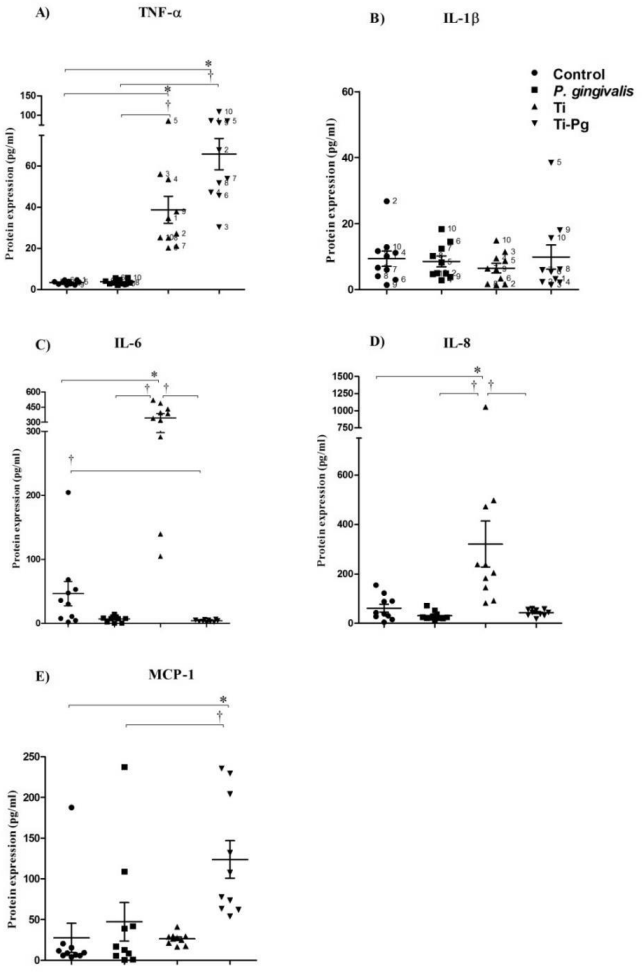

Figure 3. Protein production of proinflammatory mediators by PIGFs

The levels of secreted (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-8 and (E) MCP-1 in culture supernatant from non-challenged and challenged fibroblasts from peri-implantitis patients (PIGF, N=10, mean±SD). PIGFs were either non-challenged (Control, circles) or challenged with *P. gingivalis* alone (*P. gingivalis,* squares), 10 TiO_2 particles/PIGF alone (Ti, upward triangles) or pre-incubated with 10 TiO_2 particles/PIGF followed by *P. gingivalis* challenge (Ti-*P. gingivalis,* downward triangles). Symbols represent the mean protein levels of each individual patient from experiments performed in duplicate. * p<0.05 represents difference between the non-challenged and challenged cells. † p<0.05 represents difference between challenged cells.

Discussion

In the current study, we report effects of rutile TiO_2 particles on the inflammatory responses of peri-implant granulation tissue fibroblasts (PIGFs) to a *P. gingivalis*-challenge. We demonstrate that TiO_2 particles are cytotoxic to PIGFs at high concentrations. Sub-toxic concentrations of TiO_2 particles did not affect viability of *P. gingivalis* and PIGFs but did elicit inflammatory responses in PIGFs.

We found that gene- and protein expression of TNF-α, IL-6 and IL-8 were increased in PIGFs treated with TiO_2 particles alone. A challenge with *P. gingivalis* alone increased the expression of all studied genes. Furthermore, gene-expression and protein production of TNF-α and MCP-1 were induced even more after a combined TiO_2 and *P. gingivalis*-challenge, compared to a challenge with *P. gingivalis* alone, indicating that the presence of TiO_2 may enhance fibroblast responses to bacterial stimuli. IL-6 and IL-8 protein levels were strongly decreased in PIGFs challenged with *P. gingivalis* alone or a TiO_2 and *P. gingivalis*-challenge. This is probably caused by activity of *P. gingivalis* proteases that degrade IL-6 and IL-8 [32-34]. Similarly, protein production of IL-1β was not affected by any experimental condition, which could also be because of proteolytic breakdown of IL-1β [34].

Cytotoxic effects of Ti on host cells have been reported earlier [35], and also the ability of sub-toxic Ti-doses to induce inflammatory responses from various host cells has been demonstrated [21, 36]. Both apoptosis and necrosis have been reported as a cause of decreased viability after Ti exposure [37, 38] as well as apoptosis [39].The reduced cell viability we report here may be a combination of necrosis and apoptosis [40]. In addition, clinical studies have reported increased levels of, IL-1β, IL-8, IL-6 and MCP-1 in the peri-implant tissues and

crevicular fluid of peri-implantitis patients [41, 42]. Results of the current study show that both TiO_2 particles and *P. gingivalis,* individually, can induce gene- and protein expression of pro-inflammatory mediators in PIGFs. Moreover, when fibroblasts had been in contact with Ti before *P. gingivalis*-challenge, an augmented effect on TNF-α gene- and protein expression existed. By inducing the release of pro-inflammatory factors from PIGFs, the interaction of TiO_2, *P. gingivalis,* and PIGFs may thereby contribute to tissue damage and bone resorption in peri-implantitis.

The up-regulation of TNF-α in response to TiO_2 particles alone, and followed by a viable *P. gingivalis*-challenge, is interesting since TNF-α is a mediator that can trigger the release of various pro-inflammatory and bone-resorbing factors [43]. Nowzari et al. [44] concluded that in spite of minimal bacterial accumulation, TNF-α and IL-1β levels in peri-implant fluid were elevated compared to natural teeth. Moreover, reduction of TNF-α levels following peri-implantitis treatment has been associated with an improvement in the clinical parameters of affected implants [45]. The importance of TNF-α in peri-implantitis is further evidenced by the fact that fibroblasts from peri-implantitis patients express multiple factors associated with peri-implant inflammation and bone loss upon TNF-α stimulation [46].

We have previously reported that PIGFs have a distinct and peculiar role in inflammation associated with peri-implantitis and that unstimulated PIGFs have a higher gene-expression of pro-inflammatory mediators compared to gingival fibroblasts from healthy individuals [47]. Furthermore, PIGFs retained a prolonged pro-inflammatory response to *P. gingivalis* compared to fibroblasts from periodontitis lesions. The present study suggests that PIGFs react to both Ti and *P. gingivalis* with a pro-inflammatory response and that the combined effect of *P. gingivalis* and Ti is even stronger.

The combined interaction of Ti and *P. gingivalis* with host cells has not been reported earlier, although it is known that LPS-coated Ti particles are able to enhance inflammatory responses [48, 49]. The mechanism of such responses may occur through stimulation of Toll-like receptors (TLRs). For instance Hirayama et al. found that expression of several TLRs in rat bone-marrow macrophages was affected by LPS-coated Ti particles [49]. Islam et al. showed in murine bone-marrow macrophages that both TLR2 and TLR4 are involved in inflammatory activity of Ti particles combined with bacterial substances [50]. Thus it seems likely that TLR activation plays a role in the combined inflammatory effects of Ti particles and *P. gingivalis* on PIGFs.

Alternatively, Ti allergy may play a role [51]. An allergic reaction is defined as an excessive immune reaction that occurs after coming into contact with a known antigen [52]. Environmental exposure to Ti is widespread, ranging from toothpastes to food (E171), cosmetics (*i.e.* in sunscreens) and paints [53]. Due to this widespread environmental exposure, the human body may contain a Ti concentration of 50 ppm [54], therefore, sensitization to Ti may have occurred even before the placement of a dental implant. Since the we used in our experiments were obtained from a sample of patients with peri-implantitis and have already been exposed to Ti [19], the possibility of sensitization of PIGFs to Ti cannot be ruled out.

Although there is lack of consensus on a specific therapy for peri-implantitis, treatment modalities currently in practice include non-surgical debridement of the implant surface, open flap debridement alone or combined with bone grafting or lasers and other surgical techniques [55-59]. It has been reported that implantoplasty (surgical debridement combined with smoothing and polishing the rough surface of titanium implants) may provide a better treatment option [58, 59]. However, it should be noted that implantoplasty generates Ti particles that might

interact with the peri-implant tissue enhance inflammation around the dental implant [60]. Therefore, effective measures should be taken during implantoplasty to reduce the amount of residual Ti in the peri-implant tissues.

To the best of our knowledge, this study reports for the first time on the influence of TiO_2 particles on the interaction between PIGFs and viable *P. gingivalis*. Our results suggest that Ti wear debris may play an important role in inflammation associated with peri-implantitis. We used the non-capsular *P. gingivalis* strain HG91 [61]. The capsular structure of *P. gingivalis* may play a role in host-pathogen interaction since non-capsular *P. gingivalis* can internalize more efficiently into gingival fibroblasts, and induce higher pro-inflammatory responses compared to their capsular variants [62, 63]. *P. gingivalis* fimbriae might also be involved in the interaction between host cells and TiO_2-Pg. Fimbriae are critical for invasion of host cells [64], and are also involved in attachment of *P. gingivalis* to titanium surfaces [65]. Fimbriae might thus enhance a close interaction between *P. gingivalis*, host cells and Ti particles, thereby contributing to the *TNF-A* response to TiO_2-Pg. However, we sought to determine effects of viable *P. gingivalis* rather than specific virulence factors. Future *in vitro* and *in vivo* studies are needed to extrapolate our results to clinical situations. Likewise, other bacteria may also influence the interaction between host cells and Ti in peri-implantitis.

In conclusion, rutile TiO_2 particles are cytotoxic to PIGFs in high doses, and in sub-toxic doses they can induce pro-inflammatory responses in PIGFs. Interestingly, incubation of fibroblasts with TiO_2 particles before a *P. gingivalis*-challenge enhanced gene expression and protein production of TNF-α even more than a challenge with *P. gingivalis* alone. Therefore, Ti wear particles in peri-implant tissues in combination with *P. gingivalis* infection, may contribute to the pathogenesis of peri-implantitis by enhancing inflammation around implants.

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Chapter 5:

Effects of non-surgical peri-implantitis treatment, a retrospective study

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Chapter 5

Abstract

We aimed to evaluate the effects of non-surgical treatment with/without antibiotics (AB) on clinical and microbiological parameters of peri-implantitis, retrospectively.

A total of 40 patients with ≥1 functional dental implant with bleeding on probing (BoP), pocket probing depth (PPD) ≥5mm, bone loss ≥3mm were selected. Data was collected from the patients' database, ACTA Amsterdam at baseline and three months after treatment. From every patient one dental implant with the deepest PPD, BoP and bone loss was selected as a target implant and the deepest site as a target site.

At target site and implant level, reduction of PPD (both $p<0.001$), greater mucosal recession [261] ($p<0.001$; $p=0.003$, respectively) and BoP ($p=0.005$; $p<0.001$, respectively) were recorded. In the AB group at implant level, mean MR ($p=0.002$) and BoP ($p=0.04$) improved compared with the no AB group. At site level mean PPD ($p=0.004$), MR ($p=0.004$) and plaque ($p=0.02$) were improved. At evaluation, 47.5% of the target implants did not need surgery. All diagnosed bacteria were found at baseline, except *Aggregatibacter actinomycetemcomitans* and at evaluation, *Porphyromonas gingivalis* was not detected in the AB group.

In conclusion, non-surgical peri-implantitis treatment improved most of the clinical parameters, and AB showed additional value for MR and BoP.

Introduction

Peri-implantitis is defined as chronic inflammation of the peri-implant tissues around dental implants with bleeding and/or suppuration on probing and crestal bone loss around a dental implant [1, 2]. If left untreated peri-implantitis may lead to loss of the dental implant. The prevalence of peri-implantitis ranges from 16%-77.4% at patient level and 6.6%-34% at implant level [3, 4].

Although multiple factors are involved in the etiology of peri-implantitis, microorganisms have an essential role [5]. The submucosal biofilm associated with peri-implantitis significantly differs from that of healthy dental implants [6, 7]). While healthy implants are predominantly colonized by Gram-positive facultative cocci [6, 8], Gram-negative mainly anaerobic species such as *Porphyromonas gingivalis*, *Prevotella intermedia* and *Prevotella nigrescens*, and *Aggregatibacter actinomycetemcomitan*s are present in the submucosal biofilm associated with peri-implantitis [9, 10]. The cultivable microbiota, associated with peri-implantitis closely resembles microbiota associated with chronic adult periodontitis [9]. Microorganisms not primarily associated with periodontitis such as, Enteric rods *Staphylococcus* and *Candida* species have also been reported in peri-implantitis [6, 11-16].

Evidence-based literature on treatment protocols for peri-implantitis is limited [17]. There is one study that reports on the clinical and microbiological effects of adjunctive systemic antibiotics (AB) to non-surgical treatment of peri-implantitis [18]. This study showed an improvement of the clinical parameters of peri-implantitis and after one year the effect still remained significant when compared to baseline. Since, the study had no control group, it is uncertain if adjunctive AB are effective to treat peri-implantitis. The additional effects of

systemic AB to non-surgical peri-implantitis treatment in comparison to non-surgical treatment alone have not been documented in literature.

We hypothesized that non-surgical treatment with AB improves the clinical and microbiological parameters of peri-implantitis. The aims of this retrospective study were 1) to evaluate the effect of non-surgical peri-implantitis treatment on clinical and microbiological parameters, and 2) to relate these parameters, and use of systemic AB to non-surgical peri-implantitis treatment response.

Materials and Methods

Patient selection

Patient data were extracted from the patient database of the Academic Centre for Dentistry, Amsterdam (ACTA), The Netherlands.

Peri-implantitis was defined as presence of bleeding on probing (BoP) and/or suppuration on probing with pocket probing depths (PPD) of ≥5mm, and crestal bone loss ≥3mm (measured from the first thread of the implant to the base of the crestal bone on peri-apical radiographs).

For patient selection, the inclusion criteria were 1) presence of at least one functional titanium screw type dental implant with peri-implantitis, 2) implants in function for ≥1 year and 3) age ≥18 years. Patients were excluded if they had used systemic AB and/or non-steroidal anti-inflammatory drug, respectively three months before and four weeks before the treatment. A total of 40 patient records were selected (Figure 1).

Data collection

The following variables were extracted from the patient records at the baseline (before the treatment): 1) age and gender, 2) medical condition according to the American Society of Anesthesiologists (ASA) classification [19], 3) number of teeth, 4) smoking and alcohol drinking habits, and 5) history of periodontitis. Further, the dental implant with the deepest pocket with BoP and bone loss was selected as target implant and the deepest pocket as target site. Data on implant type, location and screwed- or cement retained, keratinized or no keratinized mucosa was extracted.

The following full-mouth and target implant/site clinical parameters were collected at baseline and at 3 months evaluation: 1) visible plaque (yes or no), 2) BoP, 3) suppuration on probing, 4) PPD measured from the mucosal margin to the bottom of the probeable pocket, 5)

clinical attachment level (CAL, recorded from a fixed reference point using a stent) and 6) mucosal recession (MR=CAL-PPD). All measurements were performed at six sites per implant using a click probe with a standardized probing force of 0.2N (Hawe Click-Probe, Hawe Neos Dental, Switserland).

If and which systemic AB were used during the non-surgical peri-implantitis treatment was obtained from the patient records. Microbiological data was collected from the deepest implant sites at baseline and 3 months evaluation.

Data was made fully anonymous and this study was approved by the scientific committee of the ACTA, The Netherlands.

Baseline

Peri-implantitis patients were referred to departments of Implantology and Periodontology, ACTA for treatment of peri-implant infection. At the first visit, medical and dental history questionnaires and above described clinical parameters were recorded according to standard faculty procedures (DAM, PL). Subsequently, if necessary, the patients were referred to a dental hygienist (JvdH) for non-surgical treatment and AB were prescribed.

Submucosal plaque was collected from the deepest peri-implant pocket using sterile paperpoints (Absorbent Points #5-4; Henry Schein U.K. Holdings Lrd., Southall, Middlesex, UB2 4AU England), placed in an Eppendorf tube with reduced transport fluid (Syed & Loesche 1972) and stored in 4ºC until culture within 24h.

Anaerobic culture of the putative periodontal pathogens was carried out as described previously [21]. Briefly, serial dilutions of the submucosal plaque samples in sterile phosphate buffered saline were plated on 5% horse blood agar plates (Oxoid no.2, Basingstoke, UK)

supplemented with hemin (5mg/l) and menadione (1mg/l). For detection of *Aggregatibacter actinomycetemcomitans*, serial dilutions were plated on trypticase soy-serumbacitracin-vancomycin (TSBV) plates. Blood agar plates were incubated anaerobically (80%N_2, 10%H_2, and at 10%CO_2) at 37˚C while TSBV plates were incubated in air in the presence of 5%CO_2 for up to two weeks. The total number of colony forming units (CFU) was counted and converted to CFU/ml. The presence and proportions of *Porhyromonas gingivalis, Prevotella intermedia, Tannerella forsythensia, Peptostreptococcus micros, Fusobactererium nucleatum, Campylobacter rectus* and *A. actinomycetemcomitans* were recorded. The bacterial species were identified on the basis of colony morphology, microscopy, Gram-staining, anaerobic growth, the inability to ferment glucose, indole production as well as production of a set of metabolic enzymes.

Non-surgical treatment (two to three visits)

Before the mechanical non-surgical treatment, patients rinsed one minute with 0.12% chlorhexidine mouthwash (PerioAid, Dentaid, Barcelona, Spain). Implants with peri-implantitis were treated under local anesthesia (Ultracain-DS forte, Hoechst GmbH, Frankfurt am Main, Germany) using an ultrasonic device with an implant tip (EMS, Nyon, Switserland) and carbon fibred instruments (Universal Implant Deplaquer®; Hawe Neos Dental, Bioggio, Switserland). Full dental treatment was performed in case of gingivitis or periodontitis. Patients were advised to rinse with 0.12% chlorhexidine twice a day for four weeks. If necessary, patients received oral hygiene instructions every visit.

Evaluation

Three months after the completion of the non-surgical treatment the above described parameters were measured (DAM, PL). Based on the clinical parameters, patients with limited disease resolution or persisting peri-implant infection, were referred for peri-implant surgery.

Statistical analysis

Statistical analyses were performed using SPSS software (PASW Statistics version 20.0, Chicago, IL, USA). Because the data was not normally distributed, Wilcoxon signed ranks tests were used to compare means of two continue variables and McNemar tests were applied to compare means of two categorical variables. Mann-Whitney U-tests were used to study associations of continue variables and Fisher's exact tests for categorical variables. P-values<0.05 were considered statistically significant.

Results

Baseline characteristics of the patient cohort

A total of 40 individuals, aged 38–76 years (mean age 61.0 years; 21 males and 19 females), fulfilled the inclusion criteria. The baseline characteristics of the patient cohort are summarized in Table 1. A total of 18 patients received systemic AB; in all cases a combination of amoxicillin 375mg and metronidazole 250mg three times a day for 7 days. The other 22 patients did not use any antibiotics during the non-surgical treatment.

Figure 1: Flow chart of patient selection

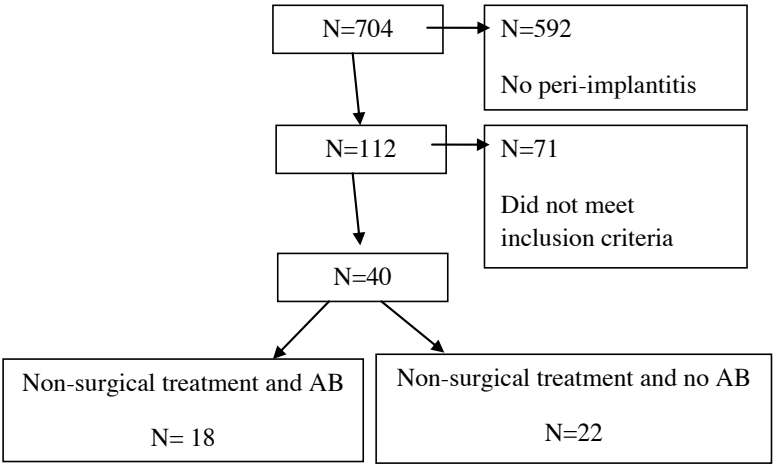

Table 1. Baseline characteristics of the patient cohort (N=40)

Variables	Subcategory	N (%)
Age (mean ±SD)	18-39 (38.7 ±0.6)	3 (7.5)
	40-59 (52.8 ±5.2)	11 (27.5)
	60-79 (67.1 ±4.6)	26 (65.0)
Total	18-79 (61.0 ±10.1)	40 (100.0)
Gender	Male	21 (52.5)
	Female	19 (47.5)
Dentate/Edentulous	Edentulous	7 (17.5)
	Dentate	33 (82.5)
Remaining number of teeth	Min.	6
N= number of teeth	Max.	28
	(Mean ±SD)	19.7 (±5.7)
Medical Condition	Healthy (ASA 1)	11 (27.5)
	Unhealthy (ASA = 2)	29 (72.5)
Current and past smoking habits	Current smoker	8 (20)
	Non-smoker	28 (70)
	Never smoker	9 (22.5)
	Past-smoker	19 (47.5)
	Unknown	4 (10.0)
Smoking habit	Heavy (>15 cig/day)	2 (25.0)
	Moderate (10-15 cig/day)	2 (25.0)
	Light (1-9 cig/day	4 (50.0)
	(Mean amount ±SD)	10.2 (±6.1)
Pack Years	1-10 pack years	4 (50.0)
	11-15 pack years	1 (12.5)
	>15 pack years	3 (50.0)
	Mean years (±SD)	27.5 (±13.8)
Alcohol (2 or more units a day)	Yes	13 (32.5)
	No	27 (67.5)
History of treated periodontitis	Yes	11 (27.5)
	No	23 (57.5)
	Unknown	6 (15.0)

Clinical parameters of the target implant sites

At baseline, no significant differences were found for the target site between the AB and no AB groups. At evaluation, the mean PPD of the target sites was significantly lower for both study groups (Table 2A, $p<0.001$). However, the mean PPD was significantly lower in the AB group ($p=0.004$) when compared to the no AB group. The mean CAL change was only

significant in the AB group (11.9mm baseline vs. 10.6mm evaluation, p=0.002). The mean MR was significantly higher at evaluation for both study groups (AB p=0.004; no AB p=0.04). And mean MR was found significantly greater in the AB group (p=0.004) than the no AB group. The mean BoP reduction showed a trend at evaluation in both groups (AB p=0.08; no AB p=0.025) when compared to baseline. The greatest PPD and MR changes were seen in deep pockets (6-8mm) regardless of the use of additional AB.

Also a trend was seen for less mean suppuration on probing and plaque-accumulation at evaluation for both groups, however not significant. Further, the mean plaque-accumulation at evaluation in the no AB group was significantly lower when compared to the AB group at the deepest sites of the target implants (p=0.02).

Clinical parameters of the target implants

At evaluation the mean PPD around the target implants was significantly lower for both AB and no AB groups (Table 2B, p=0.001 and p=0.02, respectively). The mean CAL did not differ when compared to baseline for both groups. The mean MR was only significantly greater for the AB group (5.6mm baseline vs. 6.5mm evaluation, p=0.003) and was also greater in comparison to the no AB group (p=0.002). Mean BoP was only significantly different in the AB group at evaluation (p=0.001) and showed a significantly lower mean BoP when compared to the no AB group (p=0.04). The mean suppuration on probing and plaque were lower at evaluation for the whole group (p=0.05 and p<0.001, respectively). Further, the mean plaque was also lower for the both AB and no AB groups (p=0.05; p=0.003, respectively).

In the AB group 55.5% and in the no AB group 40.9% of the target implants did not need surgery at the evaluation.

Table 2. Clinical parameters of target implant site (A) and target implant (B) at baseline and 3 month evaluation of patients treated without (N=22) or with (N=18) adjunctive antibiotics.

Variables	**Baseline**	**Evaluation**	**p-value Baseline vs. evaluation**	**p-value Evaluation AB vs. No AB**
A. TARGET IMPLANT SITE				
Mean PPD (mm ±SD)				
Total group	7.4 (1.4)	5.2 (1.4)	<0.001	
AB*	7.0 (1.5)	4.5 (1.0)	<0.001	
No AB	7.7 (1.3)	5.7 (1.5)	<0.001	0.004
Mean CAL (mm ±SD)				
Total group	11.4 (2.0)	10.5 (1.9)	0.001	
AB	11.9 (1.9)	10.6 (1.9)	0.002	
No AB	10.9 (1.9)	10.4 (1.9)	0.11	0.6
Mean MR (mm ±SD)				
Total group	4.0 (1.9)	5.1 (2.4)	0.001	
AB	4.9 (2.0)	6.1 (1.5)	0.004	
No AB	3.2 (1.4)	4.2 (2.7)	0.04	0.004
Mean BoP (%)				
Total group	100	80	0.005	
AB	100	83	0.08	
No AB	100	77	0.025	0.6
Mean suppuration on probing (%)				
Total group	20	8	0.10	

AB	22	6	0.08	
No AB	18	9	0.41	0.7
Mean plaque (%)				
Total group	38	23	0.13	
AB	44	39	0.74	
No AB	32	9	0.06	0.02
B. TARGET IMPLANT				
Mean PPD (mm ±SEM)				
Total group	5.5 (1.2)	4.6 (1.2)	<0.001	
AB	5.5 (0.8)	4.2 (0.7)	0.001	
No AB	5.6 (1.5)	4.9 (1.4)	0.02	0.06
Mean CAL (mm ±SEM)				
Total group	10.4 (2.3)	10.3 (2.2)	0.24	
AB	11.1 (2.1)	10.7 (2.0)	0.14	
No AB	9.8 (2.4)	9.9 (2.2)	0.87	0.22
Mean MR (mm ±SEM)				
Total group	4.9 (2.0)	5.5 (2.4)	0.003	
AB	5.6 (2.0)	6.5 (1.9)	0.003	
No AB	4.4 (1.7)	4.6 (2.4)	0.27	0.002
Mean BoP (0-6 sites)				
Total group	5.0 (1.3)	3.6 (1.9)	<0.001	
AB	5.1 (1.1)	2.9 (1.8)	0.001	
No AB	5.0 (1.5)	4.2 (1.9)	0.07	0.04
Mean suppuration on probing (0-6 sites)				

Total group	1.0 (1.6)	0.3 (1.2)	0.05	
AB	0.8 (1.1)	0.4 (1.4)	0.23	
No AB	0.9 (1.9)	0.3 (1.1)	0.17	0.8
Mean plaque (0-6 sites)				
Total group	2.4 (2.3)	0.8 (1.2)	<0.001	
AB	2.4 (2.1)	1.2 (1.4)	0.05	
No AB	2.5 (2.4)	0.6 (0.9)	0.003	0.2

*AB = antibiotics

Microbiological parameters

The results of the microbiological data are summarized in Table 3. All targeted bacteria except *A. actinomycetemcomitans* were found at baseline. At baseline the mean proportions and prevalence of target organisms were not significantly different between the AB and no AB group.

No difference was found in prevalence or proportions of the targeted microorganisms in the AB group between baseline and evaluation. However, in the no AB group the prevalence of *P. intermedia* and *P. micros* was significantly lower at evaluation (p=0.004; p=0.003, respectively). Further, mean proportions of *P. intermedia* were reduced at evaluation (p=0.02).

A trend for lower prevalence of *P. gingivalis* was found in the no AB group (p=0.06). The mean CFU/ml of anaerobic microorganisms in the target implant sites did not differ significantly between both groups.

Table 3. Prevalence and mean proportions (±SD) of the bacterial species at the target implant site as detected by anaerobic culture at baseline and evaluation (N=40)

Bacterial species								
		Antibiotic group (N=18)			No antibiotic group (N=22)		Evaluation AB* vs. No AB	
		Baseline	Evaluation		Baseline	Evaluation		
A. actinomycetemcomitans	Prevalence	0 (0)	0 (0)	ns†	0 (0)	0 (0)	ns	ns
	N (%)	0 (0)	0 (0)	ns	0 (0)	0 (0)	ns	ns
	Mean (±SD) proportion							
P. gingivalis	Prevalence	3 (17)	0 (0)	ns	7 (32)	4 (18)	ns	0.06
	N (%)	1.6 (5.3)	0 (0)	ns	7.2 (15.2)	39.1 (19.5)	ns	ns
	Mean (±SD) proportion							
P. intermedia	Prevalence	5 (28)	3 (17)	ns	10 (46)	4 (18)	0.004	ns
	N (%)	1.6 (4.2)	3.5 (2.3)	ns	2.2 (5.1)	1.8 (2.6)	0.02	ns
	Mean (±SD) proportion							
T. forsythia	Prevalence	8 (45)	5 (28)	ns	9 (41)	6 (27)	ns	ns
	N (%)	2.0 (4.8)	3.4 (4.1)	ns	1.1 (2.1)	3.5 (3.7)	ns	ns
	Mean (±SD) proportion							
P. micros	Prevalence	15 (84)	11 (61)	ns	18 (82)	13 (59)	0.003	ns
	N (%)	18.2 (21.7)	13.3 (15.4)	ns	18.8 (23.2)	9.2 (8.8)	ns	ns
	Mean (±SD) proportion							
F. nucleatum	Prevalence	13 (73)	13 (72)	ns	16 (73)	17 (77)	ns	ns
	N (%)	2.1 (4.7)	3.9 (8.2)	ns	2.2 (5.2)	1.6 (2.8)	ns	ns
	Mean (±SD) proportion							
C. rectus	Prevalence	1 (6)	1 (6)	ns	1 (5)	3 (14)	ns	ns
	N (%)	4.0 (0.2)	1.38 (0)	ns	2.0 (0)	1.9 (2.3)	ns	ns
	Mean (±SD) proportion							
Total N of colony forming units		**4.3x10^6 (6.0x10^6)**		**ns**	**2.7 x10^6 (3.7 x10^6)**		**ns**	

*AB=Antibiotics; †ns = not significant

Chapter 5

Discussion

This retrospective study on the non-surgical treatment of peri-implantitis with or without the adjunctive use of systemic antibiotics showed improvement for mean PPD, MR and BoP on target site and implant level between baseline and 3 month evaluation. Furthermore, non-surgical treatment with AB showed a significant improvement in PPD and MR at the deepest implant sites, and MR and BoP at implant level when compared to non-surgical treatment alone.

At this moment there is a need for evidence-based intervention studies on non-surgical peri-implantitis treatment [22]. There is one, uncontrolled cohort study that described the effect of systemic AB in combination with non-surgical treatment of peri-implantitis. They reported that combination of local debridement and systemic AB can improve clinical parameters of peri-implantitis and be maintained for at least a year [18]. Renvert et al [23] reviewed 16 peri-implantitis studies and concluded that mechanical debridement has no effect on peri-implantitis. However, they reported that BoP and PPD were reduced by mechanical debridement in combination systemic AB.

Khammissa et al [24] stated that surgical treatment will be necessary for treating peri-implantitis, and even then total elimination of peri-implantitis cannot always be achieved. In our study about 50% of the patients did not need any surgery at three months evaluation.

Thierbach et al [25] described the absence of pus as an indicator for successful non-surgical peri-implantitis treatment. Implants showing pus on probing at baseline needed surgery 3 months after non-surgical peri-implantitis treatment, and implants without pus on probing did not need surgery at evaluation. In our study we could not confirm this correlation.

Further, in the present study *P. gingivalis* tended to be absent in the AB group at the evaluation. In line with our results, effect of amoxicilline plus metronidazole on *P. gingivalis* has

been described extensively in periodontitis [21]. Interestingly, lower prevalence of *P.intermedia* and *P. micros* was found only in the no AB group. Non-surgical therapy alone has previously been described to be effective in diminishing amounts of *P. intermedia* and *P. micros* in periodontitis [26].

Peri-implantitis is an opportunistic infection and has many features in common with periodontitis; both diseases are initiated by the presence of bacteria in biofilm [2]. Therefore, the treatment of periodontitis with mechanical debridement in combination with systemic AB has been applied in peri-implantitis [2]. However, recent studies using open-ended microbial detection techniques have revealed differences between peri-implantitis and periodontitis microbiota [27, 28].

Within the limitations of the current study, the following conclusions may be drawn. Three months after non-surgical peri-implantitis treatment, almost 50% of the implants did not need surgery. No evident additional effect of the systemic antibiotics was found on the studied peri-implant bacterial species and total bacterial load.

Additional AB showed a significant improvement of PPD and MR at the deepest implant pockets. Around the implant additional AB resulted in significant greater MR, and less BoP.

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Chapter 6:

General Discussion

The success of titanium dental implants [1] has led to their increasing use in dentistry as analogs of roots of natural teeth. However, failure of dental implants can occur due to different causes, peri-implantitis being one of the major cause [2]. The incidence and prevalence of peri-implantitis is expected to increase since an increasing number of dental implants is being installed each year [3]. Although peri-implant inflammation and bone loss are used as primary indicators of the progression of disease process, diagnostic criteria for peri-implantitis are not very clear [4]. Similarly, there is lack of consensus in the scientific community on the end points for assessing the efficacy of clinical management of peri-implantitis [5]. Consequently, no evidence-based treatment protocols have yet been established for peri-implantitis [6, 7]. Therefore, peri-implantitis constitutes a major clinical problem, which needs research to give us more insight into the pathogenesis of the disease process and enable us to design evidence-based treatment protocols for peri-implantitis.

Scientific literature is unanimous on the polymicrobial nature of peri-implantitis, in which Gram-negative anaerobic bacteria dominate the peri-implant sub-mucosal microflora [8-13]. In this thesis, we have chosen *Porphyromonas gingivalis* as a model periodontopathic bacterium, to study its interaction with host cells. *Porphyromonas gingivalis* is a Gram negative bacterium, strongly associated with peri-implantitis [13]. *P. gingivalis* interacts with host cells in a variety of ways [14] and thus may play an important role in the pathogenesis of peri-implantitis. Host-pathogen interaction has been studied extensively in the context of periodontitis but studies focusing on host-pathogen interaction in peri-implantitis are scarce.

Fibroblasts are the most common cells in the peri-implant connective tissues and they provide structural support to the connective tissues by synthesizing extracellular matrix and collagen [15, 16]. Fibroblasts also respond to microorganisms by producing inflammatory

mediators. Due to their slower turnover rate compared, for example, to epithelial cells, fibroblasts may be crucial in maintaining the inflammation associated with peri-implantitis [17]. Therefore, in the current thesis fibroblasts were chosen to study host-pathogen interaction in peri-implantitis.

Non-capsular strains of *P. gingivalis* are more efficient in invading gingival fibroblasts

P. gingivalis takes part in the pathogenesis of peri-implantitis by interacting with host cells via employing its various virulence factors [18]. The polysaccharide capsule of *P. gingivalis* is considered to be an important virulence factor due to its role in evasion of host immune system [19]. In **Chapter 2** of the current thesis, we hypothesized that non-encapsulated strains of *P. gingivalis* invade gingival fibroblasts more efficiently than the encapsulated strains. It was shown that the isogenic non-encapsulated mutant of *P. gingivalis* W83 (ΔEpsC mutant) invaded gingival fibroblasts more efficiently compared to the encapsulated parent strain. Furthermore, we showed that *P. gingivalis* can survive inside gingival fibroblasts even in the presence of antibiotics (amoxicillin and metronidazole).

Invasion of host cells is an important mechanism employed by pathogenic bacteria to evade host immune system. It has been reported that the encapsulated *P. gingivalis* strains may evade host defense by reducing the host immune responses [19], while the non-encapsulated strains may do so by invading host cells. Invasion of non-phagocytic host cells such as gingival fibroblasts may render *P. gingivalis* protection against phagocytosis by primary phagocytic cells (e.g. macrophages and neutrophils). Invasion and survival of *P. gingivalis* inside gingival fibroblasts can also offer protection against antibiotics. This could potentially play a role in the recurrence of *P. gingivalis* infection in peri-implantitis and periodontitis. Higher internalization

of known invasive bacteria such as *Helicobacter pylori* has been associated with antibiotic resistance and treatment failure [20].

Bacterial adhesion of host cells is the first step in the invasion process. A previous study has reported that *P. gingivalis* adhere more to epithelial cells from periodontitis-susceptible individuals [21]. Further, non-capsular serotypes of *P. gingivalis* have been shown to adhere more to gingival pocket epithelial cells than encapsulated strains [22]. Besides the capsule, other *P. gingivalis* virulence factors such as fimbriae [23, 24] and gingipains [25] influence the host cell invasion process by *P. gingivalis*. Decreased ability of FimA deficient *P. gingivalis* mutants to invade epithelial cells has been demonstrated [23,24]. Since peri-implantitis is associated with multispecies biofilm, role of other oral bacteria in host cell invasion should also be taken into account. *Fusobacterium nucleatum,* another periodontopathic bacterium, has been suggested to facilitate invasion of *P. gingivalis* into epithelial and endothelial cells [26]. Most available studies about host cell invasion by *P. gingivalis* use epithelial cells and data on invasion in gingival fibroblasts is limited. Intracellular *P. gingivalis* in gingival epithelial cells and immortalized gingival keratinocytes can promote survival of *P. gingivalis* by activating a variety of apoptotic pathways [27, 28], and *P. gingivalis* infection induces regulation of distinctive *P. gingivalis* proteins and genes that could improve its survival inside epithelial cells [29-31]. Although the outcome of internalized *P. gingivalis* in gingival fibroblasts is not known, persistence and multiplication of *P. gingivalis* within epithelial cells has been demonstrated previously [32, 33]. The inability of commonly used antibiotics in peri-implantitis and periodontitis treatments to fully eliminate internalized *P. gingivalis in vitro* (Chapter 2), supports the possibility of host cells acting as reservoirs for re-infection of *P. gingivalis*. Furthermore, it can be speculated that infection caused by non-encapsulated *P. gingivalis* strains might be

difficult to eliminate with traditional antibiotics and might have higher chance of recurrence. Nevertheless, more *in vivo* studies are needed to confirm such an extrapolation of *in vitro* results to clinical situations. In our study, combination of metronidazole and amoxicillin was able to significantly reduce the internalized load of *P. gingivalis*. However, taking into account the concentrations of antibiotics that we used in our *in vitro* experiments and the levels of antibiotics in gingival fluids, the elimination of internalized *P. gingivalis* by antibiotics alone seems unlikely. Therefore, the invasion potential of *P. gingivalis* should be taken into account when prescribing antibiotic therapies for peri-implantitis and periodontitis patients.

Peri-implant granulation tissue fibroblasts play a role in the inflammation and tissue breakdown associated with peri-implantitis

In **Chapter 3** an *in vitro* infection model was used to determine the inflammatory and matrix-degrading responses of peri-implant granulation tissue fibroblasts (PIGFs) from peri-implantitis patients to *P. gingivalis* challenge, and compared with responses of fibroblasts from periodontitis patients (PGFs) and periodontally healthy controls (HGFs). Furthermore, the inflammatory responses of PIGFs and PGFs were determined in sustaining inflammation in peri-implantitis and periodontitis, respectively.

Our results showed that at baseline, non-challenged PIGFs and PGFs are in a pro-inflammatory state compared with HGFs. This observation is in line with previous reports [34] and reflects the ability of PIGFs and PGFs to maintain their activated state in culture. Further, *P. gingivalis* challenge resulted in significant induction of pro-inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8 and moncyte chemtactic protein (MCP)-1 in PIGFs and PGFs but not in HGFs. Although PIGFs have been shown to have a higher *in vitro* pro-inflammatory

response when stimulated with tumor necrosis factor (TNF)-α [35], the interaction of viable *P. gingivalis* with PIGFs has not been reported earlier. We did not find any significant difference in the inflammatory responses of PIGFs and PGFs to *in vitro P. gingivalis* challenge.

Extracellular matrix degrading molecule matrix-metalloproteinase (MMP)-1 was significantly up-regulated in PIGFs and PGFs in response to *P. gingivalis* challenge. This up-regulation of MMP-1 in the absence of a significant change in the levels of tissue inhibitor of metalloproteinases (TIMP)-1 and transforming growth factor (TGF)-β1 indicate a role of *P. gingivalis* in matrix breakdown in peri-implantitis and periodontitis. Bordin et al. [35] have reported decreased secretion of TIMP-1 and TGFβ-1 at baseline by PIGFs compared to PGFs, although we did not find such a difference between PIGFs and PGFs. In our study, PIGFs had significantly higher levels of TIMP-1 compared to HGFs at baseline, and there was no significant difference in the expression of TGFβ-1 between the different groups of fibroblasts either at baseline or after *P. gingivalis* challenge.

Although non-stimulated PIGFs had significantly higher levels of MMP-8 when compared to HGFs, *P. gingivalis* challenge significantly down-regulated expression of MMP-8 by PIGFs. MMP-8 was also down-regulated in PGFs by *P. gingivalis* challenge, although the differences were not statistically significant. This is an interesting finding since MMP-8-null-mice have been shown to be at greater risk for alveolar bone loss in *P. gingivalis*-induced periodontitis [36]. Moreover, in a *P. gingivalis*-induced experimental periodontitis model, MMP-8 has been associated with a reduced expression of lipopolysaccharide-induced CXC chemokines (LIX/CXCL5) [37], which are important chemokines involved in neutrophil recruitment [38, 39]. The mechanism behind the protection offered by MMP-8 is not clear, however, MMP-8 is known to breakdown and deactivate certain proinflammatory mediators [40, 41] and its anti-

inflammatory properties are recognized [42, 43]. *P. gingivalis* appears to decrease protection offered by MMP-8 against inflammation and alveolar bone loss via interactions with host-cells such as fibroblasts. On the other hand, MMP-8 is primarily produced by neutrophils and *P. gingivalis* appears to be a weak inducer of MMP-8 even in neutrophils [44].

Further, we demonstrated that after removal of *P. gingivalis*, PIGFs remained in a pro-inflammatory state compared to PGFs. This sustained pro-inflammatory response by PIGFs highlights their importance in the pathogenesis of peri-implantitis. This data suggests that inflammation and matrix-breakdown may continue in peri-implantitis even after removing the source of infection.

Animal studies have revealed important differences between experimentally induced peri-implantitis and periodontitis lesions. Compared to lesions around natural teeth, more pronounced inflammation and greater size of inflammatory infiltrate in peri-implantitis lesions have been reported [45, 46]. Clinical and radiographic signs of tissue destruction also tend to be more pronounced in peri-implantitis lesions compared to lesions around teeth [45]. Our results support the idea that disease progression and tissue destruction in peri-implantitis might follow a different course than in periodontitis. Furthermore, sustaining proinflammatory response of PIGFs to infectious stimuli may play a role in inflammation and tissue destruction in peri-implantitis. However, it is important to acknowledge that other factors such as; the difficulty to decontaminate the implant surface, absence of a physical barrier in the form of periodontal ligament around implants, decreased vascularity of the peri-implant connective tissues [47], presence of a foreign body in the form of dental implant and interaction of peri-implant tissue with the implant material are also important in the progression of inflammation and tissue destruction in peri-implantitis.

Titanium wear debris can influence host-bacterial interaction in peri-implantitis

Titanium (Ti) is the most widely used material for dental implants due to its excellent biocompatibility with living tissues [48]. Ti was previously thought to be an inert material, however, Ti ions and particles have been found in different layers of peri-implant connective tissues [49] and recently interest is growing in the immunological responses of tissues to Ti wear debris study [50-53]. Studies focusing on the role of Ti in immune responses in peri-implantitis are limited. Moreover, a role of Ti in the interaction between *P. gingivalis* and fibroblasts has not been reported previously.

In **Chapter 4** we investigated the influence of Ti microparticles on fibroblast-*P. gingivalis* interaction in peri-implantitis *in vitro*. Our results suggest that at high concentrations, Ti significantly reduces the viability of PIGFs *in vitro*. Sub-toxic concentrations of Ti and a challenge with *P. gingivalis* alone elicited pro-inflammatory reactions in PIGFs. Interestingly, a combined challenge with Ti-particles and *P. gingivalis* induced a greater increase in the expression of TNF-α and MCP-1 when compared to a challenge with *P. gingivalis* alone. This may imply that microorganisms such as *P. gingivalis* in combination with Ti-wear debris are capable of enhancing inflammation in the peri-implant tissues.

The potential of Ti to elicit inflammatory immune reactions in various host cell types has been reported in several *in vitro* studies [54-56]. Limited evidence from clinical studies shows that elevated levels of inflammatory cytokines such as TNF-α and IL-1β are found in the peri-implant sulcular fluid, in spite of minimal bacterial challenge [57, 58]. Inflammatory host response to external stimuli starts with the triggering of pattern recognition receptors (PRR) such as Toll-like receptors (TLRs). TLRs may function as receptors for microbial organisms as well

as endogenous ligands [59, 60] and increased expression of TLRs, including TLR4 and TLR9, have been found in the interface membrane around loosening total hip replacement implants, where Ti particles may exist [61]. Recently it has also been shown that Ti ions have biological and/or adverse effects on the expressions of receptor activator of nuclear factor kappa beta (NF-κB) ligand (RANKL) and osteoprotegerin (OPG) in osteoblastic cells [62]. Imbalances in the levels of RANKL-RANK and OPG are important in pathologic bone resorption as these factors are essential for the differentiation and development of osteoclasts [63]. Therefore, tissue reactions to Ti may influence the rate of bone resorption around dental implants.

A recent study reported that mRNA levels of TNF-α, IL-1β, and IL-6 increased after phagosytosis of Ti particles coated with LPS in comparison to Ti particles without LPS coating [64]. Another study showed that TLR2 and TLR4 contribute to the biological activity of titanium particles with adherent bacterial debris [65]. These studies further support our results that Ti and microbes may act in concert in the pathogenesis of peri-implantitis.

Non-surgical peri-implantitis treatment is effective in improving clinical parameters of peri-implantitis

Chapter 5 describes a retrospective study, in which we evaluated the effects of non-surgical peri-implantitis treatment on the clinical and microbiological parameters of peri-implantitis. In addition, clinical and microbiological parameters as well as use of systemic antibiotics were related to non-surgical peri-implantitis treatment response.

Our study showed that regardless of systemic antibiotics use (combination of amoxicillin 375mg and metronidazole 250mg three times a day for 7 days), non-surgical treatment of peri-implantitis was effective in improving clinical parameters such as bleeding on probing (BoP),

mucosal recession (MR) and peri-implant pocket probing depth (PPD) at three months evaluation when compared to baseline. Interestingly, adjunctive use of systemic antibiotics showed additional value in reducing BoP and improving MR compared to no use of antibiotics. The prevalence and proportions of the targeted peri-implant sub-mucosal microbiota were not significantly affected by the use of systemic antibiotics. No differences were found in the prevalence or proportions of the targeted microorganisms in the antibiotic group between baseline and evaluation. However, in the no antibiotic group, lower prevalences of *P. intermedia, P. micros* and *P. gingivalis* were found at evaluation. All diagnosed bacteria were found at baseline, except *Aggregatibacter actinomycetemcomitans* and at evaluation, *Porphyromonas gingivalis* was not detected in the AB group. 56 % of the implants in the antibiotic group and 41% of the implants in the no antibiotic group needed surgical treatment.

Treatment of peri-implantitis is aimed to decrease the load of submucosal bacteria by disinfecting the implant surface, and restoring the soft tissue around implant. While mechanical debridement alone is effective in reducing the peri-implant inflammation and bacterial loads, studies report that many implants still need surgical interventions in order to halt the progress of peri-implantitis [66]. Therefore, many different types of non-surgical and surgical methods have been proposed for the management of peri-implantitis [66, 67]. Surface decontamination of the affected implant is mainly carried out by scaling and debridement of the implant surface, alone or combined with the use of local or systemic antibacterial agents [67]. The use of lasers [68] and photodynamic therapy [69] have also been proposed for the surface decontamination of dental implants, although the evidence for the their effectiveness is still preliminary.

Studies on use of systemic antibiotics in the treatment of peri-implantitis are scarce [70, 71]. There is only one, uncontrolled study [71] that describes the effects of non-surgical peri-

implantitis treatment along with the use of systemic antibiotics (Ornidazole 1000mg for 10 days) on the clinical and microbiological parameters of the affected implants. The study reports significant reduction of PPD and BoP as a result of the intervention, which is in line with the findings of our study. In contrast to our study, the study by Mombelli et al. [71] also reports significant suppression of the submucosal anaerobic microflora in the 12 months observation period. This lack of consensus might be due to the different types of systemic antibiotics used in both the studies.

Although use of systemic antibiotics has been associated with improvement of clinical signs of peri-implantitis, the evidence is still inconclusive regarding the effects of systemic antibiotics on the microbiota associated with peri-implantitis. It should also be noted that most studies make use of traditional approaches to microbiological sampling and detection. It is possible that the optimal bacteriological sampling method has not been established yet [72]. In addition, many species in the oral cavity are found and not yet identified [73]. *In vitro* studies have also demonstrated that the surface texture and composition of dental implants can significantly influence the associated microbiota [74, 75], influencing the effect of non-surgical treatment.

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Chapter 7:

General Abstract

P. gingivalis is a Gram-negative, anaerobic bacterium associated with peri-implantitis. *P. gingivalis* induces inflammatory and immunological responses in host cells which are aimed at eliminating the infection and restoring tissue homeostasis. In susceptible hosts, interaction of *P. gingivalis* with host cells may result in aberrant inflammatory responses which lead to inflammation and tissue destruction around the dental implant. Titanium dental implants under occlusal stresses can undergo corrosion and wear. As a result, titanium wear particles (TiO_2) are present in the peri-implant soft tissues. However, influence of TiO_2-particles to host-pathogen interaction in peri-implantitis is largely unknown. Fibroblasts are important host cells, as they not only provide structural support to the implant but also take part in inflammatory host reactions to external stimuli. In the current thesis, we aimed to study the role of interaction between fibroblasts, viable *P. gingivalis* and titanium particles in the pathogenesis of peri-implantitis. In addition, effects of non-surgical peri-implantitis treatment on the clinical and microbiological parameters were studied.

We found that viable *P. gingivalis* can invade gingival fibroblasts *in vitro* and the non-encapsulated *P. gingivalis* is more efficient in invading gingival fibroblasts. It has been suggested that the predominant mode of encapsulated *P. gingivalis* strains to evade host defenses is blunting the host immune responses; the non-encapsulated strains may evade the host defenses by invading host cells. Interestingly, even high concentrations of antibiotics were not able to completely eradicate the *P. gingivalis* internalized into gingival fibroblasts. This may imply that internalization of *P. gingivalis* into fibroblasts protects it from antibiotics to which it is otherwise susceptible. We speculate that, internalized *P. gingivalis* may potentially act as a reservoir for future re-infection of peri-implantitis and periodontitis sites. Therefore, internalization of *P.*

gingivalis into gingival fibroblasts may contribute significantly to the pathogenesis of peri-implantitis.

We also studied gene expression and protein production of selected pro-inflammatory cytokines and matrix-metalloproteinases in *P. gingivalis* – fibroblast interaction. Peri-implant granulation tissue fibroblasts from peri-implantitis patients (PIGFs) were challenged with viable *P. gingivalis* and compared with gingival fibroblasts from periodontitis patients (PGFs) and periodontally healthy controls (HGFs). Our results indicate that before the *P. gingivalis* challenge, *interleukin (IL)-1β, IL-8* and *monocyte chemotactic protein (MCP)-1* are expressed at higher levels in PIGFs and PGFs compared to HGFs. After the *P. gingivalis* challenge, significant up-regulation was observed in the gene expression of *IL-1β, IL-6, IL-8, MCP-1* and *MMP-1* in PIGFs and PGFs but not in HGFs. Interestingly, the *P. gingivalis* challenge down-regulated *MMP-8* expression in PIGFs. In addition, PIGFs sustained higher induction of *IL-1β, MCP-1* and *MMP-1*compared to PGFs, after the removal of *P. gingivalis*. Our results indicate that non-challenged fibroblasts from peri-implantitis and periodontitis lesions are in a pro-inflammatory state and give higher pro-inflammatory responses when challenged with *P. gingivalis*. In addition, PIGFs may play a role in the pathogenesis of peri-implantitis by sustaining inflammation in the peri-implant tissues.

We studied the effects of TiO_2-particles alone and in combination with *P. gingivalis* on the inflammatory reactions in PIGFs. Despite the biocompatibility properties of titanium, our results suggest that TiO_2-particles can induce pro-inflammatory reactions in PIGFs. A challenge with *P. gingivalis* alone elicited pro-inflammatory reactions in PIGFs. Interestingly, a combined challenge with TiO_2-particles and *P. gingivalis* caused a stronger increase in the gene and protein expression of TNF-α and MCP-1 when compared to a challenge with *P. gingivalis* alone. Our

findings may implicate that *P. gingivalis* infection in combination with the presence of titanium wear particles has the potential to enhance inflammation associated with peri-implantitis.

Peri-implantitis is treated non-surgically as well as surgically in contemporary dental practice. Non-surgical treatment of peri-implantitis mainly consists of mechanical debridement of the implant surface with/without the use of local and/or systemic antibacterial agents. However, the effects of non-surgical treatment of peri-implantitis on the clinical outcome have not been thoroughly studied. We retrospectively studied the effects of non-surgical peri-implantitis treatment with or without the use of systemic antibiotics, on the clinical and microbiological parameters of peri-implantitis. Regardless of systemic antibiotics use, non-surgical peri-implantitis treatment was effective in improving clinical parameters such as bleeding on probing (BoP), mucosal recession (MR) and peri-implant pocket probing depth (PPD), at three months evaluation when compared to baseline. Use of systemic antibiotics showed additional value in improving BoP and MR around the implants. However, use of systemic antibiotics did not significantly affect the presence or proportions of target bacteria in submucosal plaque. 47.5% implants did not need surgery three months after non-surgical peri-implantitis treatment, regardless of antibiotic use. These findings indicate that non-surgical peri-implantitis treatment is effective in reducing peri-implant inflammation.

In conclusion, research presented in the current thesis indicates that non-encapsulated *P. gingivalis* can evade host defenses by intersnalization into gingival fibroblasts and that internalized *P. gingivalis* can survive antibiotic treatment *in vitro*. Further, *P. gingivalis* may enhance and sustain inflammation and facilitate matrix breakdown by means of interaction with fibroblasts from peri-implantitis and periodontitis lesions. Moreover, peri-implantitis fibroblasts react to titanium wear particles by releasing pro-inflammatory cytokines and this effect is

increased in presence of *P. gingivalis*. Therefore, *P. gingivalis*-fibroblast-titanium interaction may significantly contribute to the pathogenesis of peri-implantitis. Lastly, non-surgical treatment of peri-implantitis is effective in reducing inflammation in peri-implantitis.

Chapter 8

Hoofdstuk 8

Nederlandstalige samenvatting

Chapter 8

Porphyromonas gingivalis is een Gram-negatieve anaerobe bacterie die geassocieerd is met peri-implantitis. In geval van infectie reageren gastheercellen normaalgesproken op *P. gingivalis* met een ontstekingsrespons die tot doel heeft de infectie te elimineren, en normale weefselhomeostase te herstellen. In een gevoelige gastheer leidt de interactie tussen *P. gingivalis* en gastheercellen echter tot een verstoorde ontstekingsrespons, wat resulteert in peri-implantitis; overmatige ontsteking en weefselafbraak rondom een tandimplantaat.

Tandimplantaten zijn door kauwkrachten onderhevig aan corrosie en slijtage. Hierdoor kunnen er kleine titaniumdeeltjes (TiO_2) vrijkomen in de zachte weefsels rondom het implantaat.

Tot nu toe is er weinig bekend over de invloed die zulke titanium deeltjes bij peri-implantitis kunnen hebben op de reactie van gastheercellen op orale pathogenen zoals *P. gingivalis*. Gingivale fibroblasten zijn gastheercellen in de weefsels rondom het implantaat die een actieve rol spelen bij de ontstekingsrespons op externe stimuli. Daarnaast leveren deze fibroblasten structurele steun aan het tandimplantaat. Het onderzoek in dit proefschrift had tot doel om *in vitro* te bestuderen hoe de interactie tussen fibroblasten, levende *P. gingivalis*, en titanium deeltjes bijdraagt aan de het pathogenese van peri-implantitis. Daarnaast werd onderzocht of een non-chirurgische behandeling effectief bijdraagt aan het remmen van ontsteking, en verbeteren van klinische en microbiële parameters bij peri-implantitis.

Uit het onderzoek bleek ten eerste dat *P. gingivalis* gingiva fibroblasten kan binnendringen. Ongekapselde *P. gingivalis* waren daarin efficiënter dan gekapselde *P. gingivalis*. Gekapselde *P. gingivalis* ontwijken waarschijnlijk het immuunsysteem van de gastheer voornamelijk vanwege het kapsel, dat herkenning door de gastheer voorkomt. Ongekapselde stammen zouden wellicht het immuunsysteem kunnen ontwijken door in

gastheercellen binnen te dringen. In dit opzicht is het interessant dat zelfs hoge concentraties antibiotica niet in staat waren om geïnternaliseerde *P. gingivalis* volledig te doden. Door binnen te dringen in fibroblasten wordt *P. gingivalis* dus beschermd tegen antibiotica waarvoor deze bacterie normaalgesproken gevoelig zou zijn. Het is mogelijk dat *P. gingivalis*, geïnternaliseerd in fibroblasten, dient als een reservoir van waaruit her-infectie van de weefsels rondom implantaten of tanden met *P. gingivalis* kan plaatsvinden. Het binnendringen van gingivale fibroblasten door *P. gingivalis* kan hierdoor een belangrijke rol spelen bij de pathogenese van peri-implantitis.

Hierna werd de genexpressie en eiwitproductie van een aantal pro-inflammatoire cytokines en matrix-metalloproteinasen bij de interactie tussen *P. gingivalis* en fibroblasten bepaald. Hiertoe werden fibroblasten verworven uit granulatieweefsel rondom tandimplantaten van peri-implantitis patiënten (PIGFs), en vervolgens *in vitro* gestimuleerd met levende *P. gingivalis*. De reacties van deze peri-implantitis fibroblasten op *P. gingivalis* werden vergeleken met fibroblasten van parodontitis patiënten (PGFs) en van gezonde donoren (HGFs). De resultaten laten zien dat in afwezigheid van *P. gingivalis* de cytokines interleukine (IL)-1β, IL-8, en monocyte chemotactic protein (MCP)-1 meer tot expressie kwamen in peri-implantitis en parodontitis fibroblasten, dan in gezonde fibroblasten. Een stimulus met *P. gingivalis* leidde vervolgens alleen in peri-implantitis en parodontitis fibroblasten tot een verhoging van de expressie van de cytokines IL-1β, IL-6, IL-8, MCP-1, en MMP-1, maar niet in gezonde fibroblasten. Alleen in peri-implantitis fibroblasten bleek verder de expressie van MMP-8 te worden verlaagd door een stimulus met *P. gingivalis*. Bovendien bleven peri-implantitis fibroblasten gedurende langere tijd een verhoogde expressie van IL-1β, MCP-1 en MMP-1 behouden, ook nadat de stimulus met *P. gingivalis* was beëindigd. Dit wijst erop dat peri-implantitis en parodontitis fibroblasten vergeleken met gezonde fibroblasten een meer pro-inflammatoir profiel hebben, en heviger reageren op een

stimulus met *P. gingivalis*. Peri-implantitis fibroblasten kunnen daarnaast door het aanhouden van een ontstekingsreactie een belangrijke rol spelen bij de pathogenese van peri-implantitis.

Vervolgens werden de mogelijke effecten van titaniumdeeltjes, en van titaniumdeeltjes in combinatie met *P. gingivalis,* op ontstekingsreacties in peri-implantitis fibroblasten onderzocht. Hoewel titanium over het algemeen een biocompatibel materiaal is, blijkt uit onze resultaten dat titanium deeltjes ontstekingsreacties opwekken in peri-implantitis. Wanneer peri-implantitis fibroblasten werden gestimuleerd met zowel titanium deeltjes als *P. gingivalis*, leidde dit tot een nog sterker verhoogde expressie van de cytokines TNFα en MCP-1, dan bij *P. gingivalis* alleen. Dit impliceert dat de aanwezigheid van titanium afbraak deeltjes en van *P. gingivalis* de ontsteking bij peri-implantitis kan verhevigen.

Peri-implantitis kan zowel chirurgisch als non-chirurgisch worden behandeld. Een non-chirurgische behandeling bestaat voornamelijk uit het mechanisch reinigen van het implantaatoppervlak, eventueel in combinatie met lokale of systemische antibiotica. Het is echter nog onvoldoende bekend wat het klinische resultaat van een dergelijke behandeling is bij peri-implantitis. Daarom hebben wij, retrospectief, de effecten van een non-chirurgische behandeling, al dan niet in combinatie met lokaal of systemisch antibioticagebruik, op klinische en microbiële parameters bij peri-implantitis bestudeerd. Hieruit bleek dat een non-chirurgische behandeling na 3 maanden een positief effect had op een aantal klinische parameters, zoals bloeding bij sonderen, mucosale recessie, en pocket diepte rondom het implantaat. Het gebruik van systemische antibiotica leidde daarbij nog tot een extra reductie van de bloeding, pocket diepte en mucosale recessie rondom implantaten. Het gebruik van antibiotica had echter geen significant effect op de proporties van peri-implantitis geassocieerde bacteriën in sub-mucosale plaque. Bij 47,5% van de implantaten was het na 3 maanden niet nodig om ook nog een chirurgische behandeling uit te voeren. Antibioticagebruik speelde daarbij geen rol. Deze resultaten geven aan dat een non-

chirurgische peri-implantitis behandeling effectief is om ontsteking rondom een implantaat te verminderen.

Uit het onderzoek dat is gepresenteerd in dit proefschrift kan worden geconcludeerd dat ongekapselde *P. gingivalis* de defensiemechanismen van de gastheer kan ontwijken door binnen te dringen in fibroblasten, en dat *P. gingivalis* daardoor *in vitro* een antibioticabehandeling kan overleven. Ook kan de interactie tussen *P. gingivalis* en fibroblasten in een peri-implantitis of parodontitis laesie leiden tot aanhoudende ontsteking, en weefselafbraak. Bovendien reageren peri-implantitis fibroblasten ook op titanium debris deeltjes met het produceren van pro-inflammatoire cytokines. Deze reactie wordt versterkt door de gecombineerde aanwezigheid van titanium en *P. gingivalis*. Daarom is de interactie tussen *P. gingivalis*, fibroblasten, en titanium deeltjes van significant belang voor de ontwikkeling van ontsteking bij peri-implantitis. Een non-chirurgische peri-implantitis behandeling kan effectief zijn bij het reduceren van deze ontsteking.

Chapter 9

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(مننه)

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Chapter 8

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