The effect of tobacco exposure on bone healing and the osseointegration of dental implants

Clinical and molecular studies

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To Petter, Nour and Charlie

ABSTRACT

Background: The mechanisms behind the impact of smoking on osseointegration are not fully understood. **Aim:** To correlate the clinical and molecular aspects of osseointegration in smokers compared with non-smokers. **Methodology**: **Study I:** In a retrospective cohort study of smokers and non-smokers, the 5-years implant survival and marginal bone loss (MBL) of machined and oxidized implants, were assessed. **Studies II and III:** In a prospective controlled study, smokers (n=16) and non-smokers (n=16) received machined, oxidized and laser-modified implants. Pain scores, implant stability quotient (ISQ) and gene expression of peri-implant crevicular fluid (PICF) and baseline bone biopsies were analyzed during 0-90d. Clinical assessments and radiology were performed at 90d. **Study IV:** Smokers (n=24) and non-smokers (n=24), each received two mini-implants with machined and oxidized surfaces. The gene expression of selected factors was analyzed in implant-adherent cells and surrounding bone after 1d, 7d and 28d. **Results**: **Study I:** Overall implant survival rate was lower in smokers. In smokers, machined implants failed more frequently than oxidized implants. Mean MBL at 5 years was higher at machined implants in smokers vs. non-smokers. **Studies II and III:** A higher ISQ was found in smokers compared to non-smokers. Greater MBL was found in smokers than non-smokers, particularly at the machined implant. At 90d in smokers, the PICF around machined implants revealed a higher expression of pro-inflammatory cytokine, IL-6, and a lower expression of osteocalcin compared with the surface-modified implants. Multivariate regression revealed that smoking, BoP, IL-6 expression in PICF at 90d and HIF-1α baseline expression are predictors for MBL at 90d. **Study IV:** Cells adherent to machined implants revealed higher expression of pro-inflammatory cytokine, TNF-α. After 7d and 28d, the expression of bone formation gene, ALP, was higher at oxidized implants. Smoking was associated with initial inhibition of bone remodeling (CTR) and coupling (OPG and RANKL) genes in cells on machined implants. **Conclusions**: Smoking is associated with higher MBL during the early healing phase (0-90d), and an increased failure rate and MBL in the long-term (5 years). Whereas the machined implants were associated with a dysregulated inflammation, osteogenesis and remodeling, an increased MBL and failure rate in smokers, the oxidized implants appear to favor osseointegration by mitigating the negative effects of smoking. It is concluded that the local effects of smoking on osseointegration are modulated by host factors and implant surface properties.

Keywords: crevicular fluid, dental implants, gene expression, human, implant surfaces, implant survival, marginal bone loss, osseointegration, pain, periodontitis, resonance frequency analysis, smoking, titanium

SAMMANFATTNING PÅ SVENSKA

Bakgrund: De cellulära och molekylära mekanismerna för osseointegration är ofullständigt kända. **Målet** med avhandlingen var att korrelera de kliniska och molekylära aspekterna under osseointegration i rökare jämfört med icke-rökare. **Metod: Studie I**: I en retrospektiv studie av rökare och icke-rökare utvärderades 5-årig implantatöverlevnad och marginal benförlust (MBF) av maskinbearbetade och oxiderade implantat. **Studier II och III:** I en prospektiv studie (0-90 dagar) av rökare (n=16) och icke-rökare (n=16) installerades ett maskinbearbetat, ett oxiderat och ett lasermodifierat implantat i varje patient. Postoperativ smärta och implantatstabilitetskvot (ISQ) registrerades. Genuttryck analyserades i fick-exudat omkring implantat samt i det ben som implantat sattes in i (baseline). Radiologiska och kliniska bedömningar utfördes efter 90 dagar. **Studie IV:** Rökare (n=24) och icke-rökare (n=24), förses med två mini-implantat, ett maskinbearbetat och ett med oxiderad yta. Genuttrycket av utvalda faktorer analyserades i cellerna på implantatytan samt i omgivande ben efter 1 d, 7 d och 28 dagar. **Resultat: Studie I:** Efter fem år var implantat- överlevnaden generellt lägre hos rökare och i synnerhet vid maskinbearbetade implantat. MBF var högre vid maskinbearbetade implantat hos rökare jämfört med icke-rökare. **Studier II och III:** Högre ISQ-värden sågs hos rökare jämfört med icke-rökare. Efter 90 dagar var MBF var högre hos rökare än hos icke-rökare, särskilt vid maskinbearbetade implantat. Ett högre uttryck för IL-6 och ett lägre uttryck av OC, påvisades vid maskinbearbetade implantat. Multivariat regressionsanalys visade att rökning, BoP, IL-6-uttryck i fickexudat efter 90 dagar och HIF-1α-uttryck i benbiopsier (baseline) är viktiga faktorer kopplade till MBF efter 90 dagar. **Studie IV:** Högre uttryck av TNF-α påvisades i cellerna på maskinbearbetad yta jämfört med oxiderad yta. Däremot var uttrycket av ALP högre i celler på oxiderad yta. Rökning var förknippad med initial inhibition av benremodelleringsfaktorer (CTR, OPG, RANKL) i celler på maskinbearbetad yta. **Konklusion:** Rökning är associerad med högre MBF under den tidiga läkningsfasen (0-90 dagar), samt en högre MBF och ökad implantatförlust på lång sikt (5 år). Medan maskinbearbetade implantat i rökare associerades med en ökad inflammation, minskad osteogenes och remodellering, en ökad marginal benförlust och implantatförlust, så kompenserades de negativa effekterna av rökning av det oxiderade implantatets egenskaper. Sammanfattningsvis dras slutsatsen att de lokala effekterna av rökning på osseointegration moduleras av värdfaktorer och implantatets ytegenskaper.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Sayardoust S, Gröndahl K, Johansson E, Thomsen P, Slotte C. Implant survival and marginal bone loss at turned and oxidized implants in periodontitis-susceptible smokers and never-smokers: a retrospective, clinical, radiographic case-control study. *J Periodontol* 2013; 84:1775-1782.

II. Sayardoust S, Omar O, Thomsen P. Gene expression in peri-implant crevicular fluid of smokers and non-smokers. 1. The early phase of osseointegration. *Clin Implant Dent Relat Res* 2017. doi: 10.1111/cid.12486.

III. Sayardoust S, Omar O, Norderyd O, Thomsen P. Clinical, radiological and gene expression analyses in smoker and non-smokers. 2. The late healing phase of osseointegration. *Submitted for publication*.

IV. Sayardoust S*, Omar O*, Norderyd O, Thomsen P. Implant-associated gene expression in the jawbone of smokers and non-smokers. A human study using quantitative qPCR. In manuscript.

* Equal contribution

CONTENTS

ABBREVIATIONS

ALP	Alkaline phosphatase
BA	Bone area
BIC	Bone-implant contact
BoP	Bleeding on probing
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
CatK	Cathepsin K
COL	Collagen
CTR	Calcitonin receptor
FGF	Fibroblast growth factor
GI	Gingival index
HIF-1α	Hypoxia-inducible factor-1α
IGF	Insulin-like growth factor
IL	Interleukin
ISQ	Implant stability quotient
MBL	Marginal bone loss
M-CSF	Macrophage colony stimulating factor
MCP-1	Monocyte chemotactic protein 1
MSC	Mesenchymal stem cell
OC	Osteocalcin
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
PDGF	Platelet-derived growth factor
PI	Plaque index
PICF	Peri implant crevicular fluid
PPD	Probing pocket depth
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor-kappa B
RANKL	Receptor activator of nuclear factor-kappa B ligand
RFA	Resonance frequency analysis
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 Introductory remarks

The use of dental implants as a treatment for tooth loss is common practice in modern dentistry. Osseointegration, a prerequisite for treatment with titanium implants, is defined as the direct structural and functional connection between bone and the surface of an implant.[1] Successful osseointegration involves a cascade of biological events, including initial inflammation, bone formation and bone remodeling.[2] In experimental studies in animals, the cellular and molecular events that determine these biological processes have been partly unraveled, following the analysis of the gene expression, structure, ultrastructure and biomechanical conditions (stability) of the implant-bone interface.[3-9]

Although treatment with dental implants is reliable, with a reported high survival and success rate, biological complications do occur and a number of risk factors have been implicated, including the medical status of the patient, smoking, bone quality, bone grafting, irradiation therapy, parafunctions, operator experience, degree of surgical trauma, bacterial contamination and susceptibility to periodontitis.[10, 11] Smoking and periodontal disease are two known factors with potentially negative effects on treatment outcomes. In spite of this, the molecular and cellular mechanisms involved in early osseointegration and the effects of smoking and periodontitis on these mechanisms remain poorly understood.

Considerable attention has focused on the modification of implant surface properties in an attempt to influence and promote the biological events which constitute the process of osseointegration.[3, 4] Nevertheless, there is a considerable lack of understanding of the role of implant surface properties and host biological responses which distinguish osseointegration in normal conditions from that in compromised situations. The majority of the latter studies have used experimental models of systemically and/or locally induced compromised conditions.[12-16] More studies are needed to understand the molecular basis of osseointegration in these environments, particularly in humans.

By studying a group vulnerable to complications, i.e. smokers with periodontitis sensitivity, and additionally comparing different implant surfaces, an insight can be obtained into the reasons for complications associated with implant treatments. By better understanding osseointegration at molecular level, it will be possible accurately to identify relevant risk factors

and individually tailor treatments based on a patient's specific level of risk in order to reduce the occurrence of biological complications and optimize treatment outcome.

1.2 Bone

Bone has traditionally been regarded as a static tissue of little biological interest, but, over the past two decades, this view has changed. Evidence indicating that bone is a complex and dynamic organ has been accumulated.[17] It is a highly vascularized, mineralized tissue and, in addition to being a structural tissue supporting the movement of the body, it also acts as an endocrine organ,[18] as it is a reservoir for calcium and ions, as well as a storage site for growth factors. The production of red and white blood cells takes place within the bone.[17]

Bone generally consists of an outer layer of compact bone (cortical bone) and a more porous and vascularized center (trabecular bone). The main component of bone is the extracellular matrix, which is composed of an inorganic and an organic phase. The inorganic constituent is the mineral, hydroxyapatite, formed by calcium and phosphate. The organic phase consists of collagen fibers, mainly type I collagen, and other proteins such as fibronectin and osteocalcin, as well as glycosaminoglycans.[19]

Bone is formed by two different embryonic processes: endochondral (long bones) and intramembranous (flat bones: cranial and facial) ossification. Studies of fracture healing in humans have elucidated these processes.[20] Endochondral ossification starts with cartilage tissue being formed, whereas intramembranous ossification starts with mesenchymal cells directly differentiating into osteoblasts without the formation of cartilage.

1.2.1 Bone cells

Several different cell types are associated with bone. There are those of mesenchymal origin and those of hematopoietic origin. Osteoblasts are derived from mesenchymal stem cells (MSCs). MSCs are able to differentiate into several different cell types, including osteoblasts, chondroblasts and adipocytes.[21] On specific signals, MSCs differentiate into osteoprogenitors,[22] with the potential to proliferate and differentiate into preosteoblasts, and finally form mature osteoblasts.[22] The osteoblasts are the bone-forming cells responsible for the accumulation of the extracellular matrix and mineralization. During the early phase of bone formation, they express high alkaline phosphatase (ALP) and growth factor activity. As the osteoid becomes mineralized, new bone tissue develops; it contains collagen type 1, bone

sialoprotein (BSP) and osteocalcin (OC), which play an important role in bone mineralization.[23] Osteoblasts mature into osteocytes when enclosed in the bone extracellular matrix.[24] Osteocytes have the ability to communicate with one another, with other bone cells and with cells of the blood vessels, through canaliculi. Osteocytes create canalicular networks over long distances, where they are able to transmit signals.[25] It is important that osteocytes are responsible for mechanosensing, responding to mechanical stimuli and therby controlling the activity of osteoblasts and osteoclasts.[26, 27]

Osteoclasts are derived from the hematopoietic lineage. They are formed by the fusion of macrophages. Macrophages thereby play a major role in regulating bone formation and skeletal homeostasis.[28] Macrophages have an important impact on the process of bone formation apart from being an osteclast precursor.[29] Most organs/tissue contain populations of macrophages. In bone, a sub-population termed osteal macrophages, located directly adjacent to osteoblasts, has been identified and it has been suggested that it regulates bone-formation processes.[30] One main function of macrophages is the phagocytosis of apoptopic cells (efferocytosis).[31] Macrophages fuse into osteoclasts in response to macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-kappa B ligand (RANKL). Osteoclasts are responsible for bone resorption.[32] The process of bone resorption by osteoclasts is dependent on signals produced by osteoblasts. RANKL binds to a surface receptor, the receptor activator of nuclear factor-kappa B (RANK), on osteoclasts, stimulating osteoclast activitiy and bone resorption.[33] Osteoclasts bind to bone matrix via integrins and bone is resorbed in the space created between the ruffled membrane of the cell and the bone surface. The bone surface is broken down by enzymatic degradation. The osteoclasts produce hydrogen ions into this compartment, creating an acidic environment which solubilizes the organic part of the bone surface.[34] Calcitonin receptor (CTR) is a cell surface receptor exclusively expressed in osteoclasts, mainly mature ones, and it is therefore widely used as a marker of osteoclasts.[35] It has also been suggested that CTR inhibits osteoclastic activity by inducing the loss of the ruffled border and causing immobility and the arrest of bone resorption.[35] Cathepsin K (CatK) is one of the important lysosomal proteases responsible for the enzymatic degradation of organic components.[36]

In addition to these cells, the bone marrow consists of precursors of different types of leukocytes, fibroblasts and adipocytes.[37] The role of leukocytes is evident in response to trauma or infection, but their role in the steady state has not yet been clarified.

1.3 Bone healing

Bone is an organ that retains the potential for regeneration in adult life, as it possesses considerable capacities for repair. The stages of bone healing mirror the sequential stages of embryonic endochondral or intramembranous bone formation and can be divided into three overlapping, continuous phases: inflammation, bone formation and remodeling.

After the initial trauma, there is bleeding, initiating coagulation. This forms a blood clot/hematoma. Inflammatory cells are recruited to the site, making the hematoma a source of pro-inflammatory cytokines, e.g. interleukins (IL-1, IL-6), tumor necrosis factor-α (TNF-α) and also growth factors, e.g. fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and the transforming growth factor β (TGFβ) superfamily members. These molecules induce a cascade of cellular events that initiate healing [38] and start the recruiting signals for mesenchymal stem cells (MSCs). The role of IL-6 is complex, as it is also implicated as an anti-inflammatory cytokine and is not only pro-inflammatory,[39] for example, in bone, IL-6 is regarded as pro-osteoclastic, but it has also been suggested that it plays a role in osteoblast regeneration.[40]

One crucial step in the repair of the bone is vascularization, which is provided for by the early initiation of VEGF and angiopoietin 1.[20]

Bone formation occurs during the reparative phase of bone healing by intramembranous and/or endochondral ossification. Endochondral ossification begins with the formation of a cartilage template, whereas the MSCs differentiate into chondroblasts by TGF-β signaling. On the other hand, in intramembranous ossification, bone formation occurs directly without the formation of cartilage callus. MSCs proliferate and differentiate into osteoblasts via the signaling of bone morphogenic proteins (BMPs) released from the affected bone matrix.[41] Among the BMPs, BMP-2 is one of the most potent osteoblast-stimulating factors within the TGF-β family, playing important roles in the maintenance of bone mass. BMP-2 in particular plays a major role in inducing the osteoblastic differentiation of mesenchymal stem cells [42] and in bone healing.[43, 44]
Towards the end of the bone-formation phase, the expression of pro-osteogenic signals like BMPs decreases and a renewed increase in pro-inflammatory cytokines takes place instead.[45]

At the initiation of the remodeling phase, osteoblasts upregulate their expression of macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-kappa B ligand (RANKL).[38] This stimulates the

recruitment, differentiation and activation of osteoclasts, thereby starting the bone-remodeling process. The coupling process between bone formation and bone resorption is tightly controlled by the coupling triad, RANK/RANKL/OPG. Osteoblast RANKL binds to osteoclast RANK, thereby initiating osteoclast differentiation. OPG is a decoy receptor, which binds RANKL, thereby fine-tuning osteoclast differentiation.[33] In addition to the osteoclastic regulation of osteoclastogenesis, a number of cytokines are also involved in the regulation. TNF-α, IL-6 and IL-1 are some of the cytokines which modulate the bone-remodeling process by influencing the production of M-CSF and RANKL.[46]

The process of remodeling does not only occur during bone healing but is a lifelong process which is essential for calcium homeostasis and the preservation of the skeleton.[47] Bone remodeling depends not only on regulation by biological signals but mechanical stimuli are also essential. Loading has an great impact on bone mass.[34] Osteocytes are involved in these processes by so-called mechanosensing, responding to mechanical stimuli through the controling activity of osteoblasts and osteoclasts.[26, 27]

1.4 Compromised conditions of bone

Several conditions are associated with abnormalities in the bone formation and remodeling processes. They include osteoporosis, diabetes, irradiation and smoking. With respect to dental implants, whereas all these are regarded as bone-compromising conditions for dental implants, their impact on osseointegration and implant survival remains the subject of disagreement in several reports. For instance, in a meta-analysis, whereas irradiation and smoking demonstrated a significant association with an increased risk of dental implant failure, this relationship could not be confirmed with diabetes and osteoporosis,[48] while a recent systematic review based on 12 studies suggested that diabetes mellitus is associated with a greater risk of peri-implantitis, independently of smoking.[49]

Osteoporosis is a common disease in the aging population and it is placing an increasing burden on the individual and the health-care system. It is characterized by a low bone mass, due to an imbalance within the remodeling process. Both bone formation and bone resorption are affected.[13] However, the osteoclastic activity outweighs the osteoblastic activity. There are two types of osteoporosis; primary and secondary, where the latter is induced by other diseases or drugs. Primary osteoporosis is also divided into two subgroups depending on whether it is caused by estrogen deficiency (postmenopausal osteoporosis) or by aging (senile osteoporosis).[50] RANKL expression is

upregulated in the MSCs of postmenopausal women, indicating increased osteoclastic activity in postmenopausal osteoporosis.[51] In senile osteoporosis, both men and women are affected, although this type is more common in women, and estrogen is not the sole cause. Increased levels of PTH and decreased levels of vitamin D and IGF have been shown to be etiological factors.[52]

Diabetes is associated with the delay and non-union of fractures in diabetics compared with non-diabetics in clinical studies.[12, 53] Diabetic patients are also more prone to osteomyelitis.[54] Furthermore, children with type 1 diabetes and hyperglycemia have decreased bone mineral density and increased OPG expression and a low osteocalcin concentration in blood samples, indicating a risk of impaired growth.[55]

It has been suggested that osteoclasts are less sensitive to irradiation, whereas osteoblasts and osteocytes are affected by reduced cell activity and cell death.[14] However, recent insights suggest that the irradiation-induced effects on bone healing and regeneration are due to more complex biological processes affecting several cell types, where prolonged pro-inflammatory processes may be involved. For osseointegrated dental implants, there is strong clinical evidence of a high failure rate in irradiated bone, especially in the maxilla.[15, 56] Osteoradionecrosis (ORN) is one of the most severe complications of irradiation, predominantly affecting mandible bone. Originally, it was believed that ORN was caused by vascular damage and hypoxia.[57] Current evidence supports the view that ORN is a more complex process and is of fibroatrophic character.[58]

1.5 Osseointegration

Titanium is a biomaterial that is accepted and widely used in oral rehabilitation. The success of endosseous oral implants depends extensively on bone-healing mechanisms and the ability of the alveolar bone to rebuild and integrate the implant within the newly formed bone. The concept of osseointegration was first described by Brånemark and colleagues in the 1960s and 70s.[59, 60] Osseointegration is defined as ‘a direct structural and functional connection between ordered, living bone and the surface of a load-bearing implant’.[1] The clinical application of osseointegration in implant dentistry first gained global acceptance following the Toronto Conference on Osseointegration in Clinical Dentistry in 1982.

The early healing phase following implant installation is important for the long-term success of the implant. In particular, mechanical implant stability is

regarded as a prerequisite for the short- and long-term clinical success of osseointegrated implants.[61] Osseointegration is a dynamic process in which primary stability is gradually replaced by secondary stability. A series of studies on humans have described the process of osseointegration by retrieving miniature titanium implants with a moderately rough surface, together with the surrounding bone.[62-65] The samples were then analyzed using histology and morphometric measurements after one, two, four and six weeks. These studies revealed that, after one week, old bone was in close contact with the implant surface and the implant appeared to rely on mechanical stability. After two weeks, areas of bone resorption were found. The first signs of osseointegration indicated by the formation of woven bone were also found on the implant surface after two weeks. At four weeks, the healing process around the implant featured modeling and remodeling. At six weeks, the resorption areas/remodeling were minor and woven bone was found in close contact with the implant surface. Even lamellar bone was present at the interface.

Experimental studies in rabbits have demonstrated a rapid enhancement in pull-out load during the first four weeks after implantation, whereas the torsional strength started to increase after four weeks.[66]

The cellular and molecular events of osseointegration have mainly been described in experimental, uncompromised animal models.[2, 3, 5] The healing processes during osseointegration mimic those observed during fracture, consisting of successive phases of inflammation, regeneration and remodeling. However, the healing process around an implant surface is predominantly regarded as intramembranous ossification. The presence of the implant and its properties influence the cellular and molecular events involved in the recruitment of inflammatory and mesenchymal stem cells and the expression of different cytokines, matrix protein and growth factors at the implant interface, particularly in the implant-adherent cells. Multiple cell types are involved, such as erythrocytes, platelets and inflammatory cells (granulocytes and monocytes), arriving at the implantation site. These cells are influenced by the implant surface.[67] The process starts with blood clot formation and adsorbing proteins covering the implant surface. Early inflammatory cell recruitment is associated with the triggered expression of cytokines and growth factors, such as IL-1β, TNF-α, PDGF, TGF-β and BMP-2.[4] Experimental studies reveal a peak in the gene expression of pro-inflammatory cytokines in implant-adherent cells at one to three days.[4] A fibrin matrix is formed and the recruitment of MSCs and osteogenic progenitors, from the adjacent tissue, blood vessels and endosteal and periosteal surfaces, takes over.[68] These cells differentiate into bone-forming osteoblasts and also produce BMPs, which trigger the osteoblastic cells to produce woven bone in the extracellular matrix,

on the surface of the surrounding bone (appositional bone formation) or directly on the implant surface (contact osteogenesis).[69] While the process of bone formation continues, the process of bone remodeling is triggered,[70] leading to the remodeling of woven bone around the implant into more organized lamellar bone, which is also mechanically stronger. It has been shown that the remodeling activities occurring at the bone-implant interface are a tightly coupled balance between osteoclasts and osteoblasts, which is controlled by the fine-tuning of RANK/RANKL/OPG expression.[3] Although the remodeling phase has been regarded as the final phase of osseointegration, experimental studies suggest that remodeling is an essential process, starting at an early stage in conjunction with the insertion of the implant.[4, 71]

The cellular and molecular activities of the implant-adherent cells continue during the different phases of osseointegration and they are linked to the regeneration of mature, well-mineralized bone in direct contact with the implant surface. This leads to the development of a stable, functional connection between the implant surface and the recipient bone.[2]

1.6 Soft tissue in osseointegration

The transmucosal segment of a dental implant is surrounded by soft tissue called "peri-implant mucosa" which separates the peri-implant bone from the oral cavity. It has been suggested that this soft-tissue collar in contact with the implant serves as a biological seal, preventing microbial invasion and the development of inflammatory processes.[72] The soft-tissue seal around an implant thus ensures healthy conditions and the survival of the implant over time.[73] This was first studied in dogs in studies conducted by Berglundh and co-workers in 1991.[72] The anatomical and histological features of the peri-implant mucosa were compared with gingiva around teeth.

Histologically, the peri-implant mucosa consists of a highly keratinized oral epithelium connected to a thin barrier epithelium. The dimensions of the peri-implant junctional epithelium and soft-tissue margin were shown to be comparable to the biological width around a natural tooth but slightly longer. Further comparisons between teeth and implants showed that collagen fibers in natural teeth are perpendicularly oriented, attaching from the tooth cementum to the alveolar bone, serving as a barrier to epithelial down-growth and bacterial invasion.[74] Dental implants lack a cementum layer and collagen fibers are thus oriented in a parallel manner to the implant surface, making them much weaker and more prone to periodontal breakdown and subsequent bacterial invasion.[75] The lack of a periodontium is also a potential factor that allows for faster inflammation progression around implants.[75] A clinical study

comparing peri-implant vascularization with gingival vascularization demonstrated differences in both morphology and density.[76]

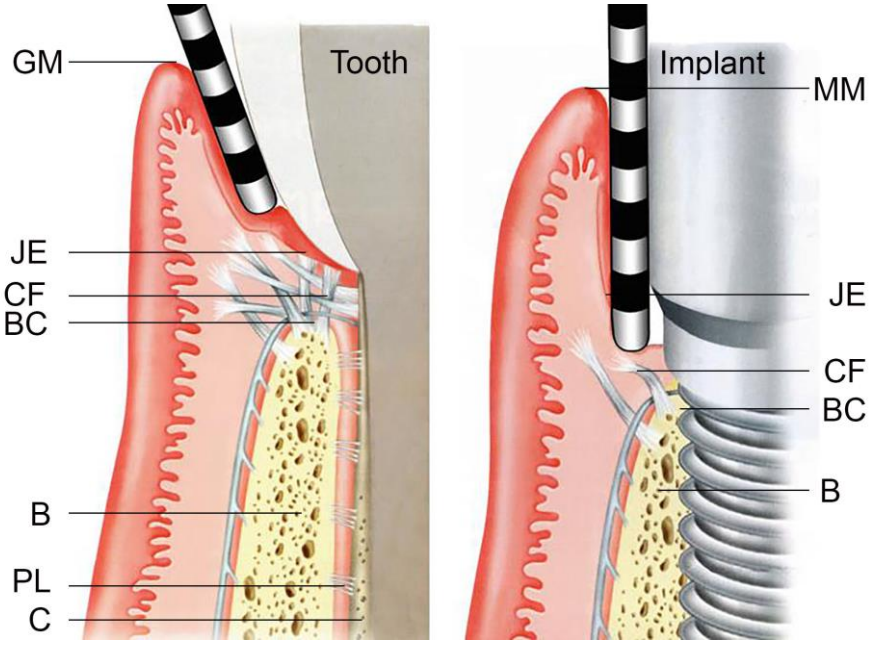

Figure 1. *Demonstrating difference of periodontal and peri-implant soft tissue.(GM-gingival margin, JE-apical end of junction epithelial, CF-collagen fibers, BC-bone crest, B-bone, PL-periodontal ligament, C-cementum) (Illustration adapted from Rose et al. [77]).*

Implant surface topography has been found to have little impact on the peri-implant mucosa, at least as judged by morphological investigations. For example, comparisons of different surfaces have not revealed any noteworthy differences in sulcus depth, peri-implant junctional epithelium or soft connective tissue contact with implant.[78-80] Implants placed in fresh extraction sockets may result in a longer dimension of the peri-implant junctional epithelium.[81]

1.7 Implant materials

Due to the favorable long-term clinical treatment outcomes of titanium implants, titanium is regarded as the golden standard material for the fabrication of dental implants.[2] Titanium has high biocompatibility, high

corrosion resistance and the modulus of elasticity is comparable to that of bone.[82, 83] The use of alloys is increasing due to their advantageous mechanical properties.[84, 85] Nevertheless, there are no clinical comparative studies that are able to determine whether there are long-term, clinical differences between the two types of bulk material.[86]

The surface properties of titanium dental implants are largely related to the titanium oxide layer. The favorable characteristics of titanium are mostly due to the surface oxide, which makes the titanium chemically stable and corrosion resistant. The surface titanium oxide can vary in thickness and may also contain different elements, depending on the method of preparation and the temperature used during fabrication.[2, 87, 88] In addition, the surface topography/surface roughness is related to the surface oxide and in some cases in combination with the bulk metal, depending on the oxide thickness.

Based on experimental evidence, it is well established that implant surface characteristics play an important role in cellular host reactions, the healing process and the osseointegration of dental implants,[89, 90] but the mechanisms by which the implant surface influences the biological processes at dental implants in humans are not as yet well clarified. Several studies demonstrate differences in clinical outcomes between different implant surfaces.[91, 92] It remains to be determined whether the surface properties of clinically functional implants influence the molecular cascade and how this relates to the actual soft- and hard-tissue healing.

1.7.1 Implant surface modifications

There are several different types of implant surface modification. From a clinical point of view, the main objective of introducing several types of surface modification was to increase the short- and long-term stability in bone, thereby ensuring a prosthetic replacement with few complications. The presence or absence of macro and micro irregularities and the shape of the implant were considered at an early stage in the design of dental implants.[93]

Implant surface roughness can generally be divided into macro, micro and nano roughness. Macro roughness can range from millimeters to microns. The macro roughness can directly improve the initial implant stability and long-term fixation through the mechanical interlocking of the rough surface irregularities and the bone.[94, 95] The micro roughness usually ranges from 1-10 microns. In a systematic review by Junker and coworkers,[96] it was emphasized that the micron-level optimal surface topography results in superior growth and the interlocking of bone with the implant interface compared with smoother implant surfaces.

Originally, the machined (smooth surface) titanium implant constituted the first generation of dental implants. Although the surface appears to be relatively smooth, scanning electron microscopy analysis reveals grooves and ridges created during the manufacturing process.[96]

There are several ways to modify the surface properties of dental implants.[88] Strong acids are used to etch the surface in order to roughen titanium implants. Acid etching removes the oxide layer of titanium implants, in addition to parts of the underlying material.[97] The higher the acid concentration, temperature and treatment time, the more of the material surface is removed. A mixture of nitric acid (HNO3) and hydrofluoric acid (HF) or a mixture of hydrochloric acid (HCl) and sulfuric acid (H_2SO_4) are the solutions most commonly used for the acid etching of titanium implant surfaces.[98]

Oxidized surfaces are conceived by anodization as a process used to alter the topography and composition of the surface by increasing the thickness of the titanium oxide layer, roughness and an enlarged surface area.[87, 99]

Sandblasted and acid-etched surface (SLA and modified-SLA) implants are produced by sandblasting with large grit particles of 250-500 μm, followed by etching with acids. Macrostructures are created after sandblasting in addition to micro-irregularities supplemented by acid etching.[100]

Most of the techniques that are currently used for the surface modification of dental implants produce surface roughness predominantly on the micron scale. Several experimental studies show that surface modification as such promotes a larger amount of bone in contact with the implant surface and higher implant stability during osseointegration.[89] Studies of the possible mechanisms *in- vivo* have revealed that surfaces modified by sandblasting and acid etching, as well as with anodic oxidization, enhance the osteoblastic gene expression at the bone-implant interface,[4, 101, 102] suggesting that the micro-scale roughness enhances osteogenic differentiation at the interface and, as a result, more bone is formed in contact with the implant surface. However, it is important to remember that these surface modification techniques do not only introduce roughness on micron scale, they also alter several surface properties, including surface chemistry and other physicochemical properties.[2] Moreover, experimental studies indicate that surface-modified implants, such as anodically oxidized implants, also influence osteoclastic molecular activities, which can be linked to the enhanced remodeling and maturation of the bone interface.[3, 4] Whether similar surface-induced effects also occur at the bone-implant interface in humans remains to be determined.

During the last decade, attention has been paid to the possible role nano-surface modification may play in the osseointegration of titanium implants. Nano-scale surface roughness is categorized in the size range of 1-100.[90] Based mainly on *in vitro* studies, this nano-scale roughness is believed to promote osteoblast cell adhesion and differentiation[103] and increased adhesion has been shown for both progenitor cells and osteoblasts on a variety of nanoscale surfaces.[104, 105] There are several surface modification techniques, including grit blasting, acid etching and anodic oxidization, that produce nano-topography on the implant surface.[106] The majority of these techniques do not provide controlled nano-topography. One surface modification technique incorporating discrete nano-features on implant surfaces is laser ablation.[107] Laser surface modification is a material processing method, where the surface is modified by heat utilized from a high-power laser source, which will melt the surface.[107] Laser parameters, such as power input, determine the maximum temperature attained and the cooling rate, while the duration of interaction determines the surface structure. So, by controlling these parameters, it was possible to achieve nano-topography, superimposed on micro-scale topography of screw-shaped titanium implants.[107, 108] The laser-modified surfaces promoted more bone formation and greater biomechanical stability than machined surfaces in an experimental rabbit model.[108] In spite of this, it is not clear whether these effects could be attributed to nano-topography or macro-topography or both. Attempts to determine the specific effect of the nano-scale features revealed that controlled nano-topography, produced by lithography, promotes bone-implant contact *in- vivo*.[109] Subsequent studies indicated that this nano-topography, *per se*, attenuates the inflammatory cell response and enhances osteogenic cell activity at the bone-implant interface in an experimental animal model.[110] However, further evidence is needed regarding the possible effects of surfaces with nano-scale topography on the processes of osseointegration in humans.

1.7.2 Role of implant surface in compromised conditions

Given the clinical[92, 111] and experimental[3, 4] evidence of improved clinical outcomes and enhanced osseointegration respectively, with surface-modified implants; a role of this kind can be of particular importance for the conditions in which the implant-recipient bone is compromised. Several systemic and local conditions are associated with compromised bone healing and regeneration; they include diabetes, osteoporosis, irradiation and smoking. One intriguing question is whether specific implant surface properties might influence the local healing events around implants in risk patients with compromised bone conditions. The question of whether or not the improvements in the process of osseointegration attributed to surface

properties may compensate for the adverse processes mentioned above is yet to be explored. A systematic review of dental implants installed in irradiated jaw bone concluded that implant surface properties may play a key role in the success of treatments with implants in irradiated patients.[56] Although diabetes mellitus is not a contraindication for implant treatment, it is regarded as a risk indicator, especially in patients with poor metabolic control.[16] In a recent systematic review of the role played by the implant surface in the implant treatment of diabetic patients, only four eligible studies were included and the heterogeneity of the studies made the review inconclusive. In spite of this, a beneficial effect from the surface-modified implants was indicated in these patients.[112] Experimental studies indicate enhanced osseointegration with CaP-coated implants, in animal models with osteoporosis.[113] Taken together, experimental evidence and clinical reports and experience suggest a potential role for surface modifications when it comes to enhancing osseointegration in compromised conditions. However, the available knowledge is fragmented and there is generally a lack of knowledge of the different biological processes at the compromised bone interface to implants and the way cellular and molecular events are influenced by specific surface properties in compromised bone conditions.

1.8 Smoking

Smoking is a well-documented health risk.[114, 115] According to the World Health Organization (WHO), the tobacco epidemic is one of the largest public health threats the world has ever faced, killing around six million people a year.[116] More than five million of these deaths are the result of direct tobacco use, while more than 600,000 are the result of non-smokers being exposed to second-hand smoke.[117] Worldwide, 40% of children, 33% of male non-smokers and 35% of female non-smokers were exposed to second-hand smoke in 2004.[117]

In all, there are more than one billion smokers worldwide, the majority of whom live in low- and middle-income countries, which makes the burden of tobacco-related illness and death heaviest in the under-developed areas of the world.[118] In 2012, the global cost of smoking-attributable diseases (excluding second-hand smoking) was 467 billion US dollars. This equals 5.7% of global health expenditure, whereas almost 40% of the costs are in developing countries.[119] The corresponding cost of smoking in Sweden is almost 30 billion SEK a year.[120] Importantly, current smokers have a shortened life expectancy of more than 10 years.[121] Most of the excess mortality among smokers is due to neoplastic, vascular and respiratory diseases.[121]

Nicotine induces pleasure and reduces stress and anxiety. Smoking improves concentration and enhances at least short-term performance. Nicotine from tobacco smoke absorbs rapidly in the lung and is transported to the brain. It binds to the nicotinic cholinergic receptors in the brain, releasing a variety of neurotransmitters such as dopamine and induces its gratifying effects within 10-15 seconds after inhalation.[122] With the long-term use of nicotine, the number of nicotinic cholinergic receptors increases in the brain, developing tolerance to many of the effects and reducing the rewarding impacts.[123, 124] Addiction to tobacco is multifactorial; they include the urge for the direct pharmacological effects of nicotine but also the relief of withdrawal symptoms and learned behavioral associations.[122]

Smoking and pain have a paradoxical relationship. Animal studies have demonstrated that nicotine induces analgesia in animal models, but still the prevalence of chronic pain is overrepresented in smokers in clinical studies.[125] The analgesic properties are likely due to the effect from nicotine acetylcholine receptors.[126, 127] However, receptor desensitization and tolerance develop rapidly after regular exposure to nicotine and may persist for a considerable time, in addition to withdrawal symptoms.[128, 129] Moreover, the relationship between smoking and pain and the effect of smoking may depend on other factors such as gender, specific pain source and the fact that smoking can produce changes in the nervous system that can persist long after smoking cessation.[130, 131]

Cigarette smoke contains over 4,000 compounds, many of which are considered toxic. They include nicotine, various nitrosamines, trace elements and a variety of poorly characterized substances.[132] The negative effects of smoking on the human body (summarized in Figure 2), such as an increased risk of cancer,[133-135] respiratory diseases, osteoporosis[136, 137] and cardiovascular effects,[133-135, 138] are well known. Current knowledge indicates that smoking also impairs the immune system[139, 140] and wound[141, 142] and fracture healing.[143,144]

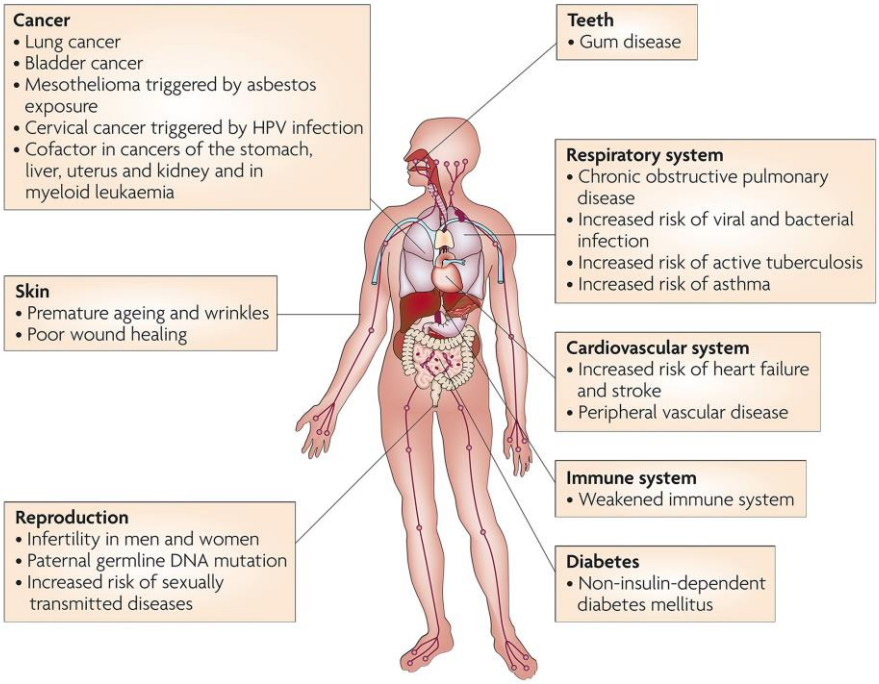

Figure 2. *Adverse effects of tobacco smoke on human health (reproduced with kind permission from Nature Publishing Group).*

1.8.1 Smoking and the oral cavity

Smoking has several effects on the oral cavity, ranging from teeth staining to cancer as the severest (Table 1). Many of the compounds of cigarette smoke are tumor initiators, tumor promoters, co-carcinogens, or direct carcinogens such as metylcholanthrene, benzo[*a*]pyrene and acrolein.[132] Cigarette smoke induces mutations that are associated with lung and oral cancers.[145] In a large-scale epidemiology research collaboration project aiming to improve our understanding of head and neck cancer (i.e. cancer of the oral cavity, cancer of the oropharynx and larynx), it was confirmed that tobacco use is one of two key risk factors for these diseases, with alcohol as the other factor.[146]

It is well documented that smokers have more tooth loss than non-smokers,[147-149] indicating poor oral health in smokers.

Table 1. Adverse effects of tobacco smoking on the oral cavity.[150]

Oral precancerous lesions
• Leukoplakia
• Erythroplakia
Oral cancers
• Squamous carcinoma
• Verrucous carcinomas
Periodontal disease
• Tooth loss
• Alveolar bone loss
Peri-implant disease
Halitosis
Stained teeth

Tobacco smoking is also regarded as a risk factor when it comes to periodontitis. Tobacco smokers were shown to be more likely to develop periodontitis compared with non-smokers.[151] Furthermore, the results after periodontal therapy are less predictable in smokers compared with non- or former smokers[152] and the risk of periodontitis recurrence appears to be higher as well.[153] The pathway of the effects of smoking on periodontal status is not fully understood, but various potential mechanisms are discussed in the literature. Smoking has been shown to affect the composition of the oral biofilm in clinical studies.[154, 155] The impairment of the immune system caused by smoking[139, 140] affects the periodontium. It appears that neutrophil migration and chemotaxis are negatively affected by smoking and it has been suggested that protease release by these cells is part of the tissue destruction in periodontitis.[156] *In vitro* studies suggest that the recruitment and adhesion of fibroblasts in the gingival and periodontal ligament are negatively affected in smokers.[157, 158] It has also been demonstrated in human gingival biopsies that non-smokers have a larger number of blood vessels in inflamed gingival tissues than non-smokers.[159]

Tobacco smoking has also been shown to represent a risk indicator for early[160] and late[161] implant loss,[151, 162] biological complications (e.g. peri-implantitis and peri-implant mucositis) and marginal bone loss.[163-165]

The list of the adverse effects of smoking/nicotine on oral tissue is long, but the mechanisms behind the effects are not clear. Readers interested in further information on the multiple effects are referred to the recent review by Agnihotri and coworkers.[166]

1.9 Smoking, bone and osseointegration

Smoking leads to an increased incidence of non-union after spinal fusion, lower bone density and increased time to union in fracture healing.[143] Skeletal effects were originally attributed to the vascular effects of cigarette smoking and increased carbon monoxide absorption.[167] However, several other mechanisms including decreased bone mineral density,[168] reduced blood supply[159] and fewer bone-forming cells[169] have been proposed. Although the exact mechanism is not fully understood, studies have shown that cigarette smoke has a negative impact on bone-forming cells and skeletal bone in

animals[170-172] and in human models demonstrating delayed fracture repair and an increased risk of non-union.[173, 174] Smoking cessation is recommended to improve bone healing in smoking patients.[175]

As for bone healing, the success of endosseous oral implants is highly dependent on the mechanisms of bone formation, bone resorption and the ability of the alveolar bone to rebuild, thus securing the dental implant in the newly formed bone. Although treatment with dental implants has revolutionized oral health care, complications do occur and a number of risk factors have been implicated, including the medical status of the patient, smoking, bone quality, bone grafting, irradiation therapy, parafunctions, operator experience, the degree of surgical trauma, bacterial contamination and susceptibility to periodontitis.[10, 11]

Bain and coworkers[176] were one of the first groups to highlight the adverse effects of smoking on the outcome of treatment with dental implants in a retrospective study of 2,194 Brånemark implants placed in 540 patients. They demonstrated that the failure rate after six years was significantly higher for smoking patients compared with non-smokers.[176] Several other clinical studies have shown that smoking has detrimental effects on treatment with dental implants, represented by implant failures.[160, 162, 177] A recent systematic review and meta-analysis, including 15 articles examining the outcomes after eight months-13 years, demonstrated an odds ratio of 1.96 for smokers, considering the failure rate of dental implants, as well as greater marginal bone loss for smokers.[178] The clinical reports on the negative effects of nicotine/smoking on osseointegrated implants have been confirmed in several experimental studies. Most of these experimental studies have focused on the histological analyses of bone in contact with the implant (BIC), bone area filling the implant threads (BA) and/or measuring the implant insertion/removal torque, in order to evaluate the detrimental effects of tobacco/nicotine on osseointegration.[179-181] A comparable approach using mini-implants in the human jaws of smokers and non-smokers showed a decrease in BIC and BA after eight weeks of healing around sandblasted, acid-etched mini-implants in smokers.[182] Conversely, in some experimental studies, no major effects on osseointegration were found when only the effect of nicotine, delivered by subcutaneous injection, was evaluated.[183-185] Further, a few animal studies have also emphasized an attenuating effect from implant surface properties on the effects induced by nicotine and tobacco.[186, 187] Interestingly, it has also been shown in rats that smoking cessation reverses the smoke-induced negative effects on osseointegration.[188, 189] Although the available clinical and experimental studies highlight the deleterious effect of smoking on osseointegrated implants, the precise mechanism, including the effect of smoking/nicotine on cells and

biological mediators involved in bone healing and regeneration at titanium implants, awaits detailed investigation.

1.9.1.1 Cellular and molecular *in vitro* studies of the effects of smoking on bone cells in the absence or presence of titanium surfaces

In vitro studies have attempted to investigate the mechanisms of the effects of nicotine on cells involved in bone healing and bone regeneration.[190] These studies have used human cell lines and, to a lesser degree, rat, rabbit and porcine cells.

With respect of inflammatory cells, nicotine, *in vitro*, appeared to attenuate pro-inflammatory activity of macrophages resulting in a down-regulation of pro-inflammatory cytokines.[191, 192] Interestingly, whereas the release of TNF-α was not affected in LPS-stimulated monocytes isolated from rheumatoid arthritis (RA) patients who are smokers, the release of TNF-α was significantly enhanced in stimulated T lymphocytes isolated from RA smokers compared to RA patients who never smoked.[193]

Regarding bone cells, nicotine has been shown to suppress osteoblast proliferation and the secretion of some key osteogenic and angiogenic mediators such as BMP-2 and VEGF.[194] Several additional *in vitro* studies have demonstrated various adverse effects on the gene expression of osteogenic differentiation markers and on bone mineralization.[194-198] Furthermore, nicotine together with LPS has been shown to stimulate the formation of osteoclast-like cells.[199] However, in absence of LPS, the effect of nicotine on osteoclast *in vitro* was not very clear.[200] Interestingly, some *in-vitro* studies have suggested a bimodal effect of smoking. Whereas high nicotine concentrations impaired osteogenic gene expression, nicotine in low concentrations enhanced osteogenic proliferation and differentiation.[201, 202]

Pereira and colleagues evaluated the effect of nicotine of different doses and tobacco compounds on the proliferation and functional activity of human bone marrow osteoblastic cells cultured on the surfaces of plasma-sprayed titanium implants. They used different doses of nicotine, low doses corresponding to levels of nicotine in the plasma of smokers and high doses corresponding to the levels in saliva in smokers. They found a dose-dependent effect, suggesting a direct modulation of the osteoblast activity in human bone marrow cells as an overall effect of nicotine.[203, 204] They also evaluated the role of nicotine in the matrix mineralization of human bone marrow, as well as Saos-2 cells on the plasma-sprayed surfaces of titanium implants, revealing a dose-dependent deleterious effect of nicotine mostly on human bone marrow cells.[205]

Furthermore, *in vitro* findings suggest a greater biofilm accumulation in response to nicotine.[206] Table 2 lists a number of *in-vitro* studies investigating the molecular activities of the effect of smoking on bone cells in the absence or presence of titanium implants.

1.9.1.2 Cellular and molecular *in-vivo* studies of the effects of smoking on bone and osseointegration

With respect to bone and bone healing, the majority of animal studies demonstrate negative effects on bone by tobacco/nicotine exposure.[190] Studies of spinal fusion revealed a lower rate of spinal fusion in rabbits to which nicotine had been administered,[207] based on histological and biomechanical testing. Bone density during distraction osteogenesis in the rabbit tibia was reduced by nicotine.[208] Nicotine has also been reported to affect angiogenesis and to delay and decrease vascularization.[209, 210] Furthermore, experimental animal studies have demonstrated that nicotine attenuates the expression of a wide range of factors involved in osteogenic differentiation and the formation of extracellular matrix and blood vessels, such as VEGF, bone morphogenic protein (BMP)-2, -4, -6 and FGF.[211, 212] It is suggested that nicotine prolongs the inflammatory response and thereby chronic inflammation *in vivo*.[213] In fact, very few experimental studies have addressed the molecular effect of smoking/nicotine with regard to osseointegration. Yamano and coworkers reported the downregulation of important osteogenic factors osteopontin, type II collagen, BMP-2 and bone sialoprotein in the peri-implant bone of rats exposed to systemic nicotine.[212] Table 3 lists a number of *in vivo* studies investigating the molecular activities of the effect of smoking on bone/bone healing and osseointegration.

1.9.1.3 Cellular and molecular studies of the effects of smoking on bone and osseointegration in humans

Relatively few human studies have explored the mechanism behind the effects of smoking on bone in humans. Chassanidis and coworkers demonstrated lower constitutive gene expressions of BMPs, especially BMP-2, in the periosteum of different long-bone sites in smokers compared with non-smokers.[214] In contrast, no difference in BMP-2 gene expression in iliac crest bone biopsies was detected between smokers and non-smokers.[215] Furthermore, molecular analysis of bone biopsies from sites planned to receive dental implants in smokers and non-smokers revealed a lower expression of OC and bone sialoprotein but a higher expression of collagen 1 in biopsies from smokers compared with non-smokers.[216]

Efforts to explore the impact of smoking on the molecular changes occurring at smokers' bone interface to implants revealed few early differences between

non-smokers and smokers.[217] Other than the latter study, there is generally a lack of knowledge of the effect of smoking on the cellular and molecular activities at the bone-implant interface in humans. Further studies are needed to survey the molecular mechanisms involved in the effect of tobacco on bone/bone healing/osseointegration.

Table 2. A number of in vitro studies investigating the molecular activities of the effect of smoking on bone cells in the absence or presence of titanium implants. (Pubmed search phrases: (osseointegration or bone or dental implants)AND(smoking or tobacco or nicotine))

Ref.	Cells	Method and analytical tools	Main findings
198	Human osteoblast like cells, MG63, human bone marrow	Cells were exposed to 0.1 pM, 1 pM, 0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM, 1 mM and 10 mM of nicotine over 72 h and cell proliferation, expression of c-fos, as well as levels of OPN in bone, were measured.	Nicotine modulated cell proliferation, upregulated the C-FOS transcription factor, and increased the synthesis of the bone matrix protein, osteopontin.
195	Human osteoblastic Saos-2 cells	Cells were exposed to nicotine concentrations of 0, 0.001, 0.01 and 1 mM over 14 days. MMPs, TIMPs, tPA, α7-nicotine receptor and c-*fos* were analyzed.	Nicotine stimulated bone matrix turnover, tPA and MMP-1, 2, 3 and 13 as detected by real-time PCR and Western blot.
199	Saos-2 cells	Cells were exposed to 1 mM of nicotine over 14 days and ALP activity, gene and protein expression of M-CSF, osteoprotegerin and PGE2 in osteoblast as well as cell proliferation and formation of osteoclast-like cells were recorded.	M-CSF and PGE2 expression increased with nicotine and LPS vs nicotine alone. OPG expression increased initially but decreased in the later stages of culture with nicotine and LPS. The conditioned medium containing M-CSF and PGE2 produced by nicotine and LPS-treated Saos-2 cells with soluble RANKL increased the TRAP staining of osteoclast precursors compared with that produced by nicotine treatment alone.
203	HBMC	Cells were exposed to nicotine concentrations between 10 ng/mL and 1 mg/mL over 35 days. Cell proliferation and ALP activity were measured.	Dose-dependent effect of nicotine on cell growth, ALP activity and matrix mineralization.
218	Osteoblast-like cells and stromal cells from rats	Cells were exposed to nicotine at concentrations of 250 μg/mL for 3, 6, 12 and 24 h, Northern hybridization, Gel mobility shift assays and Transient trans-fection assays were performed.	Nicotine suppresses BSP transcription mediated through CRE, FRE and HOX elements in the proximal promoter of the rat BSP gene.
201	Human MG63	Cells were exposed to nicotine (0 - 10,000 μM) over 72 h and cell proliferation and gene expression of type I collagen, ALP and OC were measured.	A bimodal effect on cell proliferation: low-dose nicotine increased cell proliferation and gene expression of OC, COL-I and ALP, whereas high-dose nicotine down-regulated the expression of investigated genes.

219	Human osteoblasts	Cells were exposed to 0.1 mM of nicotine over 12 days and the expression of MMPs, tPA, TIMPs, PGE2 and PAI-1, as well as cell proliferation and ALP activity were measured.	Increased expression of MMPs and tPA. Decreased expression of TIMPs. No effect on proliferation or ALP activity.
205	Human bone cells and Saos-2 cells	Cells were exposed to nicotine at concentrations between 0.0001 mg/mL and 0.5 mg/mL over 28 days and cell proliferation, ALP activity and matrix mineralization were measured.	The dose-dependent effect of nicotine on cell growth, ALP activity and matrix mineralization was not evident for Saos-2 cells, but only humen bone cells.
220	Osteoblast-like cells MG-63	Cells were exposed to 100 μM of nicotine over 24h and microarray was performed on whole human genome.	Microarray analysis revealed changes in 842 genes by nicotine. The nAChR antagonists blocked the majority of effects of nicotine.
194	Osteoblasts harvested from rabbits	Cells were exposed to 0.001, 0.1 and 10 μM and cell proliferation as well as gene expression of TGF-β1, BMP-2, PDGF-AA and VEGF were analyzed.	Nicotine suppressed osteoblast proliferation and inhibited the expression of TGF-β1, BMP-2, PDGF-AA and VEGF at concentrations of 0.1 and 10μM, but showed no effect at lower concentration.
202	BMSC	ALP activity assay, Von Kossa staining, real-time PCR (COL-I, ALP, OC, BSP, FGF1, ON) and Western Blot.	Low-dose of nicotine: increase in the expression of ALP, COL-1, BMP-2. High-dose of nicotine reduced the expression of ALP, COL-1, BMP-2. The negative effects of high-dose nicotine were reversed by Vitamin C.
196	BMSC	Cells were exposed to 0 - 5 mM nicotine over 24 h. Cell proliferation, ALP activity, and bone mineralization. Western blot and PCR.	Low nicotine dose stimulated cell proliferation and differentiation, and high nicotine dose inhibited proliferation and differentiation.
197	Human Osteoblast	Cultures were treated with sub-toxic doses of nicotine. qPCR (ALP, COL-I BSP, OC, ON, OPN, FGF and BMP-2). Von Kossa staining.	Sub-toxic nicotine concentrations may affect bone formation through the impairment of growth factor signaling system and ECM metabolism.

ALP-alkaline phosphatase, BMP-bone morphogenetic protein, BSP, bone sialoprotein, COL-collagen, FGF-fibroblast growth factor, HIF-hypoxia inducible factor, IL-interleukin, MMP-matrix metalloproteinase, nAChRs-nicotinic acetylcholine receptors, OC-osteocalcin, ON-osteonectin, OPG-osteoprotegrin, OPN-osteopontin, PDGF-platelet derived growth factor, PGE2-protaglandin E2, qPCR-quantitative polymerase chain reaction, TIMP-tissue inhibitor of metalloproteinase, tPA-tissue plasminogen activator, VEGF-vascular endothelial growth factor.

Table 3. A number of in vivo studies investigating the molecular activities of the effect of smoking/nicotine on bone. (Pubmed search phrases: (rat or rabbit or animal)AND(osseointegration or bone or dental implants)AND(smoking or tobacco or nicotine))

Ref.	Animal model	Administration/ dose	Method	Evaluated factors	Main findings
211	New Zealand white rabbits (n=28)	Osmotic mini-pumps containing either a nicotine solution or a saline solution.	Spine fusion with autogenous bone graft, fusions were harvested at 0, 2, 5, and 7 days and 2, 3, and 4 weeks after arthrodesis. Gene expression (qPCR).	COL-I and II, BMP-2,-4 and -6, VEGF	Nicotine inhibited expression of all cytokines measured.
180	Wistar rats (n=40)	Inhalation in smoke chamber, cigarette smoke of 10 cigarettes (1.3 mg nicotine, 16.5 mg tar, and 15.2 mg carbon monoxide).	Tooth extraction, tissue harvested from sockets, quantitative assessment of the mRNA levels.	ALP, BMP-2 and -7, RANKL and OPG	The expression pattern of all of the studied genes except BMP-7 was negatively affected by cigarette inhalation.
221	New Zealand white rabbits (n=30)	Nicotine- or placebo pellets implanted in the subcutaneous neck tissue of the rabbits (1.5 g 60-day time release).	Unilateral mandibular distraction, regenerated samples were harvested, qPCR.	TGF-1, PDGF-A, and bFGF	At a variety of time points the mRNA expression of TGF-1, PDGF-A and bFGF was inhibited by nicotine.
222	New Zealand white rabbits (n=48)	Nicotine pellets (1.5 g, 60-day time release) were implanted in the neck subcutaneous tissue.	Osteotomy and distraction. Time points: 5, 11 and 18 days (1 week of consolidation), respectively. Radiography, histology, immuno-histochemistry, and RT-PCR.	BMP-2, VEGF and HIF-1α	Nicotine exposure upregulated the expression of HIF-1α and VEGF and enhanced angiogenesis but inhibited the expression of BMP-2 and impaired bone healing.
212	Male Sprague Dawley rats, 4–6 weeks old (n=44)	Osmotic mini-pumps containing either a nicotine solution or a saline solution. Average 6 mg nicotine/kg/day.	The femurs were harvested. Three-point bending test. Histology and qPCR.	OPN, COL-II, BMP-2, and BSP	The bone/implant contact ratio in nicotine-delivered group was lower than control group. Higher expression of BMP-2, BSP, and COL-II in the nicotine group at 2w. At 4w, all detected genes in nicotine group decreased compared with those in controls.

223	Male Wistar rats, 10 weeks old (n=32)	Instraperitoneal nicotine injection or saline solution. 0.1 mg/kg/day, 1.0 mg/kg/day or 10.0 mg/kg/day for 21d. + rhBMP-2	Body weight measurements, radiographic evaluation, histology, immuno-localization of VEGF.	VEGF	The number of VEGF positive cells in the high-dose group was lower than in the control group. Nicotine did not inhibit the stimulatory effect of rhBMP-2 *in vitro*, but *in vivo* by adversely affecting vascularization.
224	Swiss Albino rats, (n=36)	Nicotine added to drinking water or not, 0.4 mg/kg/day or 6.0 mg/kg/day for 12 months.	Body weight measurements, plasma levels of RANKL and OPG, immuno-histochemistry.	RANKL, OPG	No difference in BMD scores of the nicotine groups. Plasma OPG levels were found to be higher in the high-dose group, in comparison to the controls and low-dose group. Tissue RANKL and OPG immunoreactivities decreased in both low- and high-dose group.

ALP-alkaline phosphatase, BMD-bone mineral density, BMP-bone morphogenetic protein, BSP, bone sialoprotein, COL-collagen, FGF-fibroblast growth factor, HIF-hypoxia inducible factor, IL-interleukin, OC-osteocalcin, ON-osteonectin, OPG-osteoprotegrin, OPN-osteopontin, qPCR-quantitative polymerase chain reaction, RANKL-receptor activator of nuclear factor-kappa B ligand, VEGF-vascular endothelial growth factor.

1.10 Methods for evaluating implants

1.10.1 Implant loss

The loss of dental implants is the most common outcome reported in the literature.[225] From a research point of view, implant loss is an objective and undisputed study outcome. Implant loss can be divided into two groups: *early* and *late* losses. Traditionally, implant installation follows a healing time of a couple of months, originally three to six months.[60] During this time, osseointegration should occur before the connection of tooth/teeth replacement. Implant loss prior to this loading of the implant is regarded as an early implant loss.[226-228] Nevertheless, there are some studies suggesting that implants also lost during the first six to 12 months of function should be regarded as early lost implants.[92, 229] Implant loss occurring after loading has mostly been regarded as late implant loss.

1.10.2 Clinical parameters

Plaque assessment: The presence of clinically detectable plaque has been correlated with peri-implant pathology. The formation of microbial biofilms at the surface of titanium implants is an important factor for the prognosis and health of peri-implant tissue.[230] Monitoring the presence of plaque around implants has been suggested as a method for evaluating dental implants.[231]

Mucosal bleeding: Mucosal bleeding is regarded as a sign of inflammation and consequently as a sign of peri-implant pathology. Mucosal bleeding has been suggested as a method for evaluating dental implants.[231] It has nevertheless been reported to have a weak correlation with marginal bone loss.[232]

Bleeding on probing (BoP): BoP appears to play a central role in monitoring peri-implant conditions. The absence of BoP has been reported to describe periodontal health with a very high predictive value [233] and BoP is denoted as one of the stronger predictors of biological complications associated with dental implants.[234]

Probing pocket depth (PPD): The physiological pocket depth of osseointegrated dental implants has been widely debated. Several factors influence the registration of pocket depth: probing force, angulation of the probe, inflammatory condition of the peri-implant tissue, extension of the supraconstruction (compromised access) and placement of the implant. Nevertheless, increasing pocket depth has been suggested as a predictor of pathology.[65, 230]

1.10.3 Resonance frequency analysis

Resonance frequency analysis (RFA) is the measurement of the frequency of a vibrating device. The measurement is made by mounting a sensor on top of the implant. The sensor is then brought to vibration by gentle magnetic pulses. If the implant stability increases, the vibration frequency of the sensor increases. ISQ is the abbreviation of "Implant Stability Quotient". The ISQ scale runs from 1 to 100 and corresponds to the resonance frequency in a close to linear manner.

Resonance frequency analysis (RFA) is one of the few tools for the objective clinical measurement of oral implant stability.[235] It has been thoroughly studied *in vitro* and *in vivo*.[236-238] However, it has still not been fully determined whether RFA provides a true measurement of osseointegration. Experimental studies suggest that RFA correlates to bone area and not to bone in contact with the implant[239] as the definition of osseointegration requires.[1] Whereas removal

torque analysis is able to discriminate between the degree of osseointegration as influenced by differences in implant surface properties, RFA at retrieval reflects only the amount of mineralized bone within the implant threads (BA) but not the actual adaptation of the bone to the implant surface contour (i.e. BIC).[108] This suggests that removal torque analysis has higher predictability for the degree of osseointegration and implant stability than resonance frequency analysis.

1.10.4 Radiology/MBL

Marginal bone loss (MBL) was specified as one of the original success criteria for treatment with dental implants and it is still regarded as an important factor for evaluating the status of dental implants, since it can potentially lead to implant failure. The definition of implant success regarding MBL has been revised over the years and it is considered to be less than 2mm after the first year.[240-242]

In the literature, it is common to determine MBL at the time of superstructure connection and to use this value as the baseline for subsequent follow-up periods. On the basis of experimental data[108] and the fluctuation of ISQ values during healing in humans,[243] it is likely that the greatest bone remodeling occurs during this very early time phase. In line with this assumption, Åstrand and coworkers[244] demonstrated, in a prospective clinical study, that the bone loss at implant placement up to prosthesis insertion was several times higher than the bone loss occurring between prosthesis insertion and the five-year follow-up.

1.10.5 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a highly sensitive method for analyzing genes in very limited biological material. In the field of dental implants, this method has been used by others[212, 245, 246] and ourselves for the analysis of different types of biological material; crevicular fluid,[247] implant-adherent cells[3-5] and peri-implant bone.[3-5]

Peri-implant crevicular fluid (PICF): Crevicular fluids of teeth and implants are exudates consisting of a mixture of serum proteins, inflammatory cells, surrounding tissue cells and oral microflora.[248, 249] The accessibility and non-invasiveness and the opportunity to analyze a wide range of factors are advantageous and make the use of PICF for analyzing the molecular activities around implants appealing. Nevertheless, the question of whether data from cells in the PICF are able to describe the cellular and molecular activities at the bone/implant interface needs to be answered. The possibility of a migration of cells between the bone-tissue interface and the PICF cannot be excluded.

Implant-adherent cells and peri-implant bone: In a series of experimental studies, using qPCR to analyze gene expression factors denoting different phases of osseointegration in normal conditions, the oxidized surface promoted the gene expression of factors involved in the recruitment and adhesion of mesenchymal stem cells, as well as the upregulation of genes involved in both osteogenic differentiation and bone remodeling.[4, 5] Studies in humans which addressed the genes expressed at different implant surfaces during early osseointegration[63, 64, 250, 251] also revealed different patterns of expression depending on the surface properties of the implants.

2 AIMS

The overall aim of this thesis was to examine the clinical and molecular aspects of treatment with dental implants in smokers compared with non-smokers.

2.1 Specific aims of the included studies

- To determine implant survival and marginal bone loss, after 90d and five years, respectively, at machined and surface-modified implants in smokers and non-smokers with a history of periodontitis
- To compare the cellular and molecular events in PICF as well as implant-adherent cells and the surrounding peri-implant bone during osseointegration of different titanium implants in smokers and non-smokers
- To evaluate the cellular and molecular events during the early (0-28d) and late (60-90d) phases of osseointegration at different titanium implant surfaces and to correlate these data to clinical and radiological observations

3 PATIENTS AND METHODS

The thesis is based on a retrospective clinical, radiographic case-control study (Study I) and prospective, randomized, blinded clinical trials (Studies II, III and IV). The retrospective study includes 80 patients and 252 implants. It focuses on clinical and radiological differences relating to implant survival and implant marginal bone loss in smokers and non-smokers treated with implants, with two distinctly different surfaces (machined and oxidized). The prospective studies are divided into two parts. Part one (Studies II and III) includes 32 patients, 16 smokers and 16 non-smokers, who each received three different implants (machined, oxidized and laser modified). Part two (Study IV) includes 48 patients, 24 smokers and 24 non-smokers, who each received two different miniature implants (machined and oxidized surface).

3.1 Ethical considerations

Studies I-IV were independently reviewed and approved by the Institutional Review Board at the University of Linköping, Sweden (doc.no: 2011/330-31 and 2011/469-31), and all the participants signed an informed consent agreement. The study was run according to good clinical practice requirements, the international Conference on Harmonization guidelines and the Declaration of Helsinki for patients participating in clinical studies.[252] CONSORT outlines for clinical studies were adopted.[253]

Each patient was thoroughly informed, both verbally and in written form, of all the procedures and requirements of the study. The study purpose was explained, as were the risks. Each patient in the smoking group was offered the chance to join a smoking cessation program before being asked to join the study. Only patients that did not want or did not manage to stop smoking were asked to join the study.

Financial disclaimer: No financial supporters influenced.

3.2 Patient selection and study design

3.2.1 Study I

A computer-generated sequence randomly selected patients from the database. The selection was based on these criteria: 1) generally healthy; 2) history of periodontal disease degree 4 or 5 according to Hugoson and Jordan criteria[254] 3) never-smokers or smokers (>10 cigarettes/day); 4) consistent type of

implant, either machined or oxidized; 5) two-stage surgery (submerged approach); 6) no complications during surgery (e.g. thread exposures and augmentation procedures) or postoperative follow-up (e.g. infection); 7) conventionally loaded implants (3 to 6 months); and 8) regular follow-up and maintenance at the Department of Periodontology. Thereafter, all patient records were checked manually to verify the database information.

Eighty patients were found to be suitable for further investigation. No separate clinical examinations were carried out as part of this study. All patient records were checked manually to verify the database information. The groups were matched regarding gender, oral hygiene and implant distribution and were then divided into two subgroups by implant type (machined or oxidized) (Figure 3).

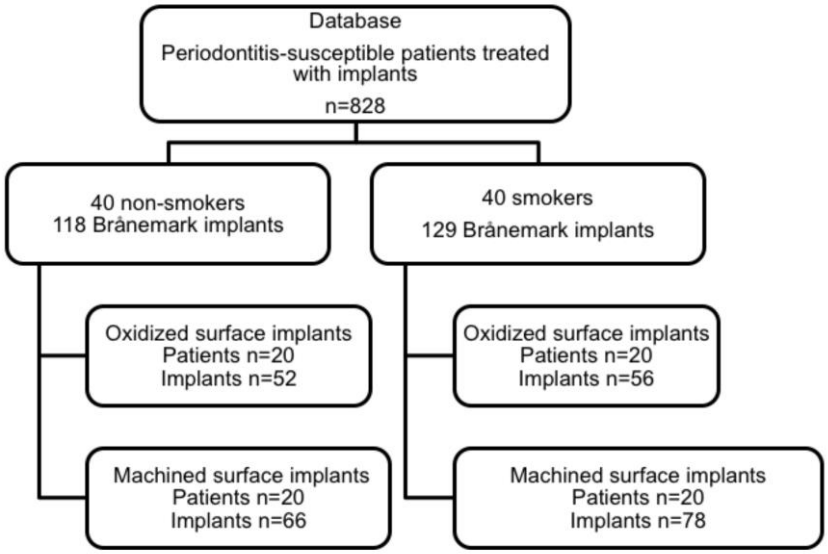

Figure 3. *Study design Study I*

3.2.2 Studies II- IV

The study subjects were selected from patients referred to the Department of Periodontology in Jönköping, Sweden, from January 2013 to June 2016.

The selection was performed according to the following inclusion criteria. Smokers were defined as individuals who had smoked an average of > 10 cigarettes/day for > 10 years. Non-smokers were defined as individuals who had never smoked. Adequate alveolar bone for implant placement without the need for grafting. Absence of risk factors that could affect levels of bone-related gene expression, including osteoporosis, chronic use of anti-inflammatory agents, use of bisphosphonates, or severe metabolic diseases

such as diabetes. At least six months between extraction and implant placement.

Thirty-two systemically healthy individuals, 16 smokers and 16 non-smokers, either partially or completely edentulous, were included in Study II and Study III (Figure 4). These patients and an additional eight smokers and eight non-smokers were enrolled in Study IV (Figure 5) to satisfy the statistical power.

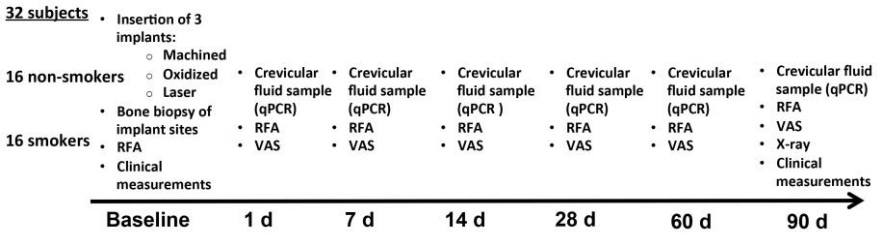

Figure 4. *Study design Studies II and III*

All patients had a history of periodontal disease and were efficiently treated at the department prior to implant insertion. All smokers were informed of the risks and the adverse effects of smoking before enrollment.

The placement of the relative order of the commercially implants and the mini-implants was randomly assigned, using a computer-generated randomization (IBM SPSS Statistics, NY, USA). The time point of retrieval of the mini-implants (1d, 7d or 28d) was also randomly assigned.

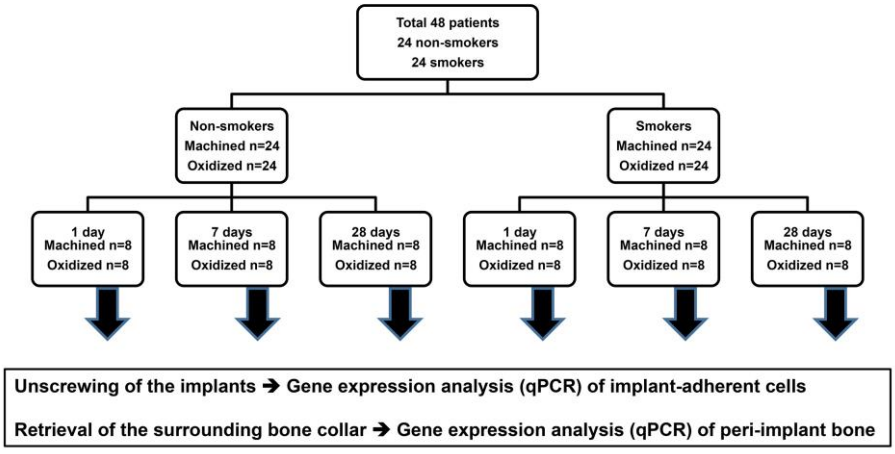

Figure 5. *Study design Study IV*

3.3 Implants and mini-implants

Study I: Brånemark System, Mark III, TiUnite (Nobel Biocare, Gothenburg, Sweden) and Brånemark System, Mark II (Nobel Biocare, Gothenburg, Sweden) were used in the study. The most commonly used length was 13mm (44%), followed by 10mm (24%), 15mm (19%) and 11.5mm (8%). A regular platform (3.75mm) was the most frequently used diameter (83%), while in the remaining cases a narrow platform (3.3mm) was used.

Studies II & III: Each patient received three commercially available dental implants (Figure 4). The three implants differed according to their surface properties and were classified as (1) machined (smooth) (Brånemark Integration, Gothenburg, Sweden), (2) oxidized (moderately rough) (Nobel Biocare, Gothenburg, Sweden) or (3) laser-modified (combination of smooth and moderately rough) (Brånemark Integration, Gothenburg, Sweden). Implant placements were randomized in order to ensure an even distribution between different sites. All the implants had the dimensions of a regular platform (3.75mm) and a length of 10mm.

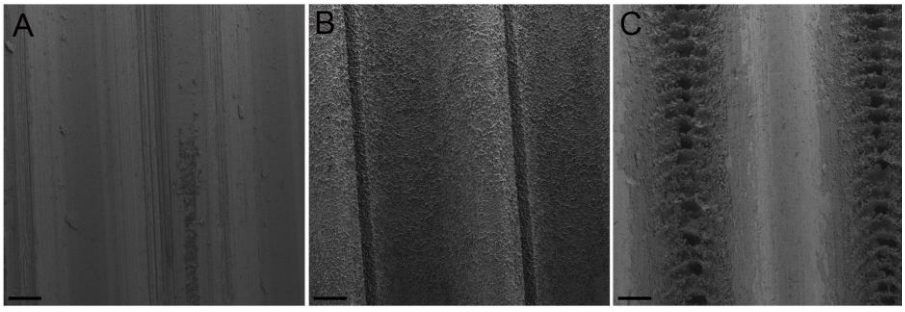

Figure 6. *Scanning electron micrographs of the different surfaces of the three implants in Study II & III; (A) machined, (B) oxidized and (C) laser modified titanium surfaces Scale bar:100µm. (Images were kindly provided by Dr A. Palmquist).*

Study IV: Each patient received two different mini-implants: one with machined surfaces and one with oxidized surface. Dimensions of the mini-implants: 2.3 x 5mm. The mini-implants were manufactured at Nobel Biocare, Gothenburg, Sweden (Figure 7).

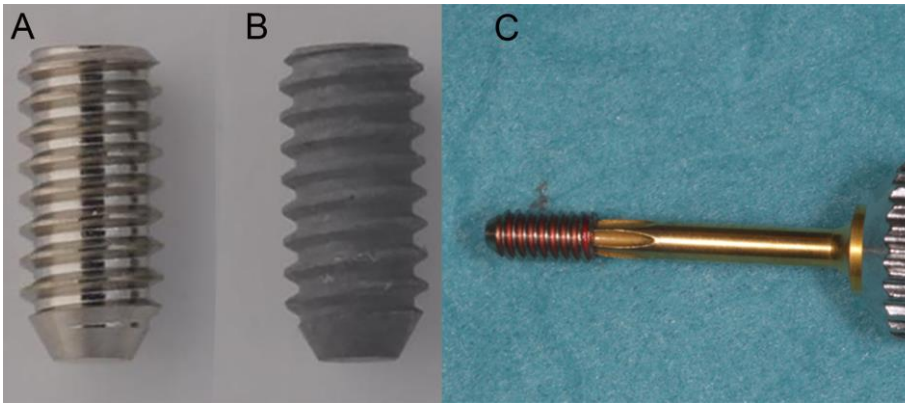

Figure 7. *A) The machined and B) oxidized mini-implants before insertion in Study IV. C) The machined mini-implant after retrieval.*

3.4 Clinical procedures

Studies II, III & IV: Surgical assessments included the evaluation of bone quality and bone quantity according to Lekholm & Zarb.[255] Plaque (PI)[256] and gingival (GI)[256] indices were assessed prior to the implant operation. Since all the patients had a history of periodontitis, it was important to ensure that they were all healthy with respect to periodontal disease before and during the study. Prior to implant site preparation, 2-mm trephines were used to retrieve

bone biopsies from the implantation sites for the subsequent analysis of baseline gene expression. Standard drilling sequences, recommended by the manufacturers, were then followed. For the mini-implants, the drilling was up to 2mm. A transmucosal healing abutment was attached to each implant at the time of installation. The mini-implants were submerged. In the mandible, all the sites were pre-threaded before the installation of the implant. After a healing time of three months, the implants were loaded with a fixed prosthesis. At the time of surgery, the patients were randomly assigned to a time point for the retrieval of the mini-implants (1d, 7d or 28). Each patient received 2 g of amoxicillin (Sandoz, Copenhagen, Denmark) 30 min prior to surgery. The surgery was performed by one operator (SS). The patients were individually informed and given standardized instructions on postoperative care. To minimize the influence of independent variables, all the patients were instructed to use 1 g of *per oral* paracetamol as required to a maximum of 4 g/day. The patients were advised to remain on a soft diet for the first postoperative week. They were also instructed to use a 0.2% chlorhexidine mouthwash twice daily for the first postoperative week.

3.5 Clinical examination and data collection

A dental hygienist, unaware of the given treatments, performed clinical measurements at 90 days and will continue to do so at one year.

At specific time points (1d, 7d, 14d, 28d, 60d and 90d), the postoperative pain experience was assessed using a 100-mm visual analogue scale (VAS) with end points 0 “no pain” and 10 “intolerable pain”.

Resonance frequency analysis (RFA) (Ostell AB, Gothenburg, Sweden) measurements were performed after the surgical implant installation and at specific time points (1d, 7d, 14d, 28d, 60d and 90d).

Plaque and gingival bleeding scores, as per Ainamo and Bay[256]: presence or absence of plaque and bleeding at the gingival margin were recorded at four sites (mesial, distal, buccal and lingual), at baseline for the dentition and at 90d for the dentition as well as for implants. The scores are expressed as %.

Pocket probing depth (PPD) and Bleeding on Probing (BoP) were measured at four points at baseline for the dentition and at 90d for dentition as well as for implants. PPD was assessed as pockets <3mm, 4-5mm or >6mm. BoP was assessed as 0=no bleeding, 1=bleeding.

Biological complications, such as dehiscence, suppuration and screw loosening were recorded at each follow-up appointment.

3.6 Radiology

Study I: After 5 years, the marginal bone loss was analyzed. Implant survival was defined as the presence of the implant in the mouth and functioning at the end of the 5-year follow-up. Most patients had been radiographically examined at the Department of Oral and Maxillofacial Radiology. Intraoral radiographs, using a long-cone paralleling technique, were obtained at the start of loading the fixed prosthesis, thus after 3 to 4 months, and at the 5-year follow-up. The distance was recorded between a reference point (implant-abutment junction or implant head–prosthetic construction) and the marginal bone level on each implant's mesial and distal sites. From these values (mesial and distal) the largest was used in the statistical analysis. If one of the sites was unreadable, the other site was chosen.

When reading film images, a magnifying lens (x7) with a measuring scale divided in tenths of millimeters was used. When reading digital images, the picture archiving and communication system's built-in measuring function, corrected for magnification, was used. One of the authors (KG) was masked to all measurements and was not aware of implant allocation.

Study III: The patients were examined at the Department of Oral and Maxillofacial Radiology. Intraoral digital radiographs, using a long-cone paralleling technique, were obtained at the start of loading the fixed prosthesis, thus after 90 days. The measurements were performed as described for Study I.

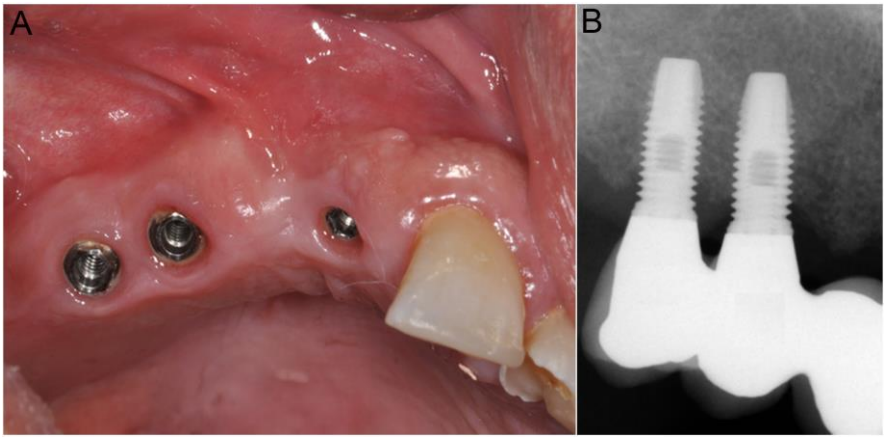

Figure 8. *Selected clinical photograph (A) and radiographs (B) from one smoking patient after 90 days of implantation.*

3.7 Gene expression analyses

3.7.1 Sampling procedure

Studies II & III: At specific time points (1, 7, 14, 28, 60 and 90 days), each implant site was gently air dried. After removing the healing abutment, the area was carefully isolated with cotton rolls. To avoid the salivary contamination of the samples, a saliva ejector was used. One paper strip (Periopaper, Amityville, NY, USA) was inserted into the crevice at the mesial midpoint until mild resistance was felt (Figure 9). After 60s *in situ*, one strip per implant and time point was transferred to a tube with RNAlater (RNAlater, Ambion Inc, Austin, TX). The extraction of RNA was performed and the samples were then stored at -70 C for subsequent gene expression analysis.

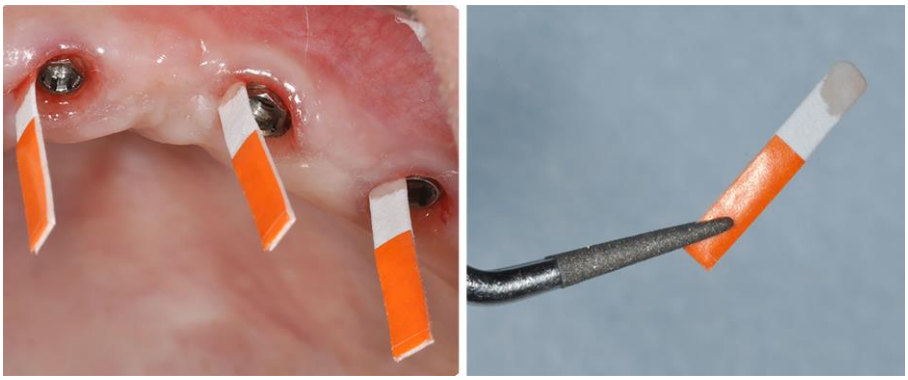

Figure 9. *Clinical photographs show the sampling of peri-implant crevicular fluid for gene expression analysis.*

Study IV: Implant retrieval at 1, 7 or 28 days following surgery, was chosen at random, re-entered and the paired (machined surface/ oxidized surface) implants removed by reverse threading and the peri-implant bone was retrieved by trephine (Ø = 4mm).

3.7.2 Quantitative polymerase chain reaction (qPCR)

In Studies II and III, RNA was extracted from filter strips. In Study IV, RNA was extracted from implant-adherent cells and peri-implant bone. After RNA extraction and purification, it was converted to cDNA. The gene panels analyzed in Studies II, III and IV are shown in Table 4.

The samples were screened for the best stable reference genes using a human reference gene panel (TATAA Biocenter). Quantities of target genes were normalized using the mean of the reference genes 18S rRNA (18S), tyrosine

3/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) (Studies II & III) and Ubiquitin C (UBC) (Study IV).
The normalized relative quantities were calculated using the delta Cq method and assuming 90% PCR efficiency ($k*1.9^{\Delta\Delta Cq}$).[257] The MIQE guidelines for the performance and reporting of the gene expression analysis were followed.[258]

Table 4. Panels of selected genes

PICF (Studies II & III)	**Baseline bone biopsies** (Study III)	**Implant adherent cells and peri-implant bone** (Study IV)
Interleukin-8 (*IL-8*)	Interleukin-6 (*IL-6*)	Interleukin-8 (*IL-8*)
Interleukin-6 (*IL-6*)	Tumor necrosis factor-α (TNF-α)	Interleukin-6 (*IL-6*)
Tumor necrosis factor-α (*TNF-α*)	Alkaline phosphatase (*ALP*)	Tumor necrosis factor-α (*TNF-α*)
Alkaline phosphatase (*ALP*)	Osteocalcin (*OC*)	Alkaline phosphatase (*ALP*)
Osteocalcin (*OC*)	Cathepsin K (*CatK*)	Osteocalcin (*OC*)
Cathepsin K (*CatK*)	Calcitonin receptor (CTR)	Cathepsin K (*CatK*)
Bone morphogenetic protein-2 (*BMP-2*)	Receptor activator of nuclear factor kappa-B *(RANK)*	Calcitonin receptor (*CTR)*
Vascular endothelial growth factor (*VEGF*)	Receptor activator of nuclear factor kappa-B ligand *(RANKL)*	Receptor activator of nuclear factor kappa-B *(RANK)*
	Osteoprotegerin *(OPG)*	Receptor activator of nuclear factor kappa-B ligand *(RANKL)*
	Bone morphogenetic protein-2 (*BMP-2*)	Osteoprotegerin *(OPG)*
	Vascular endothelial growth Factor (*VEGF*)	Bone morphogenetic protein-2 (*BMP-2*)
	Hypoxia-inducible factor-1 *α (HIF-1α)*	Vascular endothelial growth factor (*VEGF*)
		Hypoxia-inducible factor-1*α (HIF-1α)*

3.8 Statistics

All the tests had the significance level fixed at 5% and were performed using SPSS 20 (IBM SPSS Statistics, Armonk, NY).

The X^2 and Fisher's exact test were used to compare implant survival in smokers and non-smokers with the implant as the statistical unit. Student's t-test for independent samples was used for calculations of changes in marginal bone levels (Study I).

Descriptive data were analyzed with the chi-square test and ANOVA. Data normality was tested using the Kolmogorov-Smirnov Test (Studies II, III and IV). The test revealed general non-normal distributions for all genes and non-parametric analyses were therefore considered. Kruskal-Wallis and Mann-Whitney U tests were used with $p < 0.05$ as statistically significant (Studies II, III and IV).

For MBL radiological analysis in Study III, Cohen's kappa coefficient for intra-examiner agreement was used.

All the parameters provided in Studies II & III were evaluated in a bivariate correlation matrix. Further, baseline gene expression data were included in the correlation/regression analysis.

Variables that demonstrated significant correlations with MBL were subsequently entered in a multivariate linear regression model, where MBL was used as the dependent variable. In the regression model, all correlated variables were first entered as predictors and run in stepwise mode without adjustments. In the second step, the model was adjusted for age and implant site, as both showed a significant correlation with MBL in the bivariate correlation analysis. The statistical correlations and regression analyses were performed at 95% confidence intervals and the level of significance was set at $p < 0.05$.

4 RESULTS

4.1 Study I

The objectives of the retrospective study were to evaluate implant survival and marginal bone loss in periodontitis-susceptible smokers and non-smokers and to compare a moderately rough implant surface (oxidized surface) with a smooth surface (machined surface).

Overall, 17 of 252 implants were lost, producing a survival rate of 92.9% over five years. Survival rates were 89.6% for smokers and 96.9% for non-smokers, i.e. significantly lower survival in smokers ($p<0.05$). For smokers with oxidized and machined implants, the survival rates were 96.2% and 84.9% respectively, i.e. significantly lower for machined implants ($p<0.05$). For non-smokers, the survival rates were 96.1% and 96.9% for oxidized and machined implants respectively (no significant difference).

Marginal bone loss was significantly greater in smokers, 1.39 ± 0.16 mm, than in non-smokers, 1.01 ± 0.11 mm ($p<0.05$). For oxidized implants, bone loss was similar for smokers, 1.16 ± 0.24 mm, and non-smokers, 1.26 ± 0.15 mm. Significantly greater bone loss around machined implants was demonstrated in smokers, 1.54 ± 0.21 mm, compared with non-smokers, 0.84 ± 0.14 mm ($p<0.05$). Machined implants displayed significantly lower bone loss than oxidized implants in non-smokers ($p<0.05$) (Figure 10).

In smokers, the likelihood ratio for implant failure was 4.68 compared with non-smokers; after subgrouping, the ratios were 6.40 and 0.00 for machined and oxidized implants respectively.

Regression analyses were performed on all variables (age, gender, jaw, construction [partial/full-arch], bone quality, bone quantity) relating to the influence of implant failure and marginal bone loss. The only variable of significance was the influence of smoking on machined implants ($P < 0.01$, $R^2 = 0.064$, $\beta = -0.838$).

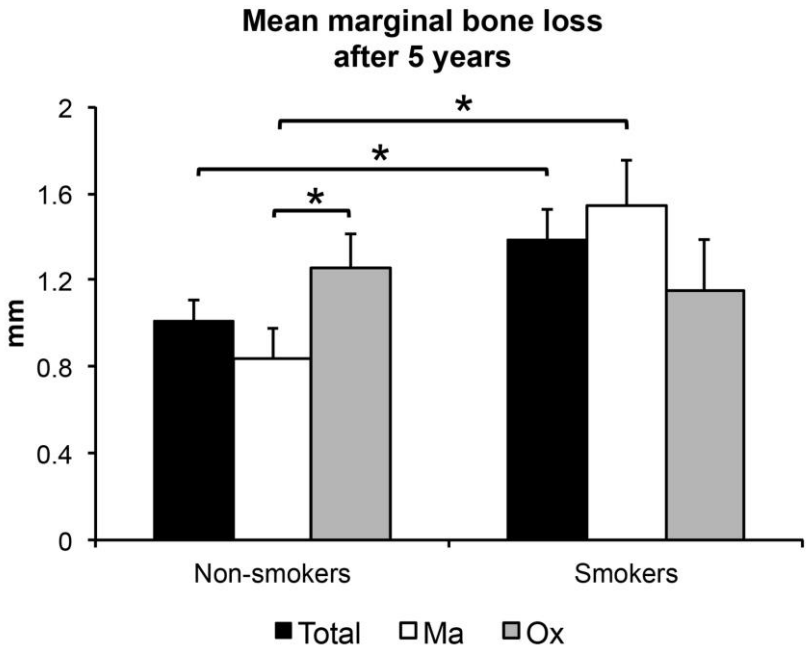

Figure 10. *Marginal bone loss (MBL) at machined (Ma) and oxidized (Ox) titanium implants in non-smokers and smokers after five years. The column graphs show the mean MBL values (in millimeters) and the standard errors of the mean. Statistically significant differences ($p<0.05$) between smokers and non-smokers or between the different implant types are indicated by asterisks.*

4.2 Study II

The objective of this randomized clinical trial was to investigate the initial clinical and molecular course of the osseointegration of different titanium implants in smokers and non-smokers.

In both groups, the highest perception of pain (as determined by the VAS) was found one day after surgery. The mean VAS values were 4.1 ± 0.62 and 3.6 ± 0.55 for the smokers and non-smokers respectively, at 1d postoperatively. During the 28d time period, the postoperative pain gradually decreased to a low level. No significant difference in postoperative pain was shown between smokers and non-smokers (Figure 11). The early high perception of pain correlated to high levels of pro-inflammatory cytokines during the first days after implantation.

A higher expression of vascularization marker VEGF was associated with higher pain scores. Both patient groups demonstrated a negative relationship between VAS score and the expression of growth factor BMP-2, i.e. the higher BMP-2 expression was associated with lower pain scores. In addition, whereas the VAS score was negatively correlated to the expression of bone remodeling factor, CatK, in non-smokers, it showed a positive association with the expression of inflammatory cytokine, TNF-α, in smokers.

The ISQ values were obtained on the day of surgery and further at 1d, 7d, 14d and 28d after surgery. Significantly higher ISQ values were demonstrated in smokers compared with non-smokers on the day of surgery, 1d, 7d and 14d (Figure 12). In the group of smokers, a significantly lower ISQ was detected for the oxidized surface at all the studied time points except 28d, in comparison with the machined implant ($p<0.01$) and the laser-modified implant at 14d ($p<0.01$) (Figure 13). No significant differences were found between the three different implant types in the group of non-smokers (Figure 15). In smokers exclusively, ISQ values correlated to harder and less atrophic bone quality and quantity respectively.
RFA revealed a positive relationship with the expression of OC in both non-smokers and smokers. Furthermore, in the smokers group, positive relationships were found between RFA and the gene expression of VEGF and TNF-α.

Smokers displayed a higher expression of osteocalcin (OC) but a later peak and a lower expression of bone morphogenetic protein (BMP-2) (at 7d) compared with non-smokers. In comparison to machined implants, surface-modified implants were associated with a higher expression of alkaline phosphatase (ALP) and cathepsin K (CatK) at 28d in non-smokers.

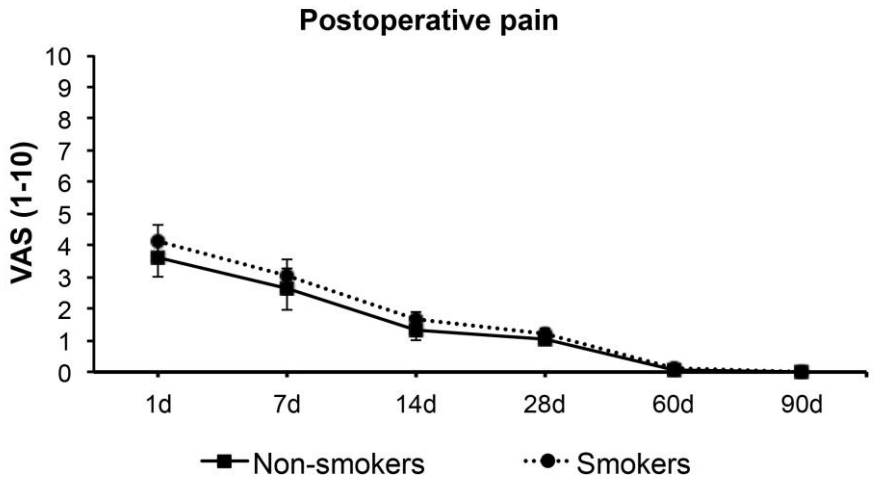

Figure 11. *Post-operative pain for smokers and non-smokers. The figure shows the combined results of Studies II and III.*

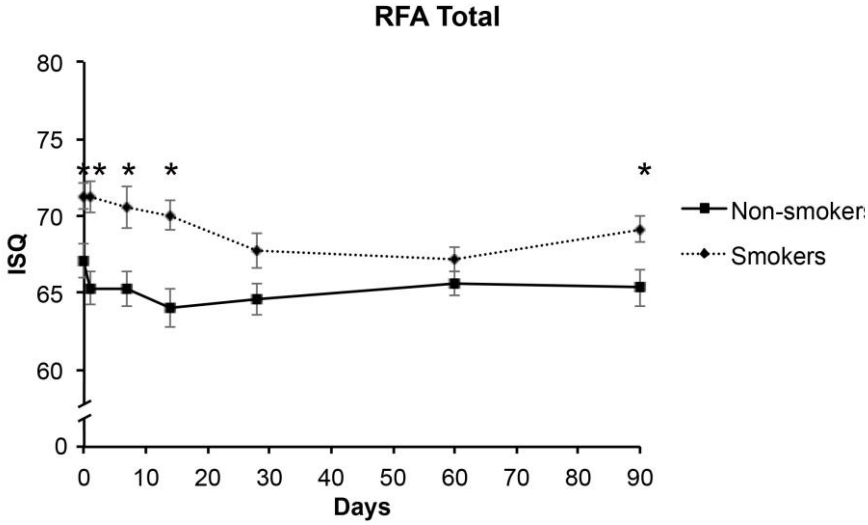

Figure 12. *RFA of smokers and non-smokers. The data are pooled with respect to implant types. Significantly lower ISQ values are detected in non-smokers versus smokers at 0-14d and 90d (P<0.05; asterisks). The figure shows the combined results of Studies II and III.*

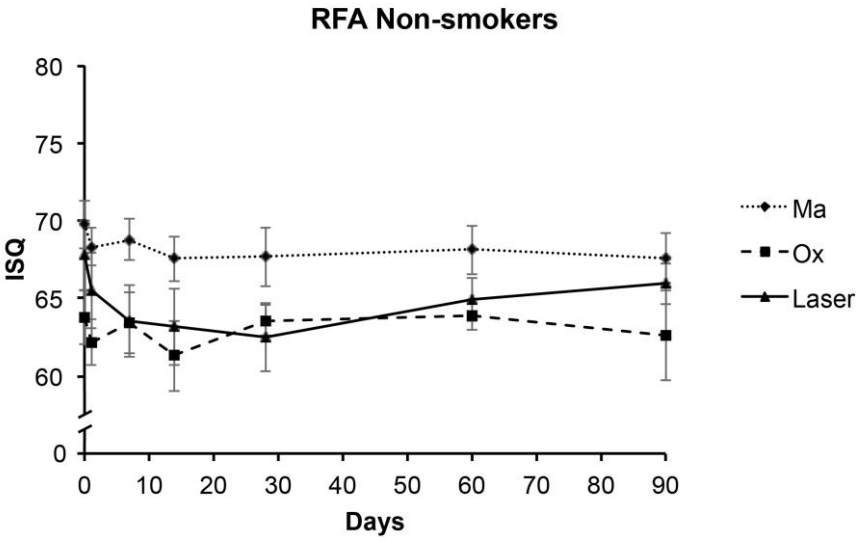

Figure 13. *RFA of the different implants in non-smokers. No significant differences are detected. The figure shows the combined results of Studies II and II.*

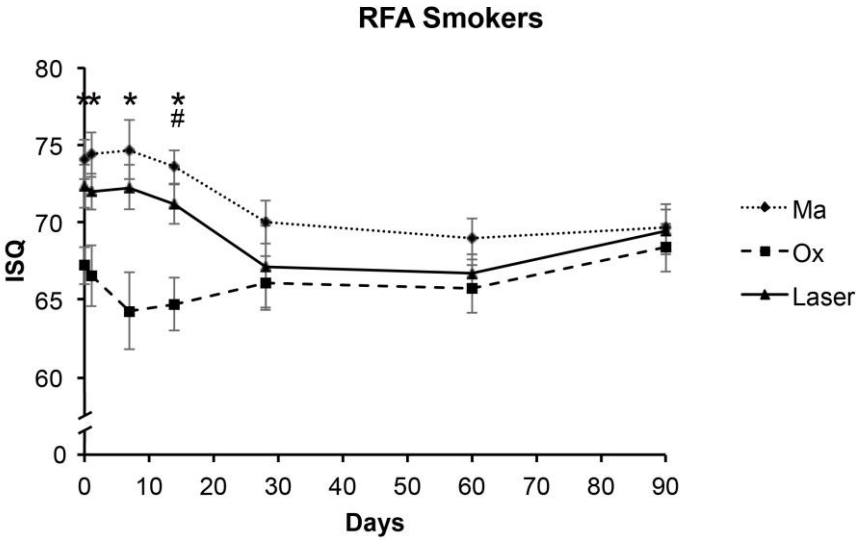

Figure 14. *RFA of the different implants in smokers. Significantly higher ISQ values are observed for the machined (at 1d, 7d and 14d) ($p<0.01$; asterisks) and laser-modified (at 14d) ($p<0.01$; hash sign) implants in comparison with those observed for the oxidized implant. The figure shows the combined results of Studies II and III.*

4.3 Study III

The objectives of this randomized, controlled clinical trial, with the same patient cohort as Study II, were to determine the cellular and molecular events during the late phase of osseointegration (after 60 and 90 days) of different titanium implants and to correlate these data to clinical and radiological observations.

The perception of pain was very low at 60d. The mean VAS score was 0.12 ± 0.08 for smokers and 0.06 ± 0.06 for non-smokers at 60d. No significant difference was demonstrated between the groups. At the end of the study period (90d), all the study subjects registered zero pain (Figure 11).

The ISQ values obtained at 60d and 90d demonstrated no significant difference between smokers and non-smokers at 60d, but at 90d the smokers showed significantly higher implant stability compared with the non-smokers (Figure 12) ($p<0.05$). When analyzing each implant type in each group (smokers and non-smokers), no significant difference was found between the different implants in smokers and non-smokers (Figures 13 and 14).

The mean marginal bone loss (MBL) after 90d was significantly higher in smokers (2.5±0.11 mm) compared with non-smokers (2.1±0.06 mm), when the data were pooled for all implant types (Figure 15A). When analyzed according to the different implant types, the mean MBL in the non-smokers was 2.0 ± 0.08 mm for machined surfaces, 2.0 ± 0.07 mm for laser-modified surfaces and 2.1 ± 0.01mm for the oxidized surfaces. No statistically significant differences were detected between the different implant types among non-smokers. In the group of smokers, the mean MBL was 2.6 ± 0.16 mm for the machined surfaces, 2.4 ± 0.21 mm for laser-modified surfaces and 2.4 ± 0.18 mm for the oxidized surfaces. A significantly higher MBL at machined surfaces was found for smokers in comparison with non-smokers (Figure 15B). There were no significant differences between smokers and non-smokers with respect to MBL at laser-modified or oxidized implants respectively.

A 13.1- and 4.4-fold higher expression of IL-6 was demonstrated at the machined and oxidized surfaces respectively, compared with the laser-modified surfaces at 90d in smokers. A significant 8.1-fold higher gene expression of OC was shown at the machined surfaces at 90d for non-smokers compared with smokers. The expression of CatK demonstrated a 3.7-fold upregulation at the laser-modified surfaces in non-smokers at 60d. OC demonstrated 4.2- and 3.9-fold higher expression at laser-modified and oxidized surfaces respectively, compared with the machined surfaces in smokers at 90d. The expression of CatK was 6.3- and 8.2-fold higher at the

machined and oxidized surfaces compared with the laser-modified surfaces at 60d for smokers.

Multivariate regression revealed the following predictors of MBL, after adjustment for age and implant location (maxilla/mandible): smoking, bleeding on probing at 90d, hypoxia-inducible factor 1 alpha (HIF-1α) expression in the recipient bone at baseline and IL-6 expression in PICF at 90d.

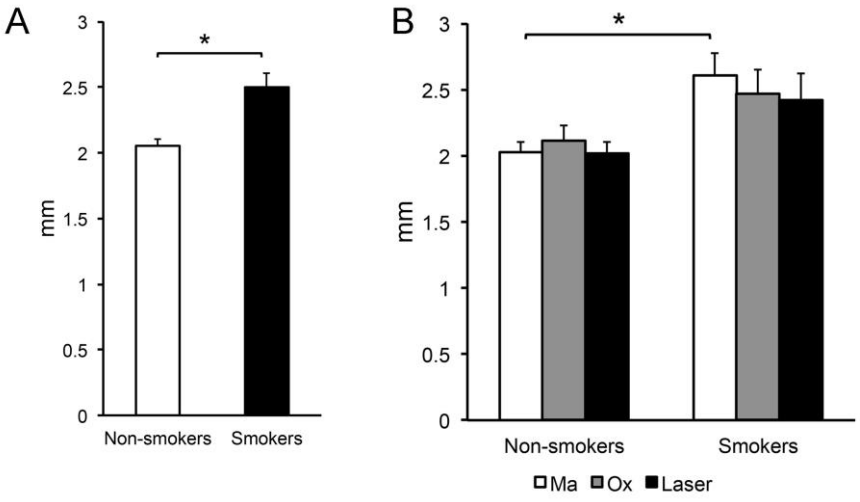

Figure 15. *(A) MBL at implants (data pooled for the different implant types) in non-smokers and smokers after 90 days of implantation. (B) MBL at machined (Ma), oxidized (Ox) and laser-(Laser)-modified titanium implants in non-smokers and smokers after 90 days of implantation. The column graphs show the mean MBL values (in millimeters) and the standard errors of the mean. Statistically significant differences ($p<0.05$) between smokers and non-smokers or between the different implant types are indicated by asterisks.*

Based on the results obtained from the regression analysis, we examined whether the MBL and the expression of HIF-1α in the baseline recipient bone differed between the maxilla and mandible in smokers and non-smokers. The results demonstrated a significantly higher MBL in the maxilla of smokers compared with the maxilla of non-smokers (Figure 16A). When HIF-1α baseline gene expression was analyzed, it was significantly downregulated 1.6-fold in the maxilla of smokers compared with the maxilla of non-smokers (Figure 16B). Moreover, in smokers, the baseline expression of HIF-1α was 2.3 times higher in the mandible compared with the maxilla.

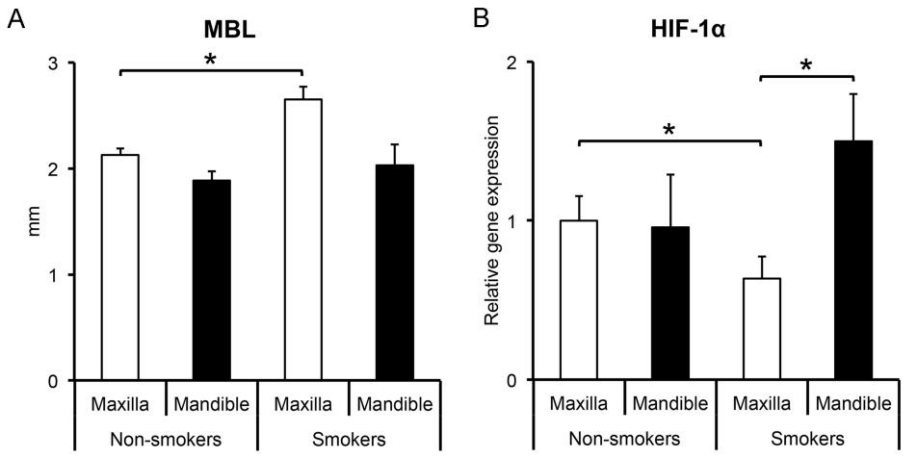

Figure 16. *Marginal bone loss (MBL) and baseline expression of hypoxia-inducible factor 1-alpha (HIF-1α) in the maxilla and mandible. (A) MBL at implants (data pooled for the different implant types) comparing the maxilla and mandible in non-smokers and smokers after 90 days of implantation. (B) Baseline gene expression of HIF-1α in the implantation sites comparing the maxilla and mandible in non-smokers and smokers. The column graphs show the mean MBL values and the mean relative gene expression respectively, together with the standard errors of the mean. Statistically significant differences ($p<0.05$) between the maxilla and mandible or between smokers and non-smokers are indicated by asterisks.*

4.4 Study IV

The objective of this randomized, controlled clinical trial was to compare the molecular events in the implant-adherent cells and in the peri-implant bone during the osseointegration of different titanium implants in smokers and non-smokers.

Differences between machined and oxidized implants in non-smokers and smokers were evident in the implant-adherent cells but not in the peri-implant bone.

When comparing implant-adherent cells in smokers versus non-smokers, a 4.5-fold lower expression of TNF-α was demonstrated at 28d in cells adhering to machined implants in smokers compared with cells adhering to machined implants in non-smokers. Comparing the implant-adherent cells with respect to the two implant types, 2- and 6.5-fold higher expressions of TNF-α were demonstrated in cells adhering to the machined implants compared with cells adhering to oxidized implants in smokers at 1d and 7d respectively. In the non-smokers, the expression of TNF-α was 1.7-fold significantly higher in cells adhering to machined implants compared with those adhering to oxidized

implants at 7d. Furthermore, at 28d in non-smokers, the expression of IL-8 was upregulated ninefold in cells adhering to machined implants compared with cells adhering to oxidized implants (Figure 17A).

In the cells adhering to oxidized implants in non-smokers, relatively high peaks of ALP and OC were detected at 1d and they did not change significantly up to 28d, whereas in smokers the peak expression of ALP and OC was observed after 7d and did not change significantly thereafter. The comparison of implant-adherent cells between smokers and non-smokers revealed a lower expression of both ALP and OC in the smokers at 1d, particularly in cells adhering to oxidized implants. However, after 7d, the expression of ALP and OC increased in the cells adhering to oxidized implants in smokers where a significantly higher expression of ALP was demonstrated at oxidized implants in smokers compared with non-smokers (Figure 17B).

The comparative analysis of osteoclastic genes between smokers and non-smokers at 7d revealed a higher expression level of CTR in cells adhering to machined implants in non-smokers compared with CTR expression (undetected) in cells adhering to machined implants in smokers (Figure 17C).

Both RANKL and OPG were triggered to higher levels at 7d, at both implant types in non-smokers and only at oxidized implants in smokers. The RANKL and OPG expressions were not detected in cells adhering to the machined implants in smokers at 7d. Their peak expressions were instead seen later after 28d of implantation. In the peri-implant bone, both RANKL and OPG already showed high levels at 1d, but they decreased to lower expressions after 28d. When comparing the two implant types, higher expressions of RANKL and OPG were detected in cells adhering to oxidized implants compared with cells adhering to machined implants after 7d in smokers. No differences in RANK, RANKL and OPG expressions were detected between the two implant types in the peri-implant bone (Figure 17D).

Comparing smokers and non-smokers, the cells adhering to machined implants in non-smokers revealed a higher VEGF expression compared with cells adhering to machined implants in smokers. Comparing the implant types in smokers, the cells adhering to oxidized implants showed a 4.5-fold higher BMP-2 expression compared with machined implants. On the other hand, a higher expression of VEGF was found at 28d in cells adhering to machined versus oxidized implants in non-smokers.

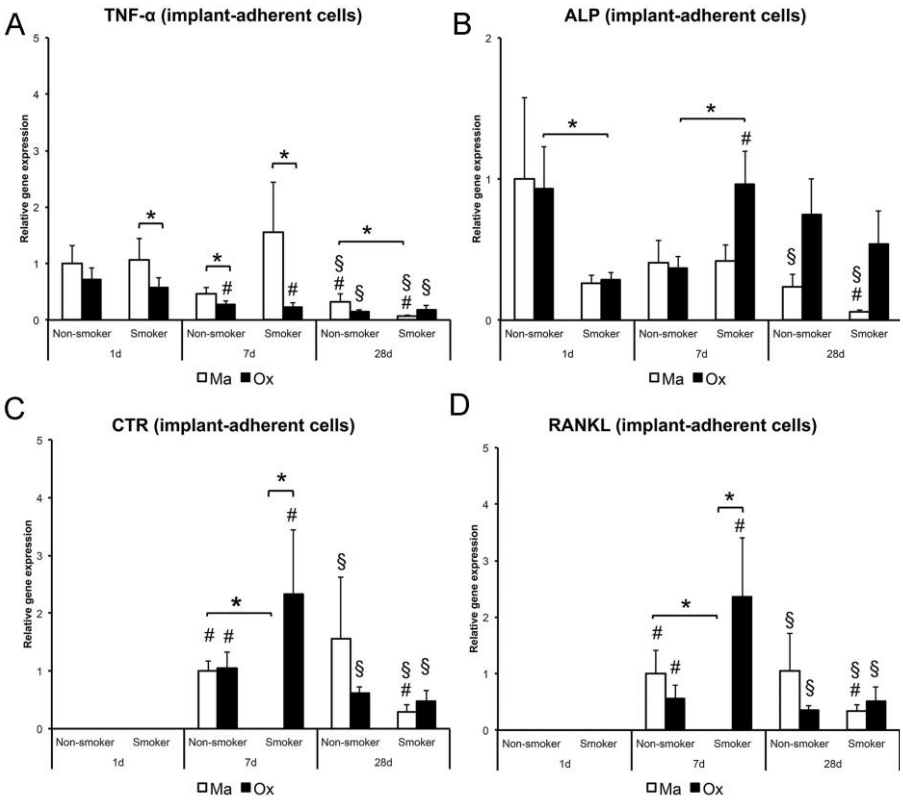

Figure 17. *The data show the expression of selected cytokines in the implant-adherent cells around machined (Ma) and oxidized (Ox) titanium implants in non-smokers and smokers after 1, 7 and 28 days. The analysis targeted: tumor necrosis factor-alpha (TNF-α)(A), alkaline phosphatase (ALP)(B) calcitonin receptor (CTR)(C) and receptor activator of nuclear factor kappa-B ligand (RANKL)(D). The column graphs show the mean relative gene expression and the standard error of the mean. Statistically significant differences ($p<0.05$) are indicated as follows: an asterisk (*) shows the significant difference when comparing the two implant types and smokers and non-smokers; the hash sign (#) shows the significant difference between two consecutive time points (1d versus 7d and 7d versus 28d) for each implant type and in each patient group; the section sign (§) shows the significant difference when comparing 1d versus 28d for each implant type and in each patient group.*

5 DISCUSSION

5.1 Methodological considerations

5.1.1 Study group and selected follow-up period

This thesis consists of one retrospective study and three prospective, randomized, clinical studies. Randomized clinical trials (RCT) are often used to measure the efficacy/effectiveness of different interventions and can provide important information about adverse effects of conditions or treatments. They are often regarded as the gold standard in research.[259] The methodological platform of the studies in this thesis consists of a combination of clinical, radiological, biomechanical and molecular analyses.

The size of the cohorts could be regarded as small. RCTs are considered to be the most reliable data provider in the field of research, but they often include small sample sizes that may reduce their scientific value.[260] However, adequate power for statistical comparative and correlative analyses is provided, in the studies in this thesis. Moreover, the allocation and randomization were computerized in all four studies, which reduced bias in selection and confounding factors in RCTs.

The majority of human studies of the effects of smoking on implant survival and MBL have used relatively long observation periods. Whereas the retrospective Study I had a five-year follow-up period, the prospective Studies II-IV had a much shorter follow-up. Both early and late time periods are important. One of the main reasons for selecting the early (< 3 months) post-implantation period is the opportunity to explore the role of early molecular, cellular and clinical parameters for the development of osseointegration/implant stability. Obviously, it is preferable to study the maintenance of osseointegration using long-term (years) postoperative follow-up periods. Moreover, the role of superstructure attachment and functional loading cannot be determined unless longer time periods are used. In this case, this would mean that the patient cohort in the prospective Studies II-IV should be followed in order to analyze the effects of smoking after loading, including late outcomes. It is the author's intention to monitor this cohort and obtain one- and five-year data.

Intra-oral radiographs using a parallel technique were obtained at 90d, before superstructure connection and implant loading (Studies I and III), and after five years of function (Study I). No customized jigs or detector holders were used

for the radiographic examinations in either Study I or Study III, since the screw-shaped implant design facilitates the accurate assessment of correct vertical projection.[261] Assessments of the radiographs were blinded and were performed by a single, experienced specialist in oral and maxillofacial radiology, who was unaware of the study groups and different implant types. High intra-examiner agreement was found and is presented in Study III.

Measurements of cotinine levels in blood, saliva or urine are considered to be the most accepted objective method of evaluating exposure to tobacco smoke.[262] In the studies included in this thesis, all smoking habits are self-reported. The prevalence of smoking based on self-reports is generally lower than estimates based on cotinine levels.[263] It is indicated that this discrepancy varies by country, cultural and socioeconomic factors.[264] Nevertheless, a study validating self-reported smoking status in Canada, a country similar to Sweden with regard to socioeconomic levels and culture, demonstrated that self-reported smoking habits have good reliability.[265]

5.1.2 Sampling and molecular analyses

In the present thesis, a sample of peri-implant crevicular fluid was taken using filter strips and a protocol largely derived from a pilot study.[247] One of the main advantages of this procedure is its non-invasiveness. Another advantage is that small sample volumes can be retrieved and used for large-scale molecular analyses.

One disadvantage is that the results are based on the entire cell population present in the PICF. We did not attempt to designate the specific gene expression to a particular cell type in this compartment. An approach of this kind would be interesting to pursue using flow cytometry and cell sorting, for example, followed by the extraction of RNA and subsequent sensitive gene and proteomic analyses. One prerequisite for an attempt like this would be a sufficient sample of biological peri-implant material, most likely utilizing a larger number of paper points than were used in the present studies.

Interestingly, several gene expression markers of osteoblasts and osteoclasts were detected in the PICF. This finding indicates the absence of a structural barrier between the implant-bone interface and the abutment-soft tissue interface, possibly allowing the passage of cells between the two compartments at least during the initial phase of healing.

In Study IV, we used a miniature implant model with two different implant surfaces: machined and oxidized. The mini-implants were retrieved after 1d, 7d and 28d for an analysis of the implant-associated gene expression of

implant-adherent cells. After un-screwing the miniature implant, the surrounding peri-implant bone was retrieved using a trephine in order to evaluate the gene expression in cells in the peri-implant bone. Although this method is invasive, it is ethically acceptable since it is performed in combination with other necessary dentoalveolar surgery. In this patient cohort (Study IV), no additional morbidity was reported. Taken as a whole, this was a safe experimental model in humans.

The molecular technique used in Studies II-IV was qPCR. All the primers were designed and validated to ensure optimal efficacy and specificity. To be able to compare gene expression between different samples, regardless of starting volume and mass of material, normalization with reference genes was performed in our studies. A panel of reference genes was screened and validated for each individual qPCR run, in order to determine the most stable reference gene(s), which may vary between the different studies and conditions. Throughout the thesis, we adhered to the MIQE guidelines.[258]

Another aspect is that the present method measures the cellular activity at RNA level (Studies II-IV). A strengthening factor would be to attempt to make measurements at protein level; however, there are still limitations to the sensitivity and the number of biological factors that can be evaluated with the current protein detection assays. In an exploratory study (to be reported separately), we have collected PICF samples (n=10) from a sub-group of the present cohort, to be analyzed using a novel technique for large-scale protein analysis.

One limitation of the present studies is the absence of morphological data, mainly pertaining to the mini-implants. This decision was made for several reasons, including our focus on gene expression, in turn limiting the number of implants available for histology. In addition, the majority of previous experimental[179, 181-184] and human[266-268] studies evaluating the effects of smoking/nicotine have used histology but not molecular analytical tools as the main analytical technique.

5.2 Implant survival

Although implant failure is a known risk when performing treatments with dental implants, it can be a dramatic event for the patient and can compromise the entire prosthetic rehabilitation. It has been suggested that early and late failures are associated with different cellular and molecular events.[11] Early failure indicates an impaired and unsuccessful osseointegration, where the normal mechanisms of bone healing have not been operative, instead leading

to the formation of a fibrous scar tissue around the implant surface. This prevents the implant from achieving osseointegration.[269] Late failures, on the other hand, take place after successful osseointegration is achieved. These failures have been suggested as the result of both a microbial challenge[270] and an inappropriate load distribution after prosthetic construction.[271] Studies of dental implants point to a higher rate of implant failure among smokers compared with non-smokers. Tobacco smoking represents a risk factor for implant loss,[160, 163, 176] biological complications, and marginal bone loss.[162, 164, 177, 272]

In agreement with previous long-term observations,[163, 176] the present thesis demonstrates that implant survival is lower in smokers after a five-year follow-up (Study I). Implant failures were recorded between three months and five years. The present retrospective data did not provide a tentative explanation for this finding. In the literature, an association between smoking and biological complications (e.g. peri-implantitis and peri-implant mucositis) and marginal bone loss[163-165] has been suggested.

One important finding after five years (Study I) was the observation of a lower implant survival for machined implants in comparison with oxidized implants in the group of smokers. It has been suggested that the oxidized surface is favorable in terms of implant survival.[92, 273] Albeit speculative, one possible explanation could be that the oxidized implants were more successfully osseointegrated and thereby less prone to adverse smoking-induced biological responses like inflammation and bacterial contamination. Support for this hypothesis is derived from experimental studies showing that oxidized implants promote bone formation and remodeling in comparison with machined implants [3-5] ultimately leading to a stronger bone anchorage.[5] This hypothesis, however, is contradicted by results of experimental studies in dogs showing that ligature exposed moderately rough implants have increased plaque accumulation and inflammation after 6 months in comparison with machined implants.[274, 275] On the other hand, our findings of greater marginal bone loss around oxidized implants compared with machined implants in non-smokers is at least in partial agreement with the latter study. Taken together, the present thesis provides additional evidence that smoking has a detrimental effect on the longevity of dental implants. Moreover, we provide novel findings that the implant surface properties play an important role in the longevity of implants in smokers.

Against this background, it is of interest to determine whether early failures are also more common in smokers, whether the cellular and molecular events of early osseointegration differ between smokers and non-smokers and whether the material surface properties influence the early biological processes

of osseointegration differently in smokers and non-smokers. In Studies II and III, we used the PICF for the molecular analysis during the process of osseointegration, whereas, in Study IV, interest focused on the implant-adherent cells at the bone-implant interface and the peri-implant bone. In fact, the implant-adherent cells are the ones that most likely represent the actual cellular compartment that governs bone healing and regeneration at the bone implant-interface.

Firstly, the reported early failures appeared before 28 days and did not differ significantly between smokers and non-smokers (Studies II and III). Secondly, in the group of smokers, higher pro-inflammatory gene expression and lower osteogenic gene expression in implant-adherent cells were associated with machined implants compared with oxidized surfaces (Study IV). Moreover, it was evident that smoking had a major inhibitory effect on the initial trigger of bone-remodeling activity in the implant-adherent cells, particularly at the machined implants. Taken together, the present human *in-vivo* observations are in agreement with a number of published articles showing inhibitory effects of smoking/nicotine on osteoblast cell proliferation, differentiation and matrix mineralization *in vitro*[194, 195, 203, 218] and the gene expression of multiple factors (e.g. ALP, collagens, bone sialoprotein and BMP-2) important for bone healing and regeneration in rat and rabbit experimental models.[180, 212, 221] Importantly, the present studies demonstrate that these inhibitory effects of smoking/nicotine are also detected in implant-adherent cells and that they are mainly expressed at the machined implant surface. Although direct proof is not provided, the possibility cannot be excluded that the differently expressed factors in relation to inflammation, bone formation and re-modeling at this early stage of osseointegration at machined implants in smokers may affect long-term osseointegration, implant stability and survival. These results vary from those in mini-implant studies comparing sandblasted implants and sandblasted/hydrofluoric acid-etched implants in smokers and non-smokers,[217, 251] demonstrating few molecular differences between the groups. This discrepancy in results could be due to the different surface properties of the mini-implants used in the studies. In contrast to the gene expression in implant-adherent cells, the analysis of PICF gene expression did not reveal major differences between smokers and non-smokers or between machined and oxidized implants until 90 days. Higher pro-inflammatory cytokine (IL-6) and lower bone formation gene (OC) expression in PICF were detected at 90 days at the machined implants compared with both oxidized and laser-modified implants in smokers. Importantly, these observations were associated with greater marginal bone loss at machined implants in smokers at 90 days (Study III).

5.3 Clinical parameters

5.3.1 PI, GI and BoP

The periodontal parameters were assessed in conjunction with implant insertion in all four studies (I-IV). In Study I, these parameters were registered in the data base used for the retrospective study and in Studies II-IV they were assessed after enrolment into the studies. The periodontal scores were generally low, indicating a well-controlled patient group, despite that all patients had a history of periodontitis. For this patient category in particular, it is extremely important to have a comparable healthy baseline for all the test and control groups within the studies, since it is well documented in the literature that periodontitis susceptibility is associated with lower survival rates and higher incidences of biological complications.[276-278]

In all four studies (I-IV) the groups of smokers and non-smokers were comparable in terms of gender, number of teeth, bone quality, bone quantity and the implant location (maxilla/mandible). Smokers were younger than non-smokers. This reflects the epidemiological view that smokers have more tooth loss at a younger age and poorer oral health.[147, 149, 279, 280]

In Study III we went beyond the baseline parameters and we assessed both clinical/periodontal parameters for the dentition and the installed implants included in the study, at 90 days. This enabled us to compare these variables of dentition at baseline and 90 days, to confirm that the minimal signs of periodontitis at baseline, are continuous and well maintained. Although all the patients had a history of periodontal disease, both non-smokers and smokers generally exhibited low clinical periodontal scores at both dentition and implants which did not progress from baseline to 90 days.

Furthermore, a comparison between the dentition and the implants was made. Here, it was shown that the comparison of the plaque index (PI) between dentition and implants and between smokers and non-smokers did not demonstrate any differences. However, the gingival index (GI) was significantly higher around the dentition than the implants in smokers. Interestingly, in a prospective study evaluating the periodontal and peri-implant status around teeth and implants in function, Zhuang and colleagues [281] did not demonstrate any difference in GI between healthy teeth and healthy implants. A possible explanation for this discrepancy in results could be due to the superstructure not yet being mounted, which facilitated the cleaning of the implants compared with the dentition, resulting in a lower GI around the implants in Study III.

Bleeding on probing (BoP) alone is indicative of soft-tissue inflammation and, when accompanied by suppuration it indicates further pathological processes.[282, 283] Although the sensitivity of BoP is low over time, the specificity is high. The absence of BoP may therefore be regarded as a reliable tool to assess periodontal health [284] and has also been demonstrated for implants.[285, 286] Although not statistically significant, there was a trend towards a higher BoP around implants in comparison to the dentition in Study III. It has been demonstrated that the soft tissue around implants is differently organized compared with the soft tissue around teeth.[72] Collagen fibers in natural teeth are perpendicularly oriented, attaching from the tooth cementum to the alveolar bone and serving as a barrier to epithelial down-growth and bacterial invasion. The collagen fibers are oriented in a parallel manner to the implant surface, due to the lack of a cementum layer at the implants. This type of fiber orientation makes the structure more prone to breakdown and subsequent bacterial invasion. Implants lack periodontium altogether, which also presents a potential risk of a more rapidly advancing inflammatory process.[75]

5.3.2 Pain

One important finding in Studies II and III was that the scores for postoperative pain peaked at 1d and rapidly decreased over the 14 first days postoperatively and to become non-existent at 90 days in both smokers and non-smokers. Consequently, no difference in the perception of pain was found between smokers and non-smokers. This indicates that treatment with dental implants is predictable and associated with little morbidity. Another important finding in Study II was that the group of patients which experienced early failure of osseointegration reported greater pain scores at 1d and 7d. Comparable findings have recently been reported, suggesting an association between pain manifestation and implant failure.[287, 288] Although not providing a mechanistic explanation, one plausible reason for the high pain scores at 1d and 7d in the failure group was the up-regulated expression of pro-inflammatory cytokines in the PICF. It is likely that excessive inflammation and attenuated regenerative signals are involved in the early failure to achieve osseointegration, indicated by the fact that the incidence of failure was correlated to a higher expression of pro-inflammatory cytokines and lower expression of BMP-2 in non-smokers. However, these assumptions should be viewed with great caution, due to the small sample size of the failed group. Further studies of the mechanisms of implant failure are needed. It has also been stressed in the orthopedic literature that the cause of excessive pain in conjunction with implant treatment, always needs to be assessed and if possible, removed.[289] The upregulation of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α has been associated with the process of pathological

pain and pain during inflammation.[290] In Study II, the link between inflammation and pain was established, indicated by the peak in postoperative pain perception corresponding to the highest expression levels of the cytokines, IL-8, IL-6 and TNF-α, at 1d and 7d, followed by a reduction after 14d. The correlation analysis in Study II, confirmed the association between pain and the expression of pro-inflammatory cytokines in both non-smokers and smokers. No major differences in the expression of pro-inflammatory cytokines were found between the different implants when comparing smokers and non-smokers in Studies II and III. However, exclusively in smokers, the expression of IL-6 at 90d (Study III) was up-regulated at machined surfaces compared with both oxidized and laser-modified surfaces, indicating a higher degree of inflammation at machined implants. In Study IV, in both implant-adherent cells and cells in the peri-implant bone, the peak of inflammation, as judged by the expression of pro-inflammatory cytokines, was observed after 1d, subsequently decreasing to the lowest levels after 28d irrespective of implant surface properties and smoking habits. The temporal findings on both sources of cells are in line with the transient inflammatory process expressed in the PICF (Study II and III) during the 90 first days of osseointegration. This suggests that successful osseointegration in non-compromised patients is associated with an initial inflammatory response which is attenuated over time and overlapping with the subsequent regenerative process.

Nicotine induces analgesia in animal models and it has been suggested that nicotine has analgesic properties, due to the effect on nicotine acetylcholine receptors.[126, 127] On the other hand, clinical studies indicate an over representation of smokers with chronic pain.[125] This may be a result of receptor desensitization and tolerance, which is developed rapidly after regular exposure to nicotine.[128, 129] In our studies, we were not able to find any differences between smokers and non-smokers regarding pain, and hence neither an analgesic nor a sensitization in pain perception in the smoking group can be verified.

5.4 Implant stability

The measurement of implant stability is an important method for the evaluating implant-success. Several methods for the assessment of implant stability are available.[291] Resonance frequency analysis (RFA) is one of the few tools for the objective clinical measurement of oral implant stability.[235] RFA has been used in the clinical setting because of the non-invasive nature of the measurement. RFA has been widely used for clinically assessing osseointegration, as well as for prognostic evaluation.[292] Nevertheless, the prognostic value of RFA has been questioned, and in a review addressing this

matter, it was stated that the prognostic value not has yet been established [293]. The question of whether RFA provides a true measurement of osseointegration at all has also been discussed. It has recently been indicated in experimental studies in rabbits, that RFA does not correlate to bone in contact with the implant,[239] which is the major structural determinant of osseointegration,[1] but instead correlating to the bone area around the implant.[108]

The results of Studies II and III, showing a higher ISQ in smokers than non-smokers seem at variance with the results of a recent study [294] comparing clinical parameters and RFA in smokers and non-smokers. Similar to the finding by Sun and co-workers,[294] a higher ISQ was detected in smokers compared with non-smokers until 14d postoperatively. In contrast, whereas the latter study revealed a significantly higher ISQ in non-smokers than smokers, three weeks post-surgery up to eight weeks post-surgery,[294] the present studies demonstrated a higher ISQ in smokers up to 90d. There are major methodological discrepancies between the two studies. Patients in the study reported by Sun and co-workers [294] were heavy smokers (>20 cigarettes or more) and all the implants were placed in the mandible. Few of the individuals in the present group of smokers (>10 cigarettes) smoked more than 15 cigarettes/day and the majority of the implants in the present study were placed in the maxilla. *In vitro* studies have indicated, that smoking affects bone in a graded manner: high concentrations have detrimental effects but low concentrations may even stimulate proliferation and osteogenic differentiation.[201, 202] The site of implants has also been a subject of discussion in the literature, with the maxilla being associated with lower implant survival.[161, 244, 295]

At present, the reason for the increased RFA in the smokers cannot be established. Taking the findings in Studies II and III together, major structural and molecular differences between the bone of smokers and non-smokers are indicated. Based on these observations, it is hypothesized that the jawbone of smokers has a different composition and organization compared with that of non-smokers. It is also suggested that the dose and duration of tobacco exposure could have important effects on the bone and the degree of stability of implants.

5.5 Marginal bone loss

5.5.1 Assessment of marginal bone loss

In Studies I and III, the MBL was assessed with a difference in the time points of MBL registration. In study I, the measurements were made at loading and

then after five years of loading, whereas, in Study III, the MBL was assessed from the insertion of the implant and after 90 days in conjunction with the loading of the implants. The time points of radiological assessments in Study I are the most common and conventional in the literature; at loading time and then after five years. In Study III, we assessed the distance between the marginal bone and the platform of the implant, revealing the MBL from implant insertion to the loading time and thereby during early healing/remodeling. On the basis of experimental data[108] and the fluctuation in ISQ values during healing in humans,[243] it is likely that the greatest bone remodeling occurs during this very early time phase, suggesting that increased MBL in smokers during this early time period might be related to different bone homeostasis in smokers, resulting in a net imbalance between the anabolic and catabolic pathways, favoring bone resorption. In line with this assumption, a prospective clinical study demonstrates that bone loss at implant placement up to prosthesis insertion is several times higher than the bone loss occurring between prosthesis insertion and the five-year follow-up.[244] The clinical relevance of MBL at this early time point (90d) could be questioned but taken together with the fact that the original and still existing criteria for implant success are defined as the amount of MBL,[240-242] the observed early MBL around implants in smokers could in fact potentially lead to implant failure. It is therefore crucial to minimize MBL in the early treatment stages. There is no single clear cause of MBL as we know it, but some factors have been discussed in the literature, such as surgical techniques, smoking and operator experience, degree of surgical trauma, bacterial contamination and susceptibility to periodontitis.

5.5.2 Marginal bone loss: smoking, implant surfaces, jawbone and molecular markers

In studies I and III, we demonstrate that the overall marginal bone loss was significantly greater in smokers compared with non-smokers after five years and as early as 90 days, respectively, following implant installation. The results from Study I corroborate previous observations of greater MBL in smokers after relatively long follow-up periods.[164, 177]

Whereas surface-modified implants demonstrated similar marginal bone loss in smokers and non-smokers, a significant difference between smokers and non-smokers was found for machined surfaces, in both Study I and III. In the non-smokers at 28d, the oxidized and laser modified implants expressed a higher level of osteoblastic gene, ALP, and bone remodeling gene, CatK, compared to machined implants in PICF (Study II). Furthermore, at 90d, smokers with machined implants exhibited a down-regulation of OC in the

PICF compared with non-smokers, as well as in comparison with the other two surfaces, oxidized and laser modified (Study III). The molecular data are in agreement with the results in experimental studies, in an uncompromised rat model, which revealed a higher expression of bone formation and remodeling factors in cells adherent to surface-modified implants compared with machined implants in the early phase of osseointegration.[4, 5] Moreover, in line with the present data, a human study demonstrated that implants with combined nano- and micro-surface modification enhanced the early expression of osteogenic factors OC and osterix, compared to micro-rough implants.[250] Taken together, these data suggest that different surface modifications trigger the early osteogenic differentiation in the implant-adherent cells in bone-implant interface zone and this appears to be mirrored in the crevicular fluid around the implant.

Bone formation and bone resorption are processes which are controlled by the coupling triad RANK/RANKL/OPG.[33] In Study IV, a low expression of RANK and no expression of CatK, RANKL and OPG were detected at 1d, in the implant-adherent cells. The expression of the genes involved in the bone remodeling (CatK, RANKL and OPG) was up-regulated at oxidized implants, at 7d, in the smokers exclusively whereas this effect was delayed on the machined surface. This finding suggests that the machined implants did not possess the same capacity as the oxidized implants to enhance osteoclastic remodeling activity in the smokers. The observations that oxidized implants rapidly trigger both osteoblastic and osteoclastic differentiation and remodeling coupling activities at the implant-bone interface (Study IV) and enhance the expression of bone formation markers, ALP and OC, in the PICF (Study III), indicate that the oxidized surface establishes a microenvironment at the implant-bone interface, which counteracts the negative effects induced by smoking. Experimental[296] and clinical[297-299] studies partly support the assumption that the oxidized surface conducts a positive effect under compromised conditions of osseointegration.

In Study III, a regression model was used in which MBL was used as the dependent variable. In this regression model, all the correlated variables were first entered as predictors and run in stepwise mode without adjustments. In the second step, the model was adjusted for age and implant site, as both showed a significant correlation with MBL in the bivariate correlation analysis. When adjusted for age and implant location (maxilla/mandible), the predictors of MBL were smoking, IL-6 expression in the PICF at 90d, BoP at 90d and HIF-1α expression in the recipient bone at baseline.

The finding of an association between MBL and BoP at 90d denotes BoP as a predictor of biological complications in relation to dental implants. It is well-known that BoP is an important indicator of an ongoing inflammatory process.[234]

The expression of IL-6 in PICF at 90d was associated with marginal bone loss as revealed by the multivariate regression analysis (Study III). IL-6 is a mediator of inflammation in various tissues and conditions[40] but also possess pro-osteoclastic[40] and anti-inflammatory[39] properties. At present, we cannot determine the mechanism whereby IL-6 contributes to marginal bone loss. A recent systematic review demonstrated evidence in the literature to support the hypothesis that implants with peri-implantitis present higher levels of pro-inflammatory cytokines in the PICF than healthy implants.[300] Since the clinical course of implant treatment proceeded in absence of major clinical signs of inflammation, the high levels of IL-6 gene expression in PICF is assumed to be an early and asymptomatic predictor of MBL and thereby of biological complications.

In Study III, an association was revealed for the first time between high marginal bone loss at 90d and a low expression of HIF-1α in the recipient bone at baseline. This indicates that HIF-1α has a positive effect on jawbone homeostasis around oral implants. HIF-1α is a transcription factor associated with the survival and differentiation of cells in hypoxic conditions.[301] It triggers a range of autocrine, paracrine and endocrine effects, when oxygen levels drop, resulting in increased oxygen delivery to the hypoxic tissue, thereby reducing its oxygen consumption.[301] HIF-1α has been suggested to regulate bone formation and bone differentiation via VEGF and placenta growth factor,[302] but the exact involvement is not yet fully known. HIF-1α promotes the osteogenesis of rat mesenchymal stem cells (MSCs) [303] and transduces MSCs to enhance osseointegration in canine mandibular defects.[304]

In the comparative analysis of the marginal bone loss in smokers and non-smokers (Study III), it was not only concluded that smokers had a higher marginal bone loss than non-smokers, but also that the maxilla, and not the mandible, accounted for this difference. Therefore, in Study III we also explored potential differences in baseline, constitutive gene expressions between the maxilla and mandible. Interestingly, the expression of HIF-1α appeared to be bone-site dependent: a lower constitutive expression of HIF-1α was detected in the maxilla than in the mandible of smokers. Furthermore, a lower expression of HIF-1α was demonstrated in the baseline maxilla of smokers compared with the maxilla of non-smokers. In contrast, no differences were observed with respect to the base-line expression in the host recipient bone between the mandibles of smokers and non-smokers.

Together with the MBL data showing a higher marginal bone loss in the maxilla of smokers compared with non-smokers these observations provide strong incentives to further examine the role of HIF-1α in the smoking-induced marginal bone loss. Furthermore, it is of interest to explore the potential role of HIF-1α as a prognostic marker of MBL in both compromised conditions and different bone sites in larger patient cohorts.

6 SUMMARY AND CONCLUSIONS

The overall aim of this thesis was to examine the clinical and molecular aspects of treatment with dental implants in smokers compared with non-smokers.

In a retrospective investigation, using a five-year follow-up (Study I), it was demonstrated that smokers had a lower survival rate compared with non-smokers, particularly in relation to machined implants. Further, a higher marginal bone loss was shown for the machined implants of smokers compared with those of non-smokers.

In prospective studies (Studies II-IV), the gene expression denoting inflammation, bone formation, remodeling, vascularization and growth factors was determined in the PICF, as well as in the implant-adherent cells and in the surrounding peri-implant bone during osseointegration. Determining the role of implant properties and the effect of smoking, the following findings were made.

- Whereas machined implants elicited a higher pro-inflammatory gene response in both the PICF and implant-adherent cells, the oxidized implants promoted a higher bone anabolic gene expression in the same compartments. Furthermore, in smokers, the oxidized implants also appeared to enhance the early bone-remodeling activity in the implant-adherent cells.
- Mainly at the machined implants in smokers, the temporal gene expression pattern suggested an initial delay in the triggering of the osteoblastic and the osteoclastic activities in the implant-adherent cells. The upregulation of the coupling factor RANKL in the cells adhering to the oxidized implants appeared to reverse the delayed effects induced by smoking.

In prospective studies, focusing on the early (0-28d) (Study II) and late (60-90d) (Study III) healing phases of osseointegration, clinical, radiological and molecular observations were correlated, showing that

- Regardless of smoking habits, the initial perception of pain gradually decreased over time, correlating with the temporal downregulation of the gene expression of pro-inflammatory cytokines. Moreover, greater, persistent pain was reported by the few patients who experienced early implant failure
- Higher initial implant stability, as determined by RFA already at baseline, was demonstrated exclusively in smokers, implying

a different (ultra)structure and composition in the recipient jawbones of smokers compared with those of non-smokers

- Already after 90d, greater marginal bone loss was demonstrated in smokers, in particular at machined implants, correlating with a higher PICF expression of the pro-inflammatory cytokine, IL-6, and a lower expression of the osteogenic gene, OC
- The greater MBL around the machined implants in smokers was more pronounced in the maxilla compared with the mandible. This was in parallel with a significantly lower baseline expression of HIF-1α in the recipient maxilla of smokers
- Using a multivariate regression model, adjusted for age and implant location (maxilla/ mandible), smoking and BoP were identified as factors of importance for MBL after 90d. Further, the baseline expression of HIF-1α in the recipient bone and IL-6 expression in PICF cells at 90d were important molecular determinants of MBL after 90 d.

On the basis of the present molecular, radiological and clinical data, it is concluded that smoking causes adverse inhibitory effects on osseointegration and increased marginal bone loss during the early healing phase (0-90d), as well as increased failure rate and marginal bone loss in the long-term (5 years). In contrast to machined implants, which were associated with a dysregulated inflammation, osteogenesis and remodeling, an increased marginal bone loss during both early and late time periods and a subsequent higher failure rate at late time periods, the surface properties of modified implants appear to favor osseointegration by mitigating the negative effects of smoking. Smoking and bleeding on probing are factors of importance for marginal bone loss during the early healing phase. Further, the IL-6 expression in peri-implant crevicular fluid and the baseline expression of HIF-1α in the recipient bone are molecular determinants of the early marginal bone loss. Together with the findings of differences between maxilla and mandible with respect to smoking-induced marginal bone loss and HIF-1α baseline expression, the results of the present thesis suggest that local effects of smoking on osseointegration are modulated by both host jawbone site and implant surface properties. Given the hazardous effects induced by tobacco on the human body, and the adverse effects on the development and maintenance of osseointegration, the cessation of smoking should be the first consideration when treating patients with dental implants.

7 FUTURE PERSPECTIVES

With the changes of demographics, i.e. an aging population, multiple challenges in health care have emerged. With an increased elderly population, follows an increased loss of teeth. The loss of teeth causes an impaired oral function and subsequently an overall poor life quality. It is indicated that implant treatment can restore some of these functions, making the research area of biomaterials and dental implants very important. In this thesis we have combined the knowledge of different molecular techniques with a clinical setting to draw conclusions and study how the molecular events during the early osseointegration mirrors the clinical events and vice versa. With this we have found some very interesting results that need to be further studied:

It is of great importance to expand the follow-ups of this patient cohort and to survey the late outcome, for example, in one- and five-year data.

Follow up the determinant factors (IL-6 and HIF-1 α) to see if the same strong association to MBL is determined after 1 year and after five-years.

A more extensive large scale RCT, preferably multicenter, validating baseline expression of HIF-1 α in recipient bone as a determinant for marginal bone loss. Likewise for IL-6 in PICF at 90d.

Studies of ultra-structure of the bone, in smokers and non-smokers.

Protein profiling of the PICF.

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