Peri-implantitis from a microbiological perspective

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Gothenburg 2013

Printed by Ineko AB, Göteborg, Sweden 2013.

ISBN 978-91-628-8635-6

http://hdl.handle.net/2077/31715

To my parents,

Ioanna & Christoforos

Contents

Abstract

Peri-implantitis from a microbiological perspective

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Background and aims: Peri-implantitis is an infectious disease affecting the supporting tissues of the dental implant. Long-term follow-up of peri-implantitis cases clinically and microbiologically is lacking and, the microbiota involved has been poorly characterized. The objectives of the present thesis were to:

i) in a patient sample identify clinical and microbiological features of peri-implantitis (Studies I & II)

ii) in a dog model, used for experimental research on periodontitis/peri-implantitis, identify the predominant subgingival species (Study III), and compare the microbiological profile of teeth/implants during spontaneous progression of ligature-induced periodontitis and peri-implantitis (Study IV)

Methods: The platform for studies I & II was 281 peri-implantitis cases selected consecutively from the Oral Microbiological Diagnostic Laboratory, Gothenburg, Sweden and analyzed by culture and/or molecular DNA-DNA hybridization method. The identification of subgingival species in the dog was based on phenotypical tests, checkerboard and DNA sequencing technology (Study III). The quantification of microbiota during experimental periodontitis and peri-implantitis was based on checkerboard methodology (Study IV).

Results: Peri-implantitis was a poly microbial anaerobic infection with increased number of Aerobic Gram negative bacilli (AGNB) in 18.6% of patients. Microbiological findings did not correlate fully to the clinical findings, probably due to accessibility problems (Study I). It was not feasible to establish peri-implant health in 54.7% of the patients. A rough surface may be associated with early disease development and inability to treat disease successfully (Study II). The subgingival microflora in dogs used for experimental purposes shows greater heterogeneity but does not differ substantially with the respective one in humans, at genus level. Nevertheless, marked differences existed at species level (Study III). Suspected subgingival bacteria, including *Pasteurella*, *Porphyromonas* and *Treponema* genera are involved in experimental periodontitis and peri-implantitis. Bacterial growth increased around teeth and implants during the period following ligature removal and established an anaerobic Gram-negative microflora (Study IV).

Conclusions: Peri-implantitis shares similar predominant species to periodontitis both in humans and experimental dog model. All studies confirm that peri-implantitis is a non-specific, polymicrobial and heterogenous disease of endogenous nature. The potential role of AGNB in disease aetiopathogenesis needs further investigation.

Keywords: Animal experiment, bacterial load, culture, checkerboard, dental implants, infection, microbiota, peri-implant disease, peri-implantitis, titanium

ISBN 978-91-628-8635-6
http://hdl.handle.net/2077/31715

Preface

This thesis is based on the following four original papers, which are referred to by their Roman numerals in the text.

I. Charalampakis G, Leonhardt Å, Rabe P, Dahlén G.
Clinical and microbiological characteristics of peri-implantitis cases: a retrospective multicentre study.
Clinical Oral Implants Research 2012; 23(9): 1045-54.

II. Charalampakis G, Rabe P, Leonhardt Å, Dahlén G.
A follow-up study of peri-implantitis cases after treatment.
Journal of Clinical Periodontology 2011; 38(9): 864-71.

III. Dahlén G, Charalampakis G, Abrahamsson I, Bengtsson L, Falsen E.
Predominant bacterial species in subgingival plaque in dogs.
Journal of Periodontal Research 2012; 47(3):354-64.

IV. Charalampakis G, Abrahamsson I, Carcuac O, Dahlén G, Berglundh T.
Microbiota in experimental periodontitis and peri-implantitis in dogs.
Submitted.

Financial support for these studies was attained by grants from TUA research.

List of abbreviations

AGNB	Aerobic Gram negative bacilli
BL	Baseline
BoP	Bleeding on probing
CCUG	Culture Collection University of Gothenburg
CFUs	Colony forming units
ICT	Inflamed connective tissue
IL-1	Interleukine 1
OMGS	Oral Microbiology, Gothenburg, Sweden
OR	Odds Ratios
PCR	Polymerase chain reaction
PPD	Probing pocket depth
RCT	Randomized controlled trial
SLA	Sand-blasted and acid-etched
SUP	Suppuration
TPS	Titanium plasma-sprayed
TVC	Total viable count
TSBV	Tryptic Soy-Serum Bacitracin Vancomycin
VMGA	Viability medium Göteborg Anaerobically prepared

Introduction

Basic concepts in health and disease around dental implants

A dental implant is an artificial metal device usually made of titanium, anchored to the bone in order to replace one or more missing teeth. The pioneering work of Dr P.I. Brånemark, introducing the concept of osseointegration, led to the development of endosseous root-formed implants, which was a 'quantum leap' in implant dentistry (Branemark et al. 1977). Osseointegration refers to the direct structural and functional bone to implant contact (Branemark 1985). One of the prerequisites to achieve osseointegration is primary stability, a result of the contact relationship or friction established between the mineralized bone at the recipient site and the metal device border. In addition to the mechanical anchorage, successful osseointegration entails a cascade of biological events, including necrosis and resorption of the traumatized bone, concomitant with new bone formation (Berglundh et al. 2003). Although the lamellar bone in primary contact with the implant should be resorbed during initial phases of healing, osseointegration is often first established in areas occupied by cancellous bone (Berglundh et al. 2003).

Despite the difference in anchorage between teeth and implants, that is, for teeth via periodontal ligament and Sharpey's fibers, soft tissues around teeth and implants i.e. gingiva and peri-implant mucosa are of similar dimensions. The outer surface of the gingiva and the peri-implant mucosa is covered by a keratinized oral epithelium. Marginally the peri-implant mucosa is continuous with a thin non-keratinized barrier epithelium facing the implant, similar to the junctional epithelium, facing the tooth surface. Thus, soft tissues at teeth and implants include a barrier/junctional epithelium about 2mm long in the 'apico-coronal' direction and one zone of supracrestal connective tissue attachment > 1 mm, separating the epithelial lining from the bone crest. In other words, the biological width encompasses similar structures and is about 3.2 mm around teeth and 3.8 mm around implants (Berglundh et al. 1991).

Soft and hard tissue reactions following a microbial challenge are similar around teeth and implants in many respects. Mucositis, similar to gingivitis, is the inflammation limited to the soft tissue surrounding the dental implant, without signs of loss of supporting bone. Peri-implantitis, similar to periodontitis, is an infectious disease, characterized by inflammatory lesion in the mucosa and additionally bone loss around the osseointegrated dental implant (Lindhe and Meyle 2008).

Peri-implantitis: A figment of imagination or a ticking bomb?

Implant therapy has undoubtedly been a very successful alternative to restore function in areas where teeth are missing, since the adoption of the biological concept of osseointegration (Branemark et al. 1977). Though excellent long-term results are often presented, biological complications may occur and pose a threat to long-term implant survival. Peri-implantitis reflects these biological complications but as a clinical entity it was not widely understood in the past. It was advocated that marginal bone loss around the neck of the implants should be considered a remodeling phenomenon, not only some months following loading, but continuously (Jemt and Albrektsson 2008). Thus, continuous bone loss was regarded to be a natural bone remodeling process rather than a pathological condition. The aforementioned concept was based on an epidemiological study showing a slow, continuous bone loss around teeth on an average of 0.1 mm annually (Hugoson and Laurell 2000). It was suggested that similar or more bone loss would be natural to occur around dental implants (Jemt and Albrektsson 2008).

Without the use of periodontal probe, soft tissue reactions to a bacterial challenge cannot be recorded in a systematic way. In addition, mere visual inspection cannot precisely assess the peri-implant soft tissue condition. Indeed, clinicians from the very beginning had as endpoint of their clinical focus the establishment of ossseointegration and were reluctant to probing, for the fear of damaging the achieved anchorage to the bone. Thus, disease remained undiagnosed for some years after having implants in function. In the absence of symptoms from the side of the patient, radiographs were not obtained to record

marginal bone level alterations of the functional implants over time. Implant loss was the most common outcome variable. Thus a 'failing' implant facing the risk of loss was never identified. Only a 'failed' implant, e.g. the terminal stage of peri-implantitis, expressed as bone loss reaching the apical part of the implant, often combined with mobility, was diagnosed by clinicians. The large number of implants installed could retain the function of a cross-arch implant-supported bridge and mask the magnitude of the problem. The disease was treated by extraction of the failed implant. It was also common practice to install long implants which, in turn, increased their longevity.

An additional reason for peri-implantitis remaining a figment of imagination was that its infectious nature was not critically appraised. Treatment with osseointegrated implants was introduced in fully edentulous patients, and in such individuals there was no biological rationale to consider post osseointegration infections, since periodontitis associated bacteria were to be automatically 'removed' from the oral cavity together with the extracted teeth. Late implant failure was explained during many years by overloading or excess loading. Although other factors have been identified, it is still considered a main reason (Mattheos, Collier and Walmsley 2012). However, a recent systematic review (Naert, Duyck and Vandamme 2012) clarified that no association between overload and peri-implant bone loss could be found in the absence of peri-implant inflammation. Yet, an overload may act as a co-existing factor as in the case of periodontitis and contribute to the already established inflammation, aggravating the peri-implant tissue breakdown.

The description of peri-implantitis as a 'ticking bomb' relates to the fact that more and more clinicians are expected to run into peri-implantitis cases. This prediction is plausible due to the widespread use of implants in modern day clinical dentistry; with the implication that the longer in function the higher the risk of peri-implantitis.

What is peri-implantitis: Prevalence and considerations

The first term used to describe pathology around dental implants was peri-implantoclasia, a general term defining disease as a catabolic condition

surrounding an implant with or without sepsis or suppuration (Boucher 1963, Jermyn 1958). However, this term did not prevail over time, as peri-implantitis was introduced and widely accepted to stress the infectious nature of the pathological conditions around peri-implant tissues (Levignac 1965, Mombelli et al. 1987). Yet, some authors were reluctant to accept the new term (Jermyn 1998).

Already in the 1st European Workshop on Periodontology in 1993 (Albrektsson T 1994), peri-implantitis was defined as an irreversible inflammatory destructive reaction around implants in function, given an achieved osseointegration. The 6th European Workshop in 2008 presented modified definitions including the collective term peri-implant disease for peri-implant mucositis and peri-implantitis. The principle has remained the same for peri-implantitis, characterized by progressive loss of supporting bone and deepening of the peri-implant pocket (Lindhe and Meyle 2008). A recent consensus report concluded that peri-implantitis is a bacterially induced inflammation of the supporting peri-implant tissues leading to non-reversible bone destruction (Lang and Berglundh 2011).

Despite the clear definition, problem emerges when the terminology is to be applied in the clinical practice. Single features are not sufficient to satisfy the diagnosis peri-implantitis and results from cross-sectional studies should be interpreted with caution. In the absence of longitudinal radiological monitoring one should be careful not to misdiagnose pseudopockets as peri-implantitis, often created by certain implant designs. Evidence for progressive bone loss should accompany clinical findings.

In addition, there is no universal approach on the cut-off points for different levels of disease. As for periodontitis (Lopez and Baelum 2003), peri-implantitis does not seem to hold any diagnostic truth, in the sense that there is no natural basis for a sharp distinction between different severity levels of disease. Most authors are prepared to accept some bone loss ranging from 1 to 1.5 mm during tissue homeostasis period the first year of function, as a result of bone remodeling (Albrektsson et al. 1986, Albrektsson T 1994, Fransson et al. 2005, Jemt and Albrektsson 2008, Roos-Jansaker et al. 2006b). However, there are greatly heterogenous thresholds for disease used in the literature. Some are

based on clinical criteria (Bragger et al. 2001, Bragger et al. 2005, Corbella et al. 2011), radiological criteria (Albrektsson T 1994, Astrand et al. 2004, Baelum and Ellegaard 2004, Cury et al. 2009, Ellegaard, Baelum and Karring 1997, Roccuzzo et al. 2010, Wennstrom J 1999), others on combination of radiological criteria with bleeding on probing (BoP) ((Dierens et al. 2012, Gruica et al. 2004, Leonhardt, Dahlen and Renvert 2003, Ravald et al. 2012, Roos-Jansaker et al. 2006b, Roos-Jansaker et al. 2006a, Roos-Jansaker et al. 2006c) and rest on combination of BOP, pocket depth and marginal bone loss, detected on x-rays (Charalampakis et al. 2012, Dvorak et al. 2011, Ferreira et al. 2006, Gatti et al. 2008, Karoussis et al. 2003, Koldsland, Scheie and Aass 2011, Maximo et al. 2008, Rinke et al. 2011, Roccuzzo et al. 2012, Rodrigo, Martin and Sanz 2012, Rutar et al. 2001, Schmidlin et al. 2010, Serino and Strom 2009, Simonis, Dufour and Tenenbaum 2010, Wahlstrom, Sagulin and Jansson 2010, Zetterqvist et al. 2010). Prevalence figures are undoubtedly affected by the above mentioned different disease definitions and various thresholds for peri-implantitis. Peri-implantitis was reported in a range of 28% to 56% of the patients (Zitzmann and Berglundh 2008). Koldsland and co-workers (Koldsland 2010) showed a substantial variance in prevalence of peri-implantitis at patient level ranging from 11.3% to 47.1% depending on whether the radiological threshold was 3mm or just detectable. In a recent review (Mombelli, Muller and Cionca 2012b) the prevalence of peri-implantitis was suggested to be in the order of 10% implants and 20% patients during 5-10 years of implants in function, but it was pointed out how greatly heterogenous the study populations were in the included studies, making a meta-analysis not feasible. Although we face similar problems with definitions and thresholds in periodontitis, in the case of peri-implantitis we have to add the diversity of implant designs, which are not genetically determined as the tooth structure. Thus, the prospect of reaching a consensus is continuously hampered and the magnitude of the incidence of peri-implantitis therefore still remains a matter of academic dispute.

Risk factors for peri-implantitis

Any factor proposed to be risk factor for periodontitis and/or peri-implantitis should not just be an extract of a statistical significant result in a paper but relate to the disease with biological plausibility. In the case of peri-implantitis we should probably talk about risk indicators because we lack the longitudinal prospective studies, which would identify true risk factors. The table below presents the risk indicators of peri-implantitis (shared and distinct from periodontitis) both at patient and implant level.

Table 1. Shared and distinct risk indicators for peri-implantitis.

Risk indicators	Periodontitis	Peri-implantitis
History of periodontitis	+	+
Poor oral hygiene	+	+
Microorganisms	+	+
Gingivitis/mucositis	+	n.i.
Smoking	+	+
Diabetes mellitus	+	+
Genetic traits	(+)	(+)
Alcohol consumption	(+)	(+)
Absence of keratinized tissue	(+)	(+)
Position in the arch	-	+
Implant design	-	(+)
Implant surface	-	(+)
Residual cement	-	+

- : negative correlation
(+): weak correlation
+: strong correlation
n.i: not investigated

History of periodontitis

Obviously current periodontitis is a risk factor but is not discussed because any implant installation on the grounds of present periodontitis in the same mouth would be malpractice. Healthy periodontium is a prerequisite for implant therapy. Nine systematic reviews have addressed the issue of a history of periodontitis as risk indicator for implant outcomes (Al-Zahrani 2008, Karoussis, Kotsovilis and Fourmousis 2007, Klokkevold and Han 2007, Ong et al. 2008, Quirynen et al. 2007, Renvert and Persson 2009, Schou 2008, Schou et al. 2006, Van der Weijden, van Bemmel and Renvert 2005). Six studies, out of which 2 prospective (Karoussis et al. 2003, Roccuzzo et al. 2010) and 4 retrospective (Aloufi et al. 2009, Carcuac and Jansson 2010, Ferreira et al. 2006, Roos-Jansaker et al. 2006c) found a significantly positive correlation of history of periodontitis to peri-implantitis. Reported odds ratios (OR) ranged from 3.1 to 4.7.

Poor oral hygiene

There is substantial evidence to support a positive correlation of poor oral hygiene with peri-implantitis. An early prospective study reported an association between poor oral hygiene and peri-implant bone loss, especially in smokers, after a 10-year follow-up (Lindquist, Carlsson and Jemt 1997). Plaque scores > 30% were related to peri-implantitis occurrence in two other studies (Ferreira et al. 2006, Marrone et al. 2012). In another study (Serino and Strom 2009) accessibility for oral hygiene was significantly correlated with peri-implant stability.

Microorganisms

It is common knowledge that microorganisms are risk factors for peri-implantitis since disease is bacterially induced. However, they have been mostly discussed as part of the biofilm, along the implant surface, starting to grow above the mucosal margin, in the absence of an optimal oral hygiene. Unfortunately the bacterial role in disease pathogenesis has been underrated because no specific bacteria have been implicated in the apical migration of the 'barrier' epithelium, equivalent to the junctional epithelium around teeth. However, microbiological research should be encouraged towards a different microbiological perspective. Bacteria should be seen as consortia and not as individual names for risk of disease. It is clear that increased anaerobic bacterial mass, as a whole, would

imply sufficient growth to exert pathology around an implant. The presence of such anaerobic microbiota associated to periodontal disease in residual periodontal pockets poses a plausible threat for future infection around dental implants (Cho-Yan Lee et al. 2012). A pocket around teeth is the ecological niche that would favor the growth of opportunistic 'pathogens' and such bacteria in adequate amounts endanger long-term peri-implant health. Bacteria in such pathological environments (i.e. deepened pockets) are clear risk factors for peri-implantitis.

Mucositis

Mucositis has not yet been investigated as a risk indicator for peri-implantitis. It is the precursor of peri-implantitis as gingivitis is the precursor of periodontitis. The proportions of the soft tissue lesions converting to irreversible hard tissue lesions both around teeth and implants in the future are not known. However, in a longitudinal study of a well-maintained Norwegian male population (Lang, Schatzle and Loe 2009), it was shown that persistent gingivitis represents risk factor for periodontal attachment loss. Based on this knowledge, we could speculate that mucositis is a potential risk factor for peri-implantitis.

Smoking

Smoking has been identified as an aggravating factor to poor oral hygiene and significantly associated to marginal bone loss around implants (Lindquist et al. 1997). A clear association between smoking and peri-implantitis has been found in 6 cohort studies (Gruica et al. 2004, Haas et al. 1996, Laine et al. 2006, McDermott et al. 2003, Rodriguez-Argueta et al. 2011, Roos-Jansaker et al. 2006c), in which adjustment for poor oral hygiene was made. The range of OR was from 3.6 to 4.6.

Diabetes mellitus

There is limited evidence stemming from retrospective studies. In a Brazilian population an increased risk for peri-implantitits was recorded in non-smoking individuals with poor metabolic control (Ferreira et al. 2006). A Belgian study (Marrone et al. 2012) recorded peri-implantitis in 42.9% of the patients with diabetes.

Genetic traits

Interleukin 1 (IL-1) gene polymorphism has been mostly investigated in the literature. Some studies showed positive correlation of IL-1 gene with peri-implantitis (Laine et al. 2006) or positive synergistic effect with smoking and bone loss (Feloutzis et al. 2003) as well as implant loss (Jansson et al. 2005). Other studies failed to find an association between IL-1 genotype and bone loss (Wilson and Nunn 1999), implant loss (Gruica et al. 2004) or peri-implantitis (Lachmann et al. 2007). Three systematic reviews (Andreiotelli et al. 2008, Bormann et al. 2010, Huynh-Ba et al. 2008) conclude the lack of robust associations.

Alcohol consumption

It has been advocated that alcohol consumption is a risk indicator for peri-implantitis but the evidence is very limited. One prospective 3-year trial (Galindo-Moreno et al. 2005) suggested that alcohol consumption of > 10 g daily resulted in significantly greater bone loss around implants. A recent case-control study recognized alcohol consumption as risk indicator for peri-implantitis (Alissa and Oliver 2012).

Absence of keratinized mucosa

Early studies (Block et al. 1996) as well as recent studies (Adibrad, Shahabuei and Sahabi 2009, Bouri et al. 2008, Kim et al. 2008, Schrott et al. 2009) encourage a wider zone of keratinized mucosa to preserve soft and hard tissue stability. However, presence of adequate keratinized mucosa could not guarantee absence of peri-implant lesions (Roos-Jansaker et al. 2006c). In two systematic reviews addressing the issue (Mehta and Lim 2010, Wennstrom and Derks 2012), it was encouraged to increase the dimensions of the keratinized mucosa in areas that the lining mucosa induces pain and discomfort to the patient, hampering optimal oral hygiene procedures. However, due to heterogeneity of the current studies, lacking longitudinal prospective design and methodological consistency, it is not possible to draw robust conclusions with regard to whether presence and amount of keratinized mucosa are critical in long-tem maintenance of interproximal bone levels around implants (Wennstrom and Derks 2012).

Position of implant in the arch

Two articles address the position of the implant in the arch as a local factor, posing a threat to long -term implant survival. Specifically, the posterior region of the maxilla was associated with higher implant loss in one study (Alsaadi et al. 2008), probably due to thin cortical bone and less dense trabecular bone. In another study, peri-implantitis was more common in the anterior region of the mandible. This position of the jaw is characterized by thin alveolar ridge, predisposing implants to marginal bone loss (Fransson C 2009).

Implant design

The focus has been placed in the literature on the comparison between long and narrow implants to short and wide implants. Three studies propagate an association of short and wide implants with increased implant loss rate (Alsaadi et al. 2008, Baelum and Ellegaard 2004, Renouard and Nisand 2006). However, a recent multi-center controlled clinical trial revealed equally reliable treatment outcomes with short 6 mm implants and long 11 mm implant in posterior areas of the mandible and the maxilla (Gulje et al. 2012).

Implant surface

There is limited evidence on the impact of the implant surface on peri-implantitis. Some studies have found a positive correlation between smooth surface and peri-implant health (Astrand et al. 2004, Esposito et al. 2007) whereas others no correlation of the implant surface on marginal bone level changes (Gotfredsen and Karlsson 2001, Wennstrom et al. 2004). A systematic review (Abrahamsson and Berglundh 2009) failed to identify any implant surface or implant system being superior in marginal bone preservation around implants being in function for at least 3 years.

Residual cement

Residual cement acts as a foreign body and there is a clear biological plausibility for a bacterial infection to evoke. In one study (Wilson 2009) clinical and /or radiographic signs of peri-implantitis were associated with excess dental cement in the majority (81%) of 39 cases. In a recent retrospective analysis (Linkevicius et al. 2012), patients with a history of periodontitis experienced peri-implantitis around all implants with residual cement.

Rationale for a more aggressive profile of peri-implantitis

Peri-implantitis is fundamentally not different from periodontitis due to some shared features. Terminology has followed the same principles and based on the much more extended knowledge from periodontitis, similar risk factors (smoking, diabetes, plaque, genetic traits, alcohol, absence of keratinized mucosa) as well as similar microbiological profiles have been easily identified and discussed in the literature. However, there are some distinct features of peri-implantitis that should be reflected, as they may serve as 'ringing bells' for clinicians to become alert and as a plausible platform to explain increased progression rate in the case of peri-implantitis.

A. Anatomical characteristics

The anatomical differences between the periodontium and the peri-implant tissues are mainly related to the structure and composition of the supracrestal connective tissue. In the periodontium, collagen fibers are arranged in groups or bundles with distinct orientations such as dentogingival, dentoperiosteal, circular and transeptal fibers (Feneis 1952, Page et al. 1974) and are attached to the root cementum inserting more or less perpendicular into the root cementum. This type of attachment does not exist between the peri-implant mucosa and the implant because the implant lacks a lining cementum. Instead, the collagen fibers of the supracrestal connective tissue at implants are invested in the periosteum of the peri-implant bone and run a course parallel to the implant surface (Abrahamsson et al. 1996, Berglundh et al. 1991a, Listgarten et al. 1992). Though not inserted into the titanium surface, collagen fibers in the supracrestal area at implants are more numerous than at teeth but the density of fibroblasts and vascular structures is lower. The absence of a healthy connective tissue fiber compartment that would wall off the peri-implant lesion from the alveolar bone creates 'open wound', which could favor the promotion of pathology around the 'fragile' barrier more rapidly. Interestingly, in a monkey experimental model (Schou et al. 1993) it was shown greater bone loss around ankylosed teeth and implants, lacking periodontal ligament, compared to control teeth. According to a recent descriptive transcriptome analysis of mucosal and gingival soft tissue

(Becker et al. 2012), transcripts associated to innate immune responses and defense responses were dominating in peri-implantitis tissues but not in periodontititis tissues. This implies that the mucogingival barrier may act as a 'scar tissue' provoking increased pathogenicity.

B. Clinical characteristics

Pus is a very common clinical finding in peri-implantitis, and though comparative clinical studies are not available, it seems to be encountered much more often while probing dental implants compared to teeth (Fig. 1). Pus should be considered as marker for ongoing infection. On the other hand, mobility is not normally present so often and with different grades at implants as it happens with teeth. The finding of mobility around implants corresponds to the terminal stage of the disease and total loss of osseointegration.

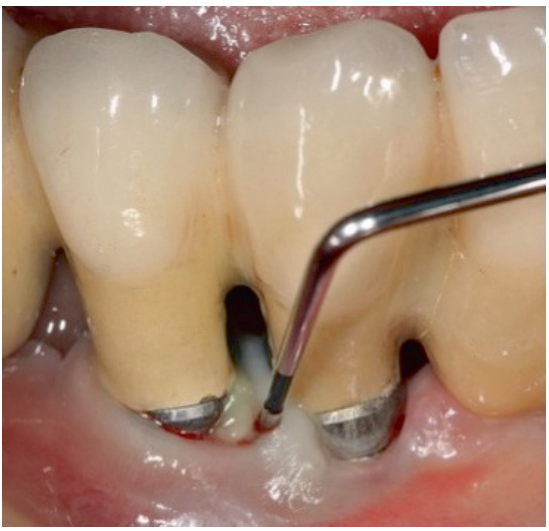


Fig. 1
Implants placed in the lower right canine and premolar region. Note the deep pocket and the pus exuding from the mesial aspect of the implant placed in the position of tooth # 44. (Courtesy of Dr Alberto Turri)

C. Radiological characteristics

Lamina dura represents the bundle bone that surrounds the tooth socket and serves as the attachment surface to which the Sharpey's fibers of the periodontal ligament invest. Due to the absence of periodontal ligament, such structure does not exist around healthy implants and is not visible in peri-apical radiographs. The peri-implant lesion is depicted as circumferential destruction of the crestal

bone, often described as crater-formed, bowl-like defect (Fig. 2). This specific form is related to the dimensions of the alveolar ridge that accommodates the implant. In sites with a narrow ridge the buccal and lingual bone will be resorbed and lost creating a crater during the progression of peri-implantitis. Two or three-wall intrabony defects are not seen in peri-implantitis lesions as bone loss seems to be symmetric at mesial, distal, buccal and lingual aspects of the implants. This difference in the configuration of the bony defect in the peri-implant lesion may be explained by the lack of ancorage of collagen fibers at implants compared to teeth.

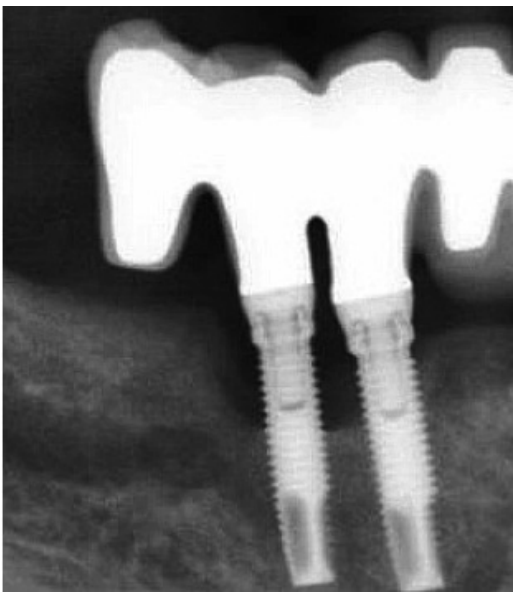


Fig. 2
Peri-apical x-ray of the same lower right region in line with the clinical findings. Note the marginal bone loss in the form of a crater around the implant placed in the position of tooth # 44 (Courtesy of Dr Alberto Turri)

D. Histological characteristics

Human biopsy material reporting histopathological data at sites with peri-implantitis have described inflammatory lesions with high proportions of B and plasma cells (Berglundh et al. 2004, Esposito et al. 1997, Gualini and Berglundh 2003). It is not the composition but the extent of the inflamed connective tissue (ICT) that differs between periodontitis and peri-implantitis lesions. In the case of a diseased implant, the infiltrate extends apically to the position of the pocket epithelium, showing clear signs of spreading i.e. inability of encapsulation of the

lesion (Berglundh et al. 2004). A recent experimental study (Carcuac et al. 2012) revealed that the ICT area was 4-6 times greater at implants than at teeth, containing larger proportions of neutrophils and osteoclasts and extending closer to the bone crest.

E. Microbial characteristics

They are discussed below in a separate section.

Microbial characteristics of peri-implantitis

Peri-implantitis has been quite early described as 'a site-specific infection yielding many features in common with chronic adult periodontitis' (Mombelli et al. 1987) or 'an inflammatory, bacterial-driven destruction of the implant-supporting apparatus' (Tonetti 1996). Both terms imply that microorganisms play a decisive role in the initiation and progression of peri-implantitis.

There are several and early lines of evidence supporting the view that microorganisms are strongly involved in peri-implantitis lesions: i) studies in humans showing that deposition of plaque on implants can induce peri-implant mucositis (Berglundh et al. 1992, Pontoriero et al. 1994) ii) demonstration of distinct quantitative and qualitative differences in the microflora associated with successful and failing implants (Augthun and Conrads 1997, Becker et al. 1990, Leonhardt et al. 1993, Mombelli 1993, Mombelli et al. 1987, Salcetti et al. 1997) iii) placement of plaque-retentive ligatures in animals leading to created pockets and accordingly shifts in the composition of the microflora and peri-implantitis (Leonhardt et al. 1992).

Microbial colonization of implants follows the same patterns as that around teeth, as followed longitudinally in various studies (Mombelli and Lang 1998, Mombelli et al. 1995a, Mombelli and Mericske-Stern 1990, Salvi et al. 2008, Sbordone et al. 1999, van Winkelhoff et al. 2000) and the microorganisms in peri-implantitis closely resemble those found in adult periodontitis (Alcoforado et al. 1991, Augthun and Conrads 1997, Listgarten and Lai 1999, Rosenberg, Torosian and Slots 1991, Salcetti et al. 1997). Attention has been focused on specific bacterial species, found in increased levels at chronic periodontitis sites

as well as diseased implant sites (Mombelli et al. 1987, Tanner et al. 1997). However, organisms not primarily associated with periodontitis, such as *Staphylococcus* spp., enterics and *Candida* spp. have also been found in peri-implant infection (Alcoforado et al. 1991, Leonhardt, Renvert and Dahlen 1999, Renvert et al. 2008b, Rosenberg et al. 1991).

Sequence of microbial colonization events and microbial profile of healthy implants

The development of an adherent biofilm on the implant surface is a process similar to the adherence of biofilm on the exposed surface of natural teeth. When an implant is inserted into the oral cavity, it provides a new and physically different 'hard tissue' surface for the colonization of microorganisms that might already be resident in the oral cavity or enter the oral cavity during biofilm formation. The effect of titanium on oral bacteria has been evaluated earlier in an in vitro model (Leonhardt and Dahlen 1995) as well as in vivo (Leonhardt, Olsson and Dahlen 1995). These studies showed that oral bacteria are forming biofilms and plaque similarly to other inert surfaces and materials e.g. hydroxyapatite and glass with the same surface structure. It has been documented that the surface free energy of titanium implants facilitates the formation of bacterial biofilms (Teughels et al. 2006). The titanium surface acts as a surface for attachment of salivary proteins and peptides and a pellicle is formed, probably quite similar to the pellicle formed on natural teeth. The pellicle provides receptors for the adhesins of specific species of oral bacteria that form the early colonizers of the implant. These species appear to be similar to those that colonize the teeth and include members of the genera *Streptococcus*, *Actinomyces* and *Veillonella*. The insertion of the implant appears to trigger the mechanisms for the development of a mature biofilm. In contrast to a cleaned tooth, which is likely to have remnants of an attached microbiota (Li et al. 2004, Socransky and Haffajee 2005), the pristine surfaces of an implant are initially devoid of an indigenous microflora and may require initial colonization by early colonizers to set the stage for the subsequent complex community (Kolenbrander et al. 2006).

For a number of years there might be a balance between the microbial challenge and the host around the implant, the biofilm that is formed is in a commensal state and this homeostatic condition is indicative of health around the implant (Marsh 1994, Marsh 2003). The microbial composition of biofilms on healthy implants may be similar to that on the surfaces of periodontally healthy teeth in the adolescent. Early reports favor this aspect, i.e. microbiota at well-maintained implants resembles the microbiota associated with healthy conditions at teeth (Apse et al. 1989, Leonhardt et al. 1992, Leonhardt et al. 1999, Meijndert et al. 2010, Mombelli and Mericske-Stern 1990, Mombelli et al. 1987, Quirynen and Listgarten 1990). Several reports have confirmed the colonization of healthy peri-implant sites by high proportions of coccoid cells, a low ratio aerobic/anaerobic species, a low number of Gram negative anaerobic species and low detection frequencies for 'periodontopathogens' (Adell et al. 1986, Bower et al. 1989, Kalykakis et al. 1994, Lee et al. 1999a, Lekholm et al. 1986, Mombelli, Buser and Lang 1988, Ong et al. 1992, Quirynen and Listgarten 1990, Sbordone et al. 1999). In later studies successful implants were colonized additionally by *Campylobacter* and *Fusobacterium nucleatum* (Lee et al. 1999a, Nowzari et al. 2008) and other putative periodontal 'pathogens' (Mombelli et al. 1995a), as well as Aerobic Gram negative bacilli (AGNB) (Leonhardt et al. 1999, Nowzari et al. 2008). The biofilm on healthy implants is confined supramucosally despite the fact that it can be in massive amounts (Heuer et al. 2007).

Table 2. Microbial findings at implants in humans; a review of the current literature.

References	Study design	Peri-implant condition	Edentulism	Microbial analyses	Microbial findings
Agerbaek 2006	Cross-sectional Prospective	Health/ Mucositis/ Peri-implantitis Maintenance	Partial	DNA-DNA hybridization 40 species	Less bacterial load on implants than teeth. No differences in complexes.

Adell 1986	Cross-sectional Prospective	Health: 80-85% Mucositis: 10-15%	Full	Dark-field microscopy	Coccoid cells, & non-motile rods 93%
Alcoforado 1991	Cross-sectional Retrospective	Peri-implantitis	Partial	Culture	Black-pigmented, *Fusobacterium*, *Campylobacter*, *P. micros*[a], *Candida*, AGNB[b]
Apse 1989	Cross-sectional Retrospective	Health/ Mucositis	Full/ Partial	Dark-field microscopy, Culture	Black-pigmented, Capnocytophaga
Augthun 1997	Cross-sectional Prospective	Peri-implantitis	Full	Culture of ICT excited during surgery	*A.a*[c], *Prevotella, Fusobacterium*, *Capnocytophaga*, *Eikenella corrodens*
Becker 1990	Cross-sectional Prospective	Peri-implantitis	Full/ Partial	DNA probe analysis 3 species	*A.a*[c], Black-pigmented bacteria
Botero 2005	Cross-sectional Prospective	Health/ Peri-implantitis	Partial	Culture	Higher amounts of AGNB[b], *P.g*[d], *Prevotella*, *Fusobacterium* at PI[e] versus health
Bower 1989	Cross-sectional Retrospective	Health	Full	Dark-field microscopy	Coccoid & non-motile rods 89% Spirochetes < 2%
De Boever 2006	Longitudinal Prospective	Health	Partial	DNA probe analysis 5 species	Implant sites positive for *A.*a, *P.g*, *P.i*[f], *T.f*[g], *T.d*[h]
Devides 2006	Longitudinal Prospective	Health/ Mucositis	Full	Polymerase chain reaction	*A.a* 13.3%-73.3% *Pg* 0%-53.3% *P.i* 46.7%-53.3%
Hultin 2002	Cross-sectional Prospective	Health/ Peri-implantitis	Partial	DNA-DNA hybridization 12 species	Higher counts *A.a*, *P.g*, *T.f*, *T.d* in PI
Kalykakis 1994	Cross-sectional Retrospective	Health/ Mucositis/ Peri-implantitis	Full/ Partial	Latex agglutination test	Black-pigmented 19.3-39% and *A.a* 12.3-17% in PI

Koyanagi 2010	Cross-sectional Prospective	Health Peri-implantitis	Partial	16S rRNA gene clone library	Less complex flora Gram-negative anaerobic, low levels *P.g*,, *A.a*
Kumar 2012	Cross-sectional Prospective	Health/ Peri-implantitis	Partial	16S rRNA pyro-sequencing	More complex flora in health and PI, Gram- anaerobic species predominant in PI
Lee 1999	Cross-sectional Prospective	Health	Partial	DNA-DNA hybridization 23 species	Greater counts of Cocci, *P. micros*, *Fusobacterium* but fewer Black-pigmented & *Campylobacter*
Lekhom 1986	Cross-sectional Retrospective	Health	Partial	Culture	Cocci & non-motile rods 69.3%, Spirochetes 0%
Leonhardt 1993	Longitudinal Prospective	Health	Partial	Culture	Similar subgingival colonization pattern at teeth and implants
Leonhardt 1999	Cross-sectional Prospective	Health Peri-implantitis	Full/ Partial	Culture	Black-pigmented 26%, AGNB 12% Black-pigmented 60%, AGNB + Staphylococci + *Candida* 55%
Listgarten 1999	Cross-sectional Retrospective	Peri-implantitis	Partial	Culture	*T.f* 59%, Spirochetes 54%, *Fusobacterium* 41%, *P. micros* 39%, *P.g* 27%
Meijndert 2010	Cross-sectional Prospective	Health	Partial	Culture	Colonization patterns similar to teeth at 1 year
Mengel 2005	Longitudinal Prospective	Health	Partial	Dark-field microscopy & DNA-DNA hybridization 3 species	Cocci 67.6%-75.3% *A.a*, *P.g*, *P.i* more frequently detected in patients with history of P[i]

Mombelli 1987	Cross-sectional, Retrospective	Health Peri-implantitis	Full	Dark-field microscopy, Culture	Cocci 82%, motile rods 1% Motile rods + spirochetes 17% Gram- anaerobic rods 24%
Mombelli 1988	Longitudinal, Prospective	Health	Full	Dark-field microscopy, Culture	Gram+ facultative cocci >80%, no spirochetes
Mombelli 1990	Longitudinal, Prospective	Health	Full	Dark-field microscopy, Culture	Cocci 70.2%, Gram- anaerobic rods 7.3%
Mombelli 1995	Longitudinal Prospective	Health	Partial	Dark-field microscopy, Culture	Colonization pattern at abutments with putative periodontal 'pathogens'
Nowzari 2008	Cross-sectional, Prospective	Health	Partial	Culture	Zero levels of *A.a*, Low levels of *A.a*,*P.g*, *P.i*, *Campylobacter* and AGNB
Ong 1992	Cross-sectional, Retrospective	Health	Full/ Partial	Culture	Anaerobic/ Aerobic ratio range: 0.01-35.9 *P.g* 0%
Quirynen 1990	Cross-sectional, Retrospective Human	Health	Full/ Partial	Phase-contrast microscopy	Cocci 65.8%, Motile rods 2.3% Spirochetes 2.1% Similar with teeth
Quirynen 2005	Cross-sectional, Prospective Human	Health	Full	DNA-DNA hybridization 40 species	Low total DNA probe count (8-18 × 10^5), Detection *A.a*, *P.g*, *T.f*
Rams 1984	Cross-sectional, Prospective	Health/ Mucositis/ Peri-implantitis	Not stated	Phase-contrast microscopy	Cocci 64.2% Spirochetes 2.3% Cocci 30.3% Spirochetes 32%
Rams 1991	Cross-sectional, Prospective	Health Peri-implantitis	Partial	Phase-contrast microscopy, Culture	Cocci 40.7% Gram- anaerobic rods 3.2% *Fusobacterium* 26.9%

Renvert 2007	Cross-sectional, Prospective	Health/ Mucositis/ Peri-implantitis	Full/ Partial	DNA-DNA hybridization 40 species	No difference in DNA probe count irrespective of edentulism/ implant status
Renvert 2008	Cross-sectional, Prospective	Health/ Mucositis/ Peri-implantitis	Partial	DNA-DNA hybridization 40 species	*Staphylococcus aureus* DNA probe count greater at implants than teeth
Rosenberg 1991	Cross-sectional, Prospective	Peri-implantitis	Full/ Partial	Phase-contrast microscopy, Culture	Motile rods + spirochetes 42%, *Fusobacterium*, *P. micros*, *Candida*, AGNB
Salcetti 1997	Cross-sectional, Prospective	Health/ Peri-implantitis	Partial	DNA-DNA hybridization 40 species	Higher detection frequency in PI of *Prevotella*, *Fusobacterium*, *P. micros*
Salvi 2008	Longitudinal, Prospective	Health	Partial	DNA-DNA hybridization 40 species	Total DNA probe count significantly higher at teeth than implants
Sanz 1990	Cross-sectional, Prospective	Health Peri-implantitis	Partial	Culture	Facultative Gram + cocci and rods Gram- anaerobic rods & Black-pigmented bacteria
Sbordone 1999	Longitudinal, Prospective	Health	Partial	Dark-field microscopy, Culture	Cocci 80.5%-81.2%, Spirochetes and Black-pigmented in very low levels
Shibli 2008	Cross-sectional, Prospective	Health/ Peri-implantitis	Partial	DNA-DNA hybridization 36 species	Higher mean count of 'red complex' in PI versus health
Tabanella 2009	Cross-sectional, Prospective	Health/ Peri-implantitis	Full/ Partial	Culture	Presence of *T.f*, *P. micros* and *Campylobacter* associated with PI

Tanner 1997	Cross-sectional, Retrospective	Mucositis Peri-implantitis	Partial Partial	Culture Culture/DNA probe analysis 8 species	Cocci, *Actinomyces*, *Veillonella* *P.g*, *P.i*, *T.f*, *Fusobacterium*, *Campylobacter*
Van Winkelhoff 2000	Longitudinal, Prospective	Health Peri-implantitis	Partial	Culture	Rapid appearance of Gram- anaerobic rods, Disease associated with *P.g*, no *A.a*

[a]: *Peptostreptococcus micros* (now designated as *Parvimonas micra*)
[b]: Aerobic Gram negative bacilli (AGNB)
[c]: *Aggregatibacter Actinomycetemcomitans*
[d]: *Porphyromonas gingivalis*
[e]: Peri-implantitis
[f]: *Prevotella intermedia*
[g]: *Tannerella forsythia*
[h]: *Treponema denticola*
[i]: Periodontitis

Transition from health to disease

With time, varying from months to years, the implant microbiota may extend submucosally and become more complex. Pockets may develop around the implant and in this ecologically changed environment increased numbers and proportions of the 'orange' and 'red' complex species (Socransky et al. 1998) will survive and thrive in a fashion analogous to the increase in these species in deep periodontal pockets adjacent to natural teeth. Rams and colleagues identified for the first time the microbiota associated with unsuccessful implants (Rams and Link 1983, Rams et al. 1984). Suspected periodontopathogenic microorganisms have been found by culture (Botero et al. 2005, Leonhardt et al. 1999, Listgarten and Lai 1999, Mombelli et al. 1987, Rams et al. 1991, Sanz et al. 1990, Tabanella, Nowzari and Slots 2009), DNA probe analysis (Becker et al. 1990, Hultin et al. 2002, Salcetti et al. 1997, Shibli et al. 2008) and pyrosequencing (Kumar et al. 2012). Only one study (Renvert et al. 2007) failed to identify significant differences of the microflora of the subgingival environment in implants with or without peri-implantitis but this result may in part be explained by differences in microbial sampling and aftercare of the samples. The study of Shibli and co-workers (Shibli et al. 2008) evaluated both the supragingival and the subgingival

microflora in diseased implants and concluded that higher mean counts of *Porphyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia* were observed in the peri-implantitis group, both at supra- and submucosal levels, implying that the supramucosal ('supragingival') biofilm in implants may play an important role as reservoir of such species for re-infecting the submucosal ('subgingival) environment in a fashion similar to the supragingival flora in periodontitis (Ximenez-Fyvie, Haffajee and Socransky 2000). However, a microbiological study using the 16S rRNA gene clone library analysis (Koyanagi et al. 2010) revealed greater bacterial diversity of submucosal biofilms compared to biofilms associated with healthy peri-implant mucosa.

The literature generally suggests that at failing implants there is an absence of homeostasis towards an ecological catastrophe (Marsh 2006), in the sense that there is a transition from a predominantly Gram-positive non-motile, aerobic and facultative anaerobic composition towards a flora with a greater proportion of Gram negative, motile, anaerobic bacteria.

Fully versus partially edentulous patients

Questions have been raised whether type of edentulism (partial/full) impacts the subgingival microflora around implants. Accumulating data suggest that microorganisms colonizing clinically healthy implants in fully edentulous subjects were very similar to the ones associated with healthy periodontal sites in periodontally healthy subjects i.e. higher frequency of Gram positive facultative cocci and lower frequency of gram-negative anaerobic rods (Adell et al. 1986, Bower et al. 1989, Mombelli et al. 1988, Mombelli and Mericske-Stern 1990, Ong et al. 1992). There was a paradigm shift with regard to the microflora of diseased implants in fully edentulous patients. Early studies did not detect *A. actinomycetemcomitans* and *P. gingivalis* in fully edentulous patients implying that the subgingival environment and no other niche can serve as primary habitat of these periodontal pathogens (Danser et al. 1994, Danser et al. 1995). However, both species were detected in peri-implantitis cases occurring five years or more after loading in edentulous subjects (Leonhardt et al. 1999). Later publications using molecular techniques managed to identify a higher prevalence of periodontal pathogens in fully edentulous patients (Devides and Franco 2006, Hultin et al. 2002, Lee et al. 1999b, Quirynen et al. 2005). These data suggested

that the soft tissues of edentulous patients harbor periodontal pathogens and are the likely source for colonization of implants after insertion in fully edentulous patients. A case report is in accordance with the above conclusions (Emrani, Chee and Slots 2008). The above thoughts have been recently confirmed with a pertinent study (Quirynen and Van Assche 2011) dispelling the myth that bacterial load is neutralized automatically by tooth extraction. Following microbial changes from the time of tooth extraction up to 1 year after abutment connection, some bacteria (*Prevotella* intermedia, P. *gingivalis*, *T. forsythia*), sampled from the saliva and the dorsum of the tongue, were not eliminated at any time point.

In partially edentulous patients the remaining dentition as a major source for colonization of implants by periodontal pathogens is fully explicit. However, the potential role of soft tissue surfaces and saliva as reservoirs for implant infection cannot be discarded (Quirynen, De Soete and van Steenberghe 2002). Since the remaining dentition has been implicated as a source of microorganisms that colonize implants, it might be surmised that higher levels of periodontal pathogens would colonize implants in subjects with a history of periodontal infection. A study that looked at partially edentulous patients with a history of severe aggressive periodontitis (De Boever and De Boever 2006) and one more focusing on maintenance patients (Agerbaek, Lang and Persson 2006) concluded that implants are immediately colonized by 'periodontopathogens' similar to the microflora of the residual pockets. Similar findings had been reported earlier (Leonhardt et al. 1993, Mengel and Flores-de-Jacoby 2005, Mombelli et al. 1995a).

Collectively, all data shown in Table 2 indicate that microorganisms colonizing implants in subjects with periodontitis are similar to that observed in the samples from periodontal pockets in the same individuals and harbor more anaerobic species than observed in fully or partially edentulous subjects with minimal or no periodontal disease. We realize that no comparison of the available studies is feasible, due to lack of homogeneity. There is tremendous variability in the study design, the inclusion criteria of patients, the sampling methods and microbiological analyses as well as in the presentation of the results. Most importantly, the majority of the studies have focused on

presence/absence of specific bacteria and even if quantitative methods have been introduced, no correlation to the total bacterial load has been demonstrated.

The dog model in experimental periodontitis and peri-implantitis

Animal models have been extensively used in oral disease research with non-human primates (apes, monkeys) and dogs being the most commonly used models, though other animals (rats, mice, rabbits, miniature pigs, ferrets, sheep) have also been used (Weinberg and Bral 1999). Already in 1899, Talbot pointed out the need for an animal model to study periodontitis and had noted the high prevalence and multi-stage characteristics of periodontitis in dogs (Talbot 1899). The dog model has been successfully used to study several aspects of periodontal disease, including prevalence, aetiological factors, clinical and histological traits (Gad 1968, Hamp and Lindberg 1977, Lindhe, Hamp and Loe 1975, Sorensen, Loe and Ramfjord 1980).

The dog has several attractive attributes that make it an appropriate model for the study of periodontitis and peri-implantitis. Periodontitis in dogs is a natural occurring disease and as such is more likely to recapitulate most aspects of disease pathogenesis of human periodontitis (Berglundh, Lindhe and Sterrett 1991b). The dog is a large animal model facilitating the space for installation of standard types of dental implants. Dogs can offer homogenous research trials, reducing confounding factors such as age, smoking or other lifestyle factors. They are bred together and controlled laboratory settings can be achieved. In addition, they share common features with humans in terms of development and aging of the immune system. They are immunologically competent at or before birth, unlike rodents. The dog is a promising clinical model for the study of diseases with breed predisposition, caused by the same gene mutations as in humans such as, adult onset insulin dependent diabetes, early onset systemic lupus erythematosus and transitional cell carcinoma of the bladder (Parker, Shearin and Ostrander 2010). It is expected that work on the dog model will

help increase our understanding of the genetic cause of many diseases shared by humans and dogs (Shearin and Ostrander 2010), including the genetic basis of periodontal disease. Understanding the genetic underpinnings of periodontitis will partly explain the difference in susceptibility/resistance to disease amongst individuals.

Periodontitis in dogs as in humans is a slowly progressive disorder. The beagle dog already presents high prevalence of periodontitis at 2 years of age but the major disease burden is carried by only a few individuals (Kortegaard, Eriksen and Baelum 2008). Thus, in order to enhance periodontal tissue destruction, it is possible to induce experimental periodontal defects by placing silk bindings (ligatures) around the teeth (Ericsson et al. 1975, Lindhe, Hamp and Loe 1973, Lindhe et al. 1975, Swenson 1947).

Despite the knowledge gap in the direct translation of results from animal research into treatment of human diseases, the role of the experimental dog model is critical as it facilitates invasive procedures rarely available to humans, i.e. histological investigation in order to profoundly understand human diseases. This model has been successfully used early in studying the role of plaque in the gingival alterations as well as in the initial phase of periodontal breakdown (Lindhe et al. 1973, Loe, Theilade and Jensen 1965, Theilade et al. 1966). The same model has been used to induce peri-implant lesions and investigate the pathogenesis of the associated disease (Lindhe et al. 1992). The latter 'classical' study was the beginning of a series of similar investigations on peri-implant tissue reactions using ligature induced dog or monkey model (Lang et al. 1993, Lang et al. 1994, Schou et al. 1993, Schou et al. 2002). Ligatures of cotton or silk were placed around properly integrated implants and adjusted in a submucosal position. The ligature being placed around the cervical area of the implant, compromised the mucosal attachment to the implant, promoted the buildup of plaque and was in turn successful in inducing peri-implantitis. In almost all studies the mandibular posterior region was chosen as the location for implants, meaning that while mandibular experimental peri-implantitis has been investigated to some extent, no scientific data are available on peri-implantitis in the maxilla. Only one study reported on the induction and treatment of experimentally induced peri-implantitis in the maxilla (Hanisch et al. 1997b).

However, this study did not reveal differences in the depth of the peri-implant defect between the maxilla and the mandible. Configurations and sizes of ligature-induced peri-implantitis bone defects in dogs seemed to well reproduce naturally occurring lesions in humans (Schwarz et al. 2007), a finding that strengthens the role of the dog model in mapping out the pathogenesis of peri-implantitis.

The model was developed one step further to investigate tissue reactions around implants during the so-called 'spontaneous progression' of experimental peri-implantitis (Zitzmann et al. 2004). This novel idea on a longitudinal follow-up of experimentally induced peri-implant lesions was based on an earlier observation (Marinello et al. 1995), where it was shown that tissue destruction continued 3 months after ligature removal. The model does not mimic accurately disease initiation in humans since a foreign body (ligatures) is introduced to create the peri-implant defects but once the tissue changes are followed longitudinally after the active breakdown period, they may recapitulate disease pathogenesis to a greater extent, as experienced in humans.

Treatment of peri-implantitis

Peri-implantitis is an infectious disease in nature and the rationale behind treatment is to reduce the bacterial load below the individual threshold level for disease. The achievement of this goal may involve various treatment strategies:

- Establishment of an optimal supramucosal plaque control both self-performed at home by means of oral hygiene instructions, motivation and professionally performed at the dental office in order to eliminate retentive factors and ensure accessibility for cleaning at the implant sites.
- Disruption of the submucosal bacterial biofilm mechanically by the use of non- surgical means.
- Reduction in metabolic activity of peri-implant pathogens and prevention of their colonization, invasion and growth in the peri-implant tissues by chemical means (antiseptics/antibiotics).
- Access of the site of infection by a surgical approach that allows the correction of plaque-retentive anatomical abnormalities.

- Prevention of recurrence of peri-implantitis by regular monitoring and supportive peri-implant therapy.

It is realized that therapies proposed for the management of peri-implant diseases appear to be largely based on the evidence available for the treatment of periodontitis, Non-surgical therapy continues to be the preliminary step in the management of peri-implant disease so as to create optimal soft tissue conditions. Based on the outcome, further decisions for surgery should be taken. Methods of non-surgical mechanical therapy that have been proposed for the treatment of peri-implantitis include the curettes, ultrasonic devices, air-abrasives and laser therapy. However, for a moderate/severe peri-implantitis lesion, non-surgical approach, irrespective of the means used, proves to be ineffective (Renvert, Roos-Jansaker and Claffey 2008c). A recent review calls for larger clinical trials with longer follow-up periods and simpler design without combinations of interventions in order to evaluate whether non-surgical treatment of peri-implantitis may establish peri-implant health (Muthukuru et al. 2012) Systemic and local antibiotics have been used in conjunction to non-surgical therapy but recurrence of the disease could not be avoided, implying the limitations in their use (Leonhardt et al. 2003, Renvert et al. 2008a).

Surgical treatment of peri-implantitis includes means that have been already used extensively in periodontal defects i.e. access flap, resective surgery and regenerative approach. Implant surface decontamination and the adjunctive use of lasers during surgery have been discussed in the literature, providing though limited evidence of superiority. Moreover, despite the plausibility to use systemic antibiotics as adjunct to surgery, due to the rapid progression of peri-implantitis in certain cases, there are presently no controlled studies in humans comparing the effect of surgical treatment of peri-implantitis with and without adjunctive use of systemic antibiotics. Among the studies included in systematic reviews on the surgical interventions (Claffey et al. 2008, Renvert, Polyzois and Claffey 2012), there is marked heterogeneity between study designs and case definitions as well as lack of control intervention (i.e. non-surgical therapy), limiting the generalization of the results and extraction of robust conclusions.

Collectively, evidence concerning the management of peri-implant diseases is in its relative infancy. Available randomized controlled or comparative clinical

trials have small sample sizes and short follow-up periods. Thus far, no evidence for an optimal treatment protocol exists making it dubious to suggest which therapeutic strategies are the most efficacious for the treatment of peri-implant lesions, based on their configuration, extent and severity.

Aims

The majority of the studies included in the literature on peri-implantitis are of small-size and cross-sectional design, focusing on single clinical and microbiological assessments at one time point only. However, without longitudinal monitoring of the disease, progressive bone loss cannot be claimed and serious queries emerge whether the recorded 'defect' reflects the true presence of a chronic disease process, a single episode of bone-remodeling or a pseudo-pocket associated to pre-installation anatomical limitations of the region. In addition, though frequent the use of an experimental model in dogs has been the latest years, very few and superficial microbiological investigations of the subgingival flora have been performed in order to validate the model from a microbiological aspect. To extend this argument a bit further, the microbiological profile of the created experimental defects remains unknown.
Thus, by conducting a series of studies with both human and animal material the following aims were pursued in this thesis:

I. Identify in retrospect clinical and microbiological features of peri-implantitis cases, followed-up longitudinally in humans (Studies I & II).

II. Identify the predominant subgingival species in dogs used for experimental periodontitis/peri-implantitis and correlate them to the respective ones in humans (Study III).

III. Analyze the microbiological profile around teeth and implants following ligature removal in experimental periodontitis and peri-implantitis in dogs (Study IV).

Materials and Methods

Ethical considerations

With regard to the human material (studies I & II), the collection of the data was originally performed for reasons other than research, meaning that ethical approval was not intended. Clinicians being responsible for the respective patients made the effort to fill in a form encompassing clinical information on implant therapy, peri-implantitis treatment and follow-up details of each case. Where additional information was needed, it was retrieved via access to paper-based and electronic patient records at appropriate working hours after written permission by the head of each clinic.

The animal experimental protocol for studies III & IV was submitted and approved by the regional Ethics Committee for Animal Research, Gothenburg, Sweden.

Subject samples

The human studies (I & II) used the same patient material, a total of 281 cases that were referred consecutively between January 2005 and January 2009 to the Oral Microbiological Diagnostic Laboratory, for microbiological analysis of samples obtained from diseased implants. Clinicians responsible for the patients were mainly periodontists but with some exceptions also oral maxillofacial surgeons and general dental practitioners. The 25 centers included in the study covered a large area of Sweden from the very south (Ystad) to the very north (Luleå).

The animal studies (III & IV) included 6 Labrador dogs, 16-months old (3 females; weight 20 kg, 3 males; weight 30 kg). During all surgical procedures general anaesthesia was induced with intravenously injected Propofol (10mg/ml, 0.6ml/kg) and sustained with N_2O:O_2 (1:1.5-2) and Isoflurane employing endo-tracheal intubation. For all radiological and clinical assessments including microbiological sampling, the animals were sedated with an i.m

injection with a combination of Butarphanol Tartrate (0.1 mg/kg; Torbugesic®, Fort Dodge Animal Health, Fort Dodge, IA, USA) and Medetomidine (25µg/kg; Dexdomitor®, Orion Corporation, Esbo, Finland). After sampling the sedation was reversed with an i.m injection of Atipamezole (125 µg/kg; Antisedan® vet., Orion Corporation, Esbo, Finland).

Study design

The human studies (I & II) were retrospective and longitudinal in their design but with different endpoints. Study I followed the patients with diseased implants from the time implants were placed up to the time of peri-implant disease diagnosis. Study II followed the same patient cases from the time of peri-implantitis treatment until some time after treatment, ranging from months to years. Several definitions were decided before retrieval of the data, as presented in Table 3, so as to ensure that identical thresholds would be followed by all different clinicians during the retrospective investigation. Baseline registrations correspond to the time point clinicians had diagnosed peri-implant disease and had obtained samples from the diseased implant sites.

Table 3. Definitions and clarifications of terms used in studies I & II.

Terms in studies I & II	Definitions and clarifications
Systemically healthy	Patient without remarkable health problems, those not suffering from cancer, cardiovascular disease, diabetes, osteoporosis, rheumatoid arthritis, depression
Suboptimal plaque control	O'Leary's Plaque Index > 20% on consecutive recall visits (O'Leary, Drake and Naylor 1972)
Light smoking	1-9 cigarettes/day
Moderate smoking	10-15 cigarettes/day
Heavy smoking	> 15 cigarettes/day
Anterior region	Incisor and canine region
Posterior region	Premolar and molar region

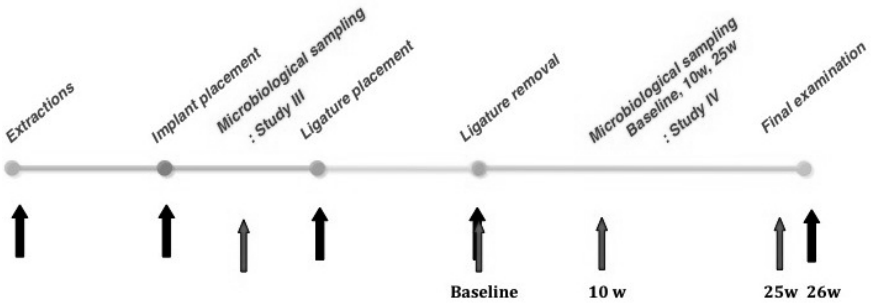
Terms in studies I & II	Definitions and clarifications
History of periodontitis	Some marginal bone loss around teeth but no SUP and/or BoP and PPD ≤ 4 mm
Peri-implantitis	SUP and/or BoP, PPD ≥ 5 mm and marginal bone loss ≥ 1.8 mm after 1 year of implants in function
Severe peri-implantitis	SUP and/or BoP, PPD ≥ 7 mm and marginal bone loss ≥ 1/3 implant length mm after 1 year of implants in function
Localized/Generalized peri-implantitis	Localized if ≤ 30% peri-implant sites were affected, otherwise generalized
Disease development	Early disease development if disease occurred < 4 years of implants in function, moderate between 4 and 6 years and late > 6 years
Treatment success	No BoP and/or SUP and PPD < 5 mm. Increased or stable marginal bone levels compared to pre-treatment levels on x-rays.
Treatment failure	BoP and/or SUP and PPD ≥ 5 mm. Progressive marginal bone loss compared to pre-treatment marginal bone levels on x-rays.

Study III was a cross-sectional microbiological study. Subgingival microbiological samples were obtained from the 4th bilateral maxillary premolars at the stage when all implants were submerged, before experimental periodontitis and peri-implantitis were induced (Fig. 3).

Study IV was a longitudinal microbiological study, describing the microbial profile around teeth and implants, at defects having been created experimentally with ligatures. The chronological outline of the experiment is illustrated in Figure 3. In brief, as already reported (Carcuac et al. 2012), the mandibular premolars and first molar and the three anterior premolars in the maxilla were extracted in the right side in all dogs. After a healing period of 3 months, 4 implants with similar geometry (MKIII NP, 3.3 x 10 mm, Nobel Biocare AB, Göteborg, Sweden) and with two different surface characteristics (implant group A; turned surface and implant group B; TiUnite surface) were placed pair-wise in a randomized order in the right side of the mandible. Three months after implant installation, oral hygiene procedures were abandoned and experimental

periodontitis and peri-implantitis were initiated with cotton ligatures, following the protocol Lindhe and co-workers introduced (Lindhe et al. 1992). The ligatures were removed 10 weeks later and this event reflected the end of the active experimental breakdown of the tissues and the start of the observation period (Baseline-BL). Plaque accumulation continued undisturbed during the subsequent 26-week follow-up period. Microbiological samples were obtained from the implants in the right side of the mandible and the 4th, 3rd and 2nd premolars in the contralateral side at three time points (BL, 10 weeks, 25 weeks).

Fig. 3. Schematic illustration of the chronological outline of the animal studies (III & IV) (m: months, BL : Baseline, w: weeks). Arrows in red indicate microbiological sampling time points.



Microbiological analysis

Before sampling sterile cotton rolls were used to isolate the experimental areas and avoid saliva contamination. Supragingival plaque was removed by wiping with sterile gauze or cotton pellets soaked in saline. Microbiological samples in all studies were obtained from the most apical part of the pocket with paperpoints and then processed for analysis with various methods.

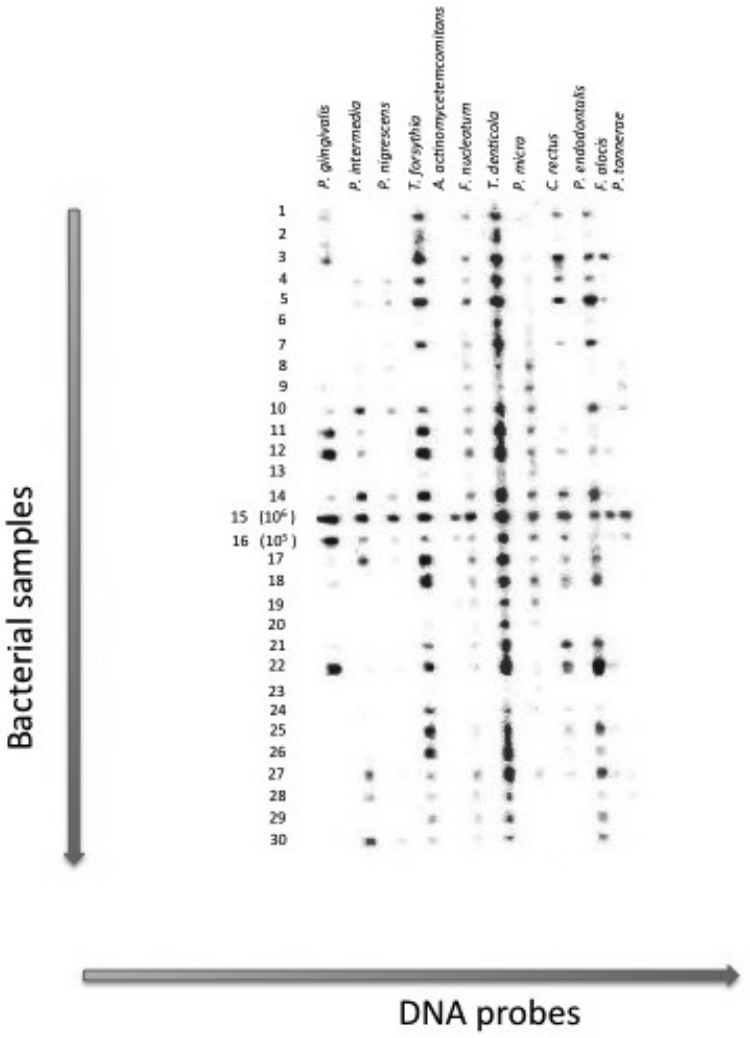
A. Culture (Studies I, II & III)

The paperpoints were placed separately for each unit (tooth/implant) in glass bottles containing 3.3 ml VMGA III (Dahlen et al. 1993) and transported to the laboratory for analysis within hours (Study III) or within two days (Study I, II). After gentle shaking with Vortex mixture, 0,1 ml of the volume of microbial suspension in the bottle was diluted into a series of 1:10 and 1:1000 (Study III) or 1:100 and 1: 10,000 times (Studies I & II) and spread with a metal loop and the streak-plate procedure evenly on a Brucella agar plate (BBL Microbiological Systems, Cockeysville, MD, USA) supplemented with 5% defibrinated horse blood, 0.5% haemolyzed horse blood and 5 mg/L of menadione, focusing on Gram-negative anaerobic bacteria. The agar plates were incubated anaerobically in jars for 6-8 days using the hydrogen combustion method (Moller and Moller 1961) i.e. 95% H_2 and 5% CO_2 at 37°C. Predominant anaerobic species were identified and in the case of dog strains (study III), they were processed for further phenotypic and genotypic characterization (see below under subtitle 'additional microbiological tests'). Total viable count (TVC) of all enumerated bacterial colonies, referred to as colony forming units CFUs, was calculated and the specific anaerobic bacteria were also expressed as % of TVC. Additional agar plates were used in studies I & II and specifically Blood agar (Difco, Detroit, MI, USA), Staphylococcus agar (Difco), Enterococcus agar (BBL) and TSBV agar plates (BBL). The relevant bacteria were incubated for 2 and 5 days, respectively, at 37°C in air with 10% CO_2. Special attention was given in human studies (I & II) to *Staphylococcus aureus*, *Staphylococcus epidermidis*, enterococci and AGNB. The latter ones were further characterized of whether they ferment lactose or not by use of eosin methylene blue agar plate (BBL). Thresholds for measure of growth of the colonies for the human studies were based on a classical study (Dahlen et al. 1982) (very sparse growth <0.1% TVC; sparse 0.1-1%TVC; moderate 1-10%TVC; heavy >10%TVC).

B. DNA-DNA hybridization (Studies I, III and IV)

Microbial samples intended for DNA-DNA hybridization were placed in sterile Eppendorf tubes separately for each implant/tooth unit and analyzed following the ''checkerboard'' methodology, as introduced by the Forsyth institute (Socransky et al. 1994), and modified by Papapanou (Papapanou et al. 1997) and Dahlén (Dahlen and Leonhardt 2006). The principle remained the same for all occasions. An equal amount of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 7.6) and 0.5 M NaOH, 100 µl each, were added in the tubes and the suspensions boiled for 5 min. The samples were neutralized by adding 800 µl 5 M ammonium acetate after boiling. Then, after samples having been lysed, the DNA from the respective samples was placed into the extended slots of a Minislot 30 apparatus (Immunetics, Cambridge, MA), concentrated onto a nylon membrane (Boehringer Mannheim) by vacuum, and fixed onto the membrane by cross-linking using UV light. The Minislot device permitted the deposition of 28 different samples in individual lanes on a single membrane, which also had two control lanes containing 10^5 and 10^6 cells of each bacterial species tested. The membrane with fixed DNA was placed in a Miniblotter 45 apparatus (Immunetics) with the lanes of DNA at 90° to the channels of the device. A 30-by-12 "checkerboard" pattern was produced for study I (see Fig. 4), whereas a 30-by-9 or 9 by 15 "checkerboard" pattern was produced for study III and a 30 by 16 ''checkerboard'' pattern for study IV.

Fig. 4. Illustration of a "checkerboard" panel 30 by 12.
The horizontal lanes represent 28 subgingival samples, adding in the middle (horizontal lanes 15, 16) the standards containing respectively10^6 (high standard) or 10^5 cells (low standard) of each test species. A signal at the intersection of the horizontal and vertical lanes indicates the presence of a species.



Each channel was used as an individual hybridization chamber for separate whole-genomic DNA probes. Bound probes were detected by anti-digoxigenin antibody conjugated with alkaline phosphatase and a chemifluorescent substrate (CSPD, Boehringer- Mannheim). The obtained signals were visualized in a LumiImager workstation (Boehringer Mannheim) and their intensity was evaluated by comparing them with signals of pooled standards corresponding to 10^5 and 10^6 cells of each species. The signals were coded on a scale from 0 to 5

(Papapanou et al. 1997). Special attention was given to score 3, which corresponded to the standard $> 10^5$ and $< 10^6$, indicating high levels of the bacterial cells. Semi-quantification was performed with the score system in human studies (I & II). Thus, score ≥ 3 corresponded to heavily colonized sites. In study IV, quantification was based on the ratio (percentage) of the DNA count of each species to the pooled standards, with the high standard reflecting the maximum percentage (100%).

C. Additional microbiological tests (Study III)

i) Phenotypic tests

All identified strains originating from the predominant cultivable subgingival microbiota in the dog were further characterized phenotypically by Gram staining, catalase production, haemagglutination capability of horse erythrocytes and API-ZYM test (API bioMérieux, Marcy l'Etoile, France) for enzyme production (Humble, King and Phillips 1977).

ii) *Genotypic test (16S rRNA gene sequencing)*

Genomic DNA was extracted and purified from bacterial cells in the mid-exponential growth phase, using a QIAamp DNA Mini kit (Qiagen Ltd, Dorking, UK). 16S rRNA gene fragments were amplified by means of a polymerase chain reaction (PCR) with universal primers. The PCR products were purified by use of a QIAquick gel extraction kit (Qiagen). The purified PCR products were sequenced directly with BigDye Terminator cycle sequencing kit (ver. 3.1) (Applied Biotech, Inc.) on an ABI 3100 Avant Genetic System (Applied Biosystems). The closest known relatives were determined by performing database searches, using the program FASTA (Pearson and Lipman 1988). These sequences and those of other related strains were retrieved from GenBank and aligned with the newly determined sequences, using the program SEQtools (Rasmussen 1995). The resulting multiple sequence alignment was corrected manually, using the program GeneDoc (Nicholas KB 1997).

Data analysis

The Statistical Package for social sciences (SPSS, version 18/19, SPSS Inc., Chicago, IL, USA) was used for both descriptive statistics and statistical analyses. Descriptive statistics alone were used only in study III for mean percentage and standard deviation of the predominant bacteria compared to TVC. The statistical computational unit was the subject in both human studies (I & II) and the dog in the experimental study (IV). The limit for statistical significance was set at p=0.05 in all studies.

In human studies (I & II) non-parametric tests were performed. Specifically, Chi-squared tests were applied to study associations between categorical variables. Kruskal-Wallis test was used to study differences between groups of a categorical variable and a continuous dependent variable. Follow-up Mann-Whitney (MW) U tests were additionally performed to investigate differences between pairs of groups and respective Bonferroni correction was applied. To evaluate any potential relationship between treatment outcome and various explanatory variables, a multiple regression model was also constructed (Study II).

In study IV, paired samples t-tests were applied to compare bacterial growth at teeth and the two implant groups, separately over time. Analysis of variance (ANOVA) and the Student-Newman-Keuls test were additionally applied to compare changes in bacterial load among the three groups (teeth, implant A, implant B).

Results

Studies I & II

The human studies (I & II) use the same pool of 281 patients from dental centers of Sweden, mostly public (80.1%). One center from Halmstad contributed to these studies with 1/3 of the cases. However, since no patient, disease and implant characteristics deviated from the overall results, this center was not considered to be an outlier. Baseline patient, dental and implant characteristics are presented below.

Table 4. Baseline patient, dental and implant characteristics.

Variables	Subcategory	N* (%)
Age	18-39	9 (3.2)
	40-59	51 (18.1)
	60-79	198 (70.5)
	80-	23 (8.2)
Gender	Male	108 (38.4)
	Female	173 (61.6)
Smoking habit	Current smokers	108 (38.4)
	Previous smokers	29 (10.3)
	Snuff	11 (3.9)
	Never tobacco users	116 (41.3)
	Unclear	17 (6.1)
Smoking dose	Heavy	28 (25.9)
	Moderate	25 (23.2)
	Light	40 (37)
	Unclear	15 (13.9)
Edentulism	Dentate	240 (85.4)

	Edentulous	40 (14.2)
	Unclear	1 (0.4)
Oral hygiene	Good	248 (88.3)
	Suboptimal	33 (11.7)
Number of implants	Single	20 (7.1)
	2-3	48 (17.1)
	4-6	146 (52)
	> 6	67 (23.8)
Arch	Maxilla	159 (56.6)
	Mandible	97 (34.5)
	Maxilla & Mandible	25 (8.9)
Implant surface	Turned (Nobel)	68 (24.2)
	TiUnite (Nobel)	45 (16)
	TPS (Straumann)	13 (4.6)
	SLA (Straumann)	49 (17.4)
	TiOBlast (Astra)	30 (10.7)
	Osseospeed (Astra)	15 (5.3)
	Unclear	61 (21.7)

**: Number of subjects in absolute count.*

All patients were diagnosed with peri-implant disease and a significant number (54.4%) had in addition periodontitis at the time of diagnosis and microbiological sampling. Most patients (91.4%) suffered from severe peri-implantitis. Details with regard to disease characteristics are presented in Table 5.

Table 5. Baseline characteristics of periodontal and peri-implant diseases.

Variables	Subcategory	N* (%)
Periodontal conditions	Healthy	7 (2.5)

	Gingivitis	24 (8.5)
	History of periodontitis	57 (20.3)
	Current periodontitis	153 (54.4)
	Unclear	42 (14.9)
Peri-implant disease	Mucositis	3 (1.1)
	Peri-implantitis	278 (98.9)
Peri-implantitis extent	Generalized	233 (82.9)
	Localized	48 (17.1)
Peri-implantitis severity	Severe	257 (91.4)
	Mild	19 (6.8)
	Unclear	5 (1.8)
Peri-implantitis development	Early (< 4 years)	116 (41.3)
	Moderate (4-6 years)	70 (25)
	Late (> 6 years)	76 (27)
	Unclear	19 (6.7)

**: Number of subjects in absolute count.*

Interestingly, implant surface was significantly correlated to the time of implants in function when disease was developed. TiUnite (Nobel Biocare, Göteborg, Sweden) and Osseospeed (Astra Tech, Mölndal, Sweden) were significantly associated with early disease development, SLA (Straumann, Basel, Switzerland) with moderate and late disease development and Turned (Nobel Biocare) with late disease development. TPS (Straumann) and TiOBlast (Astra Tech) were not correlated to the timepoint of disease development.

Microbiological analyses included culture alone in 139 (49.5%) patients and 'checkerboard' alone in 120 (42.7%) whereas samples from 22 patients (7.8%) were analyzed with both techniques. Table 6 depicts the detectability potential of species for culture and 'checkerboard' separately. Checkerboard had better potential to detect the specific species than culture (99.3% versus 81.4%). Nevertheless, both techniques, despite the use of non-identical scales, proved to detect high amounts of bacteria in 54.7% and 65% of the cases, respectively.

Table 6. Detectability potential of species for culture and checkerboard analysis.

Microbial analysis	Subcategory	N* (%)
Culture	No detection#	30 (18.6)
	Detection at high amounts‖	88 (54.7)
Checkerboard	No detection¶	1 (0.7)
	Detection at high amounts§	91 (65)

**: Number of subjects in absolute count.*

#: All species with %TVC = 0.

‖: At least one species with % TVC > 1%.

¶: All species with score = 0.

§: At least one species with score ≥ 3.

As demonstrated in Table 7, the majority of the species found by both microbiological techniques belonged to a Gram-negative anaerobic flora, primarily identified in peri-implantitis infection. *A. actinomycetmcomitans*, *S.aureus*, *S. epidermidis* and enterococci were among the least representative. However, the overall amount of the anaerobic bacteria could be regarded as low, having in mind that the majority of the cases included severely diseased implants. In addition, a significant number of AGNB were recorded in our material both at the time of diagnosis (18.6%) and at follow-up after surgery (25.9%).

Table 7 . Presence and counts of bacteria at moderately heavy/heavy amounts.

Bacteria	**Moderately heavy/ heavy** (culture)	**Score ≥ 3** (checkerboard)
	Percentage of subjects (%)	
Porphyromonas gingivalis	8.1	22.5
Prevotella intermedia/Prevotella nigrescens	27.3	25.4/28.9

Bacteria	**Moderately heavy/ heavy** (culture)	**Score ≥ 3** (checkerboard)
	Percentage of subjects (%)	
Tannerella forsythia		37.3
Aggregatibacter actinomycetemcomitans	6	4.2
Fusobacterium nucleatum		21.8
Treponema denticola		31
Parvimonas micra		9.9
Campylobacter rectus		7.7
*Porphyromonas endodontalis**		28.6
*Eikenella corrodens**		13.9
*Prevotella tannerae**		11.1
*Filifactor alocis**		7.9
*Streptococcus intermedia**		10.1
Staphylococcus aureus	1.2	
Staphylococcus epidermidis	0.6	
Enterococci	2.4	
Aerobic Gram negative bacilli (AGNB)	18.6	
Fungi	0.6	

**: reduced absolute count because of change of the panel.*

With regard to treatment and follow-up period, there was great variation, presented in Table 8. Antibiotics during surgery were administered in 170 (74.6%) patients. The most common regimen was the combination of amoxicillin and metronidazole, prescribed in 80 (47.1%). Establishment of peri-implant health was achieved in 111 (45.3%) of the cases.

Table 8. Peri-implantitis treatment-related characteristics

Variables	Subcategory	N* (%)
Type of treatment (N=274)	Non-surgical alone	46 (16.8)
	Surgical	228 (83.2)
Surgical treatment (N=228)	Access flap without AB§	48 (17.5)
	Access flap with AB	111 (40.5)
	Apical repositioned flap without AB	9 (3.3)
	Apical repositioned flap with AB	27 (9.9)
	Reconstructive surgery without AB	1 (0.4)
	Reconstructive surgery with AB	32 (17.1)
Follow-up after treatment (N=245)	9 months-1 year	96 (39.2)
	2-3 years	104 (42.4)
	4-6 years	40 (16.3)
	> 6 years	5 (2)
Treatment result (N=245)	Success	111 (45.3)
	Failure	134 (54.7)

**: Number of subjects in absolute count.*

We found a statistically significant correlation between non-smoking, late disease development, apical repositioned flap plus antibiotics and favorable treatment outcome with respective p-values 0.002, 0.047 and p=0.018. However in a final binary logistic regression model we constructed, it was only one variable (i.e. disease development) that could predict the likelihood of success of peri-implantitis treatment. Disease severity, type of therapeutic interventions and smoking did not contribute to the model. Early disease development was correlated to unfavorable treatment outcome. All final variables included in the model are presented in table 9.

Table 9. Logistic regression model predicting likelihood of treatment success.

Final independent variables	Odds ratio	95% CI for odds ratio		p-value
		lower	upper	
Age	1.016	0.986	1.046	0.308
Smoking	1.890	0.990	3.606	0.054
Number of implants	0.791	0.533	1.173	0.243
Disease severity	0.273	0.073	1.023	0.054
Non-surgical treatment	1.989	0.566	6.985	0.284
Access flap without AB§	0.664	0.201	2.195	0.502
Access flap with AB	1.141	0.451	2.885	0.780
Apical repositioned flap without AB	0.876	0.147	5.217	0.884
Apical repositioned flap with AB	3.073	0.898	10.513	0.074
Reconstructive surgery without AB	1.989	0.566	6.985	0.284
Reconstructive surgery with AB	9.833	0.000	-	1.000
Disease development	1.702	1.173	2.469	**0.005**

§: Antibiotics.

Study III

The predominant cultivable subgingival bacteria in dogs used for experimental studies are 5 to 6 species, belonging mostly to the obligate anaerobic Gram-negative flora, similar to humans. No species was identified in all six dogs, implying the great heterogeneity of the microbial profile amongst the dogs. The phenotypic characteristics of the species and their amount, expressed as mean percentage of TVC are described in table 10. Relatedness between dog and human strains was investigated by identifying cross-reactions using the checkerboard methodology. Dog strains were paired to DNA probes from human strains and some marked 'no cross-reactions' were noted. None of the dog strains belonging to *Porphyromonas* genus cross-reacted to human

Porphyromonas endodontalis and only *Porphyromonas gulae* and 1 strain *Porphyromonas* sp. (3:3) cross-reacted strongly to *P. gingivalis*. *Fusobacterium canifelinum* cross-reacted strongly with human *F. nucleatum* and *Campylobacter oricanis* but not *Campylobacter* sp. with *Campylobacter rectus*. Similarly, *Bacteroides* sp. (1:5) but not *T. forsythia* (dog) cross-reacted with human *T. forsythia.* Vice versa, human strains were cross-linked to probes from dog strains and some strong cross-reactions verified the relatedness of dog and human strains. All probes from dog strains belonging to *Porphyromonas* genus cross-reacted with human *P. gingivalis*, probes from all three *F. canifelinum* strains with *F. nucleatum* and probe from *T. forsythia* (dog) with human *T. forsythia.*

Table 10. Dog strains and their characteristics (OMGS stands for Oral Microbiology, Gothenburg, Sweden, CFU for colony forming units, TVC for total viable count, CCUG Culture Collection University of Gothenburg, API-ZYM method for semi-quantification of enzymatic activities)

Genus	Species	Dog:strain	Designation No OMGS/CCUG	CFU (%TVC)	Catalase	Haem-agglutination	API-ZYM
Porphyromonas	*P. canoris*	1:1	3832/57077	11	+	+	α-Chymotrypsin +++ Phosphatases +++ N-Acetyl-glucosaminidase +++
		2:1	3831/	15.2	+	+	α-Chymotrypsin +++ Phosphatases +++ N-Acetyl-glucosaminidase +++
	P. gulae	3:1	3860/57312	34.2	+	+	Trypsin +++ Phosphatases +++ Naphtolhydrolase +++ N-Acetyl-glucosaminidase +++
		4:1	3833/	12.8	+	+	Trypsin +++ Phosphatases +++ N-Acetyl-glucosaminidase +++

		5:1	3853/57307	5.2	-	+	Phosphatases +++ Naphtolhydrolase ++
		6:1	3837/	18.3	-	+	Phosphatases +++ Naphtolhydrolase +++
	P. cangingivalis	4:3	3862/57082	4.1	(+)	+	Phosphatases +++ α-Chymotrypsin ++ Naphtolhydrolase ++
		5:4	3851/57308	5.2	++	+	Phosphatases +++ α-Chymotrypsin +++
	Porphyromonas sp	3:3	3834/57313	36.7	+	-	Trypsin +++ Phosphatases +++ Naphtolhydrolase +++ α-Galactosidase +++ N-Acetyl-glucosaminidase +++
		5:2	3835/	33.8	+	-	Trypsin +++ α-Chymotrypsin +++ α-Galactosidase +++ N-Acetyl-glucosaminidase +++
Pasteurella	*P. stomatis*	3:6	3848/57315	2.5	+	+	Phosphatases +++
	Pasteurella sp	5:3	3852/	20.8	+	-	Esterase lipase ++ Naphtolhydrolase ++
	Pasteurella sp	6:3	3839/	24.4	++	+	Phosphatases +++ Naphtolhydrolase +++

Bacteroides	*B. tectus*	1:4	3856/57080	19.4	-	-	Phosphatases +++ α-Glucosidase +++ β-Glucuronidase +++ N-Acetyl-glucosaminidase +++
	Bacteroides sp	1:5	3857/	12.9	-	-	Phosphatases +++ N-Acetyl-glucosaminidase +++
	Bacteroides sp	2:3	3858/	12.7	-	(+)	Phosphatases +++ Naphtolhydrolase +++ β-Galactosidase +++ β-Glucosidase +++ N-Acetyl-glucosaminidase +++
	Bacteroides sp	3:4	3861	2.5	-	+	Phosphatases +++ Naphtolhydrolase +++ N-Acetyl-glucosaminidase +++
Tannerella	*T. forsythia* (dog)	2:4					Trypsin +++ Phosphatases +++ Naphtolhydrolase +++ α-Glucosidase +++ N-Acetyl-glucosaminidase +++ α-Fucosidase +++
Filifactor	*F. villosus*	1:3	3847/57079	16.1	+	+	Leucine arylamidase +++
	F. villosus	6:2	3838/57309	6.7	++	+	Leucine arylamidase +++
	F. alocis	5:5	3836/57083	10.4	-	-	Leucine arylamidase +++
Fusobacterium	*F. canifelinum*	1:2	3846/57078	5.9	-	+	Naphtolhydrolase ++

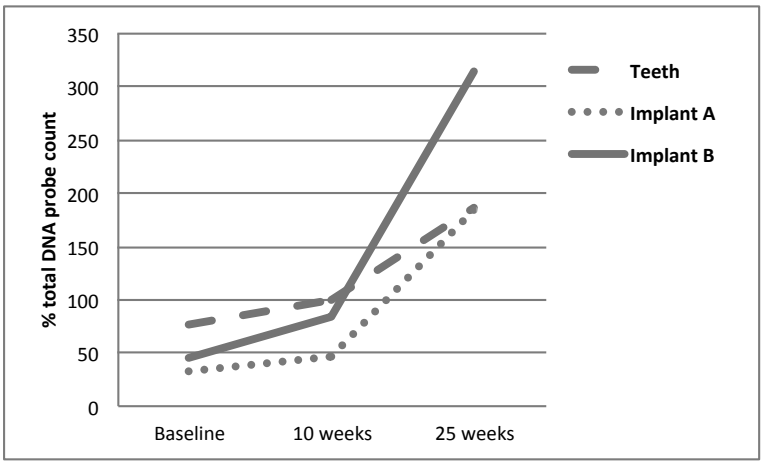
	F. canifelinum	2:2	3854/	5.7	-	+	Phosphatases +++
	F. canifelinum	4:5	3844/	2.3	-	+	Esterase +++
	F. russii	6:6	3842/57311	6.1	-	+	Phosphatases +++
Campylobacter	*C. oricanis*	4:2	3843/57305	8.1	(+)	+	Phosphatases ++ Leucine arylamidase ++
	C. oricanis	6:4	3840/57310	24.4	(+)	+	Phosphatases +++ Leucine arylamidase ++
	Campylobacter sp	6:5	3841/	7.3	(+)	+	Esterase ++ Valine arylamidase ++ Naphtolhydrolase ++
Peptostreptococcus	*P. canis*	2:5	3845/57081	3.2	-	+	α-Glucosidase +++
	P. canis	3:5	3849/57314	8.3	-	+	α-Glucosidase +++

Abbreviations: (+): weak reaction, +: positive reaction, ++: moderate activity/very positive reaction, +++: very strong activity, -: negative reaction.

Study IV

Data is reported from 5 and not 6 dogs because a male dog developed Addison´s disease and was euthanized 2 months after implant placement. Longitudinal microbiological results of the monitoring of the three groups (tooth, implant A, implant B) during 25 weeks are shown in Fig. 5. Baseline differences in bacterial load, expressed as mean percentages of total DNA probe count, between the three groups were not statistically significant. Microbial growth was ascending for teeth, implants of group A and B at all time points. The increase in bacterial load for teeth was not significant between BL and 10 weeks but was significant for the period 10 weeks-25 weeks and BL-25 weeks. For the implants in groups A and B, increase in bacterial amounts was significant for all periods (BL-10 weeks, 10 weeks-25 weeks, BL-25 weeks).

Fig. 5. Mean percentage (%) of total DNA probe count at teeth, implants in groups A and B over time. 100% corresponds to high standard DNA probe count of each species.



Changes in bacterial growth for all three groups at periods BL-10 weeks and BL-25 weeks was expressed as mean percentage of total DNA probe count difference of the 16 species between the respective time points. A comparison between the

groups with regard to bacterial growth revealed a marked but not statistically significant difference between the three groups (tooth, implant A and implant B) during BL-10 weeks and BL-25 weeks. However, a clear pattern for exponential bacterial growth at implant B was noted between 10 and 25 weeks. Figures 6 & 7 illustrate the microbial changes in DNA probe count for all 16 species used in the 'checkerboard' panel during the time spans BL-10 weeks and BL-25 weeks. The predominant bacteria accommodating the diseased implants and teeth during the tested experimental period belong mostly to *Pasteurella*, *Porphyromonas*, *Tannerella* and *Treponema* genera. The increased bacterial growth during the entire microbiological monitoring period (25 weeks) for the majority of the targeted species, especially at implants of group B is noteworthy in Fig. 7.

Fig. 6. Microbiological follow-up of teeth, implants in groups A and B during BL-10 weeks. The Y-axis represents mean percentage of DNA probe count difference of the 16 species between the two time points. Bars below zero indicate decrease of DNA probe count compared to BL.

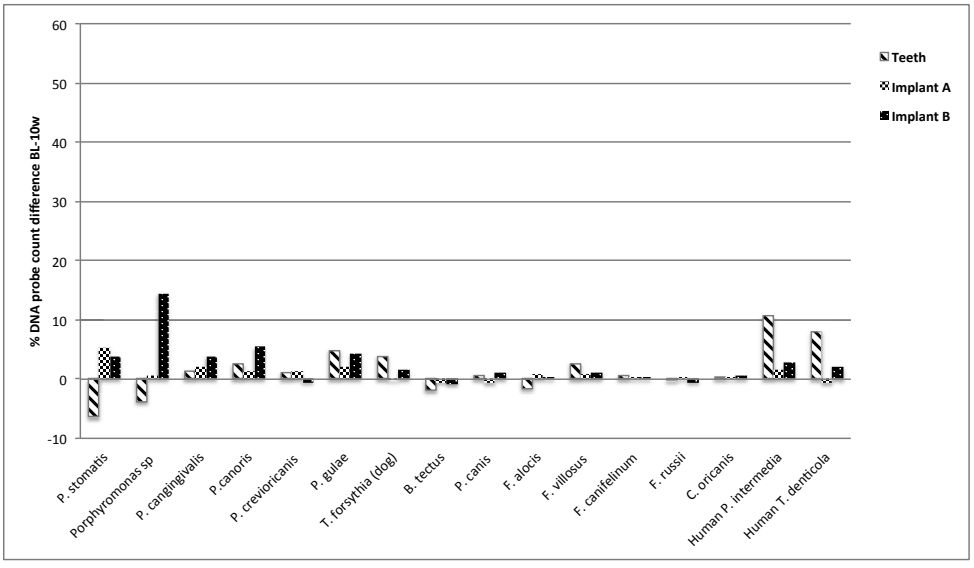
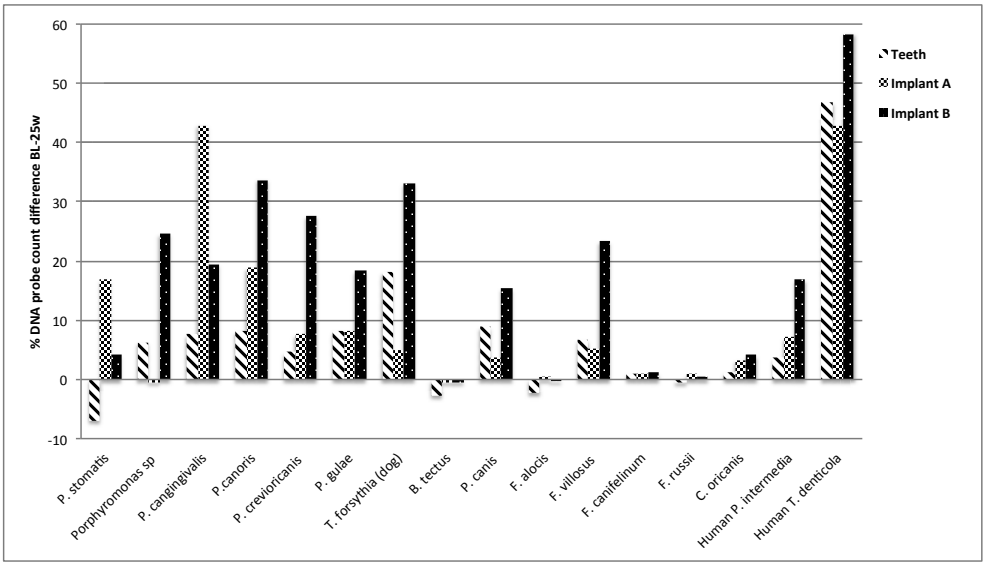


Fig. 7. Microbiological follow-up of teeth, implants in groups A and B during BL - 25 weeks. The Y-axis represents mean percentage of DNA probe count difference of the 16 species between the two time points. Bars below zero indicate decrease of DNA probe count compared to BL.



Main findings

- Patients with peri-implantitis harbored mostly Gram-negative anaerobic bacteria but high amounts of AGNB were detected in 18.6%. The microbiological burden failed to fully correspond to the severity of the disease (Study I).

- Great heterogeneity exists among clinics with regard to treatment protocols of peri-implantitis. Infection control and establishment of peri-implant health was achieved in 45.3% of the patients. Early disease development was correlated to unfavorable treatment outcome. Regression analysis could identify disease development as the only variable to be able to predict the likelihood of treatment success (Study II).

- The subgingival microbiota in dogs used for experimental purposes displayed greater heterogeneity but did not differ substantially with the respective one in humans, at genus level. Nevertheless, marked differences existed at species level (Study III).

- Suspected subgingival bacteria, including *Pasteurella*, *Porphyromonas*, *Tannerella* and *Treponema* genera were involved in experimental periodontitis and peri-implantitis in dogs. A pattern of greater increase in bacterial growth was detected at implants of group B compared to implants of group A and teeth from baseline to the end of the experiment (Study IV).

Discussion

Methodological issues on study design

This thesis is a collection of studies that vary in design. While the two experimental studies in the dog are prospective (Study III is cross-sectional and study IV longitudinal), the human studies are retrospective. Retrospective research has largely become undervalued and underutilized with the increasing singular focus on Randomized controlled Trials (RCTs). RCTs are positioned at the top of quality in research evidence but usually address only one specific question, which might not get a robust answer due to stringent criteria that in turn lead to small study samples and end up to insignificant statistical differences. Studies I & II, being retrospective but longitudinal in nature, enabled us to track a large group of patients with peri-implantitis already from implant placement to disease emergence, therapeutic approach and follow-up of treatment. They unveiled the broad course of events that took place from the very beginning to the very end, correlated disease conditions with microbiological findings and identified potential factors associated with disease development and treatment outcome. However, the material was very heterogenous including various clinics with different protocols both in treatment and aftercare of patients. Thus, interpretation of results should be made with great caution. An additional limitation was the lack of a control group with peri-implant health that would allow us to discuss prevalence of peri-implantitis and risk factors.

Study IV had a longitudinal design, which was different compared to all previous microbiological experimental studies. No focus was placed on the active breakdown of the supporting tissues with the ligaments. Previous microbiological studies on experimental peri-implantitis performed sampling during the ligature-induced disease period. In a study using the microswine model, longitudinal sampling was performed at 0, 14 and 45 days after silk ligature application (Hickey et al. 1991). Similar designs in dogs (Nociti et al. 2001, Shibli et al. 2003, Tillmanns et al. 1998) and monkeys (Eke, Braswell and

Fritz 1998) observe the microbiological changes over time during the period with ligatures. The first microbiological experimental peri-implantitis study in dogs (Leonhardt et al. 1992) tracked the microbial profile at implant sites before and after ligature placement as well as 4 weeks after ligature removal by means of culture. In another study in monkeys (Hanisch et al. 1997a) samples were obtained before ligature placement, 10 months after placement and 1 month after ligature removal. In our study, microbiological monitoring of the disease started immediately after the removal of ligatures. Rationale was that microbiological changes would mimic the clinical situation of periodontal and peri-implant lesion progression at the chronic phase of the established disease, with plaque accumulation being the only inducing factor. Thus, microbiological samples were obtained longitudinally at the time of ligature removal (BL), at 10 and 25 weeks after ligature removal.

Methodological microbiological issues

Microbiological sampling in all studies was performed with paperpoints. We aimed at the non-adherent, loosely attached bacteria, mostly those 'swimming' in the peri-implant crevicular fluid. We assume that neither the microbiota adherent to the pocket epithelium nor the one attached to the implant surface in the form of a biofilm were captured. A study comparing sampling with paper strips versus curettes at implants (Gerber et al. 2006) found higher total DNA probe counts from paper strips than curettes. Probably, the bacteria in the crevicular fluid, being in planktonic state are of more interest in terms of virulence, due to their higher metabolic activity. Within biofilms, bacteria grow more slowly and exhibit different gene expression than free planktonic bacteria. A microarray study (Resch et al. 2005) demonstrated for *S. aureus* that genes coding for many virulence factors were much more highly expressed in planktonic cells than in sessile (biofilm) cells.

Culture and checkerboard were the principal microbiological methods used for analysis of the samples in this thesis. An early comparative study between the two methods (Papapanou et al. 1997) concluded that both culture and 'checkerboard' indicated an acceptable degree of agreement. Pattern of

detectability was much more sensitive for 'checkerboard' than culture in study I (table 6). This lies in the different philosophy between the two methods.

Culture gives us a broad picture of all cultivable bacteria without any pre-targeting approach. However, the type of dispersal procedures, the culture media and the circumstances used for anaerobiosis may lead to disturbing discrepancies in the results. Limitations in the reproducibility of repeated samples at the same site have also been discussed in the literature (Mombelli et al. 1989), with a variation in recovering microscopic count, ranging from 20% to 70%. Culture has relatively low sensitivity especially when non-selective media are used and low numbers of specific bacteria in a subgingival/submucosal sample will be undetected.

DNA-DNA hybridization belongs to molecular techniques, developed by Socransky and co-workers (Socransky et al. 1994). It allows for the assessment of large amounts of plaque samples and multitude of species by hybridizing DNA samples against whole genomic DNA probes on a single support membrane. The sensitivity of the 'checkerboard' DNA-DNA hybridization assay is usually set to 10^4 cells of a bacterial species by adjusting the concentration of each DNA probe in the hybridization buffer. Whole-genomic probes have been largely criticized for their cross-reactivity with even heterologous species, leading to false-positive results (low specificity) and questioning consequently the validity of the results. While a cloned fragment of *A. actinomycetemcomitans* was not cross-reactive, a whole genomic probe of this bacteria was found to cross-react, even with *Haemophilus* bacteria, thus not being of any use in clinical samples (French et al. 1986). One study found that culture could better detect periodontal bacteria than whole-genomic DNA probes (van Steenbergen et al. 1996). Although whole genomic probes are more likely to cross-react with non-target bacteria due to the presence of homologous sequences between different bacterial species, the results of other studies suggest that this was not a common occurrence (Gunaratnam et al. 1992, Siqueira et al. 2002). Collectively, the quality of the probe and the stringency conditions incorporated in the hybridization process are critical determinants of a successful diagnostic performance of the method. Although the occurrence of cross-reactivity cannot be discarded, cross-reactions are usually limited in high-quality probes and observed within the genera, which

as an internal error is not that critical in polymicrobial anaerobic infections, where we do not expect from a specific strain to induce pathology.

Interpretation of results

More than half of patients (54.4%) in studies I & II diagnosed with peri-implantitis suffered at the same time from the counterpart disease around teeth. This implies that supportive care of partially edentulous implant patients in various clinical centers of Sweden was not optimal after implant therapy. It is highly likely that patients with periodontal disease were treated before implant therapy but were referred back to their regular dentists after implant placement and restoration. This implies that meticulous maintenance protocols were not applied at frequent intervals from general practitioners, which in turn may explain recurrence of periodontitis.

In **study I** type of implant surface was associated with disease development. One should be cautious in the interpretation of the results due to the retrospective nature of greatly heterogenous material and no robust conclusions can be drawn. The association of moderately rough surfaces (TiUnite and Osseospeed) with early disease development as well as relatively smooth (Turned) with late disease development can be partly explained by the timing they were launched in the dental market. Turned surface represents the implants with most years in function as being the first in the era of osseointegration, while the newer modified surfaces were launched after the year 2000. A rougher implant surface was the aim for the industry, since it was believed that this would speed up bone to implant contact and make the implant a feasible and convenient treatment option for all types of patients. However, a systematic review failed to identify any implant surface being more advantageous than others in preserving marginal bone levels at implants with ≥3 years in function (Abrahamsson and Berglundh 2009). There is a biologically plausible scenario in the association of specific implant surface, as for instance TiUnite, with earlier development of peri-implantitis (study I) or more progressive bone loss as shown in experimental studies in dogs (Albouy, Abrahamsson and Berglundh 2012, Carcuac et al. 2012). This highly crystalline microscopic surface has pores, which

can serve as 'nests' for bacteria to multiply and grow undisturbed. A human cross-sectional prospective study in a Belgian population (Marrone et al. 2012) found significant difference in prevalence of peri-implantitis between rough (TPS) and Turned implant surfaces (36.1% versus 19.4% respectively). However, implant surface is only one aspect of implant design and other aspects are equally important. The threaded profile of the implant alone favors bacterial accumulation, irrespective of surface. An experimental study in the dog, despite methodological concerns, failed to show significant differences amongst TPS, hydroxyapatite-coated, acid-etched and minimally rough implant surfaces with regard to bone loss (Martins et al. 2004) and TVC of bacteria (Shibli et al. 2003) over a period of 60 days after initiation of ligature-induced disease.

The threaded design of the dental implant coupled with surface roughness creates a local environment hard to access and control (Renvert et al. 2008c). This difficulty in achieving infection control is reflected both by the increased number of peri-implantitis cases with additional surgical intervention and the high number of cases with unfavorable outcome even after surgery in our material (Study II). Success in peri-implantitis treatment depends largely on the definition of successful treatment. In our study we defined success as a patient case with no bleeding and/or suppurating pockets ≥ 5 mm. In another clinical trial (Serino and Turri 2011) a clear discrepancy in the treatment outcome was shown with different thresholds. The percentage of successful cases dropped from 77% with a threshold of no pockets ≥ 6 mm to 48% with a stricter threshold of no pockets ≥ 4 mm.

Study II showed a statistically significant correlation between smoking and smoking dose with treatment outcome. Moderate and heavy smokers were significantly associated with treatment failure and non-smokers with treatment success. However, a significant amount of non-smokers (47%) had experienced peri-implantitis treatment failure, implying that additional factors, other than smoking, contribute to the unfavorable treatment outcome. The earliest relevant study in the literature was a case series of few numbers of patients, able though to demonstrate an unsuccessful treatment outcome in smokers (Leonhardt et al. 2003). The prospective study of Serino & Turri (Serino and Turri 2011) made statistical calculations at implant level due to scarce number of smokers and is

not relevant on a patient level. Another recent study (Heitz-Mayfield et al. 2012) on 36 patients showed that smoking had no significant effect on therapeutic outcome on anti-infective surgical therapy of peri-implantitis.

In the logistic regression model aiming at predicting treatment outcome, disease development at early stage after implants in function (1- 3 years) was correlated to treatment failure. The fact that early disease development was correlated to moderately rough surfaces (Osseospeed, TiUnite) may imply that bacteria express a more virulent and resistant profile in this niche, difficult to reach, giving a picture of an acute infection with a strong microbial challenge at this early stage.

Treatment protocols for peri-implantitis in study II, though heterogenous among centers, were a modification of techniques used for many years to treat bone defects around natural teeth. Access flap with antibiotics was not associated with successful treatment outcome. This finding should be interpreted with great caution because surgical protocols were greatly heterogenous among the clinical centers. A recent prospective clinical trial (Heitz-Mayfield et al. 2012) showed that access flap with antiseptics and antibiotics could arrest progression of peri-implantitis over 1- year period. However, our result is in line with an early guideline using periodontal surgery towards a change of the local environment (Mombelli et al. 1995b). Shift from anaerobic to aerobic conditions by means of resection or reconstruction, not favoring the recolonization and growth of anaerobic bacteria, seems to be critical to achieving long-term microbial homeostasis and in turn establish peri-implant health.

The antibiotic regimen most frequently described during surgery in study II was the combination of amoxicillin and metronidazole. This 'cocktail' was originally introduced for the treatment of *A. actinomycetemcomitans* periodontitis (van Winkelhoff, Tijhof and de Graaff 1992), though recently questioned by a 3-month double blind, placebo controlled, randomized longitudinal study (Mombelli et al. 2012a). Two double-blind placebo studies (Cionca et al. 2009, Winkel et al. 2001) have generalized the use of the aforementioned antibiotic scheme for all chronic periodontitis cases with favorable outcomes over a 6-month period. However, a comparative study between metronidazole and the amoxicillin + metronidazole regimen would be of interest to justify the use of a 'cocktail'. Peri-implantitis is a

polymicrobial anaerobic infection and metronidazole alone should be effective, as a broad-spectrum antibiotic against anaerobic bacteria.

In both human studies (I and II) microbiological analyses were performed with culture and/or 'checkerboard'. We used a separate quantity scale for culture and 'checkerboard' analysis to correlate microbiological findings with the clinical registrations around diseased implants. The microbiological cut-off point was >1% TVC for culture and score ≥ 3 for 'checkerboard'. The microbial load did not correspond to disease severity, since the sampling method in 35%-45% of the cases failed to attract adequate amounts of bacteria, associated with the defect. We attributed this paradox to the technical difficulties confronted by clinicians during sampling. A thick superstructure hampers accessibility of the paperpoints to the bottom of the pocket and this in conjunction with the threaded non-smooth profile of the implant may result in underdiagnosis of the peri-implant pathology from a microbiological aspect. Clinicians are advised to remove implant-supported bridgework before microbiological sampling to increase reliability of the sampling process. Optimal sampling is a prerequisite for optimal microbiological analysis, irrespective of the methods used. However, in our material culture and 'checkerboard' were still able to identify moderately heavy/heavy amounts of bacteria, reflecting pathology clinically in 54.7% and 65% of the cases respectively. Despite the significant number of false negatives (> 1/3 patients) due to sampling problematique, both methods of analysis had sensitivity to reach the respective microbiological thresholds > 50%.

The results of the microbiological analyses confirmed that peri-implantitis is a mixed non-specific anaerobic infection, with opportunistic 'pathogens' not to largely differ from what we have identified around teeth suffering from periodontitis (Augthun and Conrads 1997, Becker et al. 1990, Hultin et al. 2002, Kalykakis et al. 1994, Koyanagi et al. 2010, Kumar et al. 2012, Listgarten and Lai 1999, Mombelli et al. 1987, Rams et al. 1991, Salcetti et al. 1997, Sanz et al. 1990, Shibli et al. 2008, Tabanella et al. 2009, van Winkelhoff and Wolf 2000). In studies I and II *A. actinomycetemcomitans* was identified in significant amounts in very scarce numbers of cases both by culture and 'checkerboard' corroborating other studies negative for *A. actinomycetemcomitans* or detecting it at low amounts (Botero et al. 2005, Koyanagi et al. 2010, van Winkelhoff and

Wolf 2000). Anaerobic bacteria, not necessarily belonging to 'red complex' may play equally significant role in disease pathogenesis, as they were found in high amounts such as: *P. intermedia*, P. *nigrescens* and *P. endodontalis*. This implies that single focus on 'red complex' may underestimate the peri-implant condition. Interestingly, we found a significant number of cases with high numbers of AGNB both at the time of initial sampling before any intervention as well as at follow-up sampling after surgery. Some studies have detected AGNB in peri-implantitis cases (Alcoforado et al. 1991, Botero et al. 2005, Leonhardt et al. 1999, Rosenberg et al. 1991) as well as in healthy implants (Leonhardt et al. 1999, Nowzari et al. 2008). The critical question is if AGNB are simply 'bystanders' or play a true role in disease pathogenesis. Since AGNB are aerobic bacteria, we would assume that they do not exert virulent properties in a deep anaerobic pocket, and thus are in a symbiotic state with the anaerobic flora. However, large- scale prospective studies are needed to elaborate on this assumption. If it proves to be that diseased implants continuously harbor AGNB longitudinally after failed therapeutic interventions, they should contribute to disease. *S. aureus*, though discussed in peri-implantitis literature recently (Renvert et al. 2008b, Salvi et al. 2008), was in scarce numbers at sampling time during disease diagnosis and negative at postoperative sampling. Similar findings were presented for other staphylococci and *Candida*, although they have been detected in peri-implantitis cases (Alcoforado et al. 1991, Leonhardt et al. 1999).

Study III contributed to the existing microbiological literature on dogs used for experimental purposes by classifying dog species that had never been analyzed further from genus level. Dog strains that were previously designated as e.g. '*Porphyromonas*-like' dog species have not only obtained a name for taxonomic purposes but most importantly have been correlated to respective human strains to investigate the degree of genetic relatedness. Previous microbiological experimental studies provided a vague description of the species either by culture (Eke et al. 1998, Hanisch et al. 1997a, Hickey et al. 1991, Leonhardt et al. 1992, Shibli et al. 2003), DNA probe (Hanisch et al. 1997a, Tillmanns et al. 1998) or PCR analysis (Nociti et al. 2001). However, one can question the indisputable use of DNA probes from human strains for detection and quantification of bacteria in dogs as done before the DNA sequencing era (Hanisch et al. 1997b,

Loos and Dyer 1992, Madianos et al. 1994, Tillmanns et al. 1998) as well as after (Rober et al. 2008).

Predominant cultivable subgingival bacteria in dogs belonged to *Porphyromonas*, *Fusobacterium*, *Bacteroides*, *Campylobacter*, *Peptostreptococcus* genera with regard to strictly anaerobes and *Pasteurella* genus with regard to facultative anaerobes. The great heterogeneity, with none of the predominant species found in all 6 dogs, is striking, given the similar age, diet and environmental conditions of the dogs. Previous experimental studies in dogs have identified at baseline (before ligature placement) *Porphyromonas* (Hanisch et al. 1997a, Leonhardt et al. 1999), *P. intermedia*/*nigrescens* (Nociti et al. 2001), *T. forsythia* (previously designated as *Bacteroides forsythus*) (Hanisch et al. 1997a), Fusobacteria (Hanisch et al. 1997a, Leonhardt et al. 1999, Nociti et al. 2001), *Capnocytophaga* (Hanisch et al. 1997a, Leonhardt et al. 1992). Interestingly, AGNB were not found in the subgingival flora of any dogs used for experimental purposes but isolated from the canine oral cavity (Isogai et al. 1989, Wunder, Briner and Calkins 1976). *Prevotella* spp., *Treponema* spp. (spirochetes) and AGNB were not identified in our Labrador dogs. In addition, from the facultative aerobic flora no streptococci were isolated in contrast to other experimental studies (Hanisch et al. 1997a, Leonhardt et al. 1992, Nociti et al. 2001). However, in one study focusing on streptococci in the dogs (Takada et al. 2006), it was concluded that streptococci in dogs from an animal research center represented only 2.5% of TVC. *Pasteurella* spp. have not been detected in the aforementioned experimental dog studies but consisted almost ¼ of the mean TVC in our Labrador dogs. Interestingly, *Pasteurella* species have been frequently isolated from dog bites (Talan et al. 1999). In our material we identified 4 *Porphyromonas* strains to species level (*P. canoris*, *P.gulae*, *P. crevioricanis*, *P. cangingivalis*) and 2 remained to genus level (*Porphyromonas* sp.). Up to 7 different *Porphyromonas* species have been identified in dogs/cats (Mikkelsen et al. 2008).

Correlations of DNA probes from human strains with dog strains, as performed following 'checkerboard' methodology, showed that *P. gulae* was genetically close to human *P. gingivalis* but the rest *Porphyromonas* dog strains were rather distant (weak reactions) and even more distant from human *P. endodontalis*. Similarly, dog *F. canifelinum* was close to human *F. nucleatum*, dog *Filifactor*

villosus to human *Filifactor alocis* and dog *C. oricanis* to human *C. rectus*. This implies that the dog model is a valid tool for experimental study of periodontitis/peri-implantitis because the anerobic subgingival flora in dogs resembles the counterpart flora in humans. However, distinct differences at species level call for avoidance of the use of human DNA probes in studies of the dog flora. Not making an effort to construct dog DNA probes in dog experimental studies could easily lead to false interpretation of the results.

In **study IV** microbiological changes around diseased teeth and implants were tracked longitudinally in dogs after ligature removal, leaving plaque to be the only factor contributing to disease progression without the influence of ligatures. This microbiological study is presented separately but belongs to a series of radiological and histological investigations -presented elsewhere (Carcuac et al. 2012)- by use of the same experimental dogs. Fourteen DNA probes from dog strains were constructed, based on the predominant subgingival cultivable flora. However, the 'checkerborad' panel also included DNA probes from two human strains (*P. intermedia* and *T. denticola*) because both Prevotella (Nociti et al. 2001, Radice, Martino and Reiter 2006) and spirochetes (Nordhoff et al. 2008, Riviere et al. 1996, Syed, Svanberg and Svanberg 1981) have been isolated from the canine oral cavity.

The increased bacterial growth over time for all groups separately (teeth, Turned and TiUnite implant surfaces) can be explained by the undisturbed plaque accumulation at all time periods. Thus, more plaque is expected to form during the progression of the experimental lesions. Similarly increased growth was recorded in previous longitudinal studies, though they had focused only on the ligature-induced period (Hanisch et al. 1997a, Leonhardt et al. 1992).

Interestingly, bacterial load was greater at teeth than implants with Turned surface during the first 10-week follow-up after ligature removal. This may imply that the pristine smooth surface of an implant may accumulate submucosal bacteria at a lower rate, compared to teeth that are exposed to the oral environment for longer time. However, this trend does not continue and during the following 15-week period bacterial load at implant A is higher than at teeth, ending up to a similar growth with teeth at the final sampling time. The maximal growth for bacteria during the 10-week period following ligature removal was

noticed at implants with the modified surface and continued in the same trend to reach exponential growth at the end of the microbiological monitoring period. The increased bacterial load at TiUnite implant surface during the 25-week period compared to turned surface and teeth had a tendency to corroborate the significantly greater marginal bone loss observed around this surface compared to the rest (Carcuac et al. 2012). The greater the defect on x-rays the deeper the pocket and the more the bacteria to survive and thrive in this environment. It may be the porous surface that has a direct impact on bacterial growth, offering 'protected areas' for increased bacterial synergism and undisturbed multiplication. Indeed, in a series of previous experimental studies, TiUnite was compared to other moderately rough surfaces and both an increased bone loss (Albouy et al. 2008) and a larger inflammatory connective tissue (ICT) area (Albouy 2009) was found for TiUnite. It was suggested that characteristics other than the roughness, such as the presence of porosities may be related to different tissue reactions around this specific surface (Albouy 2011).

Total bacterial load differences between the groups failed to corroborate the corresponding findings from the radiological assessments and were not statistically significant, despite a clear tendency. This finding is explained by the great internal variation of DNA probe counts between dogs as well as within dogs. The inter- and intra-variations reflect not only aspects on power but also considerations to sampling methodology.

Diseased implants and teeth in this experimental model shared similar predominant species, a mostly anaerobic proteolytic flora. *P. stomatis* was the only predominant facultative anaerobic, the rest belonged to obligate anaerobes and primarily to the *Porphyromonas*, *Tannerella* and *Treponema* genera. All bacteria were well identified from the subgingival plaque in dogs (study III) implying that endogenous bacteria from the subgingival environment caused the progression of the lesion both around teeth and implants in the dog model. Neither teeth nor implant surfaces conferred predisposition to infection with specific bacteria. Both diseases are polymicrobial, anaerobic and to great extent Gram-negative infections, as also suggested in the clinical reality from humans (Dahlen 2006, Koyanagi et al. 2010, Shibli et al. 2008). The fact that certain species demonstrated striking changes over the 25-week observation period (e.g.

decrease of *P. stomatis* at teeth, increase of *P. cangigivalis* at Turned implant surface and increase of *T. forsythia* at TiUnite implant surface), though not significantly higher, should not be interpreted as a pattern of disease specificity because there is no biological rationale for this assumption. This implies that the tendency for increased growth of specific bacteria at the end of the experiment could be random.

General discussion

Peri-implantitis has been discussed in the literature mostly as an infectious disease, based on its bacterial aetiology. Studies have focused on mere descriptions of names of bacteria, found in the peri-implant lesion or implicated in the disease process without further investigation of the role of the bacteria. Specific bacteria will only be associated to the disease and never linked with causality. As long as researchers are thrilled by single bacteria or single inflammatory changes, subsequent to the microbial challenge, overlooking the overall microbiological component, no clear conclusions on disease aetiopathogenesis can be drawn. Instead, studying the characteristics and activities of the bacterial mass as a whole, would be of great significance, because there might be a certain threshold that the host defence cannot cope with, and disease process starts (disruption of homeostasis). Differentiating transient harmless bacteria from those having increased pathogenicity and thus true involvement in disease initiation and progression is critical, in order to assign peri-implantitis as a true infection. A relevant unanswered question from this thesis is the role of AGNB. Despite the fact that AGNB were found in high amounts in almost 1/5 of patients with peri-implantitis, we do not know if and with which mechanism they contribute to the disease.

This thesis focused on quantification of associated bacteria and correlation of quantitative thresholds to disease severity. Obviously, to assess growth and multiplication of bacteria by calculating counts is one aspect of infectious potential.

Another aspect of pathogenicity that is plausible and has not been investigated in this thesis, is invasion. The more fragile soft tissue barrier at implants, coupled

with the direct bone to implant contact without an intervening periodontal ligament, makes us postulate that bacteria with increased virulent properties could invade the bone. In such cases, peri-implantitis clinically could have some commonalities to osteomyelitis.

In studies I and II, the magnitude of bacterial mass failed to corroborate the severity of peri-implantitis. It could be the complex technical diffuculties that hampered an optimal sample of the peri-implant site, as already described. If the bacteria invade the peri-implant tissues, the presence of bacteria in the connective tissue and not only in the peri-implant crevicular fluid, would additionaly explain our false negative results.

Future focus on the invasive potential of bacteria in the peri-implant tissues woud be of interest, as it would have an impact on treatment strategies. Invasive bacterial profile would imply spreading of the infection into the adjacent tissues and use of antibiotics should be considered in order to arrest the progression of the peri-implant lesion.

Concluding remarks

In this final extract I would like to summarize the implications of my microbiological results for future research.

- Peri-implantitis is clearly a polymicrobial, principally a Gram-negative anaerobic infection, as shown from two different research fronts, both human and experimental dog material. No specific bacteria would have a more orchestrated role or become 'keystone' pathogens consistently in all cases. Focus on 'red complex' or *S. aureus* alone may be a false single-minded explanatory approach. The dog has an even more heterogenous subgingival flora compared to humans, implying even more that it would be naïve to investigate specific species. Lack of microbial specificity in the aetiology of peri-implantitis should not dampen our enthusiasm for microbiology but arouse us to look at bacterial mass (quantification) and products as a whole submucosal community.
- The dog model serves as a great tool for the comparative study of various processes and study units; naturally induced versus ligature-induced disease; teeth versus implants as well as animals versus humans. The experimental model used in study IV has been further developed to focus on events that occur after the termination of the active breakdown with ligatures. The microbiological events that occur during the progression of the lesion without ligatures, having only plaque as the contributing factor, may recapitulate to great extent the natural disease progression. The defects harbor bacteria not differing to the naturally occurring peri-implant defects and the quantity of bacteria is in line with the size of the bony defect.
- AGNB have been identified in peri-implantitis patients but not in experimental studies in dogs. Prospective large-scale longitudinal clinical studies should be conducted in order to elaborate on whether AGNB have a true role in the pathogenesis of peri-implantitis. If AGNB are continuously recovered at microbiological follow-ups of unsuccessfully treated peri-implantitis cases, probably AGNB are not simply 'bystanders' and their pathogenicity should be further investigated.

- ‘Checkerboard’ DNA-DNA hybridization has a very good potential to ‘identify’ sites and patients experiencing peri-implantitis. We have to pre-target suspected ‘pathogens’ and thus fail to detect the ‘unexpected’ but in infections around dental implants we do know more or less what to expect. I would suggest that the panel should include around 8-10 DNA probes from representative species, as too many would make the results difficult to read without any more significant contribution and too few would imply specificity, which is not the case. With regard to use of ‘checkerboard’ in experimental peri-implantitis in the dog, we should continue to use dog DNA probes. It is a matter of accuracy, as there is some degree of discrepancy from the human probes and it might be critical for correct quantification of the total DNA probe count.

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Acknowledgements

I would very much like to express my sincere gratitude and appreciation to all those who made this research journey look so exciting and rewarding.

To Gunnar Dahlén, my main supervisor, for creating by far the most inspiring and vibrant research environment for me. His deep knowledge, meaningful curiosity and open-mindness have sharply molded my thinking. Having spent so many hours with a person of unquestionable integrity, never-ending generosity and everyday simplicity, I do feel I have become a better person.

To Åsa Leonhardt, my co-supervisor, for her invaluable advice, critical thoughts and her authenticity.

To Ingemar Abrahamsson, my co-supervisor, for his pedagogic support, admirable patience and stimulating discussions.

To Tord Berglundh, for instilling in me critical thinking as well as precision and clarity in academic writing.

To Lars Heijl, for supporting my research work and being so influential in my education during my residency years in the Department of Periodontology.

To Anette Carlén, for guidance and efficient resolution of administrative issues.

To Lisbeth Bengtsson, for all her skilful technical assistance and for teaching me step by step routine microbiological methods.

To my co-authors Per Rabe, Olivier Carcuac and Enevold Falsen for constructive collaboration and valuable help with the manuscripts.

To all my colleagues at the department of Oral Microbiology and Immunology for embracing me with their warmth from the very beginning and continuously looking out for me.

To my friends, coming from all walks of life, for reminding me of the magic and wonder in human relationships.

Finally, I am indebted to my grandmother Evangelia, my parents Christoforos and Ioanna, as well as my twin-brother Vangelis for their continuous and unconditional love and support.