Titanium Corrosion is a Modifier of Peri-implant Health

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Abstract

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Dental implants are highly successful at replacing missing teeth and restoring Oral-Health Related quality of life. Despite a high rate of success, however, there remain a significant number of patients who develop peri-implant mucositis and peri-implantitis. The definition of success for implants extends beyond merely implant survival; in addition to lack of mobility "success" encompasses the maintenance of health of the peri-implant tissues and supporting bone despite the constant microbial challenge in the oral environment.

It is well documented that the titanium dioxide (TiO_2) passive layer is responsible for the biocompatibility of titanium dental implants and that weakening this layer leads to titanium dissolution and corrosion. Recent data support the implication of titanium corrosion as a factor in peri-implantitis and preliminary data support that certain oral taxa may cause bio-corrosion *in vitro*. It remains unknown whether titanium corrosion is

related to differences in the peri-implant microbiome. It is also unknown whether titanium corrosion affects a change in the peri-implant health is by affecting site-specific changes in pro-inflammatory gene expression. This thesis explores the role of titanium corrosive particles in peri-implant disease and explores pathways that have plausibility for being affected by titanium.

The subsequent pages are an examination of the prevalence and risk factors associated with peri-implantitis and the role of titanium as a modulator of the oral microbiome and host response. They will provide evidence of the significance of the problem of peri-implant disease in a United States population and the risk of implant failure associated with patient specific factors such as diabetes and periodontal disease. Next, they will provide evidence that titanium corrosion products, and not the disease status, shape the peri-implant microbiome suggesting that dissolved titanium creates a unique niche in the oral cavity causing a shift of the composition of the peri-implant microbiome. In addition, the relationship between methylation levels, titanium particles, and peri-implantitis was assessed. The results show that Global DNA Methylation levels in the peri-implant crevicular fluid are greater in peri-implantitis cases when compared to controls, and independently associated to higher plaque-adjusted titanium quantities. In summary, these findings unveil the role of titanium as a dysregulator of peri-implant health, support the consideration of peri-implantitis as a distinct entity from periodontitis and highlight the importance of maintaining titanium surface biocompatibility during maintenance, and disease management.

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This work could not have proceeded without the generous support of patients that I have known over the 30 years I have been in the Department of Periodontics. Many of them have been willing participants, offering time and invaluable clinical data. They have had genuine interest in furthering the body of knowledge in this field.

Dedication

This thesis is dedicated to Kip H. Wiebusch has always encouraged me to ask questions. His undying belief in me, and constant support, allowed me to complete this goal.

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Chapter 1: Introduction

1.1 Peri-implantitis prevalence

With over 2 million dental implants placed annually in the United States (American Dental Association, 2010), characterization of long-term dental implant outcomes is essential. The long-term survival rate of dental implants has been recently reported to be 97% (Busenlechner et al., 2014), however, survival rates do not take into account the presence of biologic complications, and despite the remarkably high survival rate of dental implants, there are increasing numbers of patients presenting with peri-implant diseases (Koldsland et al., 2010). It is essential to better understand peri-implant disease prevalence and risk factors so that peri-implant inflammation can be prevented or treated. Peri-implant mucositis and peri-implantitis may lead to discomfort, surgical and nonsurgical treatment and their associated costs (American Academy of Periodontolgy, 2013), negative impacts on systemic health or eventual loss of the implant (Charalampakis et al., 2012). Current treatment protocols for peri-implantitis are based on evidence of a primary bacterial etiology that is similar to periodontitis, but non-surgical therapy which is effective for slight to moderate periodontitis has had limited results in the treatment of peri-implantitis (Esposito et al., 2012, Renvert et al., 2009, Muthukuru et al., 2012). Determining the burden of peri-implant diseases is necessary for patient consent, clinician decision-making, and allocation of resources. No prior studies have documented the prevalence of peri-implantitis in a United States population. This thesis will present data on the prevalence and risk factors associated with peri-implant disease.

1.2 Implant associated biofilm

Culture and molecular methods have specific bacterial targets, which provide valuable information regarding known pathogens but limit data of unknown and unculturable species. Considering the more than 500 distinct oral taxa that may inhabit oral surfaces, the selective assessment of a small number is biased towards false positive associations (Ahn et al., 2011). Existing studies utilizing targeted bacterial identification studies should always be cautiously interpreted. Species identified are essentially "cherry-picked" and can only be considered as markers of microbiome transitions. On the other hand, targeted 16S rRNA gives a much broader picture of the peri-implant biofilm. It allows for an open-ended exploration of the microbial composition of dental implants.

The formation of a biofilm is thought to be essential in the initiation and progression of peri-implant diseases. (Mombelli and Lang, 1998, Salvi et al., 2012, Quirynen et al., 2006, Mombelli and Lang, 1994, Heitz-Mayfield and Lang, 2010) Dental implants are placed in an oral microbial environment of commensal bacteria, and potentially pathogenic microorganisms, or pathobionts. (Cerf-Bensussan and Gaboriau-Routhiau, 2010) Much investigative effort has gone into the study of the peri-implant biofilm, its relationship to periodontal biofilm, and the clustering of bacteria in states of health and disease (Agerbaek et al., 2006, Rakic et al., 2016, Charalampakis et al., 2012, Cortelli et al., 2013, Eick et al., 2016). Despite these efforts, there is no clear consensus for a specific bacterial complex that initiates peri-implant bone loss. (Pérez-Chaparro et al., 2016) In periodontitis, a keystone pathogen (Darveau, 2009) present in low abundance has been identified that is able to disrupt the periodontal microbiota and lead to dysbiosis. The identification of such a keystone microorganism in the peri-implant microbial community remains elusive.

Initial bacterial colonization of dental implants happens quickly in the oral cavity for both dentate and fully edentulous patients (Quirynen et al., 2006, Quirynen and Van Assche, 2011). Biofilm formation has been shown to occur within thirty minutes of implant placement in the oral cavity (Fürst et al., 2007). Forty bacterial species were targeted in this previous study. Between 30 minutes and one week, only *Veillonella parvula* had higher bacterial loads at implant sites compared to adjacent teeth. At 12 weeks the species remained similar but the bacterial load was higher at tooth sites compared to implant sites. Thus, while preexisting species may colonize an implant rapidly, a distinction in microbial load is apparent between the tooth and peri-implant niche.

Agerbaek et al. compared patterns of 40 bacterial strains using DNA-DNA hybridization in subjects with dental implants and compared species found at tooth and implant sites including probing depth as a covariate. They found similar microbial findings at tooth and implant sites, but the percentage of positive sites for *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola* was lower at implant sites $\geq$ 5 mm versus tooth sites $\geq$ 5mm, and tooth sites $\geq$ 4 mm had 3.1 fold higher bacterial load than implant sites (Agerbaek et al., 2006). The difference in findings may indicate that sites with deeper probings around implants may be related to implant diameter and crown contour or depth of placement, and may not be necessarily associated with peri-implant disease in the absence of bone loss and bleeding. It could also indicate that the red complex concept does not translate well from periodontitis to peri-implantitis due to intrinsic differences in surface composition between the two.

It is well established that periodontal disease is a risk factor for peri-implantitis (Berglundh et al., 1992, Karoussis et al., 2003, Matarasso et al., 2010, Daubert et al., 2015). Quirynen et al.

examined biofilm in patients with a diagnosis of periodontal disease (Quirynen et al., 2006). Biofilm was collected from 1-week post-abutment insertion to 18 months and analyzed, concluding that at one week the species were nearly identical among peri-implant sites and tooth sites. A complex microbiota was found at implant sites within 2 weeks, and the similarity continued between tooth and implant sites through 18 months. Because the analysis did not utilize open-ended microbial analysis, the similarity between tooth and implant sites only applies to the specific species studied, and a broader survey might better describe any differences. This study suggests transmission of bacteria from periodontally-involved teeth to implants but it cannot be concluded from this study that those species cause peri-implant disease, as the selective assessment of a limited number of species is biased towards false positive associations.

Healthy implants have been characterized as being colonized predominately by gram-positive rods and cocci in a similar manner to healthy teeth (Fürst et al., 2007). In contrast to the biofilm found at healthy implants, the microbiome at sites of peri-implant disease has generally been described as similar, but not identical to that associated with chronic periodontitis, with severe peri-implantitis characterized as a polymicrobial anaerobic infection with increased numbers of aerobic gram-negative bacilli (Charalampakis et al., 2012). Not all cases of peri-implantitis fit this pattern, however. The peri-implantitis microbiome has occasionally been linked to a biofilm associated with implanted medical devices such as *Peptostreptococci micra* or *staphylococci (S. aureus and S. epidermidis)* (Mombelli and Decaillet, 2011).

Persson et al. used DNA-DNA Checkerboard to analyze the presence of 78 bacterial species in the biofilm of 166 implants with peri-implantitis and 47 healthy implants. 19 of the 78 species were found at higher counts at implants with peri-implantitis compared to the healthy implants. In this study, a cluster of bacteria including *P. gingivalis, Staphylococcus aureus,*

Staphylococcus anaerobius, *Streptococcus intermedius*, *Streptococcus mitis*, *T. forsythia* and *Treponema socranskii* were found to be associated with peri-implantitis (Persson and Renvert, 2014). While *P. gingivalis* and *T. forsythia* are associated with chronic periodontitis, the remaining species elucidate differences in the disease entities.

The first report of implant biofilm using 16S sRNA technology was in 2010 (Koyanagi et al., 2010). Subsequent studies have provided a wealth of data pertaining to the differences between healthy and diseased implants, and implants and teeth (Kumar et al., 2012, Koyanagi et al., 2013b, Dabdoub et al., 2013, Tamura et al., 2013, Schaumann et al., 2014, da Silva et al., 2014, Zheng et al., 2015). Nevertheless, the lack of consensus on case selection, definitions, taxonomic ranking and microbiome data analyses hamper direct comparison among studies. In many cases the periodontal status of the patient was not specified, the definitions of peri-implantitis and periodontitis were not uniform, or periodontitis patients were excluded from the study entirely.

Koyanagi et al. collected submucosal biofilm samples from 3 subjects, each with a healthy implant, an implant with peri-implantitis, and a periodontally diseased tooth and compared the microbial profiles. They concluded that the peri-implant biofilm was more complex than either a periodontally healthy tooth or periodontitis, in contrast to all other studies. They reported a low prevalence of common periodontal pathogens in the peri-implant biofilm. The phyla *Chloroflexi*, *Tenericutes*, and *Synegistetes*, and *Synergistetes* along with Parvimonas micra, *Peptostreptococcus stomatis*, *Pseudoramibacter alactolyticus*, and *Solobacterium moorei* were only detected at implant sites (Koyanagi et al., 2010). Koyanagi et al. expanded on their prior data and added an additional 3 subjects. With the addition, they assessed the differences between peri-implantitis biofilm and periodontal microbiome in subjects with periodontitis. The

peri-implant composition was more complex when compared to the periodontal sites. The prevalence of periodontal pathogenic bacteria was not high at implant sites (Koyanagi et al., 2013). These results provide additional insight but are limited by the small sample size, and it is difficult to draw any broad conclusions.

In 2012 Kumar et al. utilized 16S pyrosequencing to assess the subgingival biofilm of 40 subjects with periodontitis, peri-implantitis, or periodontal and peri-implant health. Specific bacteria were found to be associated with each group. Several species were found to be unique to the peri-implant niche. They concluded that that peri-implant microbial community is significantly different from the periodontal microbial community and is less complex than periodontitis, and that there may be a different mechanism for the pathogenesis of peri-implantitis (Kumar et al., 2012). This contradiction to Koyanagi et al.(Koyanagi et al., 2010) in microbial complexity may be due to a larger sample, but may also be confounded by the fact that the current periodontal status of the implant patients was not included in the analysis and pooled samples were used in the analysis.

In 2013 Dabdoub et al. reported on a much larger group of 81 patients in a study evaluating the microbiome of implants and adjacent teeth. They assessed 4 separate groups: healthy teeth/ healthy implant, diseased tooth/healthy implant, healthy tooth/diseased implant, and both diseased. The percentage of shared species was evaluated using deep-sequencing to determine the degree of similarity between the pairs. 523 species were identified and the resulting analysis led to the delineation of two distinct ecosystems at teeth and implants, bringing into question the earlier hypothesis that teeth are a microbial reservoir for implants (Dabdoub et al., 2013).

Using 16S rDNA sequences and anaerobic culture technique Tamura et al. evaluated submucosal bacterial specimens from 30 patients and distinguished 20 genera at healthy implant sites and 31

genera at sites of peri-implantitis (Tamura et al., 2013). The peri-implantitis sites had a 10-fold higher number of colony forming units than healthy implant sites. They concluded that the conventional periodontal pathogens were not the only species active in peri-implantitis and that asaccharolytic anaerobic gram-positive rods may play an important role. They did not report the periodontal status of the patients in each of the groups.

Schaumann et al. assessed the periodontal and peri-implant microbiome in 7 patients with both generalized periodontitis and peri-implantitis and found no distinction in the biofilm between the teeth and implants in the same subjects (Schaumann et al., 2014). They found the most abundant genera on implants were *Rothia*, *Streptoccaceae* and *Porphyromonas*. The lack of distinction in the biofilm between teeth and diseased implants determined by the Principal Coordinate Analysis of weighted UniFac distances within the same patient suggests that other factors in addition to biofilm composition may account for the distinct pathology between peri-implantitis and periodontitis.

Periodontal disease is a risk factor for peri-implantitis, however healthy patients may also develop peri-implant disease. da Silva et al. evaluated microbial diversity at 20 implants with peri-implantitis compared to healthy implants, excluding patients with periodontitis and those who reported smoking. They reported marked differences in the composition of subgingival biofilm between the healthy implants and implants with peri-implantitis and also reported more orange complex periodontal pathogens present at sites of peri-implantitis compared to health (da Silva et al., 2014).

Lastly, in 2015 16S rRNA analysis was utilized to assess 24 patients with 10 healthy implants, 8 with mucositis, and 6 implants with peri-implantitis. (Zheng et al., 2015). Zheng et al. did not report on the periodontal diagnosis of the patients, but were the only group to report on mucositis

findings using 16S sRNA analysis. They concluded that the microbial communities of peri-implant mucositis were intermediate in nature between those of healthy implants and implants with peri-implantitis, and that periodontal pathogens such as *P. gingivalis, Tannerella forsythia, Prevotella intermedia,* and *Capnocyotophaga ochracea* play a role in shifting from health to disease at implant sites.

There is a wealth of information ranging from data on early colonization, longitudinal changes in the peri-implant biofilm, comparison of peri-implant biofilm with periodontal biofilm, differences in biofilm by implant surface and type, to biofilm changes in states of implant health and disease. In a leap akin to the first use of a microscope, the advent of open-ended, culture free sequencing techniques allow a much richer picture of the peri-implant biofilm including previously unculturable and / or unknown species. The results of sequencing data suggest that the periodontal and peri-implant microbiomes are less similar than previously thought and indeed, represent unique niches in the oral cavity (Dabdoub et al., 2013). In addition, there is emerging evidence of the interplay between the titanium surface and peri-implant biofilm, which may provide insight into the pathogenesis of peri-implant disease (Sridhar et al., 2015, Pozhitkov et al., 2015, Pettersson et al., 2016).

Studies have assessed peri-implant microbiome in relation to disease status of the implant (Canullo et al., 2015), by status of adjacent teeth (Eick et al., 2016), by probing depth (Karoussis et al., 2007), and by patient periodontal status (Zhuang et al., 2016). There is general agreement that the peri-implant biofilm is distinct from periodontitis, and moderate agreement that it is less complex. Otherwise, little consensus exists on a specific species associated with peri-implantitis raising the hypothesis that a factor other than the individual's periodontal status shapes the peri-implant microbiome. In light of recent information on the role of titanium corrosion in peri-

implantitis there is heightened interest in its effect on the microbiome's structure. To date no study has used 16S analysis to evaluate the peri-implant microbiome in relation to periodontal status of the patient and titanium corrosion products.

1.3 Epigenetic regulation of gene transcription in peri-implantitis

A comparison of peri-implantitis and periodontitis lesions reveals histopathological similarities between the two diseases with both lesions described as having high proportions of B cells and plasma cells (Berglundh et al., 2004). There is also evidence that the development of periodontitis and peri-implantitis lesions progress in a similar manner (Heitz-Mayfield and Lang, 2010). At the same time there are distinct differences between the two diseases. A more recent study found histopathological differences including inflammatory cell infiltrate extended more apically in peri-implantitis compared to periodontitis with more extension beyond the pocket area into the perivascular compartments in the apical area (Berglundh et al., 2011). There are reports that the rate of progression in a peri-implantitis lesion can be faster with a distinctly different bone defect pattern (Berglundh et al., 2011, Derks et al., 2016), yet it remains unknown why peri-implantitis generally presents with more extensive inflammation than periodontitis. A possible explanation may be that inflammatory mediators are activated by titanium ions released in the peri-implant sulcus (Wachi et al., 2015).

The immune response is influenced by genetic and epigenetic factors. The term epigenetic refers to changes in the genome that alter gene expression, or allow some genes to be activated and others to be silenced but do not alter the DNA sequence (Razzouk and Termechi, 2013, Lod et al., 2014). The major alterations in the genome are DNA methylation, and histone acetylation and methylation which affect the ability of transcription factors to bind to DNA (Fitzpatrick and Wilson, 2003). Environmental factors can affect the epigenetic modifications, such as stress,

diet, smoking, microbes and toxins (Barros and Offenbacher, 2009). Increased titanium dissolution in the peri-implant sulcus due to titanium corrosion may be an implant-specific factor associated with epigenetic modification, as titanium particles have been shown to stimulate an immune response more strongly than other restorative materials used in implant restorations (Jacobi-Gresser et al., 2013). These epigenetic modifications are tissue specific (Lod et al., 2014) and can be evaluated by looking at the variation in global methylation levels at implant sites. The methylations status would alter activation of genes that are associated with peri-implantitis, stimulating bone resorption. Understanding the epigenetic patterns associated with peri-implantitis could provide information on the etiology of peri-implant disease.

Epigenetic modification can silence or stimulate expression of proteins that could lead to peri-implant disease. Several different biomarkers have been studied for their expression in peri-implantitis cases. A 2004 study looked at vaso-endothelial growth factor (VEGF) which is a key stimulator of angiogenesis in biopsy samples taken from select areas of severe peri-implantitis, healthy implants, and periodontitis, and found an increase in VEGF in peri-implantitis in comparison to the two other groups in the sulcular and junctional epithelium (Bullon et al., 2004). Another group used cultured fibroblasts from subjects with healthy tissue, periodontitis, or severe peri-implantitis and found VEGF secretion from the peri-implantitis fibroblasts to be six times greater than healthy tissue, and two times greater than periodontitis fibroblasts (Verardi et al., 2011). Recently, an animal model was used to assess release of titanium ions when exposed to sodium fluoride, and the resultant titanium release stimulated expression of pro-inflammatory cytokines (Wachi et al., 2015).

Gingival crevicular fluid (GCF) or peri-implant crevicular fluid (PICF) sampling is a simple and non-invasive method that can be used to obtain biomarkers and DNA. A 2002 review of PICF

assays to predict peri-implantitis concluded that this method may be useful for future chair-side testing to monitor peri-implant health and disease but more data was needed to establish the relationship between specific clinical conditions and PICF biomarkers (Kaklamanos and Tsalikis, 2002). Mierzwinska-Natalska et al used PICF samples to look for presence of VEGF around healthy and diseased implants and confirmed the prior findings of an increase in VEGF in diseased sites when compared to healthy sites (Mierzwinska-Nastalska et al., 2010). Additionally, a recent study using PICF and plaque samples analyzed quantities of 5 periodontal bacteria and 5 protein biomarkers at healthy implants compared to peri-implantitis sites and found that the ability to diagnose disease was enhanced by *T. denticola* combined with interleukin-1 beta (IL-1β), vascular endothelial growth factor (VEGF), and tissue inhibitor of metalloproteinase-2 (TIMP-2) PICF levels (Wang et al., 2016). This minimally invasive method can be used to detect global methylation levels in oral samples and provide a broad picture of methylation patterns in health and disease.

While it has been well documented that biomarker expression is markedly different at sites of peri-implantitis when compared with healthy implants, it is not clear what leads to the differences in gene expression. Epigenetic changes affect gene expression. The subsequent chapters will present data supporting the association between increased amounts of titanium corrosive particles and increased global methylation.

1.4 Titanium material properties

Biofilm formation on implants may be dissimilar to teeth due to chemical and physical surface properties of the implant on which the biofilm is established (Lang et al., 2011). Bacterial adhesion on titanium is affected by surface roughness, free energy, chemistry, and titanium purity (Han et al., 2016). *In vivo* models using titanium disks inserted into acrylic splints that are

used for 24-72h have been used to assess the influence of surface characteristics on initial biofilm formation. John et al. reported that in healthy subjects, machine-modified acid-etched surfaces had slower formation versus acid-etched sandblasted large grit, acid-etched surface (SLA) or machined-surface (John et al., 2015). Ribeiro et al. also assessed surface treatments including machined pure titanium, acid-etched titanium, and anodized and laser irradiated disks in healthy subjects, and conversely did not find significant differences in total bacteria and *S. oralis* by surface treatment (Ribeiro et al., 2016). These studies are difficult to compare, as the surface treatments were not equivalent in the two studies, however, they shed light on the impact of titanium surface characteristics on the oral microbiome.

The presence of titanium in the oral cavity is a distinct microenvironment, and the oral microbiome has the potential to disrupt the biocompatible titanium dioxide surface layer (Rakic et al., 2016). Titanium particles have been detected both inside and outside epithelial cells and macrophages in peri-implant mucosa of patients with and without peri-implantitis (Olmedo et al., 2012). In a clinical study performing cytological analyses of peri-implant tissue samples the concentration of titanium was higher in the peri-implantitis group compared with the group without peri-implantitis; no traces of titanium were observed in controls (Olmedo et al., 2013). In addition, the metal powders of titanium have been shown to be cytotoxic (Li et al., 2010). In a review of epidemiology of peri-implant disease, titanium corrosion was suggested to have relevance for later peri-implant bone loss (Klinge, 2012). Dental implants have a surface coating of titanium dioxide (TiO_2) that is responsible for the biocompatibility of titanium implants. (Mouhyi et al., 2012b). This surface coating can be weakened or disrupted leading to titanium corrosion (Mouhyi et al., 2012). We have shown that oral bacterial taxa are capable of affecting titanium electro-conductive properties, and can lead to spontaneous generation of electrical

potential and corrosion of titanium implants (Pozhitkov et al., 2015). *S. mutans* has been shown to increase the corrosion current (Fukushima et al., 2014) and to induce titanium corrosion (Sridhar et al., 2015). It is not known whether specific microbes lead to titanium implant corrosion while titanium corrosion and other wear products stimulate growth of these microbes leading to a heightened host response, or whether the host response to titanium corrosive particles themselves might be the major factor in the peri-implant bone loss. The subsequent chapters will shed light on the role of titanium in peri-implantitis.

Chapter 2: Prevalence and Predictive Factors for Peri-implant Disease and Implant Failure: a Cross-sectional Analysis

2.1 Abstract

Background: Long-term studies worldwide indicate that peri-implant inflammation is a frequent finding and that the prevalence of peri-implantitis correlates with loading time. Implant loss, while less frequent, has serious oral health and economic consequences. An understanding of predictive factors for peri-implant disease and implant loss would help providers and patients make informed decisions.

Methods: A cross-sectional study was performed on 96 patients with 225 implants who had their implants placed between 1998 and 2003. Implant placement data was collected from patient records and patients presented for a clinical and radiographic follow-up examination. Implant status and periodontal status were determined and the data analyzed to determine prevalence of peri-implant disease or implant loss and a predictive model was tested.

Results: The mean follow-up time for the patients was 10.9 years. The implant survival rate was 91.6%. Peri-implant mucositis was found in 33% of the implants and 48% of the subjects, and peri-implantitis occurred in 16% of the implants and 26% of the subjects. Subjects with peri-implantitis were twice as likely to report a problem with an implant as subjects with healthy implants. Peri-implantitis is associated with younger ages and diabetes at time of placement, and with periodontal status at time of follow-up. Implant loss is associated with diabetes, immediate placement and larger diameter implants.

Conclusions: One in four patients and one in six implants have peri-implantitis after 11 years. The data suggests that periodontal and diabetes status of the patient may be useful for prediction of implant outcomes.

2.2 Introduction

Peri-implant diseases have been classified as either peri-implant mucositis or peri-implantitis, with both described as infectious diseases. Peri-implant mucositis has been defined as soft tissue inflammation around a functioning dental implant with bleeding on probing, and peri-implantitis is distinguished by accompanying loss of supporting marginal bone past normal bone remodeling (Zitzmann and Berglundh, 2008). Peri-implant mucositis is thought to be reversible, while peri-implantitis is more difficult to reverse (Esposito et al., 2012).

Prevalence estimates of these two entities vary widely based on study design and disease definition. A recent systematic review and meta-analysis of implants after at least 5 years of function reported a prevalence rate of peri-implant mucositis of 63.4% of subjects and 30.7% of implants and a rate of peri-implantitis of 18.8% of subjects and 9.6% of implants (Atieh et al., 2013). A consensus statement from the 2012 European Association for Osseointegration Consensus Conference has accepted these rates, suggesting that one in five patients will experience peri-implantitis within 5 years after implant placement (Klinge et al., 2012). 10-year estimates of implant level prevalence of peri-implantitis have varied widely, with groups reporting rates from 12% to 43% of implants (Zitzmann and Berglundh, 2008, Roos-Jansaker et al., 2006, Fransson et al., 2005).

Risk factors for peri-implant diseases have been identified in prior studies (Heitz-Mayfield, 2008, Karoussis et al., 2007, Mombelli et al., 2012, Koldsland et al., 2011). Strong evidence indicated that poor oral hygiene, a history of periodontitis, and cigarette smoking are associated with greater risk (Heitz-Mayfield, 2008). Additional risk factors of diabetes, alcohol consumption and genetic traits have also been proposed (Lindhe et al., 2008) and there is

growing evidence of a contributing risk from residual dental cement following restoration placement (Wilson, 2009). Additional factors that have been reported include occlusal overload (Fu et al., 2012); however, this may require further investigation to rule out other causative factors (American Academy of Periodontolgy, 2013).

The purpose of this study is to identify possible risk factors for implant loss and peri-implant diseases and to utilize those risk factors to form a predictive model for peri-implantitis and implant loss. It is also our aim to quantify the prevalence of peri-implant disease at approximately 10 years after implant placement by utilizing the best-available definitions of peri-implant diseases at the time of publication. By including a patient questionnaire, the study seeks to determine whether implant problems might affect patient perception of their implants. Most prevalence studies have been reported in Europe, where patient demographics and health care delivery models may differ from those in the United States.

2.3 Methods

2.3.1. Subject recruitment

A list of patients was generated by contacting former residents in the Department of Periodontics, University of Washington, and from an existing database of patients who had oral implants placed at the Department of Periodontics between 1998 and 2003. Inclusion criteria were: 1) patients over 18 years old at time of consent, 2) implant(s) to be evaluated placed between 1998 and 2003, and 3) radiographs taken after initial remodeling available for comparison. There were no exclusion criteria.

365 charts were screened for implant placement date, patient contact information and for verification of initial radiographs. 241 patients fulfilled the inclusion criteria. The eligible

patients were initially contacted by phone utilizing a phone script, and if there was no phone contact, letters were sent to initiate contact. 137 subjects could not be contacted due to invalid telephone numbers and/or returned mail. Of the 104 patients who were contacted, 96 presented for a follow-up examination.

A total of 225 implants were inserted in the 96 patients at Baseline. Additional implants placed before or after the baseline were not included in the study. The study implants were placed in the University of Washington Graduate Periodontics Clinic by various graduate students under the supervision of multiple faculty according to standard protocols at the time. Subsequently the implants were restored with either a cement or screw-retained restoration. 8 different implant brands were placed with 89% representing four types of implants [§] [‖] [¶] [#], and 11% by four additional implant systems[**] [Ħ] [‡] [§§] combined. See Table 1.

2.3.2 Chart review

Data regarding the conditions at the time of implant placement were recorded from the patient's chart including: date of implant placement, implant brand, implant dimensions, immediate or delayed placement, bone graft use prior to or at time of implant placement, type of bone graft, antibiotic use, smoking status at placement and health status at time of implant placement. Health status was reported by the patient in their medical history and was not verified by laboratory testing. Diabetes diagnosis was not further defined as type I or type II. The closest periodontal

[§] Biomet 3i, Palm Beach Gardens, FL, USA
[‖] Straumann, Basel, Switzerland
[¶] Nobel Biocare, Gothenburg, Sweden
[#] Branemark System, Nobel Biocare, Gothenburg, Sweden
[**] Centerpulse Dental, Inc. Carlsbad, CA, USA
[Ħ] Astra Tech, Mölndal, Sweden
[‡] Sulzer Dental Inc., Carlsbad, CA, USA
[§§] Steri-Oss, Nobel Biocare, Gothenburg,

charting to the time of implant placement was utilized to assign a periodontal diagnosis at the time of implant placement using the International Workshop for a Classification of Periodontal Diseases and Conditions criteria (Armitage, 1999).

2.3.3 Clinical follow-up examination

Examinations were performed in the Graduate Periodontics Clinic at the University of Washington between September 2011 and March 2013. A detailed health history was taken from each patient. Current health status was not verified by laboratory testing. A comprehensive periodontal examination was performed using a PCP-UNC 15 probe to record probing depth and attachment loss at 6 sites per tooth or implant, and a radiograph and photograph were taken of each implant. Bleeding on probing was recorded on a binary scale (presence/absence) for each implant surface. A gingival index and plaque index were recorded for each implant (Löe, 1967). All implants were photographed. Recession, keratinized tissue (KT), and restoration type (cement or screw-retained) were recorded for each implant.

Information was collected regarding the frequency of periodontal maintenance or prophylaxis care for the subjects since implant placement. In addition, a questionnaire was administered about each implant to gather qualitative information using closed-ended questions about patient perception of their implant including biologic or technical complications and patient satisfaction. The patients were asked if they were aware of any problems, if they had experienced pain, bleeding, or pus. They were asked if they had antibiotic treatment, surgical treatment or if the implant was removed. The patient answered separately for each implant.

2.3.4 Radiographic bone loss assessment

A baseline radiograph was obtained from the patient record as close as possible to the insertion of the final prosthesis. Digital radiographs were made of the implants at the time of the follow-up exam using film holders to ensure paralleling technique and diminish distortion of the image. Bone loss was measured using a digital radiograph viewing system which provides measurement tools calibrated to the size of the phosphor plate used for radiography[III]. The baseline radiographic measurement was taken at implant loading. If no radiograph was available from that date, the examiners used the radiograph from time of implant placement, using a threshold vertical distance of 2mm from the expected marginal bone level following remodeling (Sanz et al., 2012b). Mesial and distal bone loss was measured and recorded. A sample patient baseline and follow-up radiograph and patient photos are shown in Figure 2.

2.3.5 Definitions

For this study, peri-implant mucositis was defined as the presence of bleeding on probing and/or gingival inflammation with no evidence of radiographic bone loss beyond normal remodeling. Peri-implantitis was defined as the presence of bleeding on probing and/or suppuration, with 2mm of detectable bone loss following initial remodeling, and a probing depth of 4mm or greater. The presence of 2mm of bone loss alone without mucositis symptoms did not count as a case of peri-implantitis. Due to non-standardized radiographs at prosthetic insertion and follow-up examination, we included the case definition of a threshold of 2mm from the expected marginal bone level following remodeling post-implant placement (Sanz et al., 2012b). Implant failure was defined as a removed, lost, mobile or fractured implant (Buser et al., 2012).

2.3.6 Determination of periodontal status

[III] MiPACS Dental Enterprise Viewer 3.1.916 ,Medicor Imaging, Charlotte, NC

Full mouth periodontal charting made prior to implant placement were utilized to assign an initial periodontal diagnosis. The initial diagnosis was assigned by the examiner as healthy, gingivitis, slight, moderate, or severe chronic periodontitis, with slight periodontitis defined as 1-2mm of attachment loss and moderate and severe as 3-4mm and 5mm or more attachment loss respectively (Armitage, 1999). New comprehensive periodontal exams were performed and a follow-up periodontal diagnosis was assigned.

2.3.7 Investigator Calibration

The clinical exam was performed by two examiners (DD and BW). A calibration was performed to assess inter-examiner reliability. The first 5 subjects were completed by both to assess intra-examiner reliability for clinical measurements of probing depth, plaque and gingival index and amount of KT and periodontal status. Upon completion of the study both examiners evaluated all of the radiographs independently to measure the bone loss on the mesial and distal surface of each implant to reach agreement on the implant status. If there was a difference of opinion the examiners did an additional measurement together to attempt to reach consensus. If they did not reach consensus, the radiographs were re-examined with a third examiner (TF) to reach agreement on the peri-implant status.

2.3.8 Statistical analysis and sample size justification

A sample size of 96 patients is sufficient to yield patient-level prevalence estimates with standard error of 0.05 or less, which implies the half-width of confidence intervals for prevalences to be 0.10 or less. Analyses were conducted on implant level and patient level[¶¶] [##]. Confidence

[¶¶] SPSS for Windows, Version 21.0, SPSS, Chicago, IL
[##] R, Version 2.15.0, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org

intervals for prevalences were calculated using the large-sample normal approximation for the patient-level level outcomes and using Generalized Estimating Equations for implant level outcomes. Relative risks (RR) for both implant failure and peri-implantitis were estimated separately for each potential risk factor using Poisson log-linear regression with adjustment for length of time since implant placement. An exploratory analysis was performed using Poisson regression to assess the ability of baseline variables to predict a poor outcome defined as implant failure or peri-implantitis.

The study protocol was approved the Institutional Review Board at the University of Washington and all subjects provided written informed consent.

2.4 Results

2.4.1 Study subjects

Data from a total of 96 patients who had 225 dental implants placed between 1998 and 2003 were analyzed. 48 subjects were female and 48 were male. The mean age at the time of placement was 56.5 years (SD 10.4) and at time of follow-up was 67.6 years (SD 10.6, range: 31-86). The mean number of implants per subject was 2.31 (range 1-8). Six of the subjects were edentulous prior to implant placement and one additional subject became edentulous due to severe periodontitis prior to the follow-up examination. At time of placement seven subjects were smokers and five subjects were diabetic as previously recorded in their medical history. At follow-up exam 7 subjects were smokers and eight subjects were diabetic according to their new medical history evaluation. Mean follow-up time was 10.9 years (SD 1.5, range 8.9-14.8). 69.4% of the restorations were cement-retained and 30.6% screw-retained. Implant brands and

history of bone grafting are outlined with implant variables in Table 1, along with percentages of immediate and delayed implants.

2.4.2 Implant survival

Four subjects presented for the exam that had lost the implant(s) of interest. These subjects were included in the data on failures, but were not included in the analysis of peri-implantitis and peri-implant mucositis (hence these analyses are interpreted in terms of prevalence of disease given survival of the implant).

207 of the 225 implants were present at the time of the exam including one that had fractured below the implant/abutment junction and was buried so was considered to be a failed implant. It could not be probed and was not included in the analysis, leaving a total of 206 implants for the implant level analysis. The implant level failure rate was 19 out of 225 or 8.4% (95% CI 5.4 – 13.3%). The failures occurred in 16 of the 96 subjects, providing a patient level failure rate of 16.7% (95% CI 9.2 – 24.1 %). When examining time of implant failure, thirteen of the 19 failed implants were removed within one year of placement. One implant was buried due to mechanical problems. Five implants were removed later due to severe peri-implantitis. Of those removed due to peri-implantitis, one was removed after 4 years and the other four were removed between 9 and 10 years. When examining periodontal status of the subjects who had failed implants, 18 of the 19 implants were from subjects with periodontitis at placement. Of those 18, three were removed from subjects with severe periodontitis. Two of those failed implants were in one subject with severe periodontitis who also had all remaining teeth extracted and became edentulous. The periodontal status at time of follow-up for this patient was not included as the patient was edentulous at the follow-up exam.

There were significant associations between implant failure and diabetes at baseline (RR 4.8, $p < 0.01$) and at time of the follow-up exam (RR 3.3, $p = 0.01$) and between implant failure and immediate implant loading (RR 4.1, $p = 0.01$). In addition, the risk of implant failure was significantly greater as the implant increased in diameter (RR 2.3 per mm, $p< 0.01$). No association was found between implant failure and smoking status, restoration type, use of antibiotics at time of implant placement, or any of the other studied variables. See Table 2.

2.4.3 Peri-implant diseases

Patient level prevalence of peri-implant mucositis and peri-implantitis were 48% (95% CI 39% - 59%) and 26% (95% CI 18% - 37%) respectively. A patient was counted as a case in the prevalence calculation of mucositis if one or more of their implants met the criteria for mucositis. Likewise, a patient was considered to be a case in the prevalence of peri-implantitis if one or more of their implants fit the criteria for peri-implantitis. Therefore a patient could be counted as a case in both groups. Ten of the patients had implants that fell into both mucositis and peri-implantitis categories, 33 patients had mucositis with no implants with peri-implantitis, and 11 patients had implants with peri-implantitis and no mucositis.

The patient survey found that of the patients who were found to have peri-implantitis, 34% reported a problem with their implant(s) while only 14.6% of those without peri-implantitis reported having a problem.

The implant-level prevalence of peri-implant mucositis at follow-up exam was 33% (95% CI 26% - 43%) and peri-implantitis prevalence was 16% (95% CI 11% - 23%). Among the implants examined, the prevalence of peri-implantitis was somewhat higher in subjects with slight periodontitis (RR 3.0) at time of placement than those who were healthy or had gingivitis.

The association with slight periodontitis was statistically significant ($p = 0.05$). Associations with moderate and severe periodontitis (RR 2.2 and 2.1) were not statistically significant. Peri-implantitis was associated with subjects who were younger at time of implant placement (RR 0.8 per 10 years of age, 95% CI 0.6 – 1.0) and with diabetes at time of placement (RR 3.0, 95% CI 1.2 – 7.7) but was not associated with any other patient or implant characteristics at time of implant placement. There were differences between prevalence rates for different implant brands but these were not statistically significant possibly due to the small sample sizes in each group. The prevalence of peri-implantitis was significantly associated with severe periodontitis at follow-up (RR 7.3, 95% CI 3.0 – 17.3, $p< 0.001$) compared with healthy status or gingivitis. No significant differences were found in peri-implantitis risk for the following variables: gender, smoking status, diagnosis of diabetes, regular maintenance versus no maintenance, edentulous versus dentate, screw or cement retained restorations, various levels of plaque, bone grafting, amount of keratinized tissue or antibiotic use at the time of implant placement. See Table 3.

A predictive model was fit with patient age (RR 0.74 per 10 years, 95% CI 0.57-0.97), periodontal status at time of implant placement (slight, moderate or severe periodontitis versus healthy or gingivitis) (RR 2.3, 95% CI 1.1-5.0), presence of diabetes at placement (RR 4.1, 95% CI 2.3-7.1), and implant diameter (RR 1.6 per mm, 95% CI 1.1-2.2) (Table 4). The model provided a reasonable fit to the data but had only fair predictive value. Using the median predicted probability (0.19) as a cutpoint for defining prediction of failure or peri-implantitis resulted in a sensitivity of 76.1% and a specificity of 57.4%.

2.5 Discussion

A model to predict potential peri-implant disease and implant loss could provide practitioners and consumers information enabling them to make informed decisions regarding modification of risk factors or selection of alternative treatments. This predictive model found greater risk of peri-implantitis or implant loss associated with diabetes at time of implant placement, periodontal disease at the time of implant placement, younger subjects at time of placement, and larger diameter implants.

Implant diameter has not previously been suggested as a predictor for implant loss. We looked at the possibility that posterior/anterior position could be the factor rather than diameter. In this sample, the average diameter for molar and premolar sites was 4.40 (SD 0.67) while it was 4.03 (SD 0.54) in anterior sites, which suggests some confounding between position and diameter. Although implant position was not determined to be a significant risk factor, while implant diameter was significant, the small number of anterior implants prevented a clear separation of the associations with outcomes and these two factors.

Patients with a previous history of periodontal disease have been reported to be at increased risk for peri-implant disease (Heitz-Mayfield, 2008, Karoussis et al., 2007, Klokkevold and Han, 2007). Our association may have been stronger if we used 'history of periodontal disease' as a variable. For accuracy, we chose to assign periodontal status based on clinical findings rather than a report of previous history of periodontal disease. The association between peri-implantitis and periodontal status at follow-up was limited due to several cases that were classified as severe periodontitis at placement but had extractions during the follow-up period thus improving their periodontal status or changing their status to edentulous. We did find that severe periodontitis at follow-up was significantly associated with an increased prevalence of peri-implantitis.

Implant failure rates found in this study of 8.4% are similar to previous reports (Karoussis et al., 2007, Hardt et al., 2002, Pjetursson et al., 2007), with the significant risks of failure being diabetes, immediate implant placement and larger diameter implants. An association between periodontitis (at Baseline or follow-up) and implant failure was not significant, possibly due to small sample size in the periodontal disease groups. This association was weakened in cases of severe periodontitis in which the periodontal status changed after extraction of periodontally involved teeth making the status at time of exam improve or change to a status of edentulous, therefore obscuring the risk of implant failure and periodontitis.

Increasing attention in the dental literature has been focused on peri-implant disease (Klinge, 2012). Systematic reviews providing information on the prevalence of peri-implant disease report difficulty in comparing individual studies due to variation in study design and case definition (Zitzmann and Berglundh, 2008, Karoussis et al., 2007, Mombelli et al., 2012, Pjetursson et al., 2007, Ong et al., 2008, Faggion et al., 2010). Differences are reported in particular with the definition of peri-implantitis and the amount of bone loss required. One review reported studies including 8 different definitions of the amount of radiographic bone loss used as peri-implantitis threshold (Tomasi and Derks, 2012).

Another difficulty in comparing this study with other studies is that some authors consider a subject to have "peri-implantitis" if only one of several implants had peri-implantitis and do not include the subject in the mucositis analysis (Marrone et al., 2013). Thus, if that subject had multiple implants with both peri-implantitis and peri-implant mucositis the subject would not be considered to have mucositis which would then be under-reported. This study took into account in the subject level data that an individual subject could have implants with both peri-implantitis

and peri-implant mucositis. The findings of the present study are similar to previous reported findings with variation explained by differences in definitions.

Excess cements have been identified as a risk factor that may lead to the progression of peri-implant disease (Wilson, 2009). The presence of excess cement can be difficult to diagnose which raised the question of whether cement-retained restorations could be a risk factor for peri-implantitis. The selection of a cement-retained restoration over screw-retained did not account as a risk factor in our subjects. A possible explanation is that retention type does not influence peri-implant status, but rather the presence of excess cement – which may not be common enough in our sample to change the proportions – is what influences peri-implantitis status. By recording restoration type and not examining the cement-retained restorations for excess cement, the connection between excess cement and peri-implantitis may have been overlooked.

Previous systematic reviews have found smoking to be a significant risk factor for peri-implantitis (Heitz-Mayfield, 2008, Atieh et al., 2012, Hinode et al., 2006). While this study does not confirm these findings, it may be explained by the low number of smokers included in the study and a different demographic from other studies. The prevalence of smokers in this study was 7.3%. The prevalence of smokers in the State of Washington has been reported to be 14.9% for all adults (MMWR, 2010) but only 7.3% for those age 65 and above (WA Dept. of Health, January 2014), which is lower than that reported for the average United States adult population of 19% (CDC, 2011). Both figures are significantly lower than the smoking prevalence in the European Union which averages 25.1% among adults (Bogdanovica et al., 2011). However, a study in a Belgian population with a similar smoking prevalence of 19.4% of those studied also found no association in their study population between smoking and peri-implant disease (Marrone et al., 2013).

Questions also remain regarding how much bone loss is considered normal after implant placement. The criteria proposed in 1993 allow for 1mm of bone loss within the first year after placement and an additional 0.1mm of annual bone loss thereafter (Albrektsson and Zarb, 1993). However, more recently a study demonstrated the possibility that peri-implant bone loss may progress at an accelerating rate, yielding higher incidence rates over time (Heitz-Mayfield, 2008). This suggests that early diagnosis and treatment of peri-implantitis may prevent advanced tissue destruction later.

Periodontitis as a risk indicator for peri-implantitis may be due to the fact that the two disease entities share common host factors or common microbiota. Further research is needed to clarify the relationship between the disease entities.

2.6 Figures

Table 1: Implant Data

Implant Data	**N**	**Percentage**
Implants placed	225	100
Implants failed	19	8.4
Mandibular	129	57
Brand		
Straumann	69	30.7
Nobel Biocare™	39	17.3
Brånemark System®	15	6.7
Centerpulse™	10	4.4
Astra Tech	6	2.7
Sulzer	5	2.2
Steri-Oss®	3	1.3
Bone graft	59	26.2
Cemented Restoration	150	69.4
Immediate Loading	11	4.9
Posterior	183	83.1

Table 2: Relative risks for implant failure. (univariate analysis)

Risk Factor*	RR	Lower 95% Confidence Limit	Upper 95% Confidence Limit	P
Number of Implants	1.0	0.8	1.2	0.70
Age (per 10 years)	0.9	0.6	1.3	0.42
Male Gender	0.8	0.3	2.1	0.69
Smoker at Placement	NAǂ			
Smoker at Exam	0.5	0.1	2.3	0.40
Pack Years History	1.0	0.9	1.0	0.31
Periodontal Status at Baseline				
Healthy/Gingivitis (reference group)	1.00			
Slight Periodontitis	2.4	0.7	8.0	0.14
Moderate/Severe Periodontitis	1.3	0.3	4.8	0.71
*Periodontal Status at Follow-up*ǂ				
Healthy/Gingivitis (reference group)	1.00			
Slight Periodontitis	1.3	0.3	4.6	0.73
Moderate/Severe Periodontitis	1.5	0.5	4.9	0.49
Maintenance Therapy	2.9	0.4	22.9	0.31
Diabetic at Baseline	4.8	1.8	12.9	< 0.01
Diabetic at Follow-up exam	3.3	1.3	8.6	0.01
Edentulous	0.6	0.1	2.4	0.44
Brand				
Astra, Centerpulse, Steri-Oss, Branemark or Sulzer (reference)	1.00			
3i	1.5	0.3	6.4	0.60
Nobel	3.0	0.7	12.4	0.14
Straumann	0.5	0.1	3.2	0.49
Diameter	2.3	1.4	4.1	< 0.01
Length	1.1	0.9	1.5	0.28
BoneGraft	1.4	0.8	2.3	0.26
Antibiotics	0.9	0.6	1.4	0.61
Cement Restoration	0.7	0.2	2.6	0.60
KT	1.0	0.7	1.6	0.84
Immediate Loading	4.1	1.4	11.9	0.01

* Relative risks for each risk factor are estimated using separate models without adjustment for the other factors.

ǂ Relative risk not reported due to small sample size in the smoker group (n=13 with 0 failures).

ǂ Moderate and severe categories of periodontal status combined because of 0 failures in one of the groups.

Table 3: Relative risks of peri-implantitis (univariate analyses).

Risk Factor*	RR	Lower 95% Confidence Limit	Upper 95% Confidence Limit	P
Number of Implants	0.9	0.8	1.1	0.22
Age (per 10 years)	0.8	0.6	1.0	0.03
Male Gender	1.4	0.7	3.0	0.36
Smoker at Placement	1.4	0.5	4.0	0.55
Smoker at Exam	1.5	0.5	4.0	0.44
Pack Years History	0.99	0.98	1.01	0.43
Periodontal Status at Baseline				
Healthy/Gingivitis (reference group)	1.00			
Slight Periodontitis	3.0	1.0	9.2	0.05
Moderate Periodontitis	2.2	0.7	6.9	0.20
Severe Periodontitis	2.1	0.5	8.0	0.28
Periodontal Status at Follow-up				
Healthy/Gingivitis (reference group)	1.00			
Slight Periodontitis	2.2	0.8	6.0	0.12
Moderate Periodontitis	1.0	0.3	3.6	0.99
Severe Periodontitis	7.3	3.0	17.3	<
Regular Maintenance	1.2	0.4	3.9	0.78
Diabetic at Baseline	3.0	1.2	7.7	0.02
Diabetic at Follow-up Exam	1.2	0.3	4.5	0.81
Edentulous	1.2	0.3	5.5	0.81
Brand				
Astra, Centerpulse, Steri-Oss, or	1.00			
3i	1.1	0.4	2.9	0.79
Nobel	0.2	0.0	1.4	0.10
Straumann	0.4	0.1	1.3	0.12
Branemark	1.7	0.4	6.9	0.46
Diameter	1.3	0.7	2.3	0.43
Length	1.0	0.9	1.2	0.84
BoneGraft	0.8	0.5	1.5	0.58
Antibiotics	1.0	0.7	1.3	0.86
Cement Restoration	1.1	0.4	2.5	0.90
KT	0.6	0.3	1.3	0.23
Immediate Loading‡	NA			

* Relative risks for each risk factor are estimated using separate models without adjustment for the other factors.

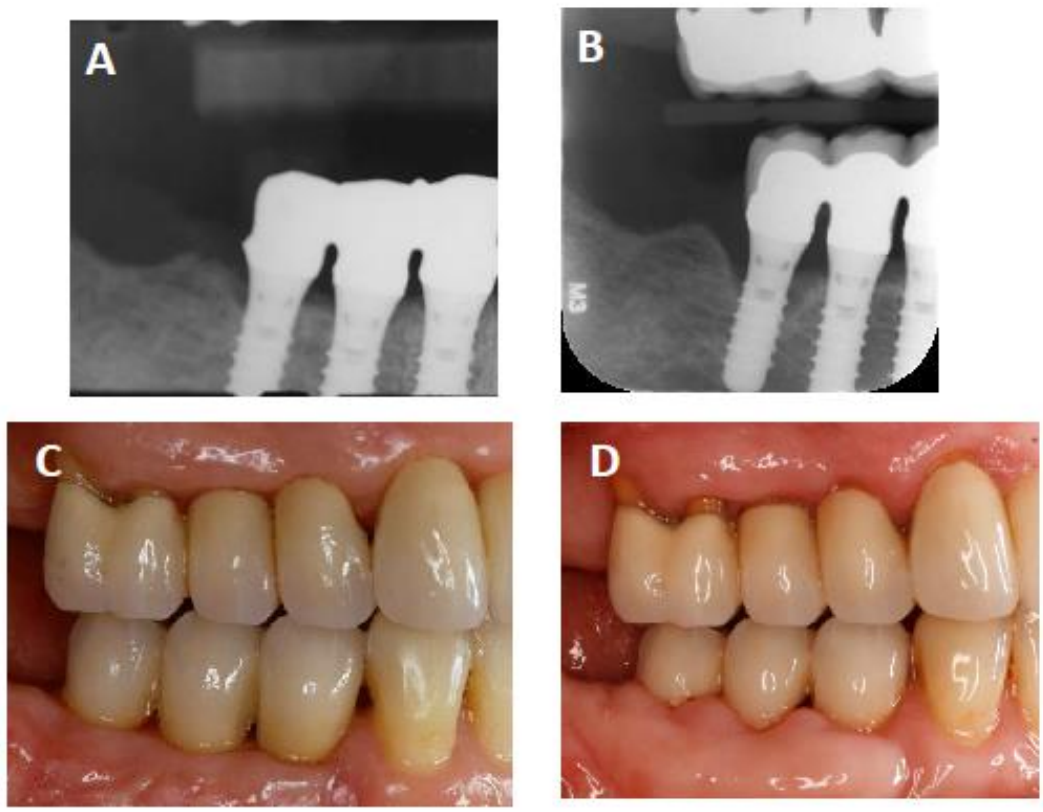
‡ Relative risk not reported due to small sample size in the immediate loading group (n=8 with 0 failures).

Table 4: Relative risks for predictive model of failure or peri-implantitis.

Risk Factor*	RR	Lower 95% Confidence Limit	Upper 95% Confidence Limit	P
Age (per 10 years)	0.74	0.57	0.97	0.03
Periodontal Disease at Placement	2.3	1.1	5.0	0.03
Implant diameter (per mm)	1.6	1.1	2.2	0.01
Diabetes at Placement	4.1	2.3	7.1	< 0.01

* Relative risks for each risk factor are estimated from one model containing all four predictor variables.

Figure 1: Sample patient radiograph at prosthesis insertion and at follow-up exam (a. and b.) and photograph taken at insertion and follow-up exam (c. and d.)



Chapter 3: Titanium as a Modifier of the Peri-implant Microbiome Structure

3.1 Abstract

Objective: Recent data support the implication of titanium corrosion in peri-implantitis. This study assessed, for the first time, the relationship between the peri-implant microbiome, titanium levels, and peri-implantitis in order to dissect the implications of titanium corrosion in peri-implantitis.

Methods: A cross-sectional study was designed to collect clinical and microbiome data from periodontitis patients having implants in function for 10 years. Periodontal and peri-implant data were assessed via clinical examination, and submucosal plaque samples were collected from 9 subjects with 15 implants from the deepest site per implant. An aliquot of the sample was used for DNA extraction and purification, while the remainder was analyzed for titanium quantity using mass spectrometry. 16S rRNA genes were sequenced by 454 pyrosequencing. Sequences were trimmed from raw data and, following quality control, were clustered into species-level taxonomic units at 97% minimum sequence similarity using QIIME pipeline. Species abundance data were log transformed and analyzed using descriptive statistics, multivariate ordination methods and correlation analyses.

Results: Six implants had a diagnosis of peri-implantitis; nine were healthy. The genera *Streptococcus*, *Prevotella* and *Fusobacterium* characterized peri-implant health. Peri-implantitis was associated with a marked increase in *Veillonella*. Quantities of dissolved titanium were identified in 6/15 sites. Titanium presence was associated both with the microbial community composition and the peri-implantitis status of implants, and inversely correlated to the 1st principal component of the microbiome (rho=-0.36) and its alpha-diversity (rho=-0.30). Permutation tests based on Canonical analysis of Principal Coordinates (CAP) found that

titanium levels (p=0.045*), and not peri-implantitis status (p=0.317), were significantly associated with the microbiota composition.

Conclusion: Our study unveils the presence of titanium as a community activist that modulates the peri-implant microbiome structure. These findings support the consideration that in patients with periodontitis, there is clear separation of the microbiome when titanium corrosion is included in the analysis, and highlight the importance of maintaining titanium surface biocompatibility during disease management.

3.2 Background

Titanium biocompatibility and clinical implant survival have been well established and yet peri-implantitis affects a significant number of patients who have dental implants with the required treatment leading to increased time, expense and possible loss of the implant (Daubert et al., 2015, Derks and Tomasi, 2015). Despite titanium's biocompatibility, titanium corrosion has been previously suggested as one of the causes for implant loss (Olmedo et al., 2012, Olmedo et al., 2013, Rodrigues et al., 2013). Pre-clinical data demonstrate that while large titanium particles are biocompatible, fine titanium particles below 10 µm can be phagocytized by leukocytes and are cytotoxic both *in vivo* and *in vitro* (Kumazawa et al., 2002). It was also suggested that titanium corrosion products elicit a foreign-body-reaction causing bone resorption (Mouhyi et al., 2012), and that bone cells are sensitive to the currents produced during corrosion events of metallic implants (Gittens et al., 2011). Our group recently discovered that the electrical conductivity of a titanium implant plays a crucial role in the extent of titanium corrosion as well as shaping the microbial community of the implant-associated biofilm

(Pozhitkov et al., 2015). Further understanding of this interplay between the periodontal microbial community and titanium corrosion may provide insight into the prevention of peri-implant disease.

In addition to titanium material properties, implants are placed into an oral cavity that is colonized by hundreds of bacterial species. Furthermore, it is difficult to assess peri-implantitis outside the context of periodontal disease and the associated biofilm. While implants may be placed in patients who have a healthy dentition, many patients with implants have periodontitis, which is recognized as a significant risk factor for peri-implantitis (Daubert et al., 2015, Berglundh et al., 1992, Karoussis et al., 2003, Matarasso et al., 2010, Ferreira et al., 2006), and bacteria are considered to be an etiologic factor for both disease entities (Kumar et al., 2012, Dabdoub et al., 2013, Koyanagi et al., 2013, Mombelli and Lang, 1998, Salvi et al., 2012, Quirynen et al., 2006). To illustrate, in a recently published 10-year retrospective study including 301 patients with 504 dental implants, 86% of these patients had periodontitis (Eick et al., 2016). Prior publications associate gram negative bacteria with periodontal disease as well as peri-implantitis (Bower et al., 1989, Mombelli et al., 1987, Charalampakis et al., 2012). Specifically, similar bacterial species have been found around implants with peri-implantitis and teeth that are diagnosed with chronic periodontitis (Salvi et al., 2012, Pontoriero et al., 1994, Lang et al., 2011). Conversely, healthy implants have been reported to be colonized by predominately gram positive rods and cocci in a similar manner to healthy teeth (Fürst et al., 2007). These observations are consistent with the fact that dental implants are not placed in a sterile environment and bacterial colonization occurs from tooth to newly placed implant within thirty minutes of implant placement and remains stable at the implant site for an extended period of time (Fürst et al., 2007, Heitz-Mayfield and Lang, 2010). It is not clear if the bacteria occupy

the peri-implant niche by proximity or whether they are associated with the etiology of peri-implant disease.

Extensive effort has gone into the characterization of the peri-implant biofilm. While recent advances in technology allows for a rich exploration of the microbial composition of both healthy versus diseased implants, and healthy versus diseased teeth, there remains a lack of consensus reporting both the complexity and the distinction of the microbiome at teeth and implant sites (Kumar et al., 2012, Koyanagi et al., 2013b, Eick et al., 2016, Schaumann et al., 2014). It is difficult to compare prior studies as either the periodontal status of the patient was not specified in reporting on the implants, the definitions of peri-implantis and periodontitis did not include any loss of attachment, or periodontitis patients were excluded from the study entirely (Kumar et al., 2012, Koyanagi et al., 2010, da Silva et al., 2014, Dabdoub et al., 2013, Zheng et al., 2015). A recent systematic review of the microbial profile associated with dental implants confirms that the lack of homogeneity of the reviewed studies did not allow for any direct comparison (Rakic et al., 2016). The lack of a clear distinction of the biofilm associated with diseased implants suggests that unknown factors in addition to biofilm composition may account for the distinct pathology between peri-implantitis and periodontitis (Schaumann et al., 2014).

The aim of this study was to determine whether factors in addition to peri-implant status might shape the peri-implant microbiome. The bacterial species in periodontitis patients who have healthy or diseased implants was determined using 16s rRNA analysis, and the quantity of titanium particles in the peri-implant plaque was quantified. The relationship between titanium corrosion, peri-implant microbiome and peri-implant status was analyzed.

3.3 Methods

3.3.1 Study population

The Institutional Review Board at the University of Washington approved the study protocol and all patients provided written informed consent. STROBE Guidelines were followed. The participants presented for a clinical and radiographic examination, and a prior publication provides details of the cross-sectional study of 96 patients with 225 implants placed between 1998 and 2003, regarding the conditions at time of placement including: the periodontal status of the patient, brands of implants, type of restoration, patient factors, and the subsequent clinical findings at follow-up exams (Daubert et al., 2015). Those findings include data on the prevalence of peri-implantitis and peri-implant mucositis. A modification was approved by the Review Board that allowed a subset of these participants who had a scheduled appointment to re-consent for a plaque sampling. Edentulous participants and those who had taken antibiotics in the last three months were excluded from the microbial sampling. Implants were included if they had a diagnosis of health or peri-implantitis. Implants with a diagnosis of mucositis were excluded from the microbial analysis in order to have a more distinct separation of health and disease.

3.3.2 Definitions

For this study, peri-implant mucositis was defined as the presence of bleeding on probing and/or gingival inflammation with no evidence of radiographic bone loss beyond normal remodeling (Albrektsson and Zarb, 1993). Peri-implantitis was defined as the presence of bleeding on probing and/or suppuration, with 2 mm of detectable bone loss following initial remodeling, and a probing depth of 4 mm or greater (Daubert et al., 2015). The presence of 2 mm of bone loss alone without mucositis symptoms did not count as a case of peri-implantitis. Due to non-

standardized radiographs at the prosthetic insertion and follow-up examination, we included the case definition of a threshold of 2 mm from the expected marginal bone level following remodeling post-implant placement (Sanz et al., 2012a).

3.3.3 Determination of periodontal status.

Full mouth periodontal chartings were utilized to assign a periodontal diagnosis at the date of the subsequent plaque sampling. The examiner gave each patient a diagnosis of healthy, gingivitis, slight, moderate, or severe chronic periodontitis, with slight periodontitis defined as 1-2 mm of attachment loss and moderate and severe as 3-4 mm and 5 mm or more attachment loss respectively (Armitage, 1999). For consistency, all exams were performed by one examiner (DD).

3.3.4 Sample collection

After supragingival plaque removal, the subgingival plaque sample was collected from the deepest probing site at each dental implant utilizing a sterile 1/2 mini gracey curette. The curette was carefully inserted to the base of the pocket with the blade facing the gingival side, away from the implant surface, and plaque was removed in an upward motion facing the gingiva. This method was used to obtain an adequate volume of plaque for microbial analysis on an individual implant basis. The plaque was transferred to a screw-cap tube with 500µl of sterile water. A 350 µl was set aside for titanium and the remaining for DNA isolation. Both were frozen at -80° C for future processing.

3.3.5 DNA isolation

DNA isolation was done according to our previously published protocol (Pozhitkov et al., 2015). Specifically, plaque was isolated using Chelex-100™ (Bio-Rad, USA), a styrene divinylbenzene

copolymer containing paired iminodiacetate ions, which act as chelating groups in binding polyvalent metal ions (Bassetti et al., 2014). A 150 µl aliquot of suspended plaque was placed into a tube containing 10 mg Chelex 100 followed by addition of 50 µl of 120 mM Tris HCl pH 8.0 followed by addition of 10 µl of 10 mg/mL proteinase K. Proteinase K was dissolved in 30 mM Tris HCl, pH 8.0. The mix was incubated at 55 ^{o}C for 30 min followed by vortexing and incubation in a boiling-water bath for 8 min. Upon removal from the boiling water bath, the tubes were centrifuged at 10,000–15,000×g for 3 min and the supernatant was transferred to a clean 1.5 ml microcentrifuge tube. Prokaryotic 16S rRNA genes were amplified using universal primers (27F and 1392R) using the GemTaq kit from MGQuest (USA) (Cat# EP012). The PCR program involved a pre-amplification step of 10 cycles with annealing temperature of 56^{o}C followed by 20 amplification cycles with annealing temperature 58^{o}C. In each cycle, elongation time was 1 min 10 s, at 72^{o}C. PCR was finalized by extended elongation for 5 min. PCR products were purified with DNA Clean & Concentrator columns (Zymo Research, USA) and quantified using the NanoDrop (Agilent, USA).

3.3.6 DNA Sequencing

DNA sequencing was done according to our previous publication (Pozhitkov et al., 2015). Briefly, each purified PCR product, 1µg, was labeled with a Multiplex Identifier (MID) during the Roche Rapid Library preparation step. Six MID-tagged sequences, representing six samples, were combined in equimolar concentrations and subjected to emPCR and DNA sequencing protocols as specified by the manufacturer's recommendations for the Roche 454 Jr instrument.

3.3.7 Titanium Quantification

The aliquot of microbial samples collected from patients were transferred to the lab for inductively coupled mass spectrometry as previously reported (Pozhitkov et al., 2015). Samples were transferred to digestion vessels (50 mL polypropylene centrifuge tubes) with four 1 ml rinses of digestion solution (50:50 (V/V) concentrated nitric acid trace-metal grade[§]: deionized (DI) water[ǁ] with a trace amount of hydrofluoric acid[¶] and 10 ppm terbium[¶] as recovery standard. Each sample was brought to 5 ml with the digestion solution. Open vessel microwave[#] digestion was used (power 800W, 100%, ramp 15 min to 100°C, hold for 45 min). After the digestion, samples were brought to 25 ml with DI water. Analysis for Ti was conducted by ICP-MS with a detection limit of 0.5 ng.

3.3.8 DNA data analysis

Sequences were trimmed from raw data and those with less than 300 bp were discarded and ambiguous base pairs were removed (non-ACGT). The rest were clustered into species-level taxonomic units (s-OTUs) at 97% minimum sequence similarity and assigned a taxonomic identity by alignment to Human Oral Microbiome Database (NIDCR) using QIIME pipeline (Caporaso et al., 2010). The pipeline annotated the sequences and allowed the integration of the data with previous metagenomic and genomic samples.

3.3.9 Statistical Analysis

Species abundance data were log transformed and analyzed using descriptive statistics, multivariate ordination methods and correlation analyses. In brief, principal components of the

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§ Fisher, trace-metal grade, USA
ǁ Fisher, Barnstead Nanopure, USA
¶ BDH, Aristar plus, USA
¶ BDH, Aristar plus, USA
# Mars Xpress, CEM, Berkerley, CA

microbiome data were extracted using centering at 0 and scaling by standard deviations. A mahalanobis dissimilarity matrix was utilized for principal co-ordinate analysis (PCoA) of the microbiome data. Exploratory examination of associations between implant disease status, quantities of dissolved titanium, and the microbiome structure (alpha-diversity, principal components of relative abundance data) were performed using correlation analyses. Permutation tests based on Canonical analysis of Principal Coordinates (CAP) were utilized to assess the association between microbiome structure, titanium levels and peri-implantitis status. All statistical analyses were restricted to participants with periodontal disease (Table 1).

3.4 Results

3.4.1 Study Subjects

36 participants of our previously characterized cohort (Daubert et al., 2015) returned for an additional visit between July and November 2014. Inclusion and exclusion criteria were applied and patients signed an informed consent. After exclusion criteria were applied, DNA extraction, purification and sequencing were completed on 17 individual implant samples from 11 patients. Nine subjects with 15 implants had a diagnosis of periodontitis. Of these, 6 implants had a diagnosis of peri-implantitis and 9 implants were diagnosed as healthy. The two remaining implants were healthy implants in periodontally healthy patients and were not included in our analysis due to the small sample size. The patient and implant data collected at the clinical examination along with the titanium and DNA quantities collected in the samples are presented in Table 1.

3.4.2 Peri-implant disease and microbial signature

A total of 295 different taxa were identified. A taxonomic summary of the top 20 most abundant genera is shown in Figure 1. Differences were found when comparing individual genera in peri-implant health and disease. The genera *Streptococcus*, *Prevotella* and *Fusobacterium* were more prevalent in peri-implant health. Peri-implantitis was associated with a marked increase in *Veillonella*.

3.4.3 Titanium levels and microbial signature

The first principal component accounts for the largest variability and converts the data set into a set of linearly uncorrelated variables. The second principle component is computed under the constraint of being orthogonal to the first component (Abdi, 2010). Higher levels of titanium in the surrounding plaque were significantly associated with the first principal component from the microbial signature ($p = 0.01$), which accounts for the as much of the variability as possible in a large set of possibly correlated variables A negative association was found between titanium and the second principal component, demonstrating that there is a decrease in diversity as the titanium level increases (Fig. 2A) In addition, there is a decrease in the alpha diversity of implant microbiome with lower adjusted titanium levels (Fig 2B).

When titanium corrosion products are not included in the analysis, there is overlap between healthy and diseased implant sites when plotted on the first two principal components of the microbiome (Fig. 3A). In contrast, when titanium corrosion is included in the model, the relationship between titanium and microbiome was evaluated using a principle coordinate analysis (PCoA) using a dissimilarity matrix of the microbial communities. Titanium corrosion products and not disease status shape the peri-implant microbiome (Fig. 3B), suggesting that titanium creates a unique niche in the oral cavity causing a shift of the alpha and beta diversity of the peri-implant microbiome. Principal component analysis (Mahalanobis distance) was used to

assess the clustering of microbiome samples. This results in a clear separation of health and peri-implantitis, with large quantities of titanium dominating in disease (Fig 3C).

3.4.4 Titanium levels and peri-implant disease

A significant association was found between titanium particles in the plaque surrounding a dental implant and the peri-implantitis disease status of the implant (Table 2). The observed difference between groups in titanium levels was approximately one order of magnitude (means on log-scale of -0.02 versus 0.92, p=0.02).

3.5 Discussion

This study used 16S rRNA analysis to provide an analysis of the microbial community found around implants in patients with periodontitis and assess the interplay of the amount of titanium corrosion and implant status on the microbial community We have demonstrated that titanium corrosion is a strong predictor of the microbes detected around dental implants. Our prior *in vitro* study (Pozhitkov et al., 2015) found a relationship between bacteria and increased implant corrosion but to our knowledge, no prior *in vivo* study has investigated this question. This relationship between dissolved titanium particles, a corrosion product, and peri-implant microbes and peri-implantitis sheds light on the distinction between implant disease and periodontitis. This finding has important ramification in the design of implant-specific peri-implantitis treatment strategies that maintain titanium surface biocompatibility.

Thus, far there has been indirect evidence that the pathogenesis of peri-implantitis may differ from periodontitis based on its faster rate of progression and defect configuration (Lang et al., 2011). The differences between periodontitis and peri-implantitis may lie in the material properties of titanium. It is well-established that titanium corrosion can occur in the presence of

oral bacteria (Fukushima et al., 2014). We previously determined that the electrical conductivity of titanium implants in the presence of biofilm is a key factor responsible for the biocorrosion process (Pozhitkov et al., 2015). Titanium corrosion has also been found to cause inflammation at implant sites (Olmedo et al., 2012, Flatebo et al., 2006) and may be an independent risk factor for peri-implant disease. In addition, titanium particles were recently shown to incite the release of the pro-inflammatory mediator IL-1β in human macrophages (Pettersson et al., 2016). The presence of titanium ions were found to be greater in cases of peri-implantitis compared to healthy controls in a case-control study (Safioti, 2016). Titanium ions be a factor that induces the pathogenic process in peri-implant lesions.

Next generation sequencing provides a method to examine the microbial signatures around dental implants and obtain information on the peri-implant microbiome that is not dependent on prior knowledge of bacterial species. Prior studies have examined the microbial biofilm around implants using 16s rRNA technology (Koyanagi et al., 2010, Kumar et al., 2012, Koyanagi et al., 2013, Dabdoub et al., 2013, Tamura et al., 2013, da Silva et al., 2014, Schaumann et al., 2014, Zheng et al., 2015). Each has used different parameters in patient selection and disease definition and in all cases the previous publications did not define the periodontal status of the implant patients, excluded patients with periodontitis, or used definitions of periodontitis and peri-implantitis that did not include attachment loss of 2mm or more. Most concur that the microbial community is distinct from the periodontal microbiome. It is difficult, however, when assessing these 8 studies to find any consensus on specific genera or species that characterize peri-implant disease. For example, one study found that health was characterized by *Treponema, Prevotella,* and *S. mutans* and disease was associated with *Veillonella, fusobacterium,* and *non-mutans Streptococcus* (Kumar et al., 2012), while others specified that *Veillonella* was lower in

peri-implantitis compared to healthy implants (da Silva et al., 2014, Tamura et al., 2013). Fürst et al. had identified *Veillonella* as the genera that increase in bacterial load from 30 minutes after placement to 1 week (Fürst et al., 2007). We found *Veillonella* associated with peri-implant disease. There may be an association between *Veillonella* and titanium that warrants further research. The lack of consensus provides additional evidence that there may be another factor shaping the peri-implant microbiome.

In contrast to prior reports, we did not find a unique signature for peri-implant disease compared to peri-implant health in our study population. While our sample size is small, one prior publication reported on a subject number of 3 and concluded that the biofilm is more complex in a condition of peri-implantitis versus health (Koyanagi et al., 2010), and another reported data based on 7 subjects, finding no distinction in the microbiome between teeth and implants within subjects (Schaumann et al., 2014). The findings reported in this chapter used a representative group of implant patients and a large enough sample to allow for significance in the findings. We present the possibility of titanium corrosion as a major contributor to both the microbial signature and the disease status of the implant. In the future it would be necessary to determine if results are similar in a larger sample size. In addition, it would be beneficial to assess titanium corrosion and microbiome on failed implants.

This study was funded by the University of Washington Hack Estate.

3.7 Figures & Tables

Table 1. Patient and implant characteristics**.** The first four columns show patient characteristics and the last 4 columns show implant-level characteristics.

Patient Characteristics				**Implant-Specific Characteristics**			
Patient Number	**Patient Gender**	**Patient Age (yrs)**	**Periodontal Disease Status**	**Tooth Number**	**Implant Disease Status**	**Titanium (ug)**	**DNA (ng/ul)**
1	Male	71	Periodontitis	2	Healthy	0.0	29.2
				3	Healthy	0.0	17.7
				14	Healthy	0.0	37.5
2	Male	61	Periodontitis	5	Healthy	10.8	42.3
				12	Healthy	0.0	31.7
				7	Peri-implantitis	31.4	71.5
				10	Peri-implantitis	74.4	75.2
3	Male	62	Periodontitis	3	Peri-implantitis	0.0	65.3
4	Female	76	Periodontitis	19	Healthy	0.0	35.2
				20	Healthy	0.0	66.1
5	Male	67	Periodontitis	28	Healthy	7.0	5.3
6	Female	75	Periodontitis	19	Peri-implantitis	0.0	49.7
7	Male	81	Periodontitis	6	Healthy	0.0	33.0
8	Male	68	Periodontitis	31	Peri-implantitis	6.0	27.1
9	Female	77	Periodontitis	20	Peri-implantitis	90.2	70.9

Table 2. Comparisons of titanium levels and principal components by implant disease status.

	Healthy	Peri-implantitis	P-value
Titanium (log-transformed), mean (SD)	-0.02 (0.56)	092 (1.03)	0.02
PC1, mean (SD)	0.02 (5.14)	-0.04 (8.37)	0.79
PC2, mean (SD)	0.17 (4.52)	-0.32 (8.56)	0.74

Figure 1: Taxonomic summary - species level per implant: Healthy and peri-implantitis

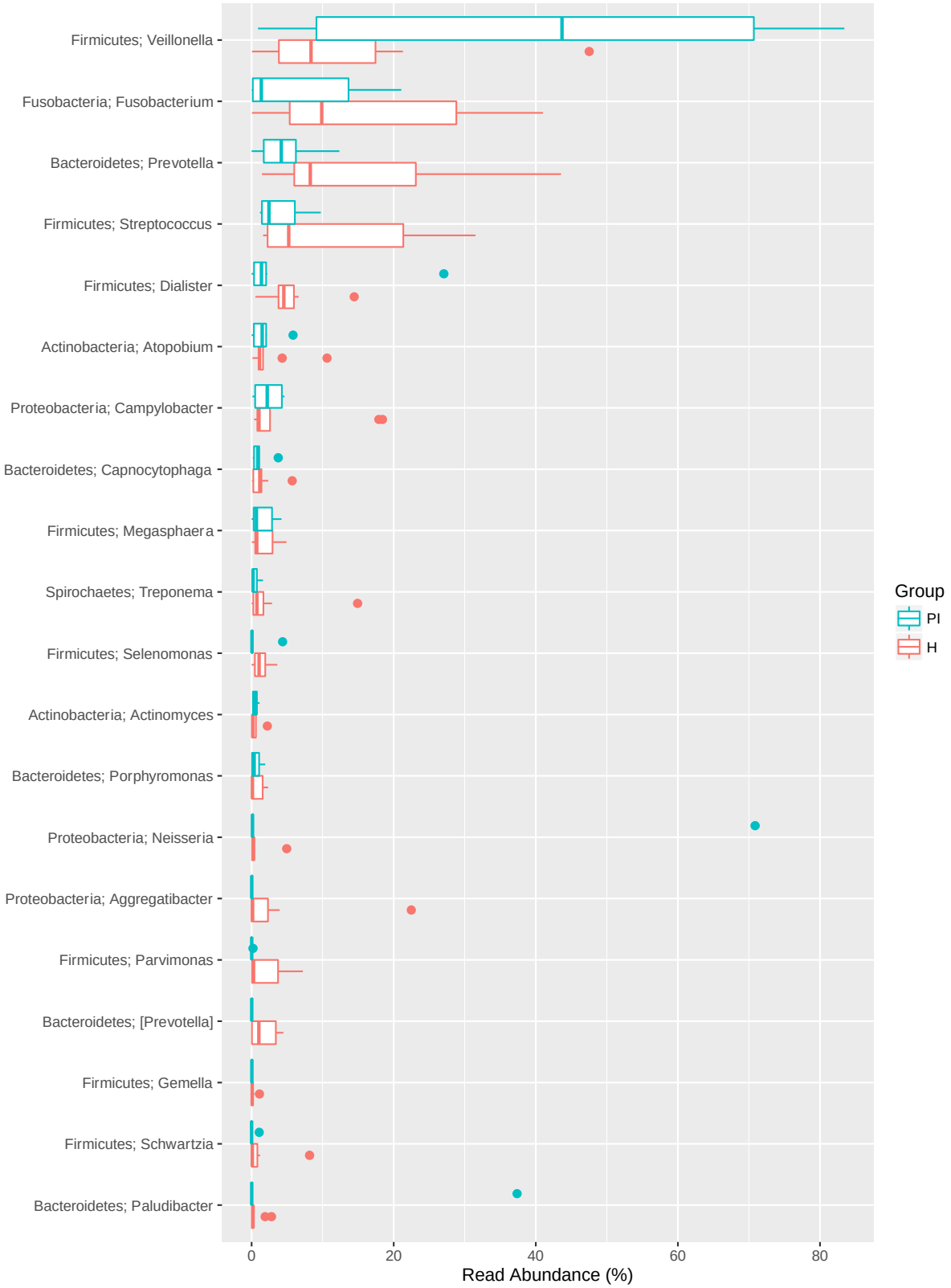


Figure 2A. Scatterplot of Titanium levels (on log-scale) versus the first principal component. There is a decrease in diversity as titanium levels increase.

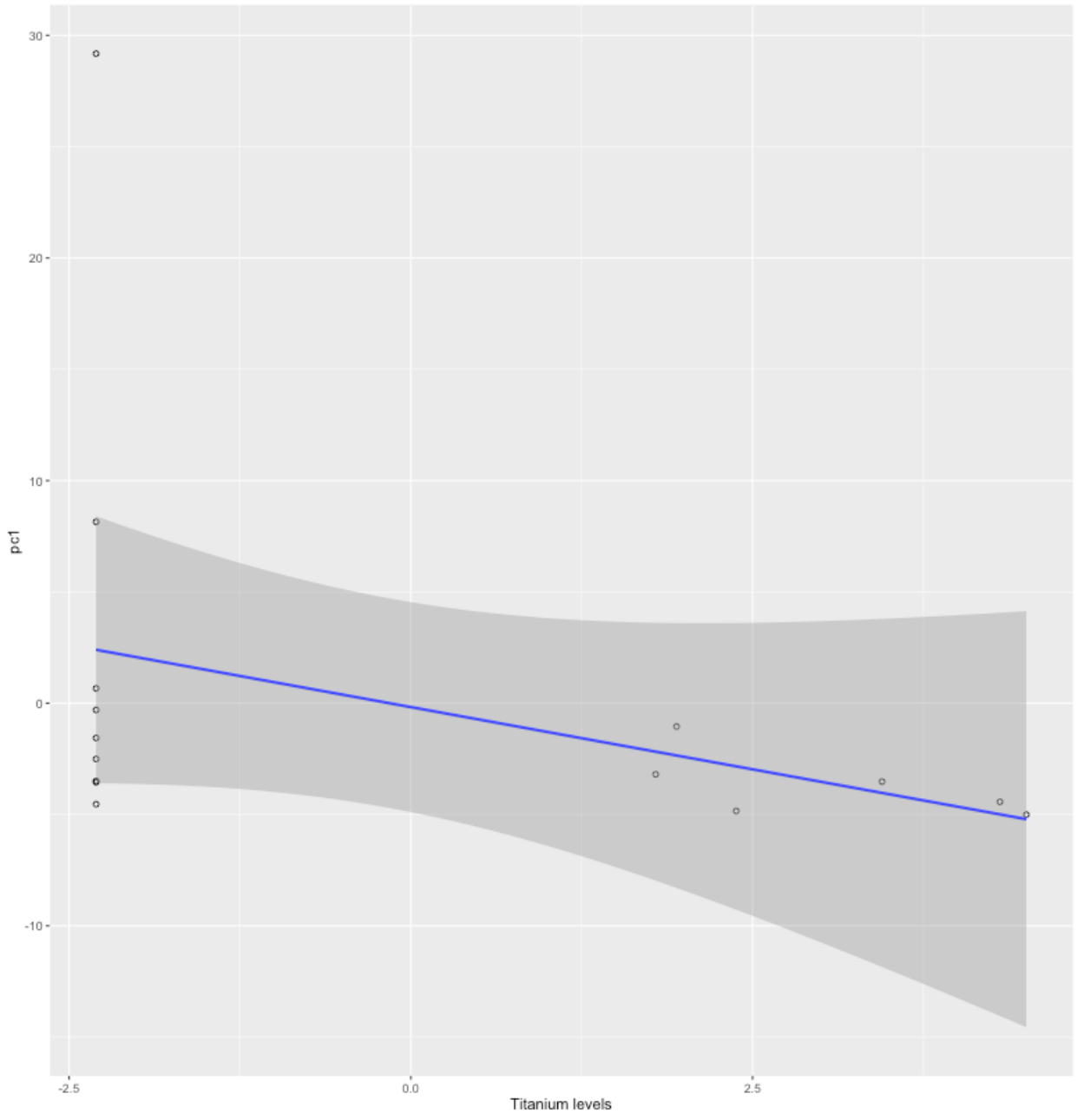


Figure 2B: Alpha diversity of implant microbiome and adjusted titanium levels.

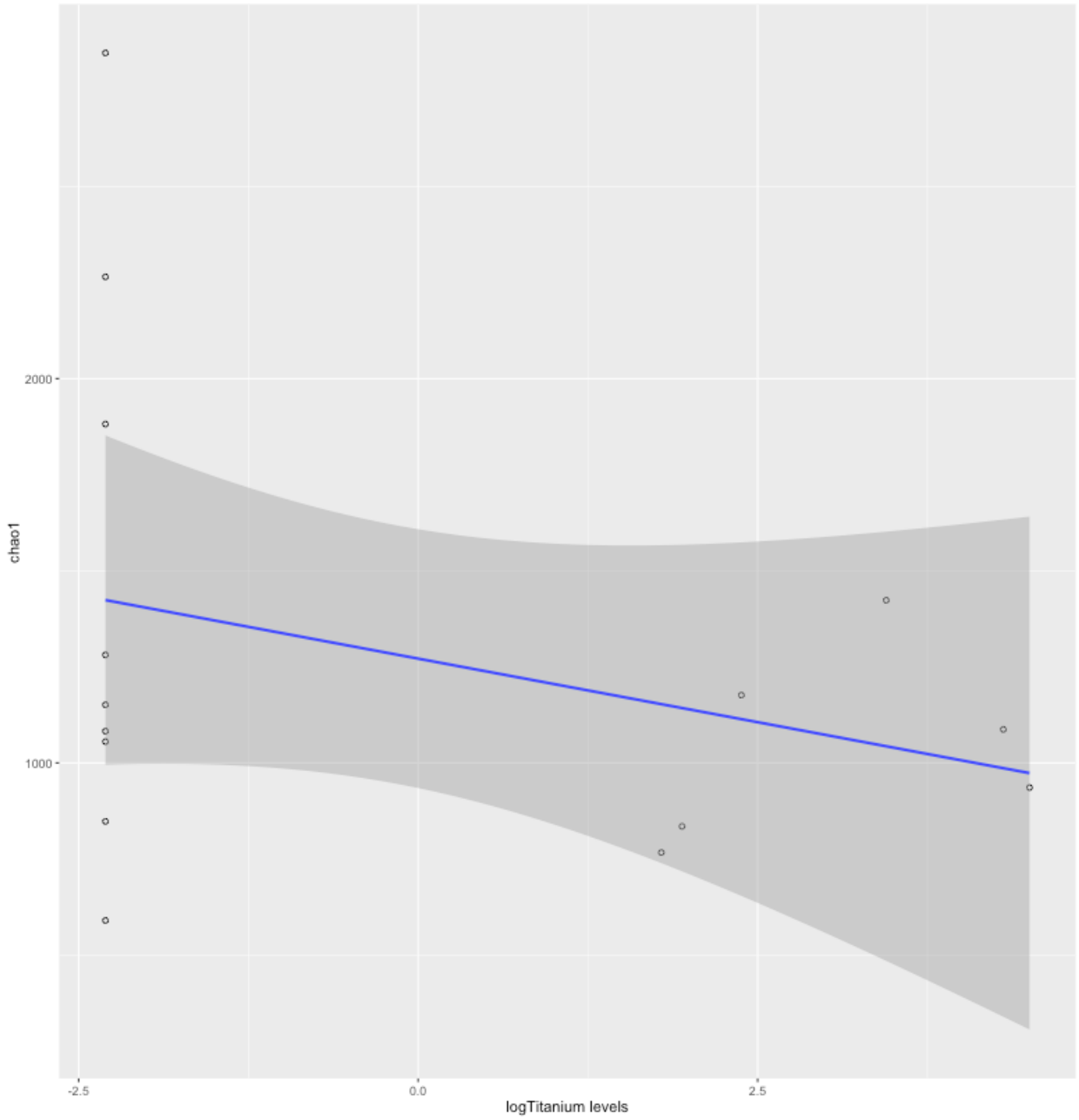


Figure 3A: Principle components of the microbiome in healthy implants (green) and peri-implantitis (orange)

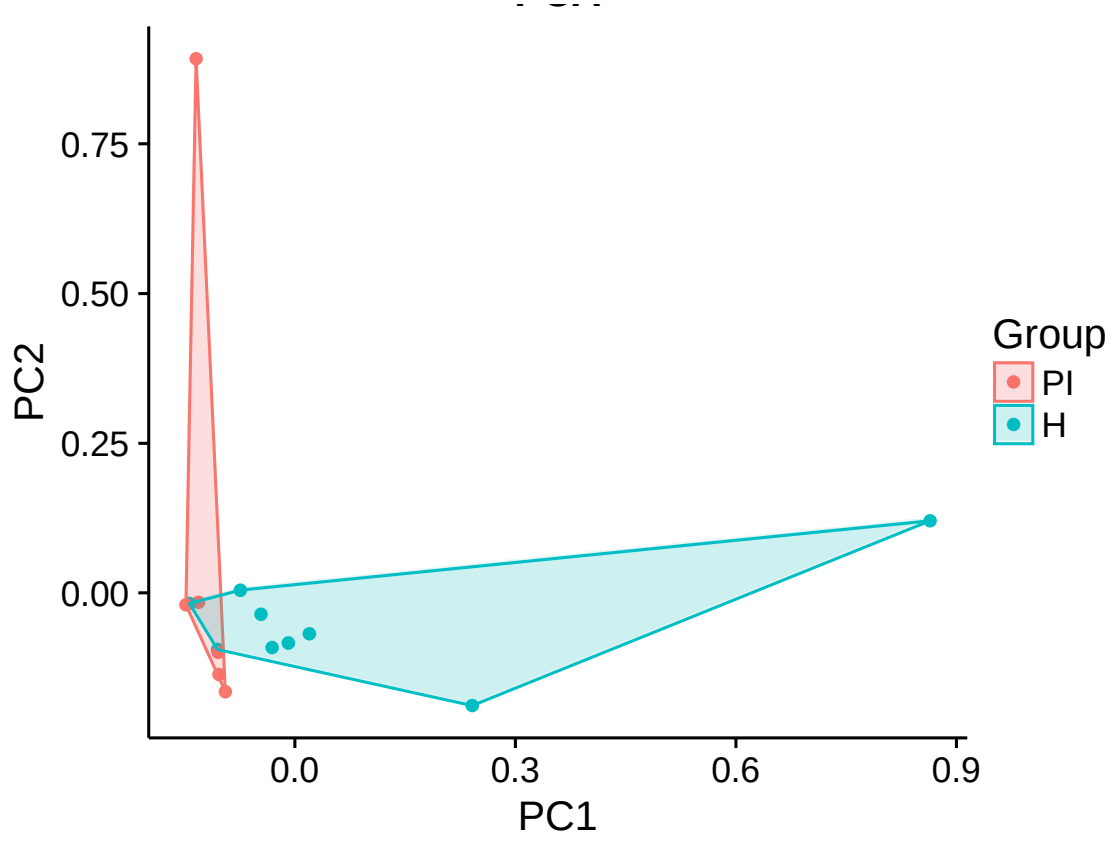


Figure 3B: Canonical Analysis of principal coordinates showing titanium levels per site in embedded bubble plots

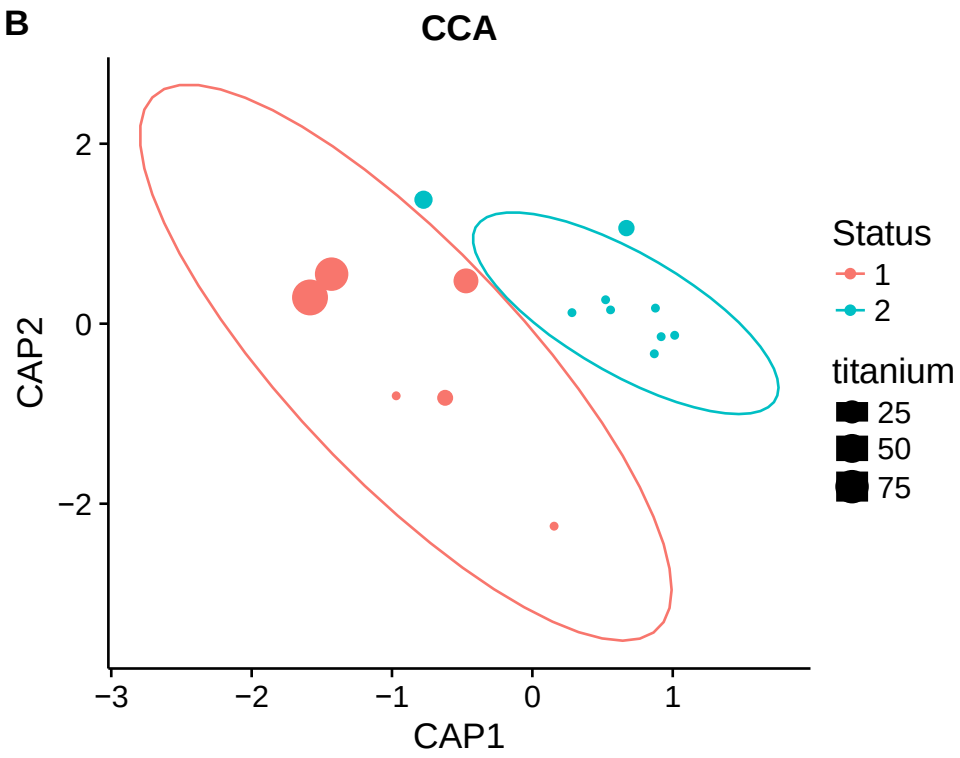
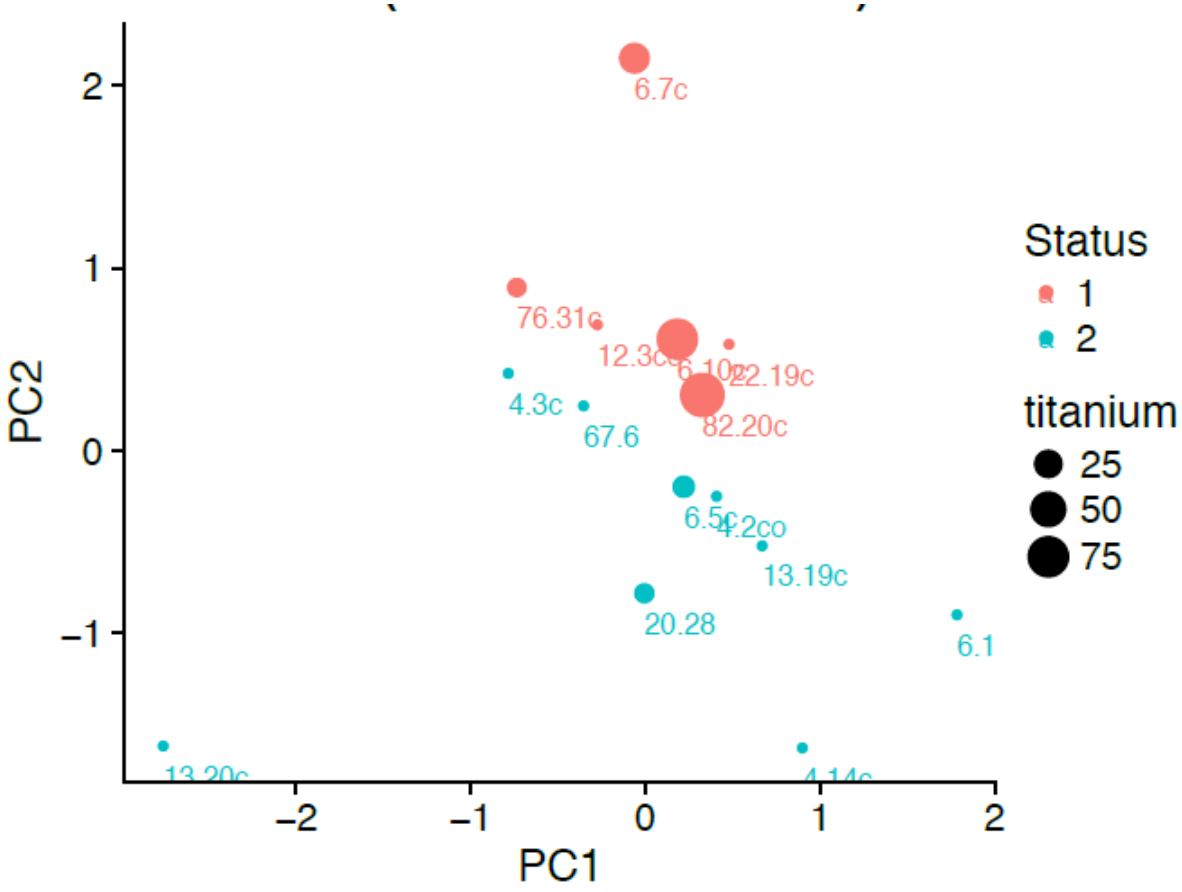


Figure 3C: 1st and 2nd Principal co-ordinate analysis (Mahalanobis distance) showing the clustering of microbiome samples analyzed from healthy (green) and disease (orange) implants. Embedded bubble plot shows titanium levels per site.



Chapter 4: Epigenetic regulation of gene transcription and its correlates in peri-implantitis: a case-control study

4.1 Clinical Relevance

Scientific rationale for study: Epigenetic changes have been associated with increased risk for inflammatory diseases and are influenced by environmental factors. Titanium corrosion, which is associated with peri-implantitis, may affect gene signaling at dental implant sites.

Principle findings: Our results show that global methylation is greater in peri-implantitis cases when compared to controls, and that implants with higher adjusted titanium quantities have higher Global DNA Methylation levels.

Practical implications: The present data shows for the first time that epigenetic regulation of gene-transcription is affected by the amount of titanium corrosion products. This finding mandates treatment of implants that focus on preservation of the titanium dioxide surface layer.

4.2 Abstract

Aim: The primary purpose of this study was to assess global methylation patterns, a method of epigenetic regulation, as they relate to the disease status of dental implants. The secondary aim was to assess the quantity of titanium particles in submucosal plaque surrounding the implants and evaluate the relationship between titanium corrosion and methylation.

Methods: Cases were defined as implants with a clinical diagnosis of peri-implantitis according to established guidelines and that were in function for at least two years, while controls were sampled from the same population and had no peri-implantitis. Submucosal plaque samples were collected and used for titanium analysis and peri-implant crevicular fluid samples were

collected and used for global methylation analysis. Global DNA methylation level was assessed using MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit. Data were analyzed with generalized estimating equation (GEE) models to account for multiple implants per participant.

Results: 21 implants with peri-implantitis and 24 healthy implants in 40 participants were analyzed. Epigenetic alterations in global gene methylation were significantly more pronounced in peri-implantitis cases as compared to controls ($p = 0.002$). Adjustment for smoking status further strengthened the observed association ($p = 0.0079$). Higher adjusted titanium quantities had significantly higher % 5-mC ($p < 0.001$).

Conclusions: Titanium corrosion is associated with aberrant DNA hypermethylation in peri-implantitis compared to healthy implants.

4.3 Introduction

Dental implants are commonly used to replace missing teeth, and while in most cases osseointegration is successful (Albrektsson et al., 2012, Busenlechner et al., 2014), dental practitioners are faced with significant numbers of patients with bone loss around implants that have been restored and are in function. Long-term studies indicate that peri-implantitis affects as many as 1 in 5 implants (Klinge et al., 2012, Atieh et al., 2013, Derks and Tomasi, 2015, Daubert et al., 2015). The primary etiologic factor for peri-implantitis is believed to be an inflammatory response induced by bacteria (Lang et al., 2011). Additional risk factors associated with peri-implant disease include a history of periodontitis, smoking, diabetes, excess cement, and genetics (Daubert et al., 2015, Heitz-Mayfield, 2008a, Mombelli et al., 2012, Wilson, 2009). Genetic studies have evaluated implant sites for the expression of specific biomarkers such as RANK,

sRANKL and OPG (Rakic et al., 2013, Rakic et al., 2014), IL 1β/TNF-α (Jacobi-Gresser et al., 2013), and VEGF (Bullon et al., 2004, Verardi et al., 2011, Mierzwinska-Nastalska et al., 2010). Gene specific analysis is valuable but does not shed light on the overall gene expression in a multi-factorial disease process. Epigenetic regulation of gene expression related to peri-implantitis may be an important factor in the peri-implantitis disease etiology and may provide new methods to approach peri-implant disease treatment in a personalized manner which addresses these differences.

The immune response is influenced by genetic and epigenetic factors. The term epigenetic refers to changes in the genome that alter gene expression, or allow some genes to be activated and others to be silenced but do not alter the DNA sequence (Razzouk and Termechi, 2013, Lod et al., 2014). The major alterations in the genome are DNA methylation, and histone acetylation and methylation which affect the ability of transcription factors to bind to DNA (Fitzpatrick and Wilson, 2003). Environmental factors can affect the epigenetic modifications, such as stress, diet, smoking, microbes and toxins (Barros and Offenbacher, 2009). Epigenetic changes are associated with aging, cancer and other diseases (Yang et al., 2004, Jones and Takai, 2001, Bork et al., 2010). These epigenetic modifications are tissue specific (Lod et al., 2014). There has been a shift in epigenetic research toward genome-wide assessment (DeAngelis et al., 2008). This can be evaluated from DNA extracted from peri-implant crevicular fluid (PICF) samples by looking at the addition of a methyl group at the 5-carbon of the cytosine ring, which results in a 5-methylcytosine (5-mC).

Titanium in various forms may be a factor leading to site-specific epigenetic modification, as titanium particles have been shown to stimulate an immune response more strongly than other restorative materials used in implant restorations (Jacobi-Gresser et al., 2013). Titanium particles

have been detected both inside and outside epithelial cells and macrophages in peri-implant mucosa of patients with and without peri-implantitis (Olmedo et al., 2013). In addition, titanium powder has been shown to be cytotoxic (Li et al., 2010, Kumazawa et al., 2002). We have found greater levels of titanium in submucosal plaque around implants with peri-implantitis when compared to healthy implants, indicating an association between titanium corrosion and peri-implantitis (Safioti, 2016). The titanium particles may influence a site-specific change in the methylation status of genes that express proteins that have been associated with peri-implant bone loss**.**

In this paper we report a simple and non-invasive method for sample collection and processing that provides adequate DNA for global DNA methylation analysis and insight into the epigenetic regulation of gene transcription and its correlates in peri-implantitis. In particular, we hypothesized that Ti particles in the peri-implant sulcus are associated with increased DNA methylation.

4.4 Methods and Materials

4.4.1 Study population

The Institutional Review Board at the University of Washington approved the study and all patients provided written informed consent. STROBE Guidelines were followed.

Cases were selected from patients who were referred to the Graduate Periodontics Clinic at the University of Washington for evaluation of a dental implant and whose implant upon examination met the definition of peri-implantitis according to established guidelines. Inclusion criteria also included the availability of a baseline radiograph and a minimum of 2 years in function for the implants of interest. Exclusion criteria consisted of history of systemic or local

antibiotics in the last three months.

For control selection, if the patient also had a healthy implant, that implant served as control. In the case of no matched healthy implant, patients from the same sample population (Graduate Periodontics clinic) with a healthy implant were recruited. Exams were conducted between June 2015 and July 2016.

4.4.2 Patient examination

The study visit consisted of a clinical examination including a detailed medical history and a periodontal examination of the implant(s) and teeth. Clinical parameters recorded at each implant site were the gingival index and plaque index scores (Löe, 1967), probing depth, bleeding on probing and suppuration at six sites per implant. Data was collected from the patient record including age, gender, and diabetes and smoking status, along with the implant brand and years in function. For this study peri-implant diagnosis was based on clinical and radiographic examination using established criteria (Zitzmann and Berglundh, 2008, Sanz et al., 2012). Briefly, a diagnosis of peri-implantitis was made for implants having a probing depth $\geq$ 5mm, bleeding on probing and/or suppuration, and bone loss $\geq$ 2mm. In the absence of previous radiographic records, a vertical distance of 2mm from the expected marginal bone level following post-surgical remodeling was used to make the diagnosis according to current recommendations (Sanz et al., 2012). Implants that had absence of bleeding on probing and/or suppuration, probing depth < 5 mm and no evidence of radiographic bone loss were determined to be healthy. Additional details have been published in Daubert et al. 2015 (Daubert et al., 2015). Full mouth periodontal chartings were utilized to assign a periodontal diagnosis. The examiner gave each patient a diagnosis of healthy, gingivitis, slight, moderate, or severe chronic

periodontitis according to Armitage 1999 as modified by the AAP taskforce in 2015 (Armitage, 1999). A consensus among the examiners was required to make the diagnoses (DD, LS).

4.4.3 PICF and Plaque Collection

Following supramucosal plaque removal, a plaque sample was taken using a sterile mini 1/2 Gracey curette at the deepest site for each included implant. The plaque sample was placed in 500 µl of sterile water in a screw-cap tube. Following submucosal plaque removal, a PICF sample was taken using fine sterile paper strips (PerioPaper, Oraflow, Smithtown, NY) inserted into the sulcus at 4 sites per implant of interest for 30 seconds as previously described in Rakic et al., 2014 (Rakic et al., 2014). The strips were placed in a second screw-cap tube and were frozen at -20°C for later processing.

4.4.4 Extraction of host DNA

The PICF samples were suspended in 300 µl Phosphate-buffered saline solution for 10 minutes at room temperature and vortexed for 1 minute. The cells were precipitated by centrifugation at 3000g for 10 minutes to separate the cells and debris, and the fluid was transferred to a clean micro-centrifuge tube. The resulting supernatant was centrifuged at 13,000g for 10 minutes and the supernatant was removed and placed in another micro-centrifuge tube as the debris-free fraction (Thaweboon et al., 2010). The cell free DNA was extracted using a phenol/chloroform extraction method, specifically an equal volume of phenol was added to the aqueous phase of the DNA and vortexed for 1 minute, then centrifuged for 5 minutes at 13,000g. The upper phase was removed to a new tube and an equal volume of chloroform was added and vortexed for 1 minute. This was again centrifuged for 5 minutes at 13,000g. The upper phase was removed and placed in a clean tube. The volume was determined and $1/10^{th}$ of the volume of 3M Na Acetate

was added along with 1.5 volume of isopropyl and incubated for 1 hour at -20°C. The sample was centrifuged at 13,000g for 10 minutes at 4°C. The liquid was discarded and the pellet was rinsed with 500 µl ice cold 80% ethanol and the centrifuged at 13,000g for 10 minutes at 4°C. The liquid was discarded and placed upside down on a paper towel to remove excess ethanol. The pellet was air dried for 1 hour. The pellet was dissolved in 30 µl of 1x TE for 10 minutes.

DNA concentration and purity were determined by spectrophotometer (Namedrop, Agilent), and the samples were stored at -20°C until they were processed for biochemical analysis using enzyme-linked immunosorbent assay (ELISA).

4.4.5 Methylation assay

50 ng of input DNA with a 260/280 ration > 1.6 was assayed in duplicate along with positive and negative controls using commercially available ELISA kits to measure the Global DNA Methylation (5-mC) (Epigentek, Farmingdale, NY). The minimal detection limit was 0.05% methylated DNA. The optical density was read on a microplate reader at 450 nm within 5-10 minutes. Concentrations were expressed as % 5-mc/sample.

4.4.6 Extraction of Bacterial DNA

A 150 µl aliquot of the plaque sample was used for DNA isolation and DNA quantification, in order to be able to quantify the amount of titanium per µg of plaque. DNA was isolated using a styrene divinylbenzene copolymer containing paired iminodiacetante ions (Chelex-100, Biorad). The isolation was done according to a previously published protocol (Pozhitkov et al., 2015). The amount of bacterial DNA per sample was quantified using 2 µl of each sample with Nanodrop (Agilent). The total titanium per sample was divided by the amount of bacterial DNA in that sample in order to normalize the titanium quantities.

4.4.7 Titanium analysis

The samples were processed as previously described (Pozhitkov et al., 2015). Analysis for Ti was conducted by ICP-MS with a detection limit of 0.5 ng.

4.4.8 Data Analysis

Summary statistics were reported as means (SD) or frequencies (%). For primary outcome assessment generalized estimating equation (GEE) models (identity link) were constructed to account for multiple implants nested in each participant. Global methylation levels were the dependent variable and a series of models with ascending levels of confounder adjustment were built to assess the association between the global methylation levels and peri-implantitis:

Model 1; independent variables: Peri-implantitis (yes / no), Titanium levels
Model 2; model 1 + smoking status
Model 3; model 2 + age
Model 4; model 3 + gender

Titanium levels were adjusted for amount of bacterial plaque collected per site as previously described (Safioti, 2016). A sensitivity analysis was performed by restricting the analysis to non-smokers to eliminate confounding by this important covariate. All analyses were performed at $\alpha=0.05$ using R statistical software (R Core Team, Vienna, Austria).

4.5 Results

4.5.1 Participants

40 patients participated in the study, which included 21 implants with a diagnosis of peri-

implantitis (cases) and 24 healthy implants (controls). One 80-year-old subject with peri-implantitis was excluded as an outlier who had 10-fold greater titanium levels than the rest (6 standard deviations larger than the mean). The mean patient age was 68.25 ± 8.98 in the healthy group and 64.29 ± 11.22 in the peri-implantitis group. 62.5 percent of the implants in the healthy group were placed in females, while 81.0 percent of the implants in the peri-implantitis group were placed in females. The number of years of implants in function was 7.76 ± 3.92 in the healthy group and 7.8 ± 4.62 in the peri-implantitis group. Number of smokers and persons with diabetes did not significantly differ between groups (p-values 0.225 and 0.625, respectively). The Plaque Index and the Gingival Index were significantly greater in the peri-implantitis group (p-values 0.019 and < 0.001, respectively) Summary statistics are given in Table 1.

4.5.2 Global DNA Methylation (5-mC) and Peri-implantitis

The results of the study indicated significantly higher % 5-mC at implants with peri-implantitis versus healthy implants (0.32 ± 0.31, 0.13 ± 0.09, $p < 0.008$). (Fig. 1). The strength of the association between global methylation and peri-implantitis remained even after confounder adjustment despite the limited sample size (Models 2-4). Results were also robust in a sensitivity analysis limited to non-smokers (N=33). GEE-derived estimates from model 1 in nonsmokers found 0.238 (SE=0.089) greater methylation levels in peri-implantitis cases versus controls (p=0.008), while a 1 unit increase in titanium levels was positively associated with a 0.09 (SE=0.038) increase in methylation units (p=0.017*).

4.5.3 Correlates of Global DNA Methylation

Univariate analysis did not find smoking status, diabetes, or years in function, or gingival index to be significantly associated with the methylation status (all p-val > 0.05). Titanium levels

normalized to plaque levels were significantly associated with methylation levels (p-val<0.001) and this association remained after adjusting for disease status (p-val < 0.001, Model 1) and smoking (p-val = 0.007, Model 2), (Fig. 2A). The trend remained after adjusting for age but not for gender (Model 3 and 4), however neither age nor gender were associated with methylation. Given the consistent effect size noted for titanium across all adjusted models, lack of association in model 4 was likely due to lack of power for such extensive adjustment (Table 2). A sensitivity analysis in non-smokers (N=33) found the association between titanium levels and global methylation to be robust (p-val=0.016), while adjusting for disease status (p-val=0.013). Smoking, years in function, and gingival index were not associated with global methylation levels (Fig. 2B-D).

4.6 Discussion

It is a challenge to find specific gene mutations that can account for a complex chronic disease. It has been suggested that it is time to move beyond the search for specific genes and also examine the alteration in levels of gene expression through the study of epigenetics (Williams, 2012). Epigenetic modifications have been associated with many chronic inflammatory diseases, ranging from neoplastic disorders including bone tumors (Delgado-Calle et al., 2012), arthritis (Wilson, 2008), oral cancer (García and García-García, 2012), and periodontal disease (Larsson et al., 2015, Zhang et al., 2010). To our knowledge, altered methylation patterns and peri-implant disease have not been examined.

A significant strength of this study is the dual evaluation of global methylation levels and titanium corrosion. Along with heritable changes in altered gene expression, environmental factors induce epigenetic changes (Mathers et al., 2010). Titanium may play a central role in

epigenetic gene regulation and provide a key to understanding the inflammatory response to biofilm around dental implants. These results demonstrate that hypermethylation quantified by increased levels of 5-mC % is significantly associated with peri-implantitis and that furthermore; the local environmental factor of titanium corrosive particles is associated with peri-implantitis.

Current treatment protocols for peri-implantitis are based on evidence of a primary bacterial etiology that is similar to periodontitis, but non-surgical therapy which is effective for slight to moderate periodontitis has had limited results in the treatment of peri-implantitis (Esposito et al., 2012, Renvert et al., 2009, Muthukuru et al., 2012). It is important to understand that although highly biocompatible, titanium may be impacted by common treatment protocols that could affect the biocompatibility. Recently, it was shown that common antimicrobial agents used in treatment of periodontitis when used on titanium surfaces leave residues that altered the titanium surface and showed some cytotoxic effect compared to controls (Kotsakis et al., 2016). The fact that methylation pattern changes are associated with increased titanium particles is additional evidence of a potential biologic impact of titanium corrosion on the local environment. It may be time to shift treatment protocols for peri-implantis toward those with minimal disruption of the TiO_2 layer.

Epigenetic patterns of periodontitis-related genes have been studied in the context of periodontal disease, resulting in application of treatment with epigenetic drugs used to treat periodontitis in an animal model (Lindroth and Park, 2013). A better understanding of epigenetics and peri-implantitis could lead to targeted treatments such as DNA methylating agents, which are specific for implants in contrast to using treatment protocols that work for periodontally involved teeth but have limited success when applied to dental implants.

4.7 Figures

Table 1: Summary statistics

Variable	**Healthy**	**Peri-implantitis**	**p-value**‡
N	24	21	
Age ± SD	68.25 ± 8.98	64.29 ± 11.22	0.239
Females (%)	62.5	81.0	0.205
Smokers (%)	8.3	23.8	0.225
Diabetes (%)	8.3	14.3	0.625
Years in function mean ± SD	7.76 ± 3.92	7.8 ± 4.62	0.825
Global Methylation % 5-mC	0.13 ± 0.09	0.32 ± 0.31	0.008*
Titanium (ng): mean ± SD	2.36 ± 5.73	6.33 ± 15.64	0.084
Plaque Index ± SD	0.63 ± 0.65	1.14 ± 0.73	0.019*
Gingival Index ± SD	0.37 ± 0.71	1.71 ± 0.84	<.001*
Probing depth mean ± SD	2.88 ± 0.68	5.94 ± 1.39	<.001*

‡ p-values for continuous variables arise from t-tests, while for categorical variables from Fischer's exact tests.

Table 2: Results of Methylation Models.

	Model 1*		Model 2*		Model 3*#		Model 4*#	
Predictors	**Methylation Levels [mean (SE)]**		**Methylation Levels [mean (SE)]**		**Methylation Levels [mean (SE)]**		**Methylation Levels [mean (SE)]**	
Peri-implantitis	0.21 (0.069)	p-val=0.002*	0.21 (0.078)	p-val=0.007*	0.22 (0.080)	p-val=0.005*	0.21 (0.076)	p-val=0.005*
Health	ref		ref		ref		ref	
Titanium levels	0.12 (0.030)	p-val<0.001*	0.10 (0.037)	p-val=0.007*	0.08 (0.042)	p-val=0.051	0.07 (0.047)	p-val=0.142
Smoking	-		-0.13 (0.080)	p-val=0.116	-0.12 (0.079)	p-val=0.130	-0.12 (0.076)	p-val=0.130

*Footnote:

Model 1; independent variables: Peri-implantitis (yes / no), Titanium levels (N=39)

Model 2; model 1 + smoking status (N=39)

Model 3; model 2 + age (N=39)

Model 4; model 3 + gender (N=39)

age and gender were not significant predictors of global methylation levels in multivariable analyses (all p-values>0.145)

Figure 1: Global Methylation level boxplots per group; (green: healthy implants and orange: peri-implantitis) $p < 0.008$.

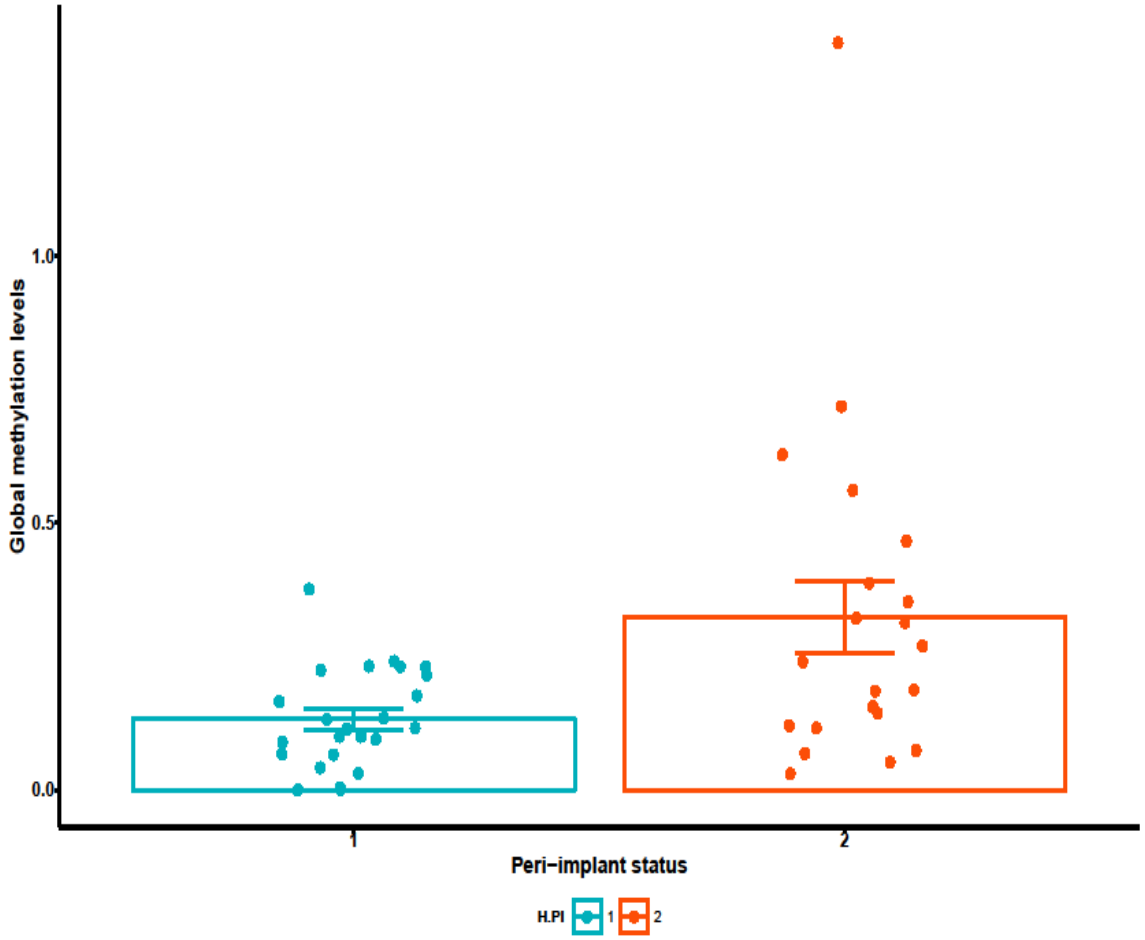
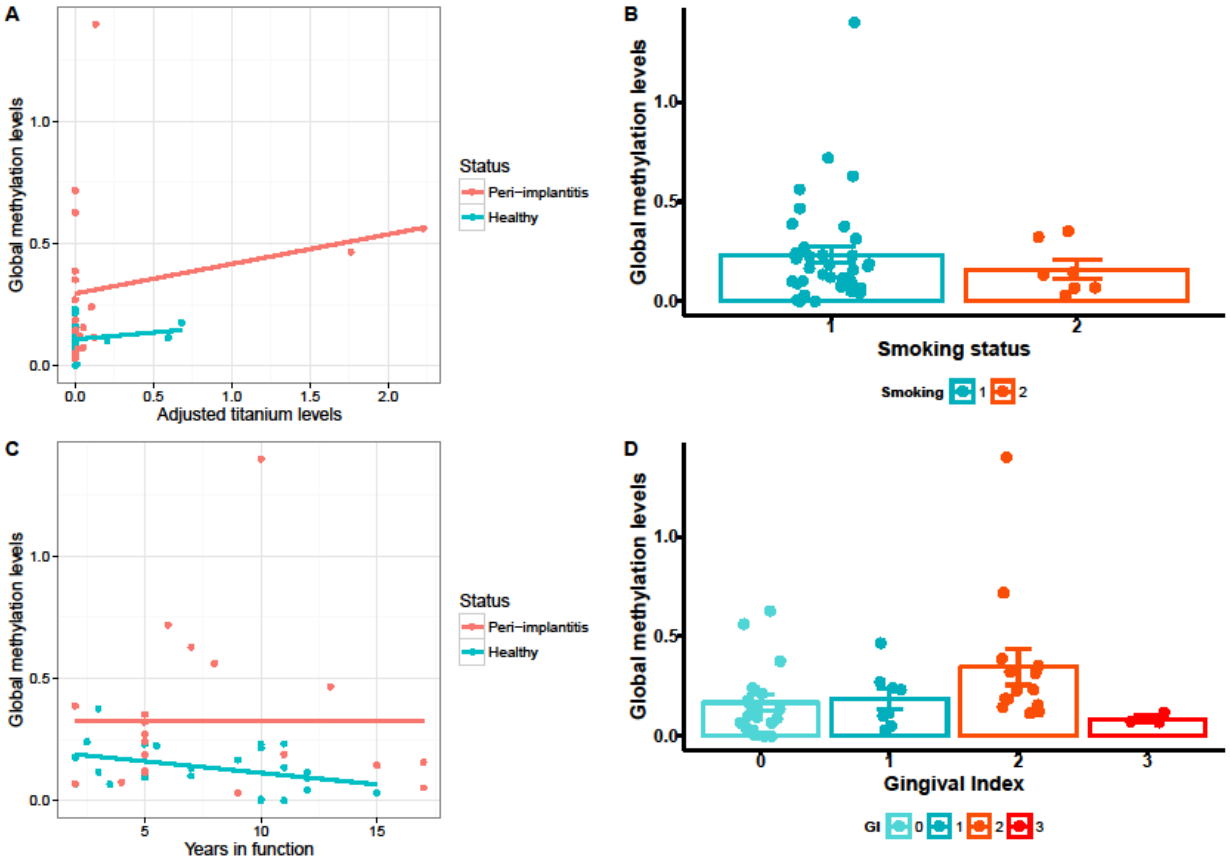


Figure 2: Global methylation per group: healthy (green) and peri-implantitis (orange)

A. Global methylation increases as adjusted titanium increases. B. no significant difference in methylation between smokers (orange) and non-smokers (green). C. no significant difference in methylation by years in function. D. no significant difference in methylation by gingival index.



Chapter 5: Additional Data

5.1 Restoration contour is a risk indicator of peri-implantitis: A cross-sectional radiographic analysis

While assessing the 225 radiographs of dental implants for Chapter 2 in order to make a determination of radiographic bone loss, it became apparent that restoration contours vary widely. This is due to a variety of reasons, including variations in size of the implant that is placed, the depth of placement, length of abutment, and the space between adjacent teeth. There is a void in the literature on this topic: the contour of an implant-supported restoration and its relationship to peri-implantitis was unclear. We utilized the data collected in the cohort included in Chapter 2 to address this question. Implants were divided into bone level and tissue level groups. The radiographs were cropped to blind the examiner, and the emergence angles and profiles were calculated using imaging software. Using the diagnosis made at the time of the clinical exam done previously, the data was analyzed to determine if the emergence angle and profile were associated with the prevalence of peri-implantitis. Eighty-three patients with 168 implants were included in this analysis, excluding implants that had failed, implants with a fixed-detachable restoration or removable overdenture, and one implant with an obvious ill-fitting restoration with a significant open-margin. The prevalence of peri-implantitis was significantly greater in the bone level group when the emergence angle was >30 degrees compared to an angle ≤30 degrees (31.3% vs 15.1%, P=0.04). In the tissue level group, the angle was not associated with prevalence. Emergence profiles were not associated with the prevalence in either group. However, for the bone level group, when a convex profile was combined with an angle of ≥30 degrees, the prevalence of peri-implantitis was 37.8% with a statistically significant interaction between emergence angle and profile ($p = 0.003$). For the bone level group, emergence angle of

>30 degrees is a risk indicator for peri-implantitis and convex profile creates an additional risk. Further radiographic and statistical analysis revealed that restoration contour is associated with increased prevalence of peri-implantitis for bone level implants and indicated that an over-contoured restoration is a risk indicator of peri-implantitis. This data is presented in full in a manuscript that will be submitted to the Journal of Clinical Periodontology in December 2016 (Katafuchi et al., 2016).

This provides additional information that may help clinicians in treatment planning and restoration design. With knowledge of the maximum tolerable emergence angle, clinicians can adjust their implant selection and placement depth, as well as their restoration design to reduce the risk of peri-implantitis.

Supplemental Figure 1. Prevalence of peri-implantitis by emergence angle and profile.

Implant Type	Mesial and/or distal EA >30	Both EA 30 degrees or Less	Difference (95% CI), p-value
Bone Level, n (%)	15/48 (31.3%)	8/53 (15.1%)	16.2% (0.5%, 31.8%), 0.04
Tissue Level, n (%)	3/39 (7.7%)	2/28 (7.1%)	0.5% (-14.5%, 15.6%), 0.94
	Mesial and/or distal Convex	Both Straight or concave	
Bone Level, n (%)	15/52 (28.8%)	8/49 (16.3%)	12.5% (-3.4%, 28.5%), 0.12
Tissue Level, n (%)	2/34 (5.9%)	3/33 (9.1%)	-3.2% (-13.0%, 6.6%), 0.52

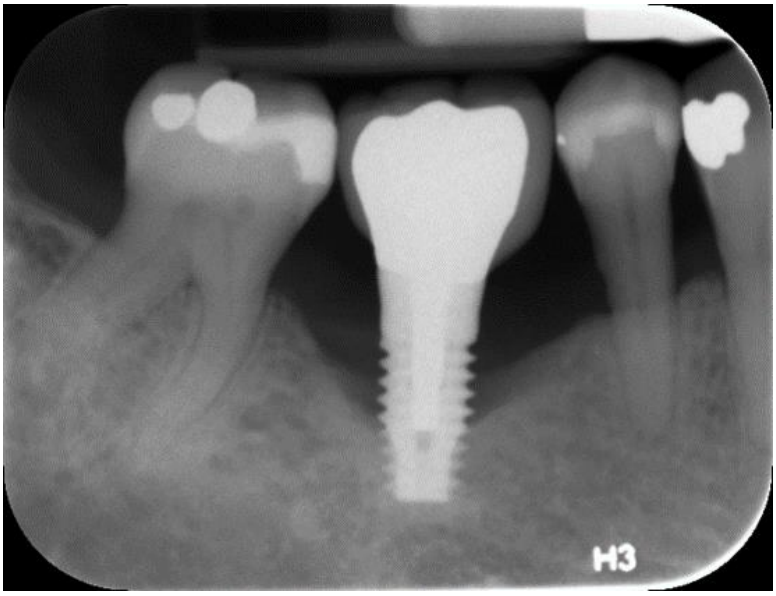
5.2 Increased levels of dissolved titanium are associated with peri-implantitis – a case-control study.

While assessing the levels of titanium in relation to the methylation status, we also hypothesized that there is an association between the dissolution of titanium from dental implants and peri-implantitis. This data is drafted in a separate paper submitted to the Journal of Periodontology. In summary, greater levels of dissolved titanium were detected in submucosal plaque around implants with peri-implantitis when compared to healthy implants, indicating an association between titanium dissolution and peri-implantitis.

A subset of samples completed in March 2016 for Chapter 4 were analyzed. Submucosal plaque from N=20 implants with peri-implantitis and N=20 healthy implants was collected with sterile curettes (N=30 participants). (Fig. 2A and 2B) Levels of titanium were quantified using inductively coupled plasma mass spectrometry (ICP-MS) and normalized for mass of bacterial DNA per sample to exclude confounding by varying amounts of plaque per site. Statistical analysis was performed utilizing Generalized Estimated Equations (GEE) to adjust for clustering of implants per subject.

Results. Implants with peri-implantitis harbored significantly higher mean levels of titanium (0.85 ± 2.47) versus healthy implants (0.07 ± 0.19) after adjusting for amount of plaque collected per site (p-value=0.033). (Fig. 2C) An 8-fold increase in levels of dissolved titanium were detected in submucosal plaque around implants with peri-implantitis when compared to healthy implants, indicating an association between titanium dissolution and peri-implantitis. Factors triggering titanium dissolution as well as the role of titanium corrosion in the peri-implant inflammatory process warrant further investigation. These data support the utilization of treatment methods that prevent disruption of titanium oxide surface (Safioti, 2016).

Supplemental Figure 2 A: Representative implant with peri-implantitis used in the study.



Supplemental Figure 2 B. Example of a healthy implant used in the study.

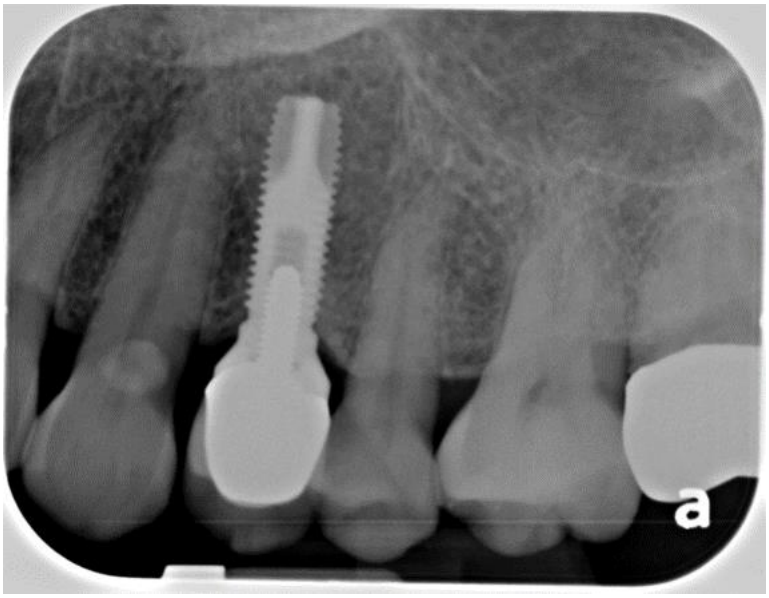
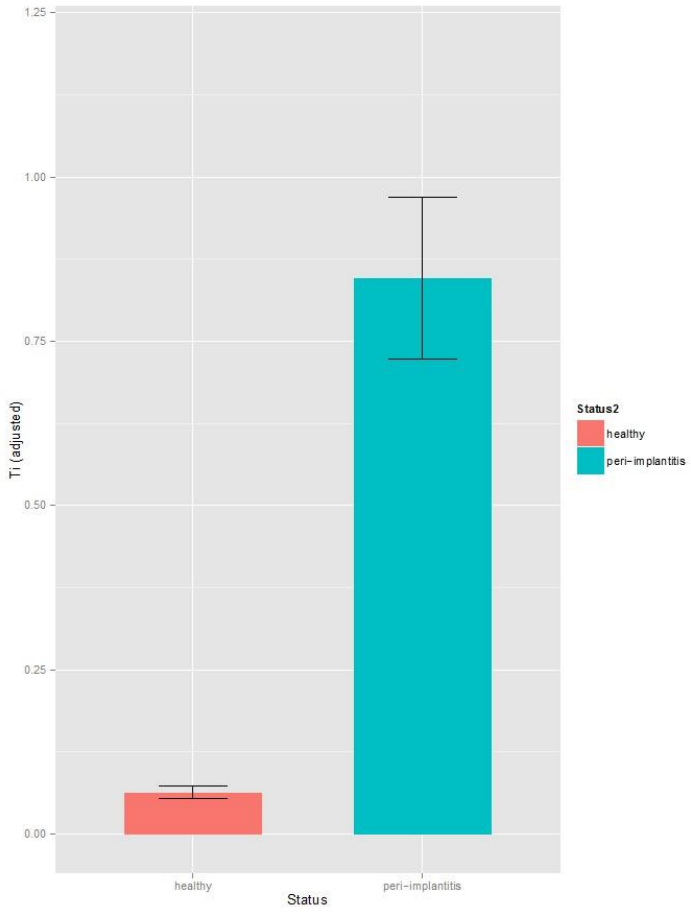


Figure 2 C: Mean adjusted titanium levels in healthy and peri-implantitis groups.



Chapter 6: General Discussion and Future Directions

6.1 General Discussion

We have demonstrated that peri-implantitis is a significant problem. Our data confirms what has been presented in other studies, and is the first long-term study to look at a United States population. The predictive model for implant failure finds diabetes to be the most significant factor in pre-implant planning, and can be utilized to support stringent management of blood sugar levels for patients interested in pursuing dental implant treatment. The prevalence data was used to assess restoration contour and show a significant association between implant supported crowns with an emergence angle of > 30 degrees and peri-implantitis. These findings provide new data that can immediately help with implant treatment planning.

The peri-implant niche is a distinct niche from the periodontal pocket. We have presented groundbreaking data showing that the microbial community in peri-implant health and disease is shaped by titanium residues in the peri-implant pocket. This information stands to change peri-implant microbial research in the future, as the effect of titanium on the microbiome has heretofore been overlooked. It will lead to more concerted efforts to preserve the titanium dioxide surface layer in an effort to decrease titanium corrosive particles.

Furthermore, we have demonstrated that titanium is an independent predictor of an increase in global methylations at dental implant sites. An increase in methylation is associated with gene-silencing, and ties the titanium corrosion to a change in host response. This opens the door to further research pursuing quantification of pro-inflammatory and anti-inflammatory cytokines in relation to titanium corrosion.

Peri-implantitis will continue to be a significant problem as more implants are placed in the future. A major paradigm shift needs to occur regarding treatment of peri-implant disease. Current treatment regimens are based on bacterial removal duplicating strategies for the treatment of natural teeth, yet the titanium surface is quite dissimilar to the roots of teeth. Treatments for peri-implantitis must be designed keeping in mind titanium material properties.

6.2 Challenges

There is lack of an accepted classification system for peri-implantitis that would differentiate the range of bone loss from minor to severe. This limits the ability to compare studies, and also to present information in a standardized manner. For example, in Chapter 2 we did a cross sectional study and anyone who had bone loss of 2mm or greater was classified in the peri-implantitis group. The majority in that group of patients had bone loss under 4mm. Those subjects were used for the plaque samples in Chapter 3. On the other hand, the cohort that was recruited for Chapter 4 was much more severe. By intention, all of the implants in the peri-implantitis group were recruited from patients who were referred for surgical treatment of peri-implantitis, and all were in the severe range. Unfortunately, it is not apparent when reading the data that the two studies had significantly different amount of peri-implant bone loss. Classification systems were proposed in 2007 (Schwarz, 2007) and in 2012 (Froum and Rosen, 2012), yet neither is commonly used in reporting. It is expected that the American Academy will include a classification system for peri-implant disease from the workshop to be held in 2017. This will enable clear communication regarding disease severity.

It was imperative in order to establish the baseline data for this work, to be able to recruit a large enough sample of patients who had implants placed 10 years ago. This was no small task, as no

database existed of patients who had implants placed in the Graduate Periodontics Clinic between 1998 and 2003. In addition, Electronic Health Records did not exist in 1998 to use as a resource for data mining for patients who had implants placed in the period of interest. The School of Dentistry changed its billing system 3 times in that time period, which precluded doing a search of billing codes to find patients who were seen for dental implant placement. In the end, former residents were contacted via email to obtain an initial list of potential patients. However, many names may have been left off the list and thus were not contacted. From that point, charts were requested from Records Retention if the patients were no longer active patients, and letters were sent to contact them. The response from the patients who were contacted was tremendous, but many potential candidates were not contacted due to the lack of a database.

Plaque samples were collected from the 225 patients who presented for the implant exam per the first Human Subject Application. This sample collection was done using one paper point per implant. After multiple attempts with different methods of DNA extraction, it was apparent that one paper point was not adequate. This led to a Human Subjects Modification to allow for additional plaque sampling. The IRB did not allow me to contact patients again unless they had an appointment scheduled in the School of Dentistry clinics, which limited the secondary samples to a small number.

A larger number may have provided additional data and potentially additional categories of implants to sample, for example - peri-implantitis in periodontally healthy patients. Periodontitis is a significant risk factor for peri-implantitis confirmed by our data and others. Therefore, finding periodontally healthy patients with a diseased implant is difficult without significant numbers of patients and implants. This problem may have been avoided by contacting researchers who had done 16S rRNA sequencing using plaque samples from implant sites.

Subsequent to the small sample size due to limited secondary samples, there were problems with sending the samples off-site for DNA sequencing. An entire batch of samples was lost at the sequencing site, and they later asked for pooled samples. In the end, the number of samples, while adequate for the power analysis, was not what we had originally intended. Access to sequencing on site would have greatly facilitated the project.

6.3 Future Directions

6.3.1 Future directions based on findings in Chapter 3

We found that peri-implantitis is associated with a marked increase in *Veillonella*. Conversely, *Fusobacterium, Prevotella,* and *Streptococcus* characterize peri-implant health. The association may indicate an effect from titanium corrosion versus a cause of titanium corrosion. In fact, it was demonstrated in vitro that *S. mutans* could lead to a chemical attack on the surface of titanium implants leading to surface dissolution by immersion in *S. mutans* for 60 days (Sridhar et al., 2015). In addition, Rodrigues et al. suggested that the acidic environment caused by biofilm triggered breakdown of the titanium surface layer (Rodrigues et al., 2013). There remains a gap in knowledge regarding the effect of other species on titanium surface corrosion. Future research could assess the differential effects of *Veillonella* in comparison to *Streptococcus, Prevotella* and *Fusobacterium* to assess surface corrosion. At the same time, growth of those species should be assessed with and without exposure to titanium to determine if titanium corrosion stimulates or suppresses growth. This could help to elucidate whether bacterial differences are a result of titanium corrosion versus a cause.

In addition, with the implementation of an oral biofilm model versus single species, the effect of titanium on the microbiome could be assessed to determine if the entire microbiome shifts when exposed to titanium versus no titanium.

6.3.1 Future directions based on findings in Chapter 4

Titanium surface dissolution may be triggered by mechanisms other than biofilm on the titanium surface. Recent reports highlight that teeth-driven treatment modalities not only yield inadequate biofilm removal from the implant surface but may also damage the TiO_2 passive layer that is responsible for the biocompatibility of titanium dental implants (Mouhyi et al., 2012). Weakening of this protective TiO_2 layer triggers a chain reaction leading to titanium dissolution and accelerated titanium corrosion (Mouhyi et al., 2012).

A gap in knowledge exists regarding the potential triggers of increased titanium dissolution from the implant surface and the mechanisms by which titanium dissolution products amplify peri-implant inflammation. Further research is needed that will clinically assess the effect of standard therapeutic treatments on the biocorrosion of titanium. We have proposed a study in which we will assess whether standard aggressive treatments which are currently used to treat peri-implantitis, disturb the biocompatibility of titanium implants leading to amplification of the disease.

Now that we have demonstrated an increase in global methylation associated with greater quantities of titanium particles, further work is needed to assess specific differences in inflammatory cytokine methylation in order to explore the mechanisms involved in peri-implant bone loss. As previously stated, increased amounts of cytokine expression have been associated with titanium in peri-implant disease. Recently titanium in physiological solutions was shown to

stimulate release of IL-1β from human macrophages (Pettersson et al., 2016). There is also data from a medical case report of a 54-year-old woman who developed rheumatoid arthritis after a spinal titanium implant placement was published in Nature Rheumatology (Dorner et al., 2006). When the patient's peripheral blood mononuclear cells were exposed to titanium dioxide the production of TNF-α increased nearly 10-fold as compared to healthy controls. A gap in knowledge exists regarding the epigenetic regulation of specific cytokines. Future research is needed to look at the effect of titanium on methylation of patients who have peri-implantitis compared to healthy controls. This could be done as we have recently proposed using whole blood from patients who have peri-implantitis and healthy implants and assessing methylation patterns.

In an attempt to predict peri-implant disease, cross-sectional data has been used to assess specific biomarkers and peri-implant microbiome. It is imperative that prospective trials are initiated that analyze the microbiome using 16S rRNA analysis in conjunction with titanium quantification and pro-inflammatory biomarker analysis. We have access to a population that would enable us to initiate a prospective 5-year translational study that could help give us real-time data to predict future disease and change implant therapy.

6.4 Conclusion

Peri-implant disease is prevalent and does not respond well to conventional therapy. It will not be resolved without innovative research plans. Titanium as a modulator of peri-implant disease is a fascinating topic to continue to explore. A deeper understanding of the causes and ways to prevent titanium corrosion has the potential to make a significant difference in the way we approach implant therapy.

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Zhang, S., Crivello, A., Offenbacher, S., Moretti, A., Paquette, D. W. & Barros, S. P. (2010) Interferon-gamma promoter hypomethylation and increased expression in chronic periodontitis. *J Clin Periodontol* **37,** 953-961. doi:10.1111/j.1600-051X.2010.01616.x.

Zheng, H., Xu, L., Wang, Z., Li, L., Zhang, J., Zhang, Q., Chen, T., Lin, J. & Chen, F. (2015) Subgingival microbiome in patients with healthy and ailing dental implants. *Sci Rep* **5,** 10948. doi:10.1038/srep10948.

Zhuang, L. F., Watt, R. M., Mattheos, N., Si, M. S., Lai, H. C. & Lang, N. P. (2016) Periodontal and peri-implant microbiota in patients with healthy and inflamed periodontal and peri-implant tissues. *Clinical Oral Implants Research* **27,** 13-21. doi:10.1111/clr.12508.

Zitzmann, N. U. & Berglundh, T. (2008) Definition and prevalence of peri-implant diseases. *Journal of Clinical Periodontology* **35,** 286-291. doi:10.1111/j.1600-051X.2008.01274.x

Curriculum vitae

DIANE M. DAUBERT

Education:

University of Washington	May 2016	PhDc	Oral Biology
University of Washington	June 2009	MS	Oral Biology
University of Washington	June 1982	BS	Dental Hygiene
Cum laude graduate			

Professional License: Washington, 1982; Registered Dental Hygienist license # DH00002622

Employment:

Institution	Department	Position	Years
University of Washington	Periodontics	Clinical Assistant Professor	2014-present
University of Washington	Periodontics	Affiliate Instructor	2001-2014
University of Washington	Periodontics	Dental Hygienist	1987-present
University of Washington	Regional Clinical Dental Research Center	Dental Hygienist	1997-2002
Private Practice	Seattle, WA	Dental Hygienist	1982-1987

Teaching Responsibilities:

University of Washington Department of Periodontics

Perio 525 -526: Prevention/Periodontics, 2001-2004, Clinical Instructor
Perio 525 -526: Prevention/Periodontics, 2005-present, Co- Course Director
Dent 610: Introduction to Clinical Dentistry, 2002-2005, Clinical Instructor
Dent 610: Introduction to Clinical Dentistry, 2006-present, Course Director
Perio 661: Advanced Root Instrumentation 2006- present, Course Director

Perio 575: Immunologic Aspects of Oral Diseases, 2008-present, lecturer
Perio 530: Principles of Periodontics, 2010-present, lecturer
DENTPC 511: Introduction to Periodontics, 2011-present, lecturer
DENT FN 500: Early Clinical Immersion, 2015-present, Course Director
American Heart Association Basic Life Support Instructor 2002-present

Committees:

Continuing Dental Education Advisory Committee: 2005-present
Instructional Resources Committee: 2009-present

Publications:

Safioti, L, Kotsakis G, Pozhitkov A, Chung W, **Daubert D**. Increased levels of dissolved titanium are associated with peri-implantitis – a case-control study. J. Perio. Accepted 21-Oct-2016 [Epub ahead of print]

Daubert D, Kelly J, Udod Y, Habor C, Kleist C, Furman I, Tikonov I, Swanson W, and Roberts F. Human enamel thickness and *ENAM* polymorphism. IJOS. 2016; 8(2): 93-97.

Bidra A, **Daubert D,** Garcia L et al. Clinical Practice Guidelines for Recall and Maintenance of Patients with Tooth-Borne and Implant-Borne Dental Restorations. J Prosthodontics. 2016; 25 S32-S40.

Bidra A, **Daubert D,** Garcia L et al. A Systematic Review of Recall Regimen and Maintenance Regimen of Patients with Dental Restorations. Part 1: Tooth Borne Restorations. J Prosthodontics. 2016; 25 S2-S15.

Bidra A, **Daubert D,** Garcia L et al. A Systematic Review of Recall Regimen and Maintenance Regimen of Patients with Dental Restorations. Part 2: Implant Borne Restorations. J Prosthodontics. 2016; 25 S16-S31.

Pozhitkov A**, Daubert D,** Brochwicz Donimirski A, Goodgion D, Vagin M, Leroux B, Hunter C, Flemmig TF, Noble P, Bryers J. Interruption of electrical conductivity of titanium dental implants suggests a path towards elimination of corrosion. Plos one, October 2015.

Daubert D, Weinstein B, Bordin S, Leroux B, Flemmig T. Prevalence and Predictive Factors for Peri-implant Disease and Implant Failure: A Cross-sectional Analysis. J Perio, March 2015; 86: 337-347.

Daubert D, Subgingival Air Polishing, Dimensions of Dental Hygiene, December 2013; 69-73.

Flemmig TF, Arushanov D, **Daubert D**, Rothen M, Mueller G, Leroux BG, Randomized Controlled Trial Assessing Efficacy and Safety of Glycine Powder Air Polishing in Deep Periodontal Pockets. J Perio. 2012; 83:4: 444-452.

Seale NS, **Daubert D**, The Use and Efficacy of Professional Topical Fluorides, Penwell September 2010.

Abstracts:

Daubert D, M. J., Leroux B, Pozhitkov A, and Kotsakis G. in *IADR Meeting* (San Francisco, March 2017)

Katafuchi M, Chen Y, Leroux B, Dixon D, and **Daubert D**
A cross-sectional radiographic analysis of implant-supported restoration contour related to peri-implantitis. Academy of Osseointegration February 2016

Daubert DM, Udod, Y, Kleist CG, Habor C, Furman, IK, Kelley JL, Swanson WJ and Roberts FA. Adaptive Evolution in Enamelin and Human Tooth Enamel Thickness. IADR 2013 #171983

Tikhonov I, **Daubert DM**, Bamashmous S, Habor C, Kleist C, Khosh I, Kelley J, Swanson W and Roberts FA
Genetics of Tooth Enamel Thickness. IADR 2010 #128620

Daubert DM, Kleist CG, Habor C, Khosh I, Kelley JL, Swanson WJ and Roberts FA
Adaptive Evolution in Enamelin and Human Tooth Enamel Thickness. ADHA Center for Lifelong Learning 2009 #5

Daubert DM, Kleist CG, Nguyen K, Khosh I, Kelley JL, Swanson WJ and Roberts FA
Adaptive Evolution in Enamelin and Human Tooth Enamel Thickness. IADR 2008 #108123

Wang IC, **Daubert DM**, and Phillips KM
Success of Immediately Placed and Immediately Restored Single Implants. IADR 2008

Wang IC, Phillips KM, **Daubert DM**, and O'Neal RB
Success of Immediately Loaded Single Implants After 1-Year Follow-up. J Dent Research Special Issue 2005

Professional Presentations:

1991: Tashkent Medical Institute, Uzbekistan, U.S.S.R.
"Preventive Dentistry in the United States"
1992: Greater Seattle Dental Hygienists' Association
"Dentistry in Tashkent, Uzbekistan"
1993: Lake Washington Dental Hygienists' Association
"Dentistry in Tashkent, Uzbekistan"
1998: University of Washington Continuing Dental Education
"Implant Maintenance: Theory, Technique and Tools"
1999: Shoreline Community College Dental Hygiene Program
"Implant Maintenance for the Dental Hygienist"

2000: University of Washington Continuing Dental Education
"Update for Dental Hygienists"
2001: Seattle Study Club for Dental Hygienists
"Implant Maintenance"
2003: University of Washington Dent 610
"Fluoride: State of the Ion 2003"
2005: University of Washington Continuing Dental Education
"Focus on the Future of Periodontics- Communication
to Facilitate Periodontal Maintenance" November 19, 2005
2006: University of British Columbia Continuing Dental Education
"Implant Supportive Therapy" January 28, 2006
Lake Washington Dental Hygienist's Association
"Fluoride: State of the Ion" January 19, 2006
University of Washington Continuing Dental Education
"Implant Supportive Therapy" February 11, 2006
University of Washington Continuing Dental Education
"Prosthetic Periodontics: Supportive Therapy" May 5, 2006
2007: University of Washington Continuing Dental Education
"Top Ten Things that Used to Be True in the Practice of Dental Hygiene"
2008: Kent, Washington study club May 8, 2008
"Implant Supportive Therapy: an Evidence Based Approach"
Kaiser Permanente Dental Group October 15, 2008
"Implant Supportive Therapy: an Evidence Based Approach"
2009: Blue Herron Study Club
"Implant Supportive Therapy: an Evidence Based Approach"
2010: University of Washington Continuing Dental Education
"Update for Dental Hygienists" December 10, 2010
2011: Lake Washington Dental Hygienists' Association, January 2011
"Top Ten Changes in Dental Hygiene Practice"
Upper Island Dental Society, British Columbia, Canada October 2011
"Top Ten Changes in Dental Practice"
Bellingham Dental Hygiene Association, November 2011
"Top Ten Changes in Dental Practice"
2012: Lake Washington Dental Hygienists'Association February 2012
"Vaccinations
University of Washington Continuing Dental Education
"Top Ten Changes in Dental Practice"
2013: University of Washington Student Research Group January 2013
"Enam Gene and Enamel Thickness: Adaptive Evolution in Humans"
Lake Washington Dental Hygienists' Association October 2013
"Powered Instrumentation: Maximizing Technology/Maximizing Outcomes"
2014: University of Washington Continuing Dental Education: Update in Periodontics April
2014 "Biofilm Removal During the Periodontal Maintenance Appointment"
Air-Polishing Consensus Conference, Las Vegas June 2014.
"Efficacy of Subgingival Air Polishing: Review of the Literature"
University of Oklahoma Faculty Retreat, Oklahoma City, July 2014

"Subgingival Air Polishing 2014"
University or Washington Oral Health Sciences Research Symposium September 2014
"Prevalence and Predictive Factors for Peri-implant Disease and Implant Loss: A Cross-sectional Analysis"
2015: University of Washington Continuing Dental Education: Update in Periodontics April 2015 "Air Polishing 2015: Supra and subgingival biofilm removal"
Washington State Dental Hygiene Association Symposium April 2015
"Periodontitis/Peri-implantitis: Non-surgical treatment update for the dental hygienist"
Lake Washington Dental Hygienists' Association May 2015
"Next Generation Air Polishing"
Southshore Study Club September 2015
"Peri-implantitis: definition, diagnosis, prevalence and non-surgical treatment"
2016: Academy of Osseointegration Annual Meeting San Diego February 2015
"Peri-implantitis: Prevalence, Etiology, and Non-surgical Treatment"
Southshore Study Club April 2016
"Periodontal Update for the Dental Hygienist"
Western Society of Periodontists August 2016
"Maintenance of Patients with Implant-borne Restorations"

Research Activities:

#3 P30 De09743 Page, R.C. (PI)
National Institute of Dental Research, "Clinical Dental Research Core Center
Role: Examiner on multiple clinical trials from 1997 – 2002.

Clinical trial testing for FDA approval of an anesthetic, PI, R.C. Page, F. Roberts.
The goal of this project was to test the safety and efficacy of an anesthetic agent placed in the periodontal sulcus for pain control during scaling and root planning.
Role: Research Dental Hygienist, 1997.

Clinical trial RCDRC for FDA approval of a therapeutic antibiotic delivery system, PI, R. Persson.
FDA phase III trial for approval of Minocycline Microspheres
Role: Research Dental Hygienist and Examiner. 1998-1999.

Zimmer Dental O'Neal, R.B. (PI) Clinical Assessment of Immediately Loaded Single Tooth Restorations Using the Tapered Screw-Vent Implant System
The goal of this project was to test for safety and efficacy of an immediate placement implant
Role: Research coordinator and examiner, 2002-2006.

Astra Tech AB Phillips, K (PI)
An open, prospective study to evaluate implant stability, marginal bone adaptation and the survival rate of Astra Tech Dental Implant System, Fixture Osseospeed^{TM}, in patients with tooth loss in the posterior mandible in an early loading protocol.

The major goal of this project is to look at early loading on implants treated with Osseospeed surface.
Role: Research Coordinator and examiner, 2003-2009.

Hack Memorial Fund Grant Daubert, D (PI)
Genetics of Tooth Enamel Thickness
The major goal of this project is to investigate a single nucleotide polymorphism that may be associated with tooth enamel thickness. This is a collaborative project with Genome Sciences.
Role: Principle Investigator 2006-2009

Nobel Biocare AB. Wang, I.C. (PI)
Comparison of Immediately Placed Scalloped and Flat Dental Implants: A Long –term Follow-up Study The major goal of this study is to compare the tissue response with scalloped vs. flat Nobelbiocare implants.
Role: Examiner and Research Coordinator, 2006-2010.

OraPharma, Inc. Flemmig, T. (PI)
Multi-Center Trial of Minocycline HCl 1mg Microspheres for the Use in Subjects with Peri-Implantitis.
Role: Examiner and Research Coordinator, 2008-2009

Elector Medial Systems, Inc. Flemmig, T (PI)
Glycine Powder Air Polishing in Deep Periodontal Pockets
Role: Examiner, 2009-2010

Hack Memorial Fund Grant Daubert, D (PI)
Cross-sectional clinical trial assessing implants placed 10 years ago in the UW Periodontal Clinic 2011-2013

OraPharma, Inc. Flemmig, T. (PI)
Multi-Center Phase Three Trial of Minocycline HCl 1 mg Microspheres for the Use in Subjects with Peri-Implantitis
Role: Examiner, 2012-2013

Straumann, O'Neal R. (PI)
A Randomized, Controlled, multi-center clinical study evaluating the crestal bone level change of Straumann Bone Level 3.3mm NC SLActive Roxolid Implants compared to Struamann Bone Level 4.1 mm RC SLActive Roxolid Implants for single tooth replacement in the anterior and pre-molar region
Role: Study Coordinator 2014-2016

ITI Reseach Grant, Pozhitkov A. (PI)
Submucosal microbiome and titanium corrosion in peri-implantitis.
Aim: to compare submucosal microbiomes of patients with and without peri-implantitis, to evaluate concentrations of titanium leached into the plaque surrounding an implant, to test

whether submucosal microorganisms create an environment facilitating electrical current and titanium corrosion
Role: Researcher and examiner 2014-2016

Hack Memorial Fund Grant Daubert, D (PI)
Epigenetic Influence on Peri-implant Disease
This is a translational research project evaluating global methylation patterns in subjects with and without peri-implantitis.
2015-2016

Awards:

University of Washington School of Dentistry Distinguished Staff Award 2003
University of Washington Distinguished Staff Award Nominee 2008
Dentsply/ADHA Graduate Student Research Award 2009
Hu-Friedy Nevi Scholarship Award 2012
Lake Washington Dental Hygiene Society Lifetime Achievement Award 2012

Professional/Scientific Organization Memberships:

Omicron Kappa Upsilon National Dental Honor Society
University of Washington Dental Alumni Association
Board Member 2005 -present
American Dental Education Association
International Academy of Dental Research
Academy of Osseointegration
American Dental Hygienists' Association
Washington State Dental Hygienists' Association
Membership Chair 1991-1994
Lake Washington Dental Hygienists' Association
Charter Member
President-elect 1996-1997
President 1997-1998
Past-president 1998-1999
HOD Chairman 2004

Certification:
American Heart Association Basic Life Support for Healthcare Providers Instructor, 2002-present