GENE EXPRESSION ALTERATIONS IN CHRONIC PERI-IMPLANITIS SITES

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ABSTRACT

Nicole Dominique Luedin: Gene Expression Alterations in Chronic Peri-Implantitis Sites
(Under the direction of Ingeborg de Kok)

Dental implant success is based on the biologic integration of an alloplastic device in both endosseous and transmucosal tissues. The health of the transmucosal tissues adjacent to the implant and abutment are essential to success. Inflammation leading to alveolar bone loss (peri-implantitis) is a risk factor influencing a significant proportion of implants and patients. To better understand the molecular pathogenesis of peri-implantitis, a within patient comparison of gene expression within tissues at healthy and affected implants was performed using the Affymetrix Human ST1 gene array platform. RNAs isolated from tissues surrounding healthy and affected implants of 21 participants were evaluated. GeneSpring and IPA software revealed significant up-regulated genes related to inflammation, B-cell function and tissue detruction, and significant down regulated genes related to desmosome function and keratinized epithelium development and function. Peri-implantitis is associated with molecular changes that implicate epithelial barrier dysfunction as a potential key aspect of pathogenesis.

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Abbreviations for Remodeling and Differentiation

Abbreviation	Marker	Main biological process
BGLAP	Gene for osteocalcin	Bone remodeling (osteoblast differentiation)
BMP-2	Bone morphogenetic protein 2	Bone development
BMP-7	Bone morphogenetic protein 7	Bone development
BRAF	erine/threonine-protein kinase B-Raf	Cell differentiation
CD19	B-lymphocyte antigen CD19/ Cluster of differentiation 19	Lymphocite differentiation
CD31	Platelet endothelial cell adhesion molecule/ cluster of differentiation 31	Leukocyte transmigration, angiogenesis, integrin activation
cFn	Cellular fibronectin	Cell differentiation
COL9A1	Gene for Collagen alpha-1(IX) chain	Collagen IX
DDK-1	Dickkopf-related protein-1	Bone remodeling (osteoblast differentiation antagonist)
FGF18	Fibroblast growth factor 18	Cell growth, tissue repair
GAPDH	Glyceralaldehyde-3- phosphate dehydrogenase	Reference gene
Hp-Hb	Haptoglobin-Hemoglobin	Access for enzymes to hemoglobin
IL-2	Interleukin-2	T-cell differentiation
IL-4	Interleukin-4	B-cell differentiation
IL-5	Interleukin-5	B-cell growth
IL-7	Interleukin-7	T-cell maturation
IL-13	Interleukin-13	Anti-inflammation
MMP-2	Matrix metalloproteinase-2	Tissue remodeling

MMP-3	Matrix metalloproteinase-3	Tissue remodeling
MMP-9	Matrix metalloproteinase-9	Bone remodeling (osteoclast differentiation)
OC	Osteocalcin	Bone remodeling (osteoblast differentiation)
OPG	Osteoprotegerin	Bone remodeling (osteoclast differentiation antagonist)
OPN	Osteopontin	Bone remodeling (osteoclast anchoring)
PAI-2	Plasminogen activator inhibitor	MMP antagonist
PDGFA	platelet-derived growth factor a	Stimulating factor for cell growth
PGE2	Prostaglandin E2	Bone remodeling (osteoblast differentiation)
PPARγ	Peroxisome proliferator-activated receptor γ	Anti-inflammation
PTH	Parathyroid hormone	Bone remodeling (osteoclast differentiation)
RANK	Receptor activator of NF-κB	Bone remodeling (osteoclast differentiation)
RANKL	Receptor activator of NF-κB ligand	Bone remodeling (osteoclast differentiation)
RUNX2	Runt-related transcription factor 2	Osteoblast differentiation
SPARC	Gene for osteonectin	Bone formation
SPP1	Gene for osteopontin	Bone remodeling (osteoclast anchoring)
sRANKL	soluble receptor activator of NF-κB ligand	Bone remodeling (osteoclast differentiation)
TGF-α	Transforming growth factor-alpha	Epidermal growth factor
TGFβ-1	Transforming growth factor-beta	Fibrogenesis and vascular homeostasis
TIMP-1	Tissue inhibitors of metalloproteinases	MMP antagonist
VEGF-1	Vascular endothelial growth factor	Angiogenesis

Abbreviations for Inflammation and Destruction

Abbreviation	Marker	Main biological process
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9	Defence response to virus
AST	Aspartate aminotransferase	Tissue destruction
CatK	Cathepsin K	Bone resorption
cC1qR	C1q receptors for collagen	Pro-inflammation
CD3	Cluster of differentiation 3	T-cell activation
CD4	Cluster of differentiation 4	Antigen presentation
CD8	Cluster of differentiation 8	Antigen presentation
CD14	Cluster of differentiation 14	Antigen presentation
COLEC12	Collectin sub- family member 12	Innate immune response
CRP	C-reactive protein	Pro-inflammation
gC1qR	C1q receptors for globular domains	Pro-inflammation
GM-CFS	Granulocyte-macrophage colony-stimulating factor	Immune/inflammatory cascade
HBD1	Human Beta-defensin 1	Immune/inflammatory Cascade
HBD2	Human Beta-defensin 2	Immune/inflammatory Cascade
HCN2	Hyperpolarization-activated cyclic nucleotide-gated 2	Inflammatory pain
HMGB1	High mobility group chromosomal protein B1	Pro-inflammation
HMGN2	High mobility group chromosomal protein N2	Pro-inflammation
ICTP	C-pelopeptide pyridinoline crosslinks of type I collagen	Bone resorption and collagen degradation
IFN-γ	Interferon γ	Immune/inflammatory cascade
IKKI	Inhibitor of κB kinase	Pro-inflammation

IL-1α	Interleukin-1α	Pro-inflammation
IL-1β	Interleukin-1β	Pro-inflammation
IL-6	Interleukin-6	Pro-inflammation
IL-8	Interleukin-8	Neutrophil chemotaxis and angiogenesis
IL-10	Interleukin-10	Pro-inflammation
IL-12	Interleukin-12	Pro-inflammation
IL-17	Interleukin-17	Pro-inflammation
IL-22	Interleukin-22	Pro-inflammation
IL-22R	Interleukin-22 receptor	Pro-inflammation
IL-23	Interleukin-23	Pro-inflammation
MCC-1	Mast cell chymase	Pro-inflammation
MCP-1	Monocyte chemo- tactic protein	Pro-inflammation
MCT-1	Mast cell tryptase	Pro-inflammation
MiR146a	microRNA 146	Regulation of inflammation
MiR499	microRNA 499	Regulation of inflammation
MMP-1	Matrix metalloproteinase-1	Pro-inflammation
MMP-7	Matrix metalloproteinase-7	Pro-inflammation and tissue remodelling
MMP-8	Matrix metalloproteinase-8	Pro-inflammation and tissue remodelling
MMP-13	Matrix metalloproteinase-13	Pro-inflammation
MMP-25	Matrix metalloproteinase-25	Pro-inflammation
MMP-26	Matrix metalloproteinase-26	Pro-inflammation
PPP2R2B	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B beta isoform	Cell death
SRGN	Serglycerin/ hematopoetic proteoglycan core protein	Mediator for apoptosis

TANK	TRAF family member-associated NF-κB activator	Pro-inflammation
TNC	Tenascin-C	Pro-inflammation
TNF-α	Tumor necrosis factor α	Pro-inflammation
TRAP	Tartrate-resistant acid phos- phatase	Bone resorption

CHAPTER 1: LITERATURE REVIEW OF PERI-IMPLANTITS

Over a 40-year period, clinical development and research efforts established dental implant therapy as a successful treatment modality for tooth replacement. Success was clearly defined in the late 1980s to include features of survival, marginal bone levels and the absence of infection (Albrektsson, Zarb et al. 1986). These guidelines helped to objectively define endosseous implant therapy as a reliable and safe means of tooth replacement for complete and partial edentulism. Since the mid 1980's, numerous clinical studies, clinical reports and experience extending across millions of individuals treated with dental implants worldwide have provided additional insight into both the success and failure of dental implants.

Recently, the definition of survival was re-affirmed as an implant that remains *in situ* with or without modification during the observation period (Jung, Zembic et al. 2012). The reported survival rate varies from 73.4% to 100%, with a mean of 94.6% after up to 20 years (Moraschini, Poubel et al. 2014). Implant success has most frequently been assessed by survival rate, prosthesis stability, radiographic bone loss and absence of infection. Due to the heterogeneity of the definitions of success in implant dentistry, Papaspyridakos et al. (Papaspyridakos, Chen et al. 2012) suggested four categories to evaluate success: (1) at the implant level, (2) peri-implant soft tissue condition, (3) prosthetics and (4) patient satisfaction level. This group found that for the success at the implant level most papers reported mobility, pain, radiolucency, and peri-implant bone loss of ≥1.5mm as criteria. The criteria for the soft tissue success included both suppuration and bleeding. The occurrence of technical complications, maintenance, function and esthetics comprised success

criteria at the prosthetic level. Finally, to evaluate patient satisfaction, discomfort, paresthesia, satisfaction with appearance and ability to chew and taste were parameters used. Papaspyridakos et al. (Papaspyridakos, Chen et al. 2012) concluded that "success in implant dentistry should ideally evaluate a long-term primary outcome of an implant-prosthetic complex as a whole." The success rate ranged from 34.9% to 100%, with a mean success rate of 89.7%, during a mean follow-up of 15.7 years (Moraschini, Poubel et al. 2014). Clearly, continued reporting of dental implant outcomes has revealed that among the predominant successes, implant prostheses incur complications and do fail, that marginal bone levels may change over time and that infection is reported. Given that inflammatory disease is known to affect implant success, much more emphasis is now focused on peri-implantitis. Peri-implantitis is a condition with inflammation in the soft tissue around an implant with loss of bone.

Marginal bone level assessment with radiographs is one of the most important reference criteria for evaluating the long-term success of dental implants. Changes in bone occur in the first 6 months after implant placement due to a physiological healing process (Sanz and Chapple 2012, De Bruyn, Vandeweghe et al. 2013). The healing process include re-establishment of the junctional epithelium and the supra-alveolar connective fibers independent from the surgical technique (Abrahamsson, Berglundh et al. 1999) and the implant system (Abrahamsson, Berglundh et al. 1996). Berglundh and Lindhe stated "that once the implant is exposed to the oral environment and in function, a mucosal attachment of a certain minimum dimension is required to protect osseointegration" (Berglundh and Lindhe 1996). Hence, it was found that a stabilization of a crestal bone level of 1.5-2.0mm below the implant abutment interface normally occurs one year after loading (Cochran, Nummikoski et al. 2009).

In health, the marginal bone changes at implants are self-limiting and minimal. For example, Moraschini et al. (Moraschini, Poubel et al. 2014) reported a mean marginal bone loss of 1.3mm

over a time period of up to 15.7 years. Marginal bone loss is encountered with some frequency. For example, in full-arch implant-supported prostheses bone loss ≥ 2mm were found in 16-29% of the patients after 12-15 years of function (Ravald, Dahlgren et al. 2013). Renvert et al. (Renvert, Lindahl et al. 2012, Renvert, Polyzois et al. 2013) have found that bone loss was greater in the first 7 years of function compared from 7 to 13 years of function. Tomasi et al. (Tomasi, Wennstrom et al. 2008) compared the longevity of teeth and implants. They found that in well-maintained patients the survival rate of teeth is greater than the one of implants and with regular maintenance the bone loss is small around teeth and implants. However, comparison between the survival rate of teeth and implants is difficult due to the heterogeneity of the study design and the patient population. Rasperini et al. (Rasperini, Siciliano et al. 2014) compared bone levels on teeth adjacent to implants and found that the bone levels around teeth were more stable than around implants in a 10 year follow-up.

While there exist differences among implant systems, a consistent observation has been the stabilization of bone changes relative to the implant abutment interface (Laurell and Lundgren 2011). An important hallmark of peri-implantitis is the progression of marginal bone loss beyond these accepted adaptive changes in health.

It has been demonstrated that inflammation is a main cause of alveolar bone loss at dental implants and that the extent of inflammation is associated with marginal bone level changes (Schou, Holmstrup et al. 1992). In a foundational study in the dog model, the abundance of inflammatory cells in the inflammatory cell infiltrate at implants was correlated with the extent of marginal bone loss (Broggini, McManus et al. 2006). In clinical studies, the extent of marginal bone loss at implants was correlated with the degree of clinical inflammation (Kehl, Swierkot et al. 2011). This inflammatory process is related to implant/ abutment exposure to the oral cavity and presumably in relationship to biofilm and not specifically to loading. In a simple and informative study, dental

implants placed by a two-stage procedure were examine after oral exposure following abutment connection of selected implants. Unexposed implants did not demonstrate bone loss during the period of time, while implants with abutments that were exposed to the oral environment did show bone loss (Naert, Gizani et al. 1999). The investigators demonstrated that marginal bone loss at implants was not a result of surgical placement and bone remodeling, but attributable to abutment connection and further biological integration.

As indicated above, the process of marginal bone loss is preceded by peri-implant mucosal inflammation. Peri-implant mucosal inflammation other biological complications are the most common problem encountered with dental implants. Peri-implant mucositis is the number one biological complication reported (Moraschini, Poubel et al. 2014), followed by alveolar bone loss around implants. For example, Jung et al. (Jung, Zembic et al. 2012) found a 5-year cumulative peri-implant soft tissue complication rate of 7.1%, and a 5.2% cumulative complication rate of alveolar bone loss ≥2mm around single dental implants. It is commonly observed; this is underscored by a recent systematic review, indicating the prevalence of peri-implant mucositis (with varying definitions) was 42.9% (95% CI 32-54%) (Derks and Tomasi 2014).

According to Mombelli et al. (Mombelli, Muller et al. 2012) the prevalence of peri-implantitis seemed to be 10% in 5-10 years of function. In a recent systematic review, the prevalence of peri-implant mucositis ranged from 16-65%, the one for peri-implantitis from 1-47%. In the same study meta-analysis estimated a mean prevalence of peri-implantitis of 22% (Derks and Tomasi 2014). Renvert et al. (Renvert, Lindahl et al. 2012) looked at the incidence of peri-implantitis of two different systems over a 13-year period. They found an incidence of 26.2% - 30.4% in the first seven years and a 7.1%-11.5% incidence from year 7 to 13. Information about the microbiota at year 7 did not hold up for a prognosis of peri-implantitis at year 13. In the Table 1.1

there are the different prevalences of peri-implantitis summarized. It changes on an implant level between 1% up to 47% and on a subject or patient level up from 11.2% to 56%

Publications	Incidence	patient/implant level	Follow-up time
(Renvert, Lindahl et al. 2012)	26.2- 30.4% 7.1-11.5%	implant implant	1-7 years 7-13 years
(Periodontology 2013)	11.2-47.1% 6.6-36.6%	subject implant	mean 5.7-10.8 years
(Atieh, Alsabeeha et al. 2013)	18.80% 9.60%	subject implant	5- >10 years
(Zitzmann and Berglundh 2008)	28 - ≥56% 12 -43%	subjects implant	≥ 5 years
(Ravald, Dahlgren et al. 2013)	16-29% 5-6%	subject implant	Up to 15 years
(Jung, Zembic et al. 2012)	5.20%	implant	5 years
(Derks and Tomasi 2014)i	1-47%	implant	3.4-11 years
(Mombelli, Muller et al. 2012)	10% 20%	implant subject	5-10 years

Table 1.1. Prevalence of Peri-implantitis

Existing reports affirm that not all patients treated with implants and not all implants within a mouth succumb to peri-implantitis. Intuitively, there must exist various risk factors for peri-implantitis. These risk factors may include local factors including tissue architecture, bone quality and biotype; features of the prosthesis and abutments; the local and systemic factors; existing peri-implant and periodontal infection, and habits (e.g. smoking) that influenced tissue responses to biofilm and function. Sanz et al. (Sanz and Chapple 2012) found that “there is heterogeneity in the risk indicators investigated across the broad categories of host-derived,

lifestyle, environmental and local factors" and therefore additional and adequately powered research needs to be done.

The effect of history of periodontitis on implant success revealed no differences in terms of survival.; nevertheless, patients with a history of periodontitis had a lower implant success rate (Ramanauskaite, Baseviciene et al. 2014). Systemic disease and a history of periodontitis have been considered risk factors for peri-implantitis (Renvert, Polyzois et al. 2013). However, systematic reviews on the effects of periodontitis on dental implant survival showed a great variability (Faggion and Giannakopoulos 2013).

When considering the existing data published and summarized in the present literature regarding peri-implantitis, it may be concluded that peri-implantitis and the associated profound interfacial bone loss that occurs at dental implants is the result of inflammation. Factors contributing to the initiation and progression of this inflammation as well as factors that preclude its control or elimination represent risk factors influencing peri-implantitis. While the extent to which individuals are susceptible to or harbor one or another risk factor influencing peri-implantitis has not been fully elucidated, it may be further concluded that inflammatory events are likely modulated by local tissue architecture, prosthesis and abutment factors, local and systemic biology that influence the accumulation, nature and response to an adherent biofilm. A biofilm-associated inflammatory response leading to bone loss can progress to implant failure.

Currently, there exist important unanswered questions regarding the inflammatory processes that surround dental implants. First, what is the precise etiology of this inflammatory process? Second, how is peri-implantitis defined and separated from peri-implant mucositis? Third, how is the inflammatory process diagnosed, measured and/or monitored? Fourth, what is the

knowledge regarding the pathophysiology of peri-implantitis. The goal of this review was to identify the present information regarding molecular mediators of inflammatory processes in peri-implantitis and their measurement in clinical therapy.

Bacterial etiology of peri-implantitis

Mombelli (Mombelli and Lang 1998) proposed the hypothesis that "microbial colonization of dental implants and infection of the peri-implant tissues can cause peri-implant bone destruction and may lead to implant failure." The author based the hypothesis on five lines of evidence, which were (1) human trials showing that peri-implant mucositis can be induced by deposition of plaque, (2) the microbiota associated with healthy and infected implants showed qualitative and quantitative differences, (3) shifts in the microbiota and peri-implantitis could be found in animal models with plaque-retentive ligatures, (4) clinical status of peri-implantitis patients could be improved with antimicrobial therapy and (5) "the level of oral hygiene has an impact on the long-term success of implant therapy."

Secondarily, Mombelli also assumed that "peri-implant infections are amenable to treatment just as periodontal infections are" (Mombelli 2002). Therefore, the author inferred that the disease is a combination of a bacterial colonization of the implant surfaces and that the immune response causes bone destruction around the implant.

Dental implants provide a target for biofilm formation. The biofilm derives from various micro-ecological niches, including the neighboring natural dentition, saliva and mucosa. The microbiota of peri-implantitis shares many species found in periodontitis with important distinctions. In peri-implant pockets, 41% of the microbiota found were gram-negative anaerobic rods, among them were *P. intermedia, Fusobacterium* spp, and *P. gingivalis*. A high amount of spirochetes were associated with peri-implantitis patients, whereas healthy implants were mainly

colonized by gram-positive facultative anaerobic cocci in significant lesser counts (Mombelli 1997, Mombelli 2002). *T. forsythia* and *T. denticola* were increased in peri-impantitis and periodontitis. Distinct for peri-implantitis, when compared to periodontitis, the microbiota included *S. aureus, S. epidermidis,* and the aerobic gram-negative bacilli *E: aerogenes, E. cloace, E. coli, H. pylori, P. micra, Pseudonomas* ssp and *Candida* ssp fungi (Koyanagi, Sakamoto et al. 2013, Belibasakis 2014, Smeets, Henningsen et al. 2014, Belibasakis, Charalampakis et al. 2015). It has been reiterated that peri-implantitis lesions harbor bacteria that are typically found in periodontitis lesions. *Staphylococcus aureus* is predominant in peri-implantitis and may provide a high positive (80%) and negative (90%) predictive value (Rasperini, Siciliano et al. 2014, Salvi and Zitzmann 2014, Smeets, Henningsen et al. 2014). Thus, a unique bacterial infection in peri-implantitis may induce unique inflammatory sequelae.

Interestingly, fully edentulous patients had a different composition of the microbiota with a smaller amount of spirochetes and *P. gingivalis* than partially edentulous patients. That led to the hypothesis that "a transmission of periodontal pathogens and partially edentulous patients with a history of periodontitis are at an elevated risk of developing peri-implantitis" (Mombelli 1997) (Mombelli 2002). It is widely accepted that oral bacteria derived biofilm is a primary etiology of peri-implantitis.

The pathophysiology of peri-implantitis has been explored and an early focus was directed at bacterial etiology. As summarized in the preceding section, an adherent oral biofilm on the implant, abutment, prosthesis surface and in peri-implant tissues invokes a host inflammatory response that in some cases leads to bone loss. The progression from peri-implant mucositis exemplified by a biofilm containing cocci, motile bacilli and spirochetes, at proportions comparable to gingivitis to the emergence of a biofilm containing gram-negative, motile, and anaerobic species

commonly found in periodontitis. Furthermore, microorganisms unique to peri-implantitis have been identified and include *S. aureus, S epidermidis, E. aerogenes, E cloace, E. coli, H. pylori, P. micra, Pseudomonas* and *Candida* spp (Belibasakis 2014).

Initial investigations identified gram-negative anaerobic bacteria and *S. aureus* as potential pathogens (Mombelli, van Oosten et al. 1987). Other periodontal pathogens, *F. nucleatum, A. actinomycetemcomitans, P. gingivalis,* and *P. intermedia*, have been repeatedly found in peri-implantitis. These and other lead bacteria have been implicated in the process however a comprehensive assessment of the microbial population using 16S rRNA based-assessment underscored a diverse bacterial population in peri-implantitis that is more complex than that formed in periodontitis (Koyanagi, Sakamoto et al. 2013). The microbial diversity associated with peri-implantitis continues to be illustrated by molecular assessment of the biofilm (da Silva, Feres et al. 2014).

Definitions of peri-implant mucositis and peri-implantitis

Peri-implantitis is distinct from peri-implant mucositis. Withdrawal of oral hygiene with resulting plaque formation on teeth and implants results in a local inflammatory response that is clinically evidenced by redness and bleeding on probing and evidenced histologically by the time-dependent increase of the inflammatory cell infiltrate adjacent to the implant/abutment (Zitzmann, Berglundh et al. 2001). This peri-implant mucosal response is termed peri-implant mucositis. According to the American Academy of Periodontolgy (AAP) (2013) and the VIII European Workshop (Sanz and Chapple 2012) on Periodontology the definition for peri-implant mucositis is the presence of inflammation confined to the soft tissues surrounding a dental implant with no signs of loss of the alveolar bone following initial bone remodeling during healing. In contrast, the AAP stated that peri-implantitis is characterized by an inflammatory process around an implant, that involves both soft tissue inflammation and progressive loss of supporting bone beyond biological bone remodeling. Due to differences in the threshold level of radiographic bone loss for

defining peri-implantitis, as well as for the time point when the bone loss occurred, Sanz et al. (Sanz and Chapple 2012) agreed on the definition for peri-implantitis when "changes in the level of crestal bone, presence of bleeding on probing and/or suppuration, with or without concomitant deepening of peri-implant pockets are present." Furthermore, they defined the bone level of 2mm from the expected level in absence of baseline radiographs as a threshold to define peri-implantitis. With existing baseline radiographs, they set threshold of bone loss is 1-1.5mm for the definition of peri-implantitis (Sanz and Chapple 2012).

Diagnosis and monitoring of peri-implantitis

There are no single diagnostic parameters for peri-implantitis. The diagnosis involves clinical and radiographic parameters. Probing with a traditional periodontal probe with 0.25N for the incidence of bleeding on probing (BoP), suppuration and probing depth (PPD) and their changes over time is widely recommended (AAP) (Zitzmann and Berglundh 2008, Sanz and Chapple 2012, 2013). Additionally, plaque accumulation indexes (PlI), clinical attachment levels (CAL), and gingival recession (REC) are recommended by other authors (Graziani, Figuero et al. 2012).

BoP has a high negative predictive value, as well as a positive predictive value of a 100%. The absence of BoP is an indicator of peri-implant tissue health. However, BoP only diagnoses a peri-implant mucositiis and is not specific for peri-implantitis. Suppuration indicates an inflammatory process and is associated with peri-implantitis. However, its absence is not an indicator of health. A PPD of 3-4mm around an implant is considered healthy (Padial-Molina, Suarez et al. 2014). However, the PPD can vary due to implant and restoration type, depth of placement for aesthetic reasons, healing time, surgical and loading protocols, as well as quality of the histologic tissue seal. A baseline PPD should be taken after the restoration is placed and the soft tissue healed initially (Padial-Molina, Suarez et al. 2014). Peri-implantitis has been characterized by

the formation of a peri-implant pocket > 4 mm, bleeding or suppuration on probing, and radiographic evident saucer-shaped bone destruction around the implant (Belibasakis 2014).

Mobility is not a useful clinical sign of for peri-implantitis, since it represents complete implant failure. When mobility is presented, identification of a mobile abutment should be made, as that mobility and associated inflammation at the implant-abutment level lead to alveolar bone loss (2013). Implant mobility can be measured either manually or with a specific device such as the Periotest dental measuring instrument (Siemens, Bensheim Germany) or with the Ostell instrument (Ostell, Gothenburg, Sweden). There is a differential diagnosis for mobility that includes a) mobility of the prosthesis at the abutment interface, b) mobility of the abutment at the implant interface, and c) mobility of the implant itself. Because implants are often splinted and the mobility can be masked, it is recommended to unscrew the prosthesis and evaluate individual unsplinted implants (Todescan, Lavigne et al. 2012).

As indicated above, marginal bone level changes are a central determinant of implant success and of peri-implantitis. Radiography is an essential tool here. Initial bone healing occurs in the first 6 month after implant placement (De Bruyn, Vandeweghe et al. 2013), therefore, the current recommendations for radiographs by the AAP and Sanz et al. (Sanz and Chapple 2012, 2013) are to take one at the implant placement and one at the time of prosthetic rehabilitation. At the time of prosthetic loading the initial bone healing and remodeling is over and provides a good baseline (De Bruyn, Vandeweghe et al. 2013). According to De Bruyn et al. (De Bruyn, Vandeweghe et al. 2013) a periapical radiograph can not detect a true bone loss of <1mm. Orthopantomographs can be used to assess peri-implant bone levels, however two-dimensional periapical radiographs are the gold standard for that assessment. However, digital radiography allows for an easy standardization of the image contrast and hence help with comparing radiographs over time to

detect bone loss (De Bruyn, Vandeweghe et al. 2013). The low predictive nature of these parameters (BoP, PPD, suppuration, radiograph etc.) by itself makes it necessary to combine them and measure them over time to render the diagnosis of peri-implantitis.

Host response

As stated before, while periodontal disease and peri-implantitis appear to share common bacterial etiology and both reflect a host response to biofilm, previous reports highlight potentially important differences between dental implants and natural teeth. Biofilm associated inflammation and the innate immune response progresses faster and results in a more extensive and severe tissue destruction at implants than at teeth (Smeets, Henningsen et al. 2014, Belibasakis, Charalampakis et al. 2015). The reasons for this differential host response (beyond yet to be determined differences in biofilm) may reflect local anatomic differences between teeth and implants. A prominent difference is that Sharpey's fibers inserting perpendicular into cementum do not exist at the implant surface. The collagen fibers of the submucosal tissue connective tissue are arranged parallel to the surface of the implant, resulting in a deeper peri-implant crevice and therefore allows for potentially deeper penetration of the biofilm when compared to teeth. A more pronounced apical extension of the inflammatory cell infiltrate was found in peri-implantitis compared to periodontitis (Berglundh, Gislason et al. 2004, Berglundh, Zitzmann et al. 2011, Alani, Kelleher et al. 2014). Furthermore, there is no periodontal ligament at implants. This results in the absence of an important periodontal vascular plexus that differentiates the blood supply between implants and teeth. The peri-implant mucosa is often reported to be less vascular than the periodontium (Berglundh, Gislason et al. 2004). Additionally, the absence of a periodontal plexus of blood vessels around implants leads to the speculation that any mechanical stimuli are less likely to contribute to the inflammatory process. Only when there is mobility at the implant abutment interface is the physical stimulation of soft tissue vasculature contributory to the inflammatory process (Broggini, McManus et al. 2006). Periodontitis lesions have a wall of non-infiltrated

connective tissue toward the alveolar bone and a separation of the biofilm from the connective tissue by a pocket epithelium. These compartmentalizations are missing in the peri-implantitis lesions (Carcuac and Berglundh 2014).

Regarding the inflammatory infiltrate that exists at both healthy and inflamed implant sites, Broggini et al. (Broggini, McManus et al. 2006) found that the highest concentration of inflammatory cells were at or immediately coronal to the implant-abutment interface. The predominant peri-implantitis cell type was neutrophils. Furthermore, there is a positive correlation with the depth of the interface, and the magnitude of the peri-implant inflammation. Carcuac and Berglundh (Carcuac and Berglundh 2014) found that peri-implantitis lesions are double the size compared to periodontitis lesions. Peri-implantitis sites "contained significantly larger area proportions, numbers and densities of plasma cells, macrophages and PMN cells compared to periodontitis." PNM cells "indicate that the effector systems of the host response, such as phagocytosis, are active in peri-implantitis" (Berglundh, Gislason et al. 2004). They concluded that in peri-implantitis both cells from the innate and the adaptive immune system are playing a role (Carcuac and Berglundh 2014). The innate immune response results in an increased infiltration of neutrophils, macrophages, interstitial dendritic cells, B-and T- cells, osteoclasts and a decrease of Langerhans cells. (Berglundh, Zitzmann et al. 2011, Belibasakis 2014, Belibasakis, Charalampakis et al. 2015).

Experimental models were needed to study peri-implantitis further. Both dogs and monkeys have been used for that approach. Overloading and ligature experiments were done trying to understand the etiology of peri-implantitis. Overload simulations alone did not find a correlation with bone loss in dogs, but a significantly increased angular bone loss when combined with ligature models. In monkeys however, overload could induce bone loss or a loss of osseointegration (Pesce, Menini et al. 2014). Isidor (Isidor 1997) found that plaque accumulation can cause bone loss, but

not loss of osseointegration, while occlusal overload can determine a loss of osseointegration. The use of ligatures resulted in a foreign body reaction and induced a destructive process around the implants and therefore does not represent a good model to study the disease in humans. In the same way, overload studies showed no similarity to bone loss in humans (Pesce, Menini et al. 2014). Berglundh et al. (Berglundh, Zitzmann et al. 2011) concluded that a "self-limiting" process of the induced tissue inflammation after removal of the ligature does not occur around implants. They found signs of acute inflammation and large amounts of osteoclasts lining the surface of the bone crest after the removal of the ligature, and therefore a progression of the bone loss. A literature review was done on canine ligature models by Martins et al. (Martins, Ramos et al. 2014). This group found that most of the studies were done with Beagle dogs and implants in the premolar and molar region of the mandible. Cotton or silk ligaments were placed in a submarginal position around implants, however the methods varied widely. Their conclusion was that the defect configuration differs between humans and the dogs and that the ligature placement results in a traumatic foreign body bone loss rather than a natural one. Therefore, an "ideal canine peri-implantitis induction model would be naturally occurring peri-implantitis induction without the action of any ligature" (Martins, Ramos et al. 2014). Pesce et al. (Pesce, Menini et al. 2014) concluded that animal studies reported contrasting results depending on the model employed and are not completely representative of the human disease.

Pathogenesis

The pathogenesis of peri-implantitis has been characterized as an initial peri-implant mucositis that spreads toward the supportive bone. Compared to peri-implant mucositis, the inflammatory response was been characterized as comprised of higher proportions of neutrophils, macrophages, T- and B- cells than periodontitis. Higher numbers of osteoclasts have been observed in peri-implantitis as well. The basis for this may be differences in the innate immune response that involves pro-inflammatory cytokines including IL-1a, IL-6, IL-8 and TNF α. It has been suggested

that the inflammatory tissue destruction that occurs at implants is more aggressive than that observed at teeth (Belibasakis 2014). The details of the many aspects of peri-implantitis pathogenesis remain relatively obscure.

In the pursuit of understanding the pathogenesis of peri-implant mucositis and peri-implantitis a significant number of articles has been published. One approach has been to examine the gene polymorphisms associated with peri-implantitis. Some studies have identified gene polymorphism associations for OPG, IL-6, TNF-α, RANKL, MiR146a/ MiR499 with peri-implantitis (Cury, Horewicz et al. 2009, Kadkhodazadeh, Tabari et al. 2012, Slotte, Lenneras et al. 2012, Casado, Villas-Boas et al. 2013, Kadkhodazadeh, Ebadian et al. 2013, Kadkhodazadeh, Jafari et al. 2013). Those genes are associated with inflammatory diseases and bone resorption. Some papers showed contradictory results of IL-1 gene polymorphism, which is a general marker of inflammation and has been implicated as a genetic marker for periodontal disease. IL-1 polymorphism has been associated with peri-implantitis (Laine, Leonhardt et al. 2006, Hamdy and Ebrahem 2011, Casado, Villas-Boas et al. 2013), associated in combination with smoking (Gruica, Wang et al. 2004) or not associated at all (Lachmann, Kimmerle-Muller et al. 2007, Melo, Lopes et al. 2012). There are inconsistent findings concerning the pro-inflammatory cytokine IL-17 polymorphism (Severino, Napimoga et al. 2011, Darabi, Kadkhoda et al. 2013, Kadkhodazadeh, Baghani et al. 2013, Kadkhodazadeh, Ebadian et al. 2013). Several gene polymorphisms are found not to be associated with peri-implantitis, among them are BRAF, TANK, Hp-Hb complex and HCN2 (Kadkhodazadeh, Amid et al. 2012, Ebadian, Kadkhodazadeh et al. 2013, Kadkhodazadeh, Jafari et al. 2013, Ebadian, Kadkhodazadeh et al. 2014).

Previous reviews related to the pathogenesis of peri-implantitis focused on the levels of inflammatory cytokines previously related to periodontitis (Candel-Marti, Flichy-Fernandez et al.

2011, Javed, Al-Hezaimi et al. 2011, Petkovic-Curcin, Matic et al. 2011, Li and Wang 2014). The relationship of levels of suspect proteases was considered in three reviews (Sorsa, Tjaderhane et al. 2006, Javed, Al-Hezaimi et al. 2011, Li and Wang 2014). The possible relationships of genetic polymorphisms influencing the levels of inflammatory mediators has also been summarized previously (Andreiotelli, Koutayas et al. 2008, Bormann, Stuhmer et al. 2010, Javed, Al-Hezaimi et al. 2011, Dereka, Mardas et al. 2012, Liao, Li et al. 2014). A general conclusion from these different summaries is that peri-implantitis involves upregulation of the general mediators of inflammation including TNF-α, IL1a, IL1b, IL6 and IL10. Fewer studies investigated more specific mediators of osteoclastogenesis, an essential aspect of peri-implantitis. The recognition of OPG, RANK and RANKL up-regulation (Sorsa, Tjaderhane et al. 2006), as well as elevated levels of more general mediators, TNF-α and IL6, affirm an existing appreciation that inflammation and osteoclastogenesis are central to the pathogenesis of peri-implantitis (Table 1.2).

Publications	**Investigated**	**Findings**	**Articles included**	**Probes**
(Javed, Al-Hezaimi et al. 2011)	IL-1β, IL-6, IL-8, MMP-1, TNF-α, IL-1	*2 studies: upregulated* IL-6 ; *4 studies: upreglated* IL-1β; *1 study: up-regulated* IL-6, IL-8, MMP1, *6 studies: upredulated* TNF-α *2 studies:* IL-1 polymorphism assoc. With PI; *1 study:* TNF-α not assoc. With PI	15	PICF
(Petkovic-Curcin, Matic et al. 2011)	IL-1β,IL-6, IL-8, MIP-1α, TNF-α	*up-regulated* IL-1β in early stage PI; 3x *up-regulated* IL-1β; *Up-regulated* IL-8, MIP-1α, TNF-α	not mentioned	PICF
(Andreiotelli, Koutayas et al. 2008)	IL-1β	IL-1β polymorphism not assoc. With PI, synergistic effect oft pos. IL-1β and smoking	8	PICF
(Bormann, Stuhmer et al. 2010)	IL-1	correlation between IL-1 polymorphism and PI with additional risk factors (eg. Smoking)	27	not mentioned

(Candel-Marti, Flichy-Fernandez et al. 2011)	IL-6, IL-8, Il-10, IL-12	*5 studies: up-regulated* IL-6; *1 study: upregulated* IL-8; *1 study:* no changes in IL-8 *1 study: upregulated* IL-10*; 1 study: up-regulated* IL-12	7	PICF
(Dereka, Mardas et al. 2012)	IL-1	IL-1 polymorphism not assoc. With PI	7	not mentioned
(Huynh-Ba, Lang et al. 2008)	IL-1	not enough evidence for an association oft IL-1 polymorphism with PI	2	not mentioned
(Sorsa, Tjaderhane et al. 2006)	MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, MMP-14, MMP-25, MMP-26	no disticntion between PI and CP; MMP-9 polymorphism not assoc. With PI; promotor polymoprhism oft MMP-1/2/3 has no influence on susceptibility to PI *Up-regulated:* MMP-3/8/9/13/14/25/26, MMP-1/2 only in low levels	not mentioned	not mentioned
(Li and Wang 2014)	IL-1β, MMP-1, MMP-3, MMP-8, MMP-13, OPG, PGE2, IL-6 IL-8, Il-10, IL-12, RANK, RANKL, CathepsinK, Elastase, Phosphatase	*Up-regulated:* IL-1β (IL-6/8 neg/pos), MMP-8/13, Elastase, Phosphatase, CathepsinK *Up-regulated:* OPG, RANK, RANKL *no difference:* PEG2, IL-10/12	not mentioned	PICF
(Liao, Li et al. 2014)	IL-1	evidence oft genetic effect oft IL-1 polymorphism	13	cells and blood

Table 1.2 Review articles for Peri-implantitis Markers

This review also identified multiple markers for bone resorption and remodeling and pro-inflammatory cytokines reported to be upregulated in peri-implantitis. Included are HMGB1, HMGN2, matrix metalloproteinases, cFn, TGF-β, RANK/RANKL, IL-22/23 (Kuula, Salo et al. 2008, Duarte, de Mendonca et al. 2009, Arikan, Buduneli et al. 2011, Luo, Xie et al. 2011, Luo, Wang et al. 2013, Rakic, Lekovic et al. 2013, Wu, Cao et al. 2013, de Araujo, Filho et al. 2014, Konermann, Gotz et al. 2014, Schminke, Vom Orde et al. 2015) (Ozcakir-Tomruk, Chiquet et al. 2012, Irshad, Scheres

et al. 2013, Rakic, Nikolic-Jakoba et al. 2013, Wohlfahrt, Aass et al. 2014). Some of the pro-inflammatory cytokines were found to be unchanged or down-regulated, among them IL-10, MMP-8 (Severino, Napimoga et al. 2011, Casado, Villas-Boas et al. 2013, Irshad, Scheres et al. 2013).

In the present review, the knowledge regarding molecular basis of peri-implantitis were conveniently categorized according to the manner of investigation and are: a) peri-implant sulcus fluid (PISF) biomarkers (Table 1.3.), b) peri-implant tissue biomarkers (Table 1.4.), and c) genetic biomarkers (Table 1.5.). A rationale for this categorization is that the source of information available will reflect the manner of collecting patient-specific information (e.g., collection of PISF, Biopsy, DNA) and may further influence the data made available (e.g.; incipient disease screening vs. genetic risk assessment).

Peri-implant sulcular fluid molecular markers

Twenty-seven papers compared specific constituents of PISF among healthy and peri-implantitis sites (Table 1.3.). Monitoring of molecular mediators in sulcular fluid in periodontitis has been adopted in assessment of PISF to study peri-implantitis (Periodontology 2013, Faot, Nascimento et al. 2015). Recent data suggests that there are differences in PISF and gingival cervicular fluid (GCF) that may be important to consider and may imply a different pathogenesis for peri-implantitis and periodontitis (Recker, Avila-Ortiz et al. 2015).

Publications	**Investigated**	**Findings**	**PI patients**
(Hall, Britse et al. 2011)	TRAP, DDK-1, OPG, CatK, OC, IL-1β, TNF-α, RANKL, ALP, GAPDH, PPIA, ACTB YWHAZ, RRN18S, B2M, UBC, RPLP, HPRT1	no sign. Differences between healthy and PI for all markers ev problem with strategy	7
(Severino, Napimoga et al. 2011)	IL-6, IL-8, IL-10, IL-17	*Up-regulated:* IL-17; *no differences:* IL-6/8/10	14

		sign. Positive correlation bt. IL-6 and IL-8 in PI	
(Rakic, Nikolic-Jakoba et al. 2013)	RANK	*Up-regulated:* 9x RANK	22
Rakic, 2013 #1}	sRANKL, RANK, OPG	*Up-regulated:* SRANKL, RANK, OPG	23
Arikan, 2011 #52}	ICTP, sRANKL, OPG	*Up-regulated:* ICTP, OPG ; *up-pregulated in healthy:* OPG, sRANKL	12
Wohlfahrt, 2014 #28}	MMP-8, TNF-α, OPN, OPG, OC, IL-6, PTH, Insulin	*Down-regulation **after Tx**:* IL-6, Insulin, MMP-8 no correlation bt. Change oft bone and marker concentration	12
Irshad, 2013 #36}	IL-1β IL-6, IL-8, MCP-1, MMP-1, MMP-2, MMP-8, TIMP-1, TGFβ-1	*Up-regulated in non-challenged (P. gingivalis) cells*: IL-1β, IL-8, MCP-1, MMP-8 *Up-regulated in challenged cells:* IL-1β, IL-6/8, MCP-1, MMP-1 *Down-regulated in challenged cells:* MMP-8	7
(Lachmann, Kimmerle-Muller et al. 2007)	IL-1β, PAI-2, PGE2	no assoc. With genotypes	11
Casado, 2013 #40}	IL-1β, IL-10	*Up-regulated:* IL-1β; IL-10 in **healthy;** *down-regulated:* IL-10	10
Darabi, 2013 #35}	TNF-α, IL-17	*upregulated:* TNF-α, IL-17	24
(Ozcakir-Tomruk, Chiquet et al. 2012)	TNC, MMP-9	*Up-regulated:* MMP-9, small for TNC	18 total, PI not mentioned
Sarlati, 2010 #56}	sRANKL	no sign. Difference in concentration	26
Slotte, 2012 #59}	CatK, TNF-α, ALP, OC, IL-1β	early loading, clinical complications with TNF-α, CatK, ALP correlation with clinical parameters and complications	immediate loading: 9 test group: 9
(Ramseier, Eick et al. 2015)	IL-1β, MMP-1, MMP-3, MMP-8, MMP-1/TIMP	MMP-8 in 90% oft sites, IL-1β in 50% oft sites, in 30% oft sites MMP-1, MMP3, MMP-1/TIMP	504 implants
(Hultin, Gustafsson et al. 2002)	elastase, IL-1β, Lactoferrin	*Up-regulated:* elastase, Lactoferrin *no changes:* IL-1β	17
(Paknejad, Emtiaz et al. 2006)	AST, ALP	*Up-regulated:* ALP, AST	12 pt with 17 implants

(Arakawa, Uehara et al. 2012)	MMP-1, MMP-8, MMP-13	*Up-regulated:* MMP-8 *no changes:* MMP-1-13	4
(Basegmez, Yalcin et al. 2012)	MMP-8, PGE2	MMP-8 might be early signal of peri-implant inflammation	72 implants in 28 patients
(Xu, Yu et al. 2008)	MMP-8, collagenase-2	*Up-regulated:* collagenase-2 971%, MMP-8 highest activation	5
(Kivela-Rajamaki, Maisi et al. 2003)	MMP-8, MMP-7	*Up-regulated:* MMP-7, MMP-8; correlated significantly with each other	13 total, PI not mentioned
(Tumer, Aksoy et al. 2008)	ICTP, OC	*Up-regulated:* OC, ICTP (ICTP not statistically significant)	15
(Yaghobee, Khorsand et al. 2014)	IL-1β, IL-6	*Up-regulated:* IL-1β, IL-6	16
(Recker, Avila-Ortiz et al. 2015)	IL-1α, IL-1β,IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, TNF-α, CRP, OPG, Leptin, Adiponectin	*u-pregulated:* IL-17A, TNF-α *no changes:* IL-1α, IL-1β,IL-4, IL-6, IL-8, IL-10, IL-12, CRP, OPG, Leptin, Adiponectin	73, PI not mentioned
(Fonseca, Moraes Junior et al. 2014)	GM-CSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IFN-γ, TNF-α	*Up-regulated:* IL-1β ,IL-8, IL-12 *no changes:* GM-CSF, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IFN-γ, TNF-α	10
(Xie, Deng et al. 2011)	HMGB1, HMGN2, IL-1β, IL-6, IL-8, TNF-α	*Up-regulated:* HMGB1, HMGN2, IL-1β, IL-6, IL-8	15
(Rakic, Struillou et al. 2014)	RANK, sRANKL, OPG, CatK, sclerostin	*Up-regulated:* RANK, sRANKL, OPG, sclerostin	52
(Casado, Canullo et al. 2013)	IL-1β, IL-10	*Up-regulated:* IL-1β, *down-regulated:* IL-10	10

Table 1.3. Peri-implant Sulcular Fluid Molecular Markers

Examination of specific mediators of tissue destruction has been conducted at the histological level. While one paper has identified potentially significant changes in the epithelial compartment of the peri-implant mucosa (Becker, Beck-Broichsitter et al. 2014) and another

implicated specific cellular constituents by immunohistochemistry (Gualini and Berglundh 2003) the majority of papers focused on common mediators of inflammation and often those previously involved in periodontal disease (Yucel-Lindberg and Bage 2013).

Attemps to link peri-implantitis to specific genes has predominantly focused on general markers of inflammation (TNF-α, IL-1a and IL-1b) and inflammatory mediators (e.g. Interleukins). It is beyond the intent of this review to explore the knowledge regarding the fundamentals of tissue inflammation, however, IL1a, IL1b and TNF-α are known as central soluble mediators of PAMP mediated inflammation and have defined roles in peri-implantitis (Lindberg and Bage, 2013). Each of these are known to be produced by cells identified in the connective tissue inflammatory cell infiltrate that expands in response to plaque accumulation at implants.

The results for PISF measures of the inflammatory cytokines TNF-α and IL-1 are not consistent. Hall et al. (Hall, Britse et al. 2011) observed no difference in TNF-α or other cytokines in PISF from healthy and PI sites. Wohlfart et al (Wohlfahrt, Aass et al. 2014) noted TNF-α levels were reduced following treatment of PI sites and other studies have observe increases in TNF-α in PISF associated with increased inflammation at implants. While studies of biomarkers in peri-implantits (Hultin, Gustafsson et al. 2002) and a cross sectional comparison of teeth vs. implants (Recker, Avila-Ortiz et al. 2015) found no up-regulation of IL-1β in peri-implantits, several other investigations consistently report IL-1β levels are increased in PISF of peri-implantitis (Xie, Deng et al. 2011, Casado, Canullo et al. 2013, Fonseca, Moraes Junior et al. 2014, Yaghobee, Khorsand et al. 2014). Interestingly, the cellular response to biofilm may be modulated in disease as demonstrated by increases when tissue derived fibroblasts were challenged by P. gingivalis (Irshad, Scheres et al. 2013). The broadest interpretation of these studies is that the inflammatory mediators TNF-α, IL-1a and IL-1β are elevated in peri-implantitis. Thus, biofilm-mediated host responses at implants involve fundamental up-regulation of inflammation.

In this review, IL-17 was prominently observed among studies involving PISF. Three studies reported that IL-17 levels are increased in PISF associated with peri-implantitis (Severino, Napimoga et al. 2011, Darabi, Kadkhoda et al. 2013, Recker, Avila-Ortiz et al. 2015). IL-17 suggests an important regulatory role for TH17 t-cells. Secretion of IL-17 by these cells stimulates the production of TNF-α, IL-1β, IL-6 and IL-1β. IL-17 is speculated to play a role in bone resorption of rheumatoid arthritis as well as periodontitis (Kramer and Gaffen 2007).

Interleukin 6 is a pro-inflammatory cytokine tightly linked to osteoclastogenesis and involved in the pathogenesis of periodontitis. IL-6 is produced by numerous cell types in response to inflammatory mediators and is found in GCF and tissues of periodontitis. Three studies failed to demonstrate differences in the levels of IL-6 in PISF at healthy versus peri-implantitis sites (Severino, Napimoga et al. 2011, Fonseca, Moraes Junior et al. 2014, Recker, Avila-Ortiz et al. 2015). However, in other comparisons, IL-6 levels were increased at peri-implantitis sites in a cross-sectional studies that comparing PISF to GCF (Xie, Deng et al. 2011, Yaghobee, Khorsand et al. 2014) and in fibroblasts from peri-implant tissues compared to healthy sites (Irshad, Scheres et al. 2013). Further study is needed to determine if IL-6 levels are relatively elevated with inflammation at implant sites compared to tooth sites.

The matrix metalloproteinases in PISF were consistently elevated in peri-implantitis (Table 1.3.). MMPs are essential enzymes mediating inflammatory tissue destruction. Their tissue specificity and tightly regulated gene expression implies there may be pathology-specific roles important in peri-implantitis. PISF MMP-8 levels, for example, may have a role in the early diagnosis of peri-implantitis (Basegmez, Yalcin et al. 2012).

PISF reveals several molecular mediators of osteoclastogenesis. In particular, OPG, sRANKL, RANK and RANKL have been measured and all are reported to be increased in PISF from peri-

implantitis sites versus healthy sites. OPG levels were not consistently up regulated with peri-implantitis, perhaps reflecting both the variability in disease severity as well as the limited number of subjects enrolled in these early studies. Similarly, variable outcomes were reported for sRANKL. ICTP levels in PISF were elevated at peri-implantitis sites and reflect high collagen turnover. Although general molecular markers of tissue turnover/destruction are observed in PISF, these studies of PISF did not demonstrate a consistent ability to identify specific molecular markers of increased osteoclast activity associated with peri-implantitis. Although the present data presents a general picture of increased inflammation leading to osteoclastogenesis (mediators of osteoclastogenesis (OPG, IL-6 and RANKL, or mediators of tissue destruction (MMPs)), further study is required to identify PISF markers of increased osteoclast activity associated with this disease.

Peri-implant Immunohistology

Histological assessment of the peri-implant lesion has been provided by many investigators. The lesions are characterized predominantly by neutrophils, macrophages, T- and B-cells. Nevertheless, compared to periodontitis, peri-implantitis is marked by a more extensive inflammatory infiltrate and innate immune response, a greater severity of tissue destruction and a faster progression rate (Belibasakis 2014). Different than in periodontitis, the lesion extends to the bony crest and it progresses spontaneous and continuously (Periodontology 2013).

To date there have been 9 reports, which have utilized human histology, immunohistology, tissue mRNA expression to explore the molecular pathogenesis of peri-implantitis (Table 1.4.). These studies affirm the inflammatory characteristics of the disease. Silva et al. (Silva, Felix et al. 2014) delineated the fundamental characteristics of inflammation around dental implants. The role of T cells was explored by examination of IL-22 and IL-23 expression (Luo, Wang et al. 2013) and was aligned with the observations made regarding IL-17 both in histology (de Araujo, Filho et al. 2014) and from IL-17 PISF levels (above). The specific cellular constituents of the inflammatory

cellular infiltrate was explored by Gualini and Berglundh (Gualini and Berglundh 2003) using CD3, CD4, CD8, CD19, and elastase antibodies. They revealed the up-regulation of both CD19 and elastase, indicating the participation of B cells and PMNs in an established process of connective tissue degeneration. They further stated that the relatively high numbers of B cells represented an aggressive form of peri-implant disease.

Becker et al (Becker, Beck-Broichsitter et al. 2014) using a molecular assessment of tissue transcriptomes to compare healthy and peri-implant tissues with tissues from periodontitis lesions observed differences in several genes specific to the cornified epithelium. It was suggested mediators for apoptosis, cell death, collagen destruction and defense against viruses, reflecting the up-regulation of these markers. In addition, the analysis suggested that transcripts associated with the innate immune response were predominant in peri-implantitis. On the other hand, the adaptive immune response was predominant in periodontitis.

Publications	**Investigated**	**Findings**	**PI patients**
(Luo, Wang et al. 2013)	Il-22, IL-22R, IL-23	*Up-regulated:* IL-22/22R/23	12
(Verardi, Quaranta et al. 2011)	cC1qR, gC1qR, IL-6, IL-8, MCP-1, VEGF-1, TGFβ-1	*Up-regulated:* 4x MCP-1, 7x VEGF-1, 12X TGFβ-1, 2X IL-6/8 cC1qR genotype assoc. with PI	10
(de Araujo, Filho et al. 2014)	TGF-β, IL-17, CD31, MCC, MCT, IL-13	*Up-regulated:* TGF-β, IL-17, CD31, MCC, MCT *downregulated:* IL-13	9
(Konermann, Gotz et al. 2014)	TRAP, RANK, RANKL, OPG, CD3, TNF-	*Up-regulated:* first 12 month RANK compared to later, RANKL, TNF-α *Up-regulated:* 2.7x CD3 (2x in smokers vs. Non-smokers) neg. correlation bt. occurence of RANK and RANKL	21
(Becker, Beck-Broichsitter et al. 2014)	SRGN, PPP2R2B, ABCC9, COLEC12	*Up-regulated:* SRGN, PPP2R2B, ABCC9, COLEC12	7

Gualini, 2003 #132}	CD3, CD4, CD8, CD19, elastase	*Up-regulated:* CD19, elastase	6
Borsani, 2005 #131}	collagen I, III, IV, V, tenascin, MMP-1, MMP-3, MMP-8, MMP-13, TIMP-1	extracellular matrix showed alterations oft collagen IV, tenascin and MMP-13 no differences with collagen I-III-V, MMP-1-3-8, TIMP-1	5
(Konttinen, Lappalainen et al. 2006)	TNF-α, IL-1α, IL-6, PDGFA, TGF-α	*Up-regulated:* TNF-α, IL-1α, IL-6	10
Histology			
(Silva, Felix et al. 2014)	histopathologic changes, edema and nuclear alterations	females have predisposition for severe edema and inflammation assoc. of edema and inflammation with nuclear changes	10

Table 1.4. Peri-implant Tissue Biomarkers

Genetic markers

Twenty-two papers were identified that evaluated markers on the genetic level associated with peri-implantitis as presented in Table 1.5. Most of the authors investigated polymorphisms that could be associated with peri-implantitis. General inflammatory mediators were commonly investigated. Five papers investigated IL-1 and IL-6 polymorphisms associations with peri-implantitis (Gruica, Wang et al. 2004, Laine, Leonhardt et al. 2006, Hamdy and Ebrahem 2011, Melo, Lopes et al. 2012, Casado, Villas-Boas et al. 2013). All these publications found a positive IL-1 or IL-6 polymorphism association with peri-implantitis, except Melo et al. (Melo, Lopes et al. 2012) who found no association. Two authors looked at the TNF-α polymorphism and found an association with peri-implantitis (Cury, Horewicz et al. 2009, Rakic, Petkovic-Curcin et al. 2014). Up-reguation of major inflammatory mediators was noted in several reports (Kuula, Salo et al. 2008, Duarte, de Mendonca et al. 2009, Luo, Xie et al. 2011, Wu, Cao et al. 2013, Rakic, Petkovic-Curcin et al. 2014, Schminke, Vom Orde et al. 2015). These authors found the same markers (TNF-α, IL-1, IL-6, IL-8, IL-10, RANKL, OPG) up-regulated as the authors in the previous section of PISF and tissue samples.

Schminke et al. (Schminke, Vom Orde et al. 2015) found markers for remodeling and tissue differentiation down-regulated (see Table 1.5).

Publications	Investigated	Findings	PI patients	Probes
(Kadkhodazadeh, Tabari et al. 2012)	OPG	SNP in OPG gene assoc with PI	40	blood
(Kadkhodazadeh, Jafari et al. 2013)	BRAF	polymorphism not assoc. With PI	38	blood
(Casado, Villas-Boas et al. 2013)	IL-6	polymorphism assoc. With PI	20	buccal cells
(Cury, Horewicz et al. 2009)	TNF-α	polymorphism assoc. With PI	20	mouthwash
(Luo, Xie et al. 2011)	HMGB1, HMGN2, IL-1β, IL-6, TNF-α, IL-8	all increased in PI	25	tissue, PICF, plaque
(Kuula, Salo et al. 2008)	MMP-25, MMP-26, HBD1, HBD2	MMPs increased in PI, HBD1 increased compared to HBD2 in PI	11	tissue
(Kadkhodazadeh, Sodeif et al. 2012)	IKKI	sig. Difference between PI and CP in rs1539243, not in rs12728136	38	blood
(Kadkhodazadeh, Amid et al. 2012)	TANK	polymorphism not assoc. With PI	40	blood
(Ebadian, Kadkhodazadeh et al. 2014)	Hp-Hb complex	polymorphism not assoc. With PI	43	blood
(Ebadian, Kadkhodazadeh et al. 2013)	HCN2	polymorphism not assoc. With PI	37	blood
(Kadkhodazadeh, Ebadian et al. 2013)	RANKL	polymorphism assoc. With PI	40	blood
(Kadkhodazadeh, Ebadian et al. 2013)	IL-17	polymorphism not assoc. With PI	37	blood
(Schminke, Vom Orde et al. 2015)	MMP-7, MMP-8, BMP-2, BMP-7, RUNX2, SPP1, BGLAP, COL9A1, FGF18, SPARC, IL-8	*upregulated*: MMP-7, MMP-8, IL-8, slightly BMP2; *no change* in SPARC *downregulated:* BMP-7, RUNX2, PPARγ, SPP1, BGLAP, COL9A1, FGF18	12	bone tissue

(Duarte, de Mendonca et al. 2009)	IL-12, TNF-α, IL-4, IL-10, RANKL, OPG	*upregulated*: IL-12, TNF-α, RANKL, IL-10 *upregulated in healthy*: IL-4, OPG	22	soft tissue
(Laine, Leonhardt et al. 2006)	IL-1RN	polymorphism assoc. With PI	71	mouthwash
(Kadkhodazadeh, Baghani et al. 2013)	IL-17	polymorphism assoc. With PI	38	blood
(Hamdy and Ebrahem 2011)	IL-1A, IL-1B	polymorphism assoc. With PI	25	oral mucosa
(Gruica, Wang et al. 2004)	IL-1	synergistic effect oft IL-1 polymorphism and smoking	34 "biological complications"	buccal cells
(Wu, Cao et al. 2013)	cFn	cFn upregulated	10	soft tissue
(Kadkhodazadeh, Jafari et al. 2013)	MiR146a/MiR499	polymorphism assoc. With PI	38	blood
(Rakic, Petkovic-Curcin et al. 2014)	CD14, TNF-α, RANKL, OPG	*upregulated:* RANKL, ratio RANKL/OPG CD14 and TNF-α polymorphism assoc. With PI	180	blood
(Melo, Lopes et al. 2012)	IL-1β, IL-6	IL-1β and IL-6 polymorphism not assoc. With PI	16	PICF, tissue

Table 1.5. Genetic Biomarkers

Kadkhodazadeh et al. found in blood-derived DNA a positive polymorphism association with peri-implantitis for OPG (Kadkhodazadeh, Tabari et al. 2012), RANKL (Kadkhodazadeh, Ebadian et al. 2013), MiR146a/MiR499 (Kadkhodazadeh, Jafari et al. 2013), which are microRNAs responsible for regulation of inflammation, and IKKI (Kadkhodazadeh, Sodeif et al. 2012), an inhibitor of NFκB kinase. The group found contradictory results for IL-17 polymorphism. In one study, the authors found an association with the pro-inflammatory cytokine IL-17 (Kadkhodazadeh, Baghani et al. 2013) and in a subsequent study they found none (Kadkhodazadeh, Ebadian et al. 2013). The same author found several markers that have no polymorphism association with peri-

implantitits. Among those are BRAF (Kadkhodazadeh, Jafari et al. 2013), a gene active in cell differentiation, and TANK (Kadkhodazadeh, Amid et al. 2012), which is associated with the activation of NFκB kinase.

In association with increased levels of inflammatory mediators, several investigations have reported related down-regulation of markers of bone formation and repair. For example, Schminke et al. (Schminke, Vom Orde et al. 2015) reported about the down-regulation of BMP7, RUNX2, PPARγ, SPP1, BGLAP, COL9A1 and FGF18. Other paper reports also observed up-regulation of molecular mediators of tissue destruction, e.g. IL-1, IL-6, Il-17, MMP-8, TNF-α, OPG and RANK/RANKL.

Many of the reports include data regarding interleukin levels in peri-implantitis. While heterogeneity in the findings makes singular conclusions difficult, the majority of findings support a pro-inflammatory disease progression associated with bone resorption (Fonseca, Moraes Junior et al. 2014). It is important to note that IL-17, a pro-inflammatory cytokine expressed by T-cells, was constantly found to be up-regulated (Severino, Napimoga et al. 2011, Darabi, Kadkhoda et al. 2013, de Araujo, Filho et al. 2014, Recker, Avila-Ortiz et al. 2015). Only one study (Luo, Wang et al. 2013) looked at IL-22 and IL-23 and found an up-regulation of both. IL-2, IL-4, IL-5, IL-7 and IL-13 are involved in B- and T-cell differentiation and address more complex immune regulation in an established chronic lesion. These authors found no changes in IL-1, IL-2, IL-4, IL-5 IL-6, IL-7 IL-8, IL-10 and IL-12 as suggested by others (Hultin, Gustafsson et al. 2002, Severino, Napimoga et al. 2011, Recker, Avila-Ortiz et al. 2015). More commonly however, authors have observed the up-regulation of IL-1, IL-6, IL-8, IL-10, IL-12 (Duarte, de Mendonca et al. 2009, Verardi, Quaranta et al. 2011, Xie, Deng et al. 2011, Casado, Canullo et al. 2013, Casado, Villas-Boas et al. 2013, Fonseca, Moraes Junior et al. 2014, Yaghobee, Khorsand et al. 2014). Contradictory findings include the ones about IL-10, a

known anti-inflammatory mediator. Casado et al. (Casado, Canullo et al. 2013) found IL-10 down-regulated, whereas in another study Casado et al. (Casado, Villas-Boas et al. 2013) and Duarte et al. (Duarte, de Mendonca et al. 2009) found it up-regulated.

Different matrix metalloproteinases were found to be up-regulated, among them MMP-7, MMP-8, and MMP-9 (Kivela-Rajamaki, Maisi et al. 2003, Xu, Yu et al. 2008, Arakawa, Uehara et al. 2012, Basegmez, Yalcin et al. 2012, Ozcakir-Tomruk, Chiquet et al. 2012, Irshad, Scheres et al. 2013, Ramseier, Eick et al. 2015, Schminke, Vom Orde et al. 2015). MMP-1, MMP-7, MMP-8, MMP-13, MMP-25 and MMP-26 are known to play an active role in tissue remodeling and pro-inflammation. MMP-2-, MMP-3 and MMP-9 however, are involved in tissue and bone remodeling. Controversially, Borsani et al. (Borsani, Salgarello et al. 2005) found no changes in MMP-8. No changes were found for MMP-1, MMP-3 and MMP-13 (Borsani, Salgarello et al. 2005, Arakawa, Uehara et al. 2012).

This review failed to identify reasons for the variability in some of the relative levels of inflammatory mediators and cytokines. The studies present considerable methodological heterogeneity and comparison of data among studies may suffer further from the use of differing definitions of disease. The temporal progression of disease status may influence the cellular population and resulting molecular environment. These data, however, permit a general conclusion that affirms the concept that peri-implantitis is a chronic inflammatory process established within the peri-implant connective tissues that influences the superimposed epithelium and adjacent bony contact with the implant.

Therapy

To date, here has no been no evidence of an ideal modality of peri-implant therapy. It can be summarized that currently prevention is the most important approach, starting from planning to the implant placement and regular professional maintenance (Heitz-Mayfield and Mombelli 2014, Padial-Molina, Suarez et al. 2014, Salvi and Zitzmann 2014, Smeets, Henningsen et al. 2014). The

treatment of peri-implant mucositis lesions using mechanical therapy is predictable (Renvert, Polyzois et al. 2013). Salvi and Zitzmann (Salvi and Zitzmann 2014) found that patient adherent to recommended individual supportive periodontal therapy yielded beneficial effects with respect to the occurrence of biologic complication and implant loss. As quality of outcome measurements gingival and bleeding (e.g. PPD, BoP, CAL, REC) indexes were used at baseline and endpoint of therapy. The current literature on treatment shows a great heterogeneity among almost every parameter used and therefore has limited quality (Graziani, Figuero et al. 2012, Heitz-Mayfield and Mombelli 2014). However, it is not said that currently used interventions are not effective (Esposito, Grusovin et al. 2012). Non-surgical therapy approaches include a combination of mechanical debridement with curettes and air polishing systems and adjuvant short-term antiseptic rinses and local or systemic antibiotics. Surgical treatment options include resective and regenerative approaches with full-thickness periosteal flap for better access (Esposito, Grusovin et al. 2012, Renvert, Polyzois et al. 2013, Heitz-Mayfield and Mombelli 2014, Smeets, Henningsen et al. 2014). Thus far, surgical procedures achieve more probing depth reduction and gain in clinical attachment level when compared to non-surgical approaches (Esposito, Grusovin et al. 2012, Renvert, Polyzois et al. 2013, Heitz-Mayfield and Mombelli 2014). According to Smeets et al. (Smeets, Henningsen et al. 2014)," an "ideal peri-implantitis therapy" is a sum of approaches leading to an individual therapy regimen concerning multifactorial etiology, treatment options and study results." Patients with a previous history of peri-implantitis or periodontitis were at a higher risk of reinfection after treatment compared to patients without that history (Renvert, Polyzois et al. 2013, Salvi and Zitzmann 2014). Positive treatment results can be maintained over a period of 12 months (Heitz-Mayfield and Mombelli 2014) up to 3-5 years (Renvert, Polyzois et al. 2013).

CHAPTER 2: A WITHIN SUBJECT MOLECULAR COMPARISON OF SOFT TISSUES AT IMPLANTS WITH AND WITHOUT PERI-IMPLANTITIS

INTRODUCTION

Dental implants are subject to mechanical and biological challenges during a lifetime of use (Pjetursson, Asgeirsson et al. 2014). Biofilm-mediated challenges to dental implants include peri-implant mucositis and peri-implantitis. Experimental biofilm accumulation in humans results in an inflammatory response within the peri-implant mucosa (Zitzmann, Berglundh et al. 2001). It is widely accepted that host responses to chronic biofilm exposure at implants interface and the body may lead to peri-implant alveolar bone loss and a related diagnosis of peri-implantitis. An alternative hypothesis regarding the molecular basis of peri-implant alveolar bone loss is that it reflects an alternative foreign body reaction representing chronic inflammation. Mobelli et al. (Mombelli, Muller et al. 2012) suggest that the prevalence of peri-implantitis affects approximately 10% of all implants and 20% of patients within 10 years after implant placement. Given the wide and growing use of implants for tooth replacement, understanding the molecular pathogenesis is of present and future importance.

Peri-implantitis is an inflammatory process within tissues surrounding dental implants, that involves both soft tissue inflammation and progressive loss of supporting bone beyond biological bone remodeling. Peri-implantitis has been characterized by peri-implant pocket > 4 mm, bleeding or suppuration on probing, and radiographic evident saucer-shaped bone destruction around the

implant (Belibasakis 2014). The pathophysiology of peri-implantitis is presently suggested to involve the innate immune system response to a biofilm containing gram-negative, motile, and anaerobic species commonly found in periodontitis as well as microorganisms unique to to peri-implantitis including S*. aureus, S epidermidis, E. aerogenes, E cloace, E. coli, H. pylori, P. micra*, *Pseudomonas* and *Candida* spp (Belibasakis 2014). The host response to this implant specific biofilm may induce a pathophysiology that differs from periodontitis. Additionally, an alternative hypothesis regarding the molecular basis of peri-implant bone loss is that it reflects a foreign body reaction to the implant in which macrophage responses culminate in osteoclastogenesis and bone loss (Trindade, Albrektsson et al. 2014).

The peri-implant lesion has been described histologically and compared to the periodontal lesion. Some authors reported that the biofilm directed innate immune response progresses faster and results in a more extensive and severe tissue destruction at implants than at teeth (Smeets, Henningsen et al. 2014, Belibasakis, Charalampakis et al. 2015). The absence of collagen fiber insertion into the implant / abutment and the absence of a periodontal vascular plexus are two anatomic differences between teeth and implants that may influence the extended inflammatory response. The inflammatory infiltrate of peri-implantitis is poorly compartmentalized. The sulcular epithelium has been described as ulcerated, and the inflammatory infiltrate is rich plasma cells, macrophages and PMN cells compared to periodontitis (Carcuac and Berglundh 2014, Belibasakis, Charalampakis et al. 2015). The molecular process of the implant-related inflammatory process requires further characterization.

Efforts to understand the pathogenesis of peri-implantitis have included molecular assessments made by immunohistology, evaluation of peri-implant sulcular fluid (PISF), and by genetic screening. For example, gene polymorphism of general inflammatory mediators such as TNF-α, IL-1β, and IL-6 have been associated with peri-implantitis. Recent data suggests that there are differences in PISF and gingival crevicular fluid (GCF) that may be important to consider and

may imply a different pathogenesis for peri-implantitis and periodontitis (Recker, Avila-Ortiz et al. 2015). Measurement of the levels of proteins or mRNAs encoding proteins associated with osteoclastogenesis and tissue destruction have also painted a general picture of inflammation mediated pathophysiology in peri-implantitis.

While there have been several descriptive studies describing peri-implantitis at the molecular level, the majority of investigations have focused on particular facets of the innate immune response or at aspects of osteoclastogenesis. Becker et al. (Becker, Beck-Broichsitter et al. 2014) used a genome wide analytic approach to describing the peri-implantitis transcriptome and thereby offered additional insights into the pathogenesis of peri-implantitis that included changes in the epithelial components of the peri-implant mucosa. The aim of this preliminary investigation was to compare the transcriptomes of healthy implant versus peri-implantitis soft tissues.

MATERIALS AND METHODS

Participant Selection

A total of 31 participants were recruited under an IRB approved protocol (UNC-CH Office of Human Ethics Committee IRB# 11-1058) from the clinics of the University of North Carolina School of Dentistry. Twenty-one peri-implantitis and 10 healthy participants were enrolled based on specific inclusion and exclusion criteria. Subjects, aged 18 – 70, willing and able to follow study procedures and instructions, providing informed consent, in good general health, but otherwise present with peri-implantitis (BOP, probing pocket depth >6mm and bone loss of ≥2.5mm). Participants were excluded if they presented with other chronic disease with oral manifestations or exhibit gross oral pathology, being treated with antibiotics for any medical or dental condition within one month prior to the screening examination, chronic treatment (i.e., two weeks or more) with medications known to affect periodontal status (e.g., phenytoin, calcium antagonists, cyclosporin, coumadin, non-steroidal anti-inflammatory drugs, aspirin) within one month of the

screening examination, ongoing medications initiated less than three months prior to enrollment (i.e., medications for chronic medical conditions must be initiated at least three months prior to enrollment), smoking, and / or a diagnosis of diabetes. There was one continuance criterion: if two outpatient visits are used to complete study procedures, there must be no changes in the subject's medical status for that subject to continue in the protocol. Subjects not meeting these criteria were withdrawn.

Clinical Protocol

Consented participants completed the standard adult medical and dental health history questionnaire provided by the UNC SOD and HIPAA consent. A comprehensive oral examination and periapical radiographic assessment was made prior to intervention. Bleeding on Probing (BoP) and Probing Pocket Depth (PPD) measures were recorded. Subsequently, resective treatment of peri-implantitis tissues and experimental biopsy of healthy implant tissues within participants or clinically healthy gingival tissues of control participants yielded tissue samples that were placed into RNA preservative solution (RNA*later*, Ambion Inc., Austin, TX), and refrigerated for 24 hours. Following the 24 hours, the RNA preservative solution was carefully removed, and samples frozen and stored at -80°C until ready for gene profile analysis. Peri-implantitis patients were provided with post-operative prescription of amoxicillin 500 mg every 8 hours for 7 days and 20 Vicodin (5/500) PRN. Participants were given post-operative instructions and an appointment was made after 1 week for post-operatory assessment.

RNA Isolation and Gene Profile Data Analyses

Frozen tissues were morselized in liquid nitrogen and total RNA was isolated using the miRNeasy Micro Kit (Qiagen, Valencia, CA) according to manufacturer's specifications. Total RNA was assessed for quality and quantity using a bioanalyzer (Aligent, Santa Clara, CA) and nanodrop ND-1000 spectrophotometer (Nanodrop, Wilmington, DE), respectively. RNAs were hybridized to the Affymetrix Human Gene 2.1 ST Array (Affymetrix, Santa Clara, CA) following the manufacturer's recommended protocols and reagents.

Data analysis was performed using GeneSpring software v.12.6 Agilent Technologies, Santa Clara, CA). For genes that showed more than two-fold up- or down-regulation the T-test paired statistical analysis was applied to determine differentially expressed genes between the groups (Healthy versus Disease). A p-value of 0.05 was used as the threshold for statistical significance. Gene ontology and pathway analyses were performed using these gene lists.

RESULTS

Tissues from 21 participants with a diagnosis of peri-implantitis were obtained from both healthy and peri-implantitis implant sites. The majority of participants had a diagnosis of treated and stable chronic periodontitis and a dental history that included periodontitis (Table 2.1.).

There were 141 genes significantly up-regulated ($p<0.05$) and 91 genes significantly down regulated ($p<0.05$) in the within-subject comparison of the peri-implantitis affected implant tissues versus healthy implant tissues. Comparison of "control" tissue and healthy peri-implant mucosa revealed 14 up-regulated and 4 down-regulated genes (not shown). Four up-regulated genes in healthy peri-implant mucosa are implicated in oral mucosa formation and function and included GRN, SLPI, CST7, and CCL19. Among the top 50 up-regulated genes, 37 encode immunoglobulin genes. Others, including CD79a (5.59 fold), MZB1 (4.96 fold), and FAM46C (4.07 fold) are expressed by B cells (Table 2.2.1.). Down-regulated genes also revealed a concentration of functionally related genes. In particular, genes that contribute to the function and structure of keratinized oral

epithelium were represented here. Included were keratin 76 (-12.6 fold), keratin1 (-8.3 fold), keratin 10 (-6.7 fold), and keratin 3 (-4.0 fold). Repetin (-5.9 fold), loricrin (-5.2 fold), MUC15 (-3.6), and ALOX12B (-2.4 fold) genes involved in the protective function of epithelium were also among down-regulated genes. Genes encoding proteins that comprise the desmosome were also down regulated; included were desmoglein1 (-6.7 fold), desmocollin1 (-3.5 fold), desmocollin 3 (-2.9 fold, desmoplakin (-2.8 fold), plaktophilin1 (-2.8 fold) and desmocollin 2 (-2.7 fold). Further reductions of chemokines associated with chronic inflammatory responses included CXCL10 (-3.6 fold) CXCL14 (-2.9 fold) produced by monocytes and fibroblasts. To test whether the identified set of 141 up-regulated and 91 down regulated transcripts represents a unique molecular signature for peri-implantitis a principle component analysis was performed and revealed separation patients included in the study (Figure 2.).

Gene ontology analysis affirmed the down-regulation of processes associated with cell attachment and epidermal function or development (Table 2.3.1.). Notably, the GO terms skin development (4.83 10-7), epidermis development (1.4 x10-7), cell-cell junction (8.86x10-6), desmosome (4.04 x 10-10), cornified envelope ($p<1.48 \times 10-4$) and structural constituent of epidermis (8.1 x 10-6) were broadly represented. When reviewing the up-regulated gene responses, many immunoglobulin, B-cell, and Fc receptor linked ontogenies were identified and highly significant (Table 2.3.1.). Increased responses to stress ($p<6.64 \times 10-11$)), immune system responses ($p<1.9 \times 10-13$), the immune response ($p <3.5 \times 10-11$) and the defense response ($p<3.5$ x 10-10 were noted in the gene ontogenies prevalent in peri-implantitis vs. healthy implant tissues.

DISCUSSION

Peri-implantitis is mostly an inflammatory disease of biofilm etiology affecting the tissues adjacent to dental implants. It has been defined as a progressive and irreversible disease of implant-surrounding hard and soft tissues and is accompanied with bone resorption, decreased osseointegration, increased pocket formation and purulence (Smeets, Henningsen et al. 2014). The inflammatory lesion in peri-implantitis is a response to peri-implant microbiota that share characteristics with tooth adherent biofilm and the emergence of species found in periodontitis (Mombelli, van Oosten et al. 1987). However, a distinct peri-implant microbiome with contribution from implant surfaces and implant-abutment interfaces is now acknowledged (Belibasakis 2014).

The peri-implantitis lesion, examined histologically, is comprised of a connective tissue infiltrated with inflammatory cells and an ulcerated epithelium separating the connective tissue from the implant (Carcuac and Berglundh 2014). The infiltrate is widely distributed within the peri-implant connective tissue, and unlike the connective tissue infiltrate of periodontitis, this infiltrate is not segregated from underlying bone. The histological comparison of peri-implantitis versus periodontitis lesions also demonstrated that the peri-implantitis lesions were larger, associated with greater vascularization, and exhibited critical histopathologic differences that may underscore a dissimilar pathogenesis.

The innate immune responses in peri-implantitis likely involves a higher number of immune cells and associated inflammatory mediators and involves an expansive process that encroaches upon the junctional epithelium toward the bone (Berglundh, Zitzmann et al. 2011). The significantly up- and down- regulated genes identified by comparing healthy versus inflamed peri-implant tissues has highlighted two potentially key aspects of peri-implantitis that support these previous observations. One is an important role for the B cell / plasma cell response(s) and the other is the epithelial degeneration implied by down regulation of protective cornified epithelium-specific gene expression and desmosomal protein gene expression.

The importance of B cells in the pathogenesis of peri-implantitis was implied by immunohistochemistry previously. In the assessment of cell types, nearly two fold increases in CD138 positive (plasma) cells, a 50% reduction in CD20 (B cells) and nearly doubling of CD68 (macrophage) cells were observed in peri-implantitis versus periodontitis lesions. In an earlier report, Gualini and Berglundh (Gualini and Berglundh 2003) observed a large proportion of B cells in peri-implantitis lesions. B cells produce antibodies and function as an antigen-presenting cell. Further B cells release cytokines for signaling immune regulatory functions (Mauri and Bosma 2012). They function in the innate immune reaction by a humoral response and through pattern recognition receptors such as Toll-like receptors, which induce the production of interferons and other cytokines (Beutler 2004). The adaptive immune system also includes B-lymphocytes. The cytokines produced by B cells depends on the differentiation and activation condition. Therefore, B-cells require specific condition to produces cytokines. Among those cytokines are IL-4, IL-6, IFN-α, IFN-β and IFN-γ. These cytokines play a role in the development and life cycle of B cells (Vazquez, Catalan-Dibene et al. 2015).

The present molecular assessment of peri-implantitis versus healthy implant tissues also demonstrates the predominant up-regulated expression of immunoglobulin genes attributable to B cells and plasma cells (Table 2.2.1). In addition, other genes expressed by B cells or involved in their regulation or chemotaxis were also observed among the up-regulated gene list. The B cells' role in innate immunity include the production of antibodies and in adaptive immunity by differentiation to plasma B cells. The B cells also may play a role in regulatory functions, particularly in autoimmune and chronic inflammatory states (Rincon-Arevalo, Sanchez-Parra et al. 2015). The prominence of immunoglobulin gene expression and related B cells role in peri-implantitis may suggest the potential targeting of B cells in the treatment of this chronic inflammatory disease. For example, rituximab depletion of B-cells has been repurposed for treatment of rheumatoid arthritis with some success (Brown and Isaacs 2015). A basis for this may be the role of B cell in modulating

autoimmunity through an INF-γ dependent control of T cell function in inflammation (Olalekan, Cao et al. 2015). Although significant up-regulation of genes specific to the PMN, T cells and monocytes were not observed here, the contributions of these cells to the reactive inflammatory connective tissue infiltrate is widely acknowledged (Carcuac and Berglundh 2014, Smeets, Henningsen et al. 2014).

The spectrum of significantly down-regulated genes in this comparative study suggests that the epithelial attachment of peri-implant mucosa is altered in peri-implantitis. The soft tissue attachment to transmucosal dental implant components shares general morphological similarities with the attachment to teeth; a biologic width comprised of both an epithelial attachment and a connective tissue contact are formed at the implant/abutment. However, the collagen fibers of the connective tissue at implants are arranged parallel to - without insertion into - the implant surface (Heitz-Mayfield and Lang 2010). This imposes less of a barrier to bacterial invasion of the connective tissue. Because there is no periodontal ligament to provide proximal vascularity in this transcortical region, there may be further impairment of local immune cell function in response to implant-related inflammatory stimuli. These structural differences may challenge peri-implant health under inflammatory conditions. Here, peri-implantitis related reductions in genes related to the structure and function of the protective sulcular epithelium suggests further impairment in disease.

The peri-implant soft tissue interface is similar to the natural tooth tissue interface and consists of an oral epithelium, a sulcular epithelium, and a junctional epithelium. In the rat model, the peri-implant sulcular epithelium possesses a keratinized stratum corneum. The peri-implant epithelium appears non-keratinized and consisted of several layers of flattened cells. The apical junctional epithelium displays wide intercellular spaces and only a few desmosomes and therefore the epithelium is very permeable. Ikeda et al. (Ikeda, Yamaza et al. 2000) suggested that the direct attachment by hemi-desmosomes exist only within the basal region of the peri-implant epithelium.

The junctional epithelium functions to separate the oral cavity, biofilm colonized surfaces (tooth or implants) from underlying connective tissues. The structural and functional protein components of the junctional epithelium can be overcome by microbiological challenges that lead to damage of the epithelium and subsequent inflammatory lesion development in the connective tissues.

In the oral epithelium, desmosomes and hemi-desmosomes function to adhere the keratinocytes to one another and to the basement membrane. They create a connection of the keratin cytoskeleton and the cell surface. The stratified oral epithelium presents a cornified cell envelope, which functions as an epithelial barrier to the tissue surface. The desmosomes are composed of desmosomal cadherins, the desmogleins and desmocollins. Desmosomal connection with the cytoplasm involves plaktoglobin, desmoplakin, plakophilin, envoplakin and periplakin (Presland and Jurevic 2002). Key desmosomal protein encoding genes were down-regulated in this comparison of healthy and peri-implantitis tissues (Table 2.2.2). DSG1, DSC1, DSC2, DSC3, DSP and PKP1 were all reduced greater than 2.7 fold. The inhibition of desmosomal attachment between cells of the protective epithelium or hemi-desmosomal attachments to the basement membrane or putatively to the implant surface may be impaired in peri-implantitis. The ulceration or absence of an epithelial separation of the inflamed connective tissue from the implant that is observed histologically and aligned with the clinical features of peri-implantitis may represent a tissue that is unable to support the health-related attachment of an intact and functional epithelium to the implant/abutment surface.

The keratinized epithelial components of the protective epithelium are altered in peri-implantitis. Here, for example, key components of the cornified epithelium were diminished; RPTN, LOR, ALOX12B, Muc15 expression were reduced. Secreted proteins including SLURP1, SPINK7 (an serine protease inhibitor that protects epithelial barrier degeneration and loss of microbial containment (Wapenaar, Monsuur et al. 2007). EXPH5, CXCL10, and CXCL14 are also implicated in

epithelial barrier formation and function and were significantly reduced in peri-implantitis versus healthy peri-implant tissues.

Keratin expression within the implant junctional epithelium differs from that of the natural tooth. However, keratin 1 is expressed in all cells of the junctional epithelium (Fujiseki, Matsuzaka et al. 2003). In the present molecular evaluation of peri-implantitis versus healthy implant tissues revealed marked reductions in the expression of keratin1, keratin 3, keratin 10 and keratin 76 and suggests a reduction of the protective keratinized epithelium of the peri-implant sulcus. The molecular program of epithelial differentiation and the function of the protective sulcular epithelium may be impaired in peri-implantitis.

It is widely reported that peri-implantitis is associated with proteolysis and bone resorption (Borsani, Salgarello et al. 2005). Two matrix metallopretinases were up-regulated in peri-implantitis tissues. MMP1 is implicated in chronic inflammatory disorders such as arthritis and degrades type I, II and III collagens. MMP3 (stromelysin 1) degrades collagens II, III, IV, IX and X as well as ECM proteins. MMP1 is expressed by basal keratinocytes and MMP3 is expressed by keratinocytes and are found in conditions of chronic inflammation. Notably, both MMP1 and MMP3 levels are elevated in GCF of patients with periodontitis (Soell, Elkaim et al. 2002). Further MMP1 and MMP3 were up-regulated in refractory periodontitis (Kim, Ramoni et al. 2006). Immuno-localization of MMP 1 and 3 to the lamina propria of the peri implant soft tissue revealed modest up-regulation. The current data indicate that collagen degenerating enzyme expression in peri-implantitis targets collagens of bone and of the basement membrane and the potential source of these MMPs may include the affected epithelium.

Other enzymes involved in connective tissue degradation were not significantly reported as up-regulated by this genome wide analysis. This may reflect the method of harvesting only affecting soft tissues by therapeutic removal tissues without en bloc resection of bone and connective tissue. Clearly, others have demonstrated that the process of inflammation in peri-implantitis results in

connective tissue degradation by ECM proteases and osteoclastic enzymes (Schminke, Vom Orde et al. 2015). Slotte et al. (Slotte, Lenneras et al. 2012) identified up-regulation of Cathepsin K, while MMP8 and MMP9 levels in PISF were also increased in peri-implantitis. Irshad et al. (Irshad, Scheres et al. 2013) demonstrated that *P. gingivalis* challenge resulted in increased expression of MMP1,2 and 8. Peri-implantitis is associated with degradation of the connective tissue matrix of the peri-implant mucosa. Other histological studies of peri-implantitis have not focused on this molecular aspect of the disease process.

CONCLUSION

The molecular comparison of tissues from healthy and peri-implantitis affected implants within subjects revealed significant changes in gene expression. Besides up-regulation of immunoglobulin genes, >2-fold up-regulation of B cell functional genes was observed. Marked down-regulation of genes encoding desmosomal proteins and functional or structural components of keratinized epithelium suggests that the pathogenesis of peri-implantitis involves dimished epithelial protection in a chronic inflammatory state. Further investigation of both the role of B cell-mediated innate and adaptive immune responses within peri-implant tissues and of the junctional epithelial condition in peri-implantitis is required.

APPENDIX: TABLES AND FIGURES FOR CHAPTER 2

Number of Subjects		31
Age (years; mean)	Total	63.0
	Male	62.2
	Female	63.7
Sex	Male	14 (45.2)
	Female	17 (54.8%)
Implant Brand	Astratech	19
	Calcitek	1
	Straumann	9
	Zimmer	1
	Nobel	1
Time of loading	Not loaded	5
	Mean (Years)	7.1
Mobility	Yes	2 (6.5%)
	No	29 (93.5%)
BoP	Yes	100%
History of Periodontitis	edentulous/ unknown	1 (3.2%)
	gingivits on a reduced periodontum	8 (25.8%)
	generalized slight chronic periodontitis	2(6.5%)
	localized moderate chronic periodontitis	5 (16.1%)
	localized severe chronic periodontitis	7 (22.6%)
	generalized moderate chronic periodontitis	5 (16.1%)
	generalized severe chronic periodontitis	3 (9.7%)

Table 2.1. Demographics of study participants, implant data and periodontal status.

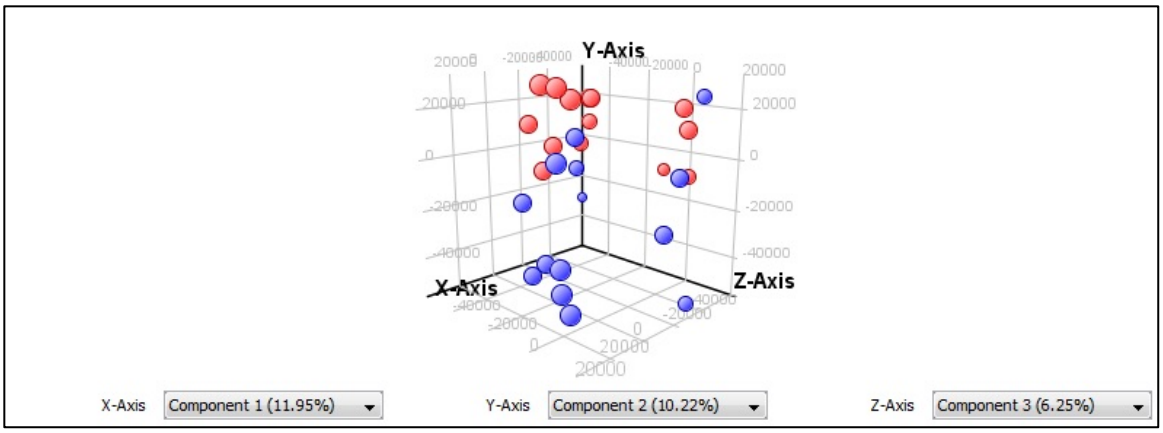

Figure 1: PCA analysis: Three-dimensional representation of principal component analysis for healthy implant tissue (blue) versus peri-implantitis tissue (red) gene expression.

Transcripts Cluster Id	FC ([Disease] vs [Control])	gene description	gene symbol
16789782	6.9628334		
16797433	6.9226146	immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavylocus|immunoglobulinheavyvariable6-1	IGHG1|IGHV6-1
16927756	6.692652	immunoglobulinlambdaconstant1(Mcgmarker)|cytoskeletonassociatedprotein2|immunoglobulinlambdavariable1-40	IGLC1|CKAP2|IGLV1-40
16797403	6.324567	immunoglobulinheavyconstantmu|immunoglobulinheavyconstantgamma1(G1mmarker)|srckinaseassociatedphosphoprotein2	IGHM|IGHG1|SKAP2
16927790	5.852411	immunoglobulinlambdavariable3-25|immunoglobulinlambdaconstant1(Mcgmarker)	IGLV3-25|IGLC1
16927806	5.775561	immunoglobulinlambdajoining3|cytoskeletonassociatedprotein2|immunoglobulinlambdavariable3-19	IGLJ3|CKAP2|IGLV3-19
16862604	5.5895634	CD79amolecule,immunoglobulin-associatedalpha	CD79A
16797520	5.341873	immunoglobulinheavyvariable3-33|immunoglobulinheavyvariable4-34|srckinaseassociatedphosphoprotein2|immunoglobulinheavyconstantgamma1(G1mmarker)	IGHV3-33|IGHV4-34|SKAP2|IGHG1
16797417	5.1481485	immunoglobulinheavyvariable4-31|immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavyconstantalpha1|immunoglobulinheavylocus|immunoglobulinheavyjoining2	IGHV4-31|IGHG1|IGHA1|IGHJ2
17000591	4.9638314	marginalzoneBandB1cell-specificprotein|NULL	MZB1
16797498	4.946259	immunoglobulinheavyconstantgamma3(G3mmarker)|immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavyvariable1-24	IGHG3|IGHG1|IGHV1-24
16797512	4.9226704	immunoglobulinheavyvariable4-31|immunoglobulinheavyconstantgamma1(G1mmarker)|srckinaseassociatedphosphoprotein2	IGHV4-31|IGHG1|SKAP2
16797504	4.913851	immunoglobulinheavyconstantgamma1(G1mmarker)	IGHG1
16797494	4.7196975	immunoglobulinheavyvariable3-20|immunoglobulinheavyvariable3-23|immunoglobulinkappalocus	IGHV3-20|IGHV3-23|IGK
16900144	4.653588	immunoglobulinkappavariable6-21(non-functional)	IGKV6-21
16797587	4.6217613	immunoglobulinheavyconstantalpha1|immunoglobulinheavyvariable3-66	IGHA1|IGHV3-66
16927734	4.601534	immunoglobulinlambdavariable9-49|immunoglobulinlambdaconstant1(Mcgmarker)	IGLV9-49|IGLC1
1679748	4.594546	immunoglobulinheavyvariable1-18	IGHV1-18

7			
16797440	4.5865674	ADAMmetallopeptidasedomain6,pseudogene|immunoglobulinheavyvariable1-2	ADAM6|IGHV1-2
16927702	4.4642377	immunoglobulinlambdavariable6-57	IGLV6-57
16927827	4.4596972	immunoglobulinlambdavariable3-9(gene/pseudogene)	IGLV3-9
16722562	4.3917747	serumamyloidA1	SAA1
16900174	4.316749	immunoglobulinkappavariable1-39(gene/pseudogene)	IGKV1-39
16927840	4.2827215	immunoglobulinlambdavariable3-1|immunoglobulinlambdaconstant1(Mcgmarker)|immunoglobulinlambdavariablecluster	IGLV3-1|IGLC1|IGLV@
16797555	4.26328	immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavyconstantmu|immunoglobulinheavyconstantalpha1|immunoglobulinheavyvariable3-48	IGHG1|IGHM|IGHA1|IGHV3-48
16932960	4.26024		
16797603	4.128261	immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavyvariable3-73	IGHG1|IGHV3-73
16797563	4.098592	immunoglobulinheavyconstantgamma1(G1mmarker)|immunoglobulinheavyvariable5-51	IGHG1|IGHV5-51
16797516	4.0843596	immunoglobulinheavyconstantmu|enhancerofpolycombhomolog1(Drosophila)|immunoglobulinheavyvariable3-33	IGHM|EPC1|IGHV3-33
16927824	4.079194	immunoglobulinlambdaconstant1(Mcgmarker)|immunoglobulinlambdavariable3-10	IGLC1|IGLV3-10

Table 2.2.1.Up-regulated genes: List of 30 highest magnitude of up-regulated genes; peri-implantitis tissue versus healthy implant tissue (n=21), there was a total of 141 up-regulated genes; table generated using GeneSpring software 12.6.

Transcripts Cluster Id	FC ([Disease] vs [Control])	gene description	gene symbol
16765056	-12.63262	keratin76	KRT76
16765029	-8.246868	keratin1	KRT1
16851708	-6.650534	desmoglein1	DSG1
16844477	-6.54742	keratin10	KRT10
16693295	-5.895377	repetin	RPTN
16671133	5.7351203	loricrin	LOR
17081945	5.2477026	secretedLY6/PLAURdomaincontaining1	SLURP1
16819099	-4.697627	calpain,smallsubunit2	CAPNS2
16990787	4.5691557	serinepeptidaseinhibitor,Kazaltype7(putative)	SPINK7
16765068	4.5518975	keratin3	KRT3
16997143	4.0056643		
17112498	3.9007287	prematureovarianfailure,1B	POF1B
16854529	-3.780263		
16706727	3.7518113	chromosome10openreadingframe99	C10orf99
17036722	-3.614411		
17032004	3.6135583		
17042000	3.6128073		
17017363	-3.610455	lymphocyteantigen6complex,locusG6C|NULL	LY6G6C
17029219	3.6073444		
17034474	3.6071198		
16977052	-3.581778	chemokine(C-X-Cmotif)ligand10	CXCL10
16736764	3.5588896	mucin15,cellsurfaceassociated	MUC15

16854509	3.4953377	desmocollin1	DSC1
17039517	3.3790529		
16693249	3.2336886	thioesterasesuperfamilymember5	THEM5
16836260	3.2146404	hepaticleukemiafactor	HLF
16872022	3.1719658	lectin,galactoside-binding,soluble,7|lectin,galactoside-binding,soluble,7B	LGALS7|LGALS7B
16713955	-3.158419	familywithsequencesimilarity25,memberG|familywithsequencesimilarity25,memberB|familywithsequencesimilarity25,memberC|familywithsequencesimilarity25,memberA|NULL|familywithsequencesimilarity25,memberHpseudogene	FAM25G|FAM25B|FAM25C|FAM25A|FAM25HP
16713779	3.0857801	familywithsequencesimilarity25,memberG|familywithsequencesimilarity25,memberB|familywithsequencesimilarity25,memberC|familywithsequencesimilarity25,memberA|NULL|familywithsequencesimilarity25,memberHpseudogene	FAM25G|FAM25B|FAM25C|FAM25A|FAM25HP
16704607	3.0457594	familywithsequencesimilarity25,memberG|familywithsequencesimilarity25,memberB|familywithsequencesimilarity25,memberC|familywithsequencesimilarity25,memberA|ankyrinrepeatandGTPasedomainArfGTPaseactivatingprotein11|NULL|familywithsequencesimilarity25,memberHpseudogene	FAM25G|FAM25B|FAM25C|FAM25A|AGAP11|FAM25HP

Table 2.2.2.Down-regulated genes: List of 30 highest magnitude of up-regulated genes; peri-implantitis tissue versus healthy implant tissue (n=21); there were a total of 91 down-regulated genes; table generated using GeneSpring software 12.6.

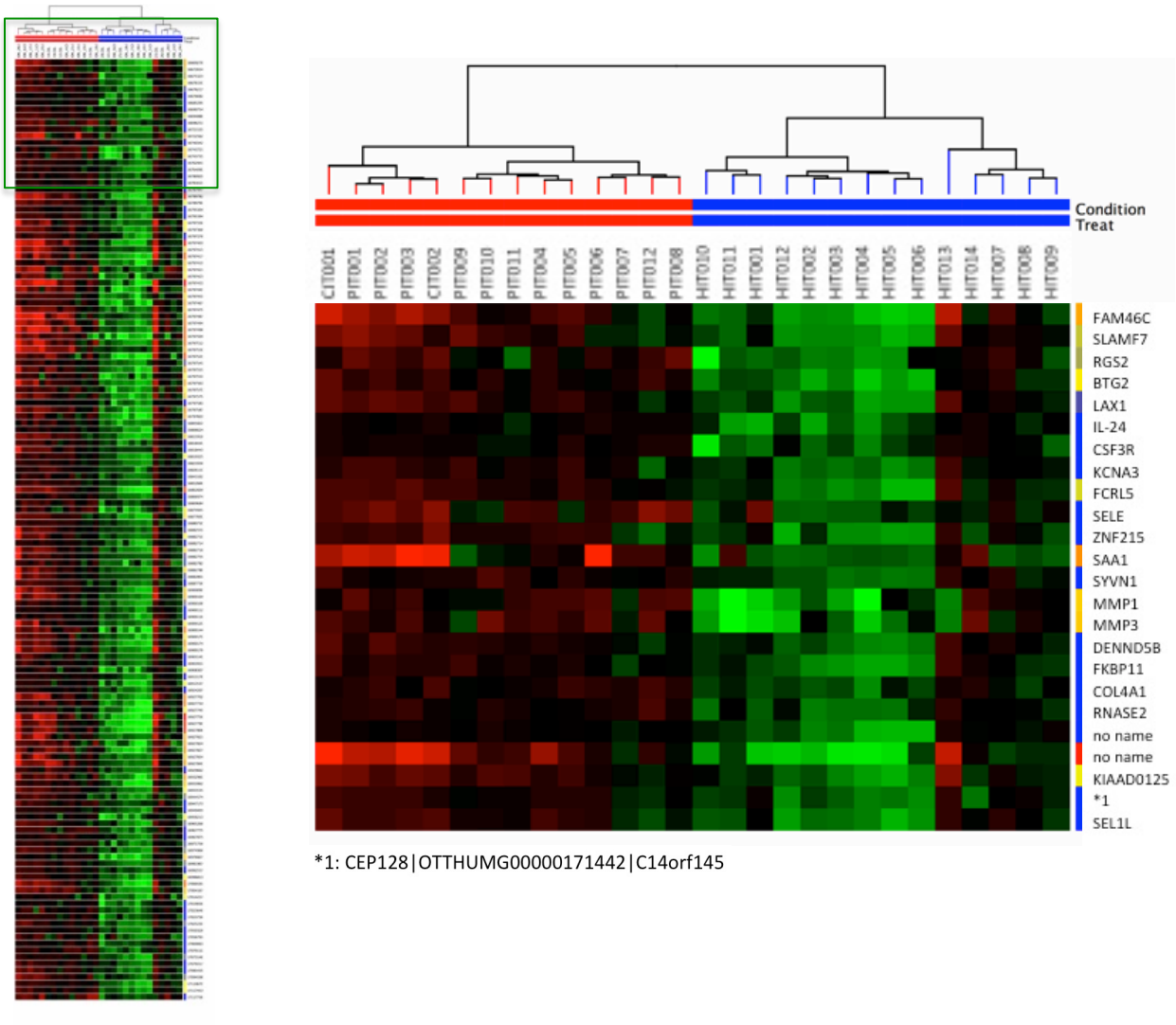

Figure 2.1. Up-regulated heat map. Molecular phenotype cluster of peri-implantitis, healthy implant and control. Samples are organized in colums and transcripts in rows. The vertical dendogram displays similarities between transcripts, while the horizontal dendogram displays similarities between samples. The heat map is colored according to the relative expression of a transcript. Supporting Information Table 2.2.1.

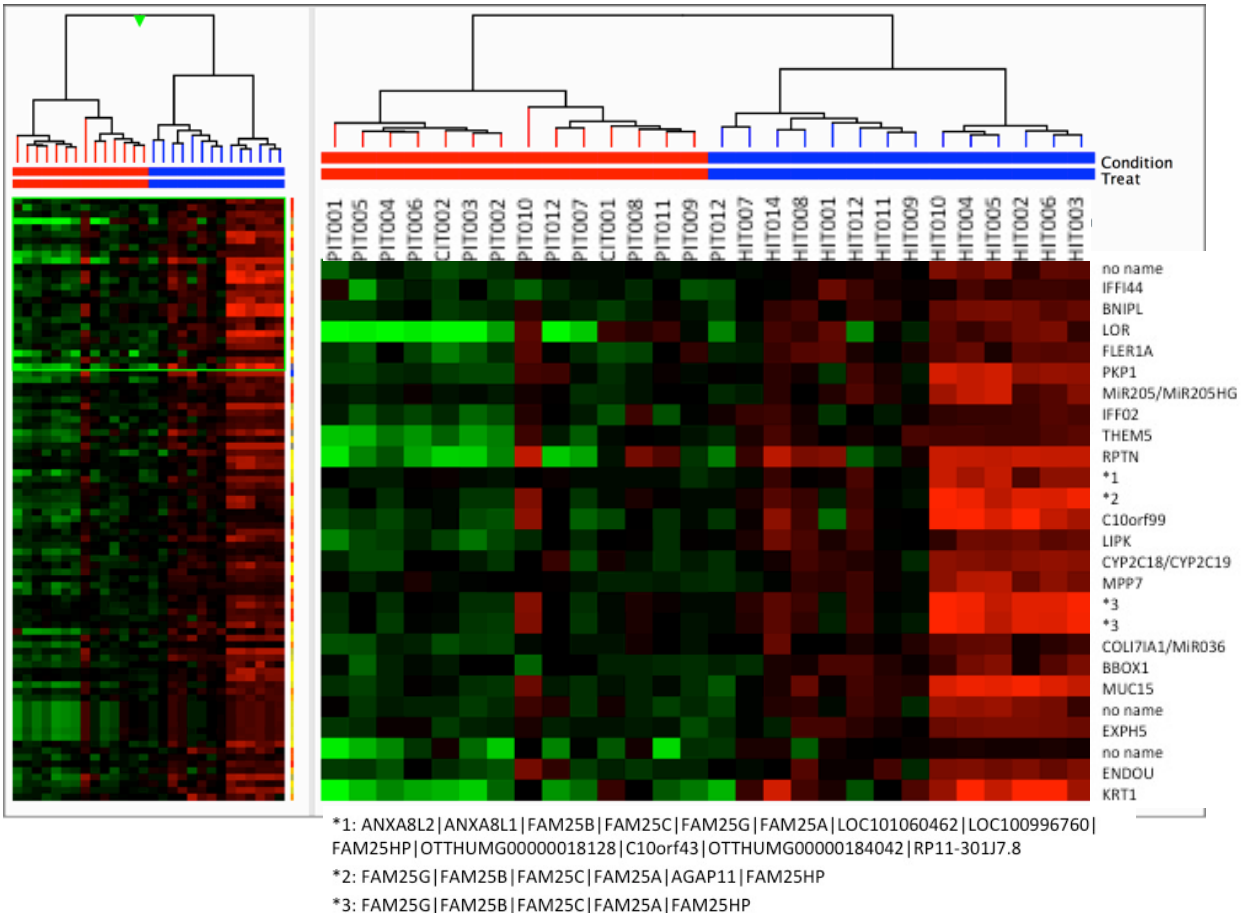

Figure 2.2 Down-regulated heat map. Molecular phenotype cluster of peri-implantitis, healthy implant and control. Samples are organized in columsand transcripts in rows. The vertical dendogram displays similarities between transcripts, while the horizontal dendogram displays similarities between samples. The heat map is colored according to the relative expression of a transcript. Supporting Information Table 2.2.2.

GO ACCESSION	GO Term	p-value	corrected p-value	Count in Selection	% Count in Selection	Count in Total	% Count in Total
GO:0044763	single-organism cellular process	1.73E-04	0.038094565	65	78.313255	11538	59.12072
GO:0065007	biological regulation	3.09E-05	0.008401043	64	77.10844	10804	55.359703
GO:0050789|GO:0050791	regulation of biological process	9.41E-06	0.003044655	63	75.90362	10244	52.490265
GO:0050794|GO:0051244	regulation of cellular process	5.33E-06	0.001903594	62	74.69879	9850	50.47141
GO:0050896|GO:0051869	response to stimulus	1.50E-08	8.58E-06	58	69.87952	7662	39.260094
GO:0016020	membrane	6.60E-05	0.016167311	54	65.06024	8515	43.630867
GO:0051716	cellular response to stimulus	2.78E-07	1.22E-04	48	57.831326	5997	30.728632
GO:0048518|GO:0043119	positive regulation of biological process	1.71E-08	9.17E-06	43	51.807228	4560	23.365444
GO:0007154	cell communication	2.51E-06	9.77E-04	43	51.807228	5382	27.577372
GO:0023052|GO:0023046	signaling	9.27E-06	0.003044655	41	49.39759	5234	26.819021
GO:0044700	single organism signaling	9.27E-06	0.003044655	41	49.39759	5234	26.819021
GO:0006950	response to stress	6.64E-11	8.14E-08	40	48.192772	3349	17.160278
GO:0007165|GO:0023033	signal transduction	3.12E-06	0.001189633	40	48.192772	4831	24.754047
GO:0071944	cell periphery	2.32E-05	0.006415528	38	45.78313	4823	24.713057
GO:0005886|GO:0005904	plasma membrane	3.63E-05	0.009299336	37	44.578312	4718	24.175035
GO:0002376	immune system process	1.91E-13	1.62E-09	34	40.963856	1981	10.150645
GO:0048583	regulation of response to stimulus	9.37E-08	4.34E-05	33	39.759037	3046	15.607706
GO:0042221	response to chemical	2.02E-05	0.005874287	32	38.55422	3666	18.784588
GO:0005576	extracellular region	5.13E-09	3.15E-06	31	37.349396	2411	12.353966
GO:0007166	cell surface receptor signaling pathway	6.29E-06	0.002202024	29	34.93976	2954	15.136298
GO:0009893	positive regulation of metabolic process	3.48E-05	0.009055521	28	33.73494	3050	15.628202

GO:0031325	positive regulation of cellular metabolic process	8.55E-05	0.019832578	26	31.325302	2854	14.6238985
GO:0006952|GO:0002217|GO:0042829	defense response	3.70E-10	2.76E-07	25	30.120481	1399	7.168477
GO:0070887	cellular response to chemical stimulus	1.19E-06	4.73E-04	25	30.120481	2102	10.77065
GO:0006955	immune response	3.46E-11	4.94E-08	25	30.120481	1251	6.4101253
GO:0048584	positive regulation of response to stimulus	1.62E-08	8.95E-06	24	28.915663	1553	7.9575734
GO:0010604	positive regulation of macromolecule metabolic process	1.36E-05	0.004012015	24	28.915663	2248	11.518754
GO:0010033	response to organic substance	7.19E-05	0.017136034	23	27.710844	2323	11.903054
GO:0009605	response to external stimulus	1.13E-05	0.003576776	21	25.301205	1769	9.064358
GO:0002682	regulation of immune system process	4.48E-07	1.83E-04	19	22.891565	1192	6.107809
GO:0071310	cellular response to organic substance	5.65E-05	0.014254508	19	22.891565	1666	8.536586
GO:0045087|GO:0002226	innate immune response	6.92E-08	3.39E-05	17	20.481928	838	4.293913
GO:0040011	locomotion	4.42E-06	0.001646827	17	20.481928	1130	5.790121
GO:0050776	regulation of immune response	1.62E-07	7.31E-05	16	19.27711	783	4.0120926
GO:0006928	cellular component movement	1.16E-04	0.025779378	16	19.27711	1315	6.738061
GO:0002684	positive regulation of immune system process	7.92E-08	3.78E-05	16	19.27711	743	3.8071327
GO:0002764	immune response-regulating signaling pathway	6.77E-10	4.84E-07	15	18.072289	452	2.3160484
GO:0050778	positive regulation of immune response	2.87E-08	1.49E-05	14	16.86747	509	2.6081164
GO:0002768	immune response-regulating cell surface receptor signaling pathway	1.69E-10	1.45E-07	14	16.86747	341	1.7472843
GO:0002757	immune response-activating signal transduction	1.34E-10	1.29E-07	14	16.86747	335	1.7165402
GO:0002252	immune effector process	3.62E-09	2.30E-06	14	16.86747	432	2.2135684
GO:0002253	activation of immune response	7.96E-10	5.46E-07	14	16.86747	384	1.9676163

GO:0001932	regulation of protein phosphorylation	1.88E-04	0.040923435	13	15.662651	956	4.898545
GO:0048870	cell motility	2.27E-05	0.006385046	13	15.662651	776	3.9762247
GO:0002429	immune response-activating cell surface receptor signaling pathway	5.18E-12	8.07E-09	13	15.662651	211	1.0811641
GO:0051674	localization of cell	2.27E-05	0.006385046	13	15.662651	776	3.9762247
GO:0016477	cell migration	8.35E-06	0.002863723	13	15.662651	706	3.6175447
GO:0006897|GO:0016193|GO:0016196	endocytosis	3.55E-07	1.52E-04	12	14.457831	442	2.2648084
GO:0042330	taxis	1.29E-05	0.003887901	12	14.457831	626	3.2076244
GO:0003823	antigen binding	1.57E-15	2.69E-11	12	14.457831	85	0.43554008
GO:0006935	chemotaxis	1.29E-05	0.003887901	12	14.457831	626	3.2076244
GO:0038093	Fc receptor signaling pathway	8.40E-09	4.97E-06	11	13.253012	249	1.2758762
GO:0006909	phagocytosis	7.22E-11	8.25E-08	11	13.253012	159	0.8147161
GO:0006959	humoral immune response	2.07E-12	7.09E-09	11	13.253012	115	0.5892601
GO:0002443|GO:0019723|GO:0042087	leukocyte mediated immunity	8.26E-11	8.86E-08	11	13.253012	161	0.8249641
GO:0001934	positive regulation of protein phosphorylation	8.78E-05	0.020081919	11	13.253012	645	3.3049805
GO:0002449	lymphocyte mediated immunity	1.43E-10	1.29E-07	10	12.048193	126	0.6456241
GO:0002431	Fc receptor mediated stimulatory signaling pathway	4.29E-12	7.35E-09	10	12.048193	89	0.45603606
GO:0050900	leukocyte migration	4.88E-08	2.46E-05	10	12.048193	230	1.1785202
GO:0002250	adaptive immune response	9.12E-10	6.01E-07	10	12.048193	152	0.7788481
GO:0038096	Fc-gamma receptor signaling pathway involved in phagocytosis	3.82E-12	7.35E-09	10	12.048193	88	0.45091206

GO:0002460	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	3.05E-10	2.38E-07	10	12.048193	136	0.6968641
GO:0034097	response to cytokine	1.92E-04	0.041269243	10	12.048193	588	3.0129125
GO:0038094	Fc-gamma receptor signaling pathway	4.29E-12	7.35E-09	10	12.048193	89	0.45603606
GO:0002433	immune response-regulating cell surface receptor signaling pathway involved in phagocytosis	3.82E-12	7.35E-09	10	12.048193	88	0.45091206
GO:0006954	inflammatory response	1.22E-05	0.003808252	10	12.048193	423	2.1674523
GO:0006958	complement activation, classical pathway	2.82E-13	1.62E-09	9	10.843373	46	0.23570403
GO:0045321	leukocyte activation	3.38E-05	0.009055521	9	10.843373	380	1.9471203
GO:0019724	B cell mediated immunity	2.10E-10	1.72E-07	9	10.843373	93	0.47653207
GO:0006956	complement activation	3.08E-12	7.35E-09	9	10.843373	59	0.30231604
GO:0016064	immunoglobulin mediated immune response	1.41E-10	1.29E-07	9	10.843373	89	0.45603606
GO:0072376	protein activation cascade	5.93E-11	7.83E-08	9	10.843373	81	0.41504407
GO:0002455	humoral immune response mediated by circulating immunoglobulin	1.58E-12	6.78E-09	9	10.843373	55	0.28182006
GO:0060326	cell chemotaxis	3.82E-07	1.60E-04	8	9.638555	159	0.8147161
GO:0030595	leukocyte chemotaxis	4.52E-06	0.001651073	6	7.2289157	101	0.51752406
GO:0034976	response to endoplasmic reticulum stress	3.44E-05	0.009055521	6	7.2289157	144	0.7378561
GO:0030968	endoplasmic reticulum unfolded protein response	6.44E-05	0.016013747	5	6.0240965	99	0.50727606
GO:0035967	cellular response to topologically incorrect protein	8.52E-05	0.019832578	5	6.0240965	105	0.5380201
GO:0006984	ER-nucleus signaling pathway	1.16E-04	0.025779378	5	6.0240965	112	0.57388806

GO:0034620	cellular response to unfolded protein	6.76E-05	0.0163 2745	5	6.024 0965	100	0.51240 01

Table 3.1. Gene ontology up-regulated genes.

GO ACCESSION	GO Term	p-value	corrected p-value	Count in Selection	% Count in Selection	Count in Total	% Count in Total
GO:0005576	extracellular region	2.79E-05	0.018125324	24	29.62963	2411	12.353966
GO:0005509	calcium ion binding	4.35E-07	7.84E-04	15	18.518518	756	3.8737447
GO:0005198	structural molecule activity	5.82E-05	0.025190806	12	14.814815	749	3.8378766
GO:0050878	regulation of body fluid levels	1.62E-05	0.01106852	12	14.814815	657	3.3664684
GO:0032787	monocarboxylic acid metabolic process	4.85E-05	0.023268873	10	12.345679	510	2.6132405
GO:0043588	skin development	4.83E-07	7.84E-04	10	12.345679	302	1.5474483
GO:0005543	phospholipid binding	1.13E-04	0.038783636	10	12.345679	565	2.8950605
GO:0007599	hemostasis	9.08E-05	0.032755584	10	12.345679	550	2.8182003
GO:0050817	coagulation	8.42E-05	0.031848434	10	12.345679	545	2.7925804
GO:0007596	blood coagulation	8.42E-05	0.031848434	10	12.345679	545	2.7925804
GO:0008544	epidermis development	1.40E-07	4.71E-04	10	12.345679	264	1.3527362
GO:0005544	calcium-dependent phospholipid binding	9.23E-14	1.20E-09	9	11.111111	42	0.21520804
GO:0005911	cell-cell junction	8.86E-06	0.006391626	9	11.111111	329	1.6857963
GO:0045111	intermediate filament cytoskeleton	9.72E-07	0.001262234	9	11.111111	251	1.2861242
GO:0006631	fatty acid metabolic process	5.78E-06	0.005362731	9	11.111111	312	1.5986882
GO:0005882	intermediate filament	2.17E-07	4.71E-04	9	11.111111	210	1.0760401
GO:0070161	anchoring junction	6.76E-05	0.02832563	7	8.641975	243	1.2451322
GO:1901568	fatty acid derivative metabolic process	3.49E-06	0.003486963	6	7.407407	99	0.50727606
GO:0006690	icosanoid metabolic process	3.49E-06	0.003486963	6	7.407407	99	0.50727606
GO:0072330	monocarboxylic acid biosynthetic process	1.13E-04	0.038783636	6	7.407407	183	0.93769217
GO:0033559	unsaturated fatty acid metabolic process	7.14E-06	0.006181973	6	7.407407	112	0.57388806
GO:0030057	desmosome	4.04E-10	2.62E-06	6	7.407407	23	0.11785202

GO:0045103	intermediate filament-based process	2.00E-07	4.71E-04	5	6.1728396	32	0.16396803
GO:0045104	intermediate filament cytoskeleton organization	1.70E-07	4.71E-04	5	6.1728396	31	0.15884402
GO:0005200	structural constituent of cytoskeleton	5.20E-05	0.023268873	5	6.1728396	97	0.49702808
GO:0045109	intermediate filament organization	6.29E-07	9.08E-04	4	4.9382715	17	0.087108016
GO:0006636	unsaturated fatty acid biosynthetic process	1.20E-04	0.03997609	4	4.9382715	61	0.31256405
GO:0006691	leukotriene metabolic process	3.29E-05	0.020374209	4	4.9382715	44	0.22545603
GO:1901570	fatty acid derivative biosynthetic process	8.58E-05	0.031848434	4	4.9382715	56	0.28694403
GO:0019370	leukotriene biosynthetic process	7.96E-06	0.006183411	4	4.9382715	31	0.15884402
GO:0046456	icosanoid biosynthetic process	8.58E-05	0.031848434	4	4.9382715	56	0.28694403
GO:0045110	intermediate filament bundle assembly	1.37E-06	0.001611926	3	3.7037036	6	0.030744005
GO:0001533	cornified envelope	1.48E-04	0.048155744	3	3.7037036	25	0.12810002
GO:0030280	structural constituent of epidermis	8.09E-06	0.006183411	3	3.7037036	10	0.05124001
GO:1902414	protein localization to cell junction	5.09E-05	0.023268873	2	2.4691358	3	0.015372003
GO:0071896	protein localization to adherens junction	5.09E-05	0.023268873	2	2.4691358	3	0.015372003
GO:0052741	(R)-limonene 6-monooxygenase activity	5.09E-05	0.023268873	2	2.4691358	3	0.015372003
GO:0019113	limonene monooxygenase activity	5.09E-05	0.023268873	2	2.4691358	3	0.015372003
GO:0018676	(S)-limonene 7-monooxygenase activity	5.09E-05	0.023268873	2	2.4691358	3	0.015372003
GO:0018675	(S)-limonene 6-monooxygenase activity	5.09E-05	0.023268873	2	2.4691358	3	0.015372003

Table 3.2. Gene ontology down-regulated genes.

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