Transcriptome Profiling of A.a

Stimulated Peri-implantitis Model

BY

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THESIS

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DEDICATION

This thesis is dedicated to my family. My loving parents, who have selflessly given me courage and hope and to my husband, who has been my constant support.

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SUMMARY

Hypothesis: Aggregatibacter actinomycetemcomitans (A.a) infection of dental implant abutments can induce peri-implant inflammation associated with peri-implant bone loss. Mutation of A.a virulence alters A.a - mediated host inflammation and peri-implant bone loss.

Objective: To define by RNA sequencing the tissue transcriptomes present in healthy and A.a-infected peri-implant tissues in the rat oral implant model.

Methods: Aa was grown on trypticase soy agar. Thirty-six female 5-month year old rats were used. Two-piece titanium implants (1.2x3.0 mm) were fabricated. The abutments were attached to the external hex of all implants. The abutments were then surface treated using grit blasting aluminum oxide (50) and hydrofluoric acid. The abutments were then inoculated with either Aa wild type (n=7), or Aa double deletion mutant of leukotoxin and cytolelthal distending (DS7S-1) (n=7) and sterile sham (n=8) in vitro for 3 days. Implants were installed in maxillary bone of rats. Micro-CT scans were taken at baseline (0), weeks 3 and 8. CT scans were then overlaid to assess for bone loss. Peri-implant tissue was harvested at 8 weeks for RNA sequencing. The transcriptomes were analyzed and compared using bioinformatic tools.

Results: Our study demonstrated the role of Aa in inducing the innate immune response at the epithelium, which ultimately resulted in peri-implantitis. Wild type Aa and mutant Aa induced responses that were associated with TLR4 receptors suggesting that they are signaling through conserved innate immunity mechanism. Mutant Aa appeared to strongly induce mucosal expression of Wig-1, a modulator of P53 function. Finally, the mutant Aa also induced upregulation of tight junction protein Claudin-2 and a component of Dynein, which controls microtubule function. The mutant Aa induced gene expression associated with rapid epithelial turnover and diminished epithelial structural integrity, was also seen with GO terms. This

implied an impact on the structural function of the epithelium. Aa was able to induce innate immune response at the peri-implant tissues, and changes that are associated with pro-inflammatory pathophysiology. Perhaps, the epithelium can be a target area for therapy.

Conclusions: Results of this study implicate Aa – related changes in the epithelial barrier function of oral mucosa in the pathophysiology of peri-implantitis

1. INTRODUCTION

1.1 Background

Endosseous implants are now widely used to replace missing teeth in order to restore health, function and esthetics due to their high level of predictability and success.[1] Zarb developed a criteria for success of an implant in 1990:

1) The implant should be immobile
2) There should be no radiolucency around an implant using a periapical radiograph
3) The mean bone loss should be <0.2mm after first year of loading
4) The patient should be asymptomatic—no pain or swelling
5) 80-85% success rate after 10 years.[1]

Misch developed a similar criteria—"Misch implant quality scale," which groups implant success on a scale of I-IV. Additionally, he recommended that anatomical variation and poor bone quality could be overcome by varying implant designs.[2]

Despite established protocols and measures to improve success, implant failure continues to be a clinical reality. Clinical signs can be seen as early, in the first 3 to 6 months, during the healing period,. These signs include swelling, fistulas, dehiscence and osteomyelitis. The early signs are critical for implant failure, but cannot be used as the only parameter for success. Implant mobility and radiographic evidence of radiolucency should also be used. Pain and or sensitivity can indicate and suggest implant mobility, but not always. Heitz-Mayfield, found mobility to be a poor clinical diagnostic tool, as it means lack of osseointegration.[3]

Osseointegration, defined as “direct the functional and structural connection between living bone and the surface of a load bearing implant”[4] was first identified in dental implants in 1965 by Branemark and then further detailed later by Davies as an ongoing progression, wherein distance osteogenesis initially begins and subsequently as tissue matures, contact osteogenesis.[5] While this type of remodeling was once considered as a normal body response, Donath described it as a foreign body reaction similar to a shrapnel found in humans.[6] In recent years, Albrektsson gave a new definition: “osseointegration is a foreign body reaction where interfacial bone is formed as a defense reaction to shield off the implant from the tissues.[7]

Implant Design:

The macro and micro-design of an implant contribute to implant success. The macro-design of an implant is the geometry of the implant. This includes the shape, depth, and pitch, which play a role in the bone-to-implant contact and the amount of stress at the crestal level.[8] Conical implants have higher primary stability. The diameter of cylindrical implants had positive correlation with primary stability.[9]

The micro-topography of an implant is an important factor to consider. Surface treatment is completed to achieve certain level of roughness. Implants can be classified as smooth, minimally rough, moderately rough and rough depending on the Sa value (Table 1).[10] Surface modification can be enhanced via subtraction such as acid etching or blasting, or addition of materials, such as fluoride or hydroxyapatite. Lang and Jepsen found moderately rough and roughened surfaces enhance osseointegration.[11] An important factor to consider with surface roughness is related to

susceptibility to bacterial buildup, "rough surface may accumulate more supra- and subgingival dental plaques than smooth ones."[12]

Table 1: Albrektsson and Wenneberg[10] Surface roughness and implant system

Surface	Value	Implant system
Smooth	<0.5 μm	
Minimally rough	0.5-1.0 μm	Branemark+Astra Tech (implants before 1995)
Moderately rough	1.0-2.0μm	Astra Tech TiOblast, Osseospeed, Nobel Biocare TiUnite, Straumann SLA
Rough	2.0μm	Plasma-sprayed titanium Dentsply Frialit-2

Some have cited that the micro-design, i.e. the surface treatment of an implant may contribute to implant failure, stating that smooth (machined) surface implants are less prone to peri-implantitis than roughened surface. However, there is limited evidence both in animal and human studies to suggest there are any differences. [12] Albouy studied: 6 Astra (Tioblast), 6 ITI (SLA), 6 Branemark-Tiunite implants and found that after 24 weeks there was a statistical amount of bone loss around Ti-unite implants, compared with SLA and Tioblast implants.[13] A follow-up histological study in 2009 concluded that all implants had similar peri-implant bone loss, however the Ti-unite implant had markedly more inflammatory cells.[14] Ti-unite surface is created by anodization using phosphoric acid electrolyte and a current is passed through it. This creates a roughened, porous surface. SLA stands for sand blasted acid etched surface. This creates a roughened surface, which will theoretically induce osteogenesis and increase attachment, seen with ITI Straumann implants. Osseospeed (Astra) is sandblasted and treated with fluoride. The fluoride ion is supposed to act like calcium and create a greater affinity for

organic compounds.[15] Implant collars can also serve as a reservoir for bacteria. Chen et al, found machined surface collars have more bone loss compared to laser treated implant collars. They found that the laser treated collars may aid in the gingival health and stability of tissue around an implant. [16]

Endosseous implants differ from teeth, especially with regard to the periodontal attachment apparatus, where the epithelium by implants is keratinized. In natural teeth, the external and internal environment is separated by enamel and junctional epithelium. The junctional epithelium is derived from reduced enamel epithelium. The junctional epithelium functions as a barrier and is structurally important in collaboration with the host immune response. The junctional epithelium runs along the cementoenamel junction. The principal connective tissue fibers run from the hard and soft tissue in all directions. They are fan shaped.

During the placement of an endossous implant, the oral mucosa is penetrated along the implant surface. A new epithelium develops and matures around an implant 8 weeks post implant installation. Lindhe stated that the down-growth of epithelial cells synthesize basal lamina and hemidesmosomes.[17] The gingival and peri-implant mucosa is lined by keratinized oral epithelium, which is continuous with the junctional epithelium and is 3-4mm long and approximately 1.0-1.55 mm high. The separates the junctional epithelium from the alveolar crest with non-inflamed, collagen-rich and connective tissue that has poor cellular proliferation. Animal research also have “revealed differences in the fibroblast to collagen ratio in the arrangement of the vasculature. The increased collagen to fibroblast ratio in the interfacial connective tissue together with a decreased vascularity of that region may have an impact on the

onset of progression." [18] Due to the keratinization, the reduced cell structure, and the parallel directionality of the collagan fibers, the sealing capability is reduced with dental implants and the connective tissue attachment is weaker than with natural teeth.[19]

Current Understanding of Implant Failures

According to the new classification on periodontal conditions and peri-implant disease , peri-implant health can be defined histologically and clinically.[20] In health, peri-implant tissue is devoid of any visible signs of inflammation and does not bleed on probing. Meanwhile, peri-implant mucositis can present with signs of inflammatory gingival changes, as well as bleeding on probing. Moreover, peri-implantitis is defined as, "a plaque-associated pathologic condition occurring in the tissue around dental implants, characterized by inflammation in the peri-implant mucosa and subsequent progressive loss of supporting bone." Peri-mucositis can lead to peri-implantitis. Peri-implantitis usually presents with circumferential bone loss at surgical entry. There are a myriad risk factors that can lead to bone loss and failure of osseointegration. Implant design and diagnostic techniques further impact early diagnosis and mitigation.

There are numerous factors that influence implant disease and failure, however a widely accepted one comes from Esposito et al, who provided factors leading to failures related to biological, mechanical, and/or iatrogenic causes. Biological factors were defined as the "as the inadequacy of the host tissue to establish or maintain osseointegration."[21] Mechanical factors related to implant components and suprastructure, such as screw loosening, implant fracture, etc. Iatrogenic factors occur during the surgical treatment often caused by malpositioning of an implant which inhibit the ability to place the anchorage unit.[21] In a systematic review of 51

human studies, Berglundh investigated the biological and technical complications across a variety of types of implant therapy including overdentures, fixed complete dentures, fixed partial dentures, single tooth replacement, immediate placement/early loading, and augmentation procedures. The review concluded biological complications were found in 40-60% of time, and technical problems were seen in 60-80% of studies.[22]

In addition to these overarching categories, implant failure can occur at different chronological points and are further identified as either early or late failures. Early failures occur before implants are loaded and may occur at the time of surgery or prior to surgery due to a response host related factors. Contributing factors to early failures include operator error, inexperience, surgical trauma, and immediate loading.[23] Studies have also suggested the potential impact of overheating during implant placement, which may cause bone necrosis and implant failure, but research is inconclusive as to the specific impacts of temperatures.[4, 24]

Recently, Jemt, et al. looked at the risk factors related to early implant failure in a total of 3448 implants installed over an eight year period and concluded that implant placement in the lower jaw had highest risk of implant failure with an odds ratio of 2.03. Additionally, overall implant failure was related to the number of implants placed an individual; the need for multiple implants placed appeared to pose a greater failure risk than a single tooth replacement.[25] This is suggested to be related to the complexity of treatment and potential loss of teeth due to periodontal disease. The loss of osseointegration can cause an implant to exhibit mobility and peri-implant radiolucency, which ultimately leads to the replacement of bone into a fibrous connection.

Late failures are attributed to prosthetic treatment or peri-implant disease, including residual cement.[26] Peri-implant disease can be described further delineated as peri-mucositis or peri-implantitis. Peri-mucositis is a reversible inflammatory disease that affects the soft tissue only. while, peri-implantitis is an irreversible disease that affects both the soft tissue and supporting bone. The prevalence of peri-mucositis and peri-implantitis was reviewed by Zitzmann, which looked at six publications between 1997-2007.[27] However, despite Zitzmann's work, peri-implantitis is not well defined and definitions remain inconsistent (Table 1). Many of the authors who describe peri-implant disease fail to include periodontal indices related to implant failure such as bleeding on probing, probing from a fixed reference point, and mucosal health. Additionally, there is inconsistency in radiographic techniques, though long-cone parallel radiographs is the established preferred practice. One potential solution to improve understanding of peri-implantitis prevalence would be utilize of a baseline measurement probing measure taken at implant placement and again at one year after loading. Perhaps, this may lead to accuracy in the epidemiological studies.

Roos-Jansaker in 2006 found 50% of implants that had been in place for 10 years had peri-mucositis.[28] Atieh et al. identified 9 studies with over a 1000 patients and 6000 implants, the estimated prevalence of peri-mucositis was 63.4% for subject based and 30.7% of implants. The estimated prevalence of peri-implantitis was 18.8% of subjects and 9.6% of implants. Smokers tended to have a higher prevalence of peri-implantitis.[29] In a recent meta-analysis Lee et al. found the prevalence of weighted mean of implant-based and subject based peri-mucositis to be 29.48% and 46.93%, respectively. The mean prevalence of peri-implantitis was 9.25% and

19.83%, again measuring implant-based and subject based. The overall the rate of peri-implantitis is 9.6% of implants and 18.8% of patients. [30]

Table 2: Prevalence of peri-mucositis and peri-implantitis

Reference	Years	(N) Subject/ Implants	Peri-mucositis	Peri-Implantitis
Fransson (2005)[2930313031]	8.6	662/3413	Not-defined N/A	Implant level: 12.4 Subject level: 27.8
Roos-Jansaker (2006)	10.8	216/987	Implant level: 16.0 Subject level: 48.2	Implant level: 6.6 Subject level: 16.2
Lee (2012)	8.1	60/117	Implant level: 9.4 Subject level: 53.3	Implant level: 10.3 Subject level: 33.3
Papantonopoulos et al. (2015)	7.1	94/340	Not defined N/A	Implant level: 26.2 Subject level: 47.9
Renvert et al. (2018)	23.3	351/86	54.7%	22.1%

Host Risk Factors for Developing Peri-Implantitis

Following successful placement of an endosseous implant, host-related factors, such as a history of periodontitis, additional endodontic treatment, smoking, lack of compliance, and systemic disease, such as diabetes, radiation therapy or use of bisphosphonates, may lead to increased late implant failures.[26] However, the issue of diabetes and smoking as potential risk factors remains inconclusive.

Research suggests that a patient's history of generalized aggressive periodontitis is likely increase the individual's risk of implant failure. In 2014, Monje et al identified a risk ratio of 4.0 for patients with an associated history of periodontitis. [32] Additional research suggests that periodontitis, poor plaque control lack of supportive therapy leads to implant failure. A history of periodontitis poses 14x greater risk of developing peri-implantitis, 5x likely to lose an implant

and 3x to develop mucositis than patients without the condition. [33] However, a history of periodontitis did result in peri-implant disease, but not in implant loss.

Peri-implantitis can also present in a unique forms. In a retrospective study, Quirynen found patients with a history of endodontic treatment can develop retrograde peri-implantitis or peri-apical peri-implantitis caused by remaining scar or granulation tissue or adjacent teeth with current endodontic involvement. [34] Typically, they present with sinus or abscess formation. Clinical signs may not present for 4-6 weeks or even after 4 years of implant placement.[32]

While extensive research confirms that smoking impairs wound healing[35], the mechanism of action is not fully understood. Despite this, both retrospective and cohort studies of smokers receiving implants have repeatedly demonstrated a strong correlative link between smoking and endosseous implant failure (See Table 3) . Heitz-Mayfield suggested that the overall odds ratio of having peri-implantitis with a history of periodontal disease and smoking is 3.6-4.6.[3] However, Stacchi conducted a meta-analysis and stated that there is limited evidence to conclude that the combination of a history of periodontitis and smoking is a risk factor for implant failure but that additional long duration prospective studies are needed.[36] One current recommendation suggested smoking cessation 1 week prior and 8 week post implant placement. [37] Lindquist found smokers presented with greater crestal bone loss than non-smokers.[38] Similarly, 18% of patients who were smokers developed peri-implantitis in a 10-year compared with 6% of patients. [39] Variation in research could be attributed to how smoking was defined in all these studies and most importantly in over-reliance on patient reported information.

Table 3: Smoking and Peri-implantitis

Author/year	Study	Year	Results
Wallace (2000)	56 patients with 187 implant (Nobel implants)	4 year period	Significant association of implant failures and cigarette smoking 16.6% in smokers compared with 6.9% in non-smokers. Shorter implants more susceptible to failures <10mm
Bain and Moy (1993)	Retrospective study on outcome of 2,194 Branemark implants placed in 594 patients		Significant association of implant failures and cigarette smoking 16.6% in smokers compared with 6.9% in non-smokers. Shorter implants more susceptible to failures <10mm Overall failure was 5.92%. Greater in smokers than in non-smokers (11.28% vs. 4.76%)
Kan et al (1999)			Decreased implant length, increased in implant failure in smokers
Lemons (1997)		6 year period	Smoking reduced density of bone in femur and vertebrate

Similarly to the interference with wound healing seen with smoking, diabetes is a metabolic disorder that alters the normal physiology in response to pancreatic beta-cell dysfunction or insulin resistance which causes long term damage to the heart, eyes, kidneys, nervous and vascular system. The global estimate of this disease is 8%. Hyperglycemia can impair wound

healing by inhibiting osteoblasts and collagen production and reducing bone formation and remodeling.[40] This can lead to impairment in healing during implant placement and increase susceptibility to implant failure. This has not been proven and is controversial in the field. Many of the studies are cross-sectional, have small sample size or even the history of diabetes is self-reported. It is commonly understood patients who present with fasting blood sugar of >126mg/dl have diabetes, however many of the studies rely on patient's self-reported glycemic measurements.

Table 4: Diabetes and Peri-implantitis

Author/year	Study	Implants	Results
Ferreira et al 2006[41]	Cross sectional study of 212 patients 183 non diabetic and 29 with diabetes in Brazilian population. Glycemic control was self reported.	Nobel BioCare implants	Odds ratio of 1.9
Aguilar-Salvatierra Et. al (2016)	85 subjects divided into three groups depending on HbA1c: 33 patients in group 1 (<6, control group), 30 patients in group 2 (6.1-8), and 22 in group 3 (8.1-10)	Each patient received one-one piece implant in anterior zone	Marginal bone loss increased in relation to HbA1c—resorption of 0.51 after 6 months to 0.72 after 2 years in comparison to 1.33 and 1.92 in group 3.
Rokn et al. 2017 [42]	Cross sectional study 134 subjects 130 non-diabetic 4 diabetic patients	Tissue level implants (Straumann) and bone level implants (Replace, Nobel BioCare), Astra Tech	No association Found association with smoking and lack of keratinized tissue OR of 2.57 and 3.89

Microorganisms in Peri-implantitis

Microorganisms play a major and primary role in initiating peri-implantitis and peri-implant disease in a susceptible host. Mombelli described peri-implantitis as a disease caused by microbiological pathogens namely, gram-negative anaerobic rods. [43] Research suggests that bacterial colonization occurs within 30 minutes after implant installation[44] though an animal study demonstrated that gingivitis may not appear for 2-21 days similar to the findings in LÖe's original study titled Experimental Gingivitis in Man.[45] Peri-implant biofilm resembles adjacent teeth, which serve as a reservoir for bacteria around an implant. Zitzmann induced peri-implant mucositis in 12 patients. Tissue biopsies were taken at various points, however at week 3, T and B cells infiltrate was more pronounced compared with natural teeth.[46]

Animal research confirms an overlap between the putative bacterial agents in peri-implant disease and those causing periodontitis, such as Porphorymonas gingivalis (Pg), Aggregatibacter actinomycetemcomitans (Aa) and Bacteriodes forsythus. However, some of the bacteria were not part of the typical periopathogenic bacteria, such as Staphlyacoccus aureus, which has been demonstrated to have a high affinity for titanium.[47] Others commonly found in periodontitis, like Pg is a black pigmented anaerobic, gram negative, non-motile, and is rod shaped. Pg has pili or fimbriae, which are useful for attachment. Pg secretes a protease called gingipain, which works to degrade the cytokines and downregulates host response. Pg has been studied and found to induce peri-implantitis in a murine model. It was noted that implant tissue sites presented with greater TNFa and a decreasing expression of Foxhead box p3 (Foxp3).[48] Foxp3 is a master regulator of T cells. Thus, the inability to stimulate T-cells suppresses the immune response leading to bone loss.

Aa is another putative pathogen for both periodontitis and peri-implantitis. It is gram negative, non-motile, and facultative anaerobe. It has LPS, polysaccharide, adherence proteins, and toxins. It has been studied extensively, especially in localized and generalized aggressive periodontitis, due to its ability to invade connective tissue and it is commonly found in the crevicular fluid of the gingival tissue.[49] The literature is inconsistent on the presence of A. a in peri-implantitis, although Valente et al. reports the inconsistencies are related to individual composition and their bacterial makeup.[50] Serotype b is elevated in localized aggressive periodontitis and produces a leukotoxin. Leukotoxins are produced by the bacteria that have an impact on disease progression. The two most common toxins are Cytolethal distenting toxin (Cdt), which targets RANKL impairs macrophage function and impairs phagocytosis,[51] and leukotoxin LtxA, which is expressed as four operons that destroy bone a, b, c, and d.

Testing for the presence of these bacteria has limitations, due PCR or DNA-DNA hybridization and a tendency for bias because bacteria must be preselected prior to probing. The 16S rRNA has been able to overcome limitations of preselection by selecting for the complete microbiome, which will include all potential bacterial genes within that community. By using this technique, Becker et al examine 28 failing implant sites and utilized DNA probe assessing for Aa, Bacteriodes intermedius, and Bacteriodes gingivalis. They concluded that greater than 10% of the aforementioned bacteria were present in peri-implant sites.[52]

Table 5: Bacterial agents and characteristics

CHARACTERISTIC					
Species	Cell morphology	Gram stain	Motility	Oxygen requirement	Virulence factors
Actinobacillus Acitnomycetemcomitans	Coccobacillus/star shaped internal morphology	Negative	Non-motile	Facultative /Capnophilic (anaerobic)	Leuokotoxin Invasion of tissue
Prevotella intermedia	Bacilus/brown black	Negative	Non-motile	Anaerobic	Pili/fimbriae Capsule LPS
Porphyromonas Gingivalis	Bacilus/brown black	Negative	Non-motile	Anaerobic	Collegenase LPS
Campylobacter Recta	Helical to straight rod shaped	Negative	Motile	Anaerobic	N/A
Spirochetes	Spirilla	Negative	Motile	Strict anaerobes	Tissue Invasion
Fusobacterium nucleatum	Bacillus, long Tapered	Negative	Non-motile	Strict Anaerobe	N/A
Bacteriodes Forysthus	Bacilus, Tapered ends	Negative	Non-motile	Anaerobe	N/A
Eikenella Corrodens	Bacilus	Negative	Translocating	Faculative anaerobe	N/A

Table 6: Micro-organisms associated with peri-implantitis

Author/year:	Study Design:	Bacteria	Results
Mombelli et al (1987)[43]	14 patients	B. gingivalis B. intermedius B. melaninogenicus Selenomonas spp Fusobacterium spp S. sanguis S. mutans A. naeslundiii A. viscous Peptostreptococcus micros Streptococcus intermdius	Implant sites were higher than peri-implant mucositis and healthy sites.
Becker (1990)[52]	36 failing implants in 13 patients clinical and DNA probe used to assess bacteria	Actinobacillus actinomycetemcomitans, Bacteriodes intennedius, Bacteriodes gingivalis	Moderate levels of bacteria found in peri-implant sites
Leonhardt et al (1999)[47]	37 patients demonstrating 1 or more implants with bone loss >3 threads, bleeding on probing, 51 patients with clinically healthy mucosa and or bone loss	Porphyromonas gingivalis Prevotella Intermedia Prevotella nigrescens Actinobacillus actinomycetemcomitans	27.8% 35.4% 37.5% Found in 60% of cases

		Staphylococus spp. Candida spp	55% of peri-implant lesions **unique bacteria and yeast found in diseased sites
Augthun (1997)	12 edentulous patients with 18 unsuccessful implants (IMZ)	Prevotella intermedia Prevotella buccae Prevotella oralis Prevotella melaninogenica Prevotella denticola Bacteroidaceae species and A. actinomycetemcomitans	Strongly dominated by gram negative bacteria And high incidence of Bacteroidaceae species and A. actinomycetemcomitans 60%
Hutlin (2002)	17 partially edentulous patients with 98 implants, 45 had marginal bone loss. 19 subjects served as controls	Actinobacillus actinomycetemcomitans, Prevotella intermedia, Bacteroides forsythus and Treponema denticola	Found greater inflammatory response around implants in partly edentulous than patients who were dentate

Histopathology:

Ivanovski found in the the presence of good oral hygiene, implant surface was devoid of inflammatory cells.[19] However, Seymour found cellular infiltrate was more profound in inflamed tissue and consisted of mainly lymphocytes, macrophages and dominated by T cells, similar to gingivitis. They were dominated by CD4:CD8 (helper: suppressor) with a ratio of 2:1 in more inflamed tissue, similar to a delayed hypersensitivity reaction. They correlated this finding to gingivitis, but they were unsure of immune response when peri-implant bone loss occurred.[53]
In a study conducted by Tzach-Nahman induced peri-implantitis using *Porphyromonas gingivals (Pg)* using mice model. It was concluded that that PG induced bone loss around implants more than teeth, with increasing levels of TNF-alpha and Foxp3 decreased around teeth.[48]

Diagnostic measures:

Radiographic evidence of bone loss is an accepted indication of peri-implantitis. In the first European workshop, it was agreed upon that the absence of mobility; radiographically measured marginal bone loss <1.5mm during first year of function;<.2mm marginal bone loss after the first

year; and absence of additional symptoms would indicate successful osseointegration. This standard measurement has been modified slightly to recognize normal bony remodeling from that of peri-implant bone loss, especially during the period of loading and function. [54]
Probing measurement allows the clinician to detect inflammation, gingival changes, probing depths relative to level of bone crest, and attachment loss. Probing around an implant has been a debatable subject amongst different clinicians. Many believe that disrupting the new epithelial connection may cause tissue harm depending on the pressure applied to probe and type of probe utilized. Etter recommends a light force of .25 Newton per centimeter (N/cm) and tip diameter of .45mm around an implant did not cause irreversible damage and that the epithelial attachment can heal after 5 days.[40] It was noted by Ericsson that a force greater than 0.5N with a diameter of .5mm resulted in damage and displaced the junctional epithelium as the probe penetrated further apically than in natural teeth, where there was more resistance.[55] Both of these studies were conducted on animal models. Froum recommends use of a smooth probe with a round diameter of 0.4-0.5mm, force less than 0.25N, clean probe using chlorohexidine to prevent contamination.[56]

Furthermore, probing should be reproducible around teeth and implants, especially angulation around pink porcelain or composite material. The absence of bleeding on probing is an important indicator for periodontal stability [56] and the absence of inflammation.[57] Jepsen sought out to determine the predictive value of BOP in periodontitis. It was concluded that BOP serves as a high negative predictive value for implant stability.[58] This was later corroborated by Luterbacher, who evaluated the accuracy of diagnostic tests of implants and teeth during rigid supportive periodontal therapy over a five year period. It was concluded that bleeding upon probing was the

best diagnostic tool to determine disease progression.[59] Despite this research, there is currently no standardized method of probing around an implant.

Other issues have been cited related to probing an implant, "risk of damage to the implant surface by the metallic probe, risk of bacterial inoculation and galvanic corrosion resulting from contact between two dissimilar metals."[56] Firstly, Fakhravar conducted in vitro study comparing metallic probes and plastic probes on 10 abutment sites. It was concluded that both metal and plastic probes, as well as metallic scalers caused surface roughness and altered the morphology of abutment surface, perhaps creating a nidus for bacterial attachment. [60] Froum's recommendation, the use of a smooth surface instrument, may eliminate this problem. Secondly, the risk of bacterial inoculation via periodontal probe has been refuted by Greenstein.[61] Although bacterial transmission can occur, the idea of pathogenicity is dependent on the individual host's susceptibility. Lastly, the potential risk for galvanism occurs when two different metals with different standard electrode potential come in the presence of an electrolyte. The more electronegative metal will become oxidized and the electropositive metal will be protected, leading to corrosion. This occurs when a metal probe touches an implant surface, exposing the oxide layer. There has been one cited example of galvanic reaction in implant leading to loss of an implant.[62,63]

Peri-implant crevicular fluid:

Biomarkers such as cytokines, enzymes and proteases have been investigated to determine disease activity as they can aid in distinguishing health from disease. Many of the studies that were conducted were cross-sectional studies, which are descriptive in nature. Therefore,

longitudinal and randomized controlled studies are needed to confirm these findings.[3]Nonetheless, the use of peri-implant crevicular fluid is a quick, repeatable, and non-invasive way to determine disease activity. (Table 2)

Table 7: Potential diagnostic biomarkers

Author/year	Study	Sample/biomarker	Results
Kao (1995)	12 subjects	PICF: IL-1β PICF	Level of IL-1β—peri-implantitis sites had 3x IL-1β was higher in peri-implantitis sites
Teronen et al. (1997)	Seven patients with peri-implantitis and 6 patients with healthy sites	MMP8 (collagenase-2)	Elevated in PICF
Panagakos et al. (1996)	13 subjects; 50 implants With healthy, early and advanced peri-implantitis	IL-1β, PGE2, MMP	
Aboyoussef et al. (1998)	29 patients, 37 healthy implants, 37 implants early peri-implantitis		Did not find a difference in PGE2 and MMP IL-1β increased 6x in early peri-implantitis
Murata (2002)	16 patients, 34 implants; 6 peri-implantitis, 8 peri-mucositis	Osteocalcin deoxypridinoline IL-1β	IL-1β levels from peri-implant sites were higher than peri-implant mucositis and healthy sites.
Fonseca et al (2014)	22 edentulous patients Divided into two groups Mucositis and peri-implantitis	IL-1β PICF	Elevated levels of IL-1β, characteristic of peri-implantitis and IL-8 was elevated in patients with peri-implantitis
Gurlek (2017)	97 implants, 19 healthy, 20 mucositis, 20 peri-implantitis And 39 natural teeth (19 healthy, 12 gingivitis, 8 periodontal disease)	Assessed GCF and PICF for IL-1β, RANKL, OPG	IL-1β was found in higher levels in PICF than in GCF And TNF-kb ligand (sRANKL) higher in gingivitis group than in peri-mucositis group.

IL-1β is commonly found in peri-implant crevicular fluid. This cytokine is a potent pro-inflammatory marker and in particular, present during cell proliferation, differentiation, and apoptosis. It is expressed in response to tumor necrosis factor-kB during the innate response, as well as, during osteoclastic activity. It is activated by macrophages and monocytes. IL-1β binds to polymorphonuclear leukocytes and stimulates the production of PGE2, increasing lysosomal activity and subsequently, bone resorption. IL-1β has been studied in periodontal disease is

known to be elevated in GICF in active periodontal sites. According to Stashenko, "IL-1β is 15-fold more potent than IL-1α, and 500-fold more potent than TNFα and lymphotoxin in mediating bone resorption in vitro."[64]

MMP-8 is a proteolytic enzyme and a marker involved with the breakdown of the extracellular matrix; it is found in connective tissue. MMP-8 has been studied in periodontal disease and is known to be a potent marker for disease activity.[3] Osteocalcin is marker for bone formation, which is sometimes utilized to assess osteoblastic activity in peri-implant crevicular fluid.

During the pathogenesis of periodontal disease, many of these biomarkers may be significant in initiating disease in response to bacterial infection, namely in relationship to gram negative bacteria. LPS begins the recruitment for the first line of defense against PMNs, macrophages and monocytes. Monocytes, in turn activate specific cytokines, i.e. IL-1β, TNF- in the recruitment of more cellular infiltrates and the destructive process. MMPs (matrix metalloproteinase) destroy mineralized tissue during the process of resorption

Despite the extensive literature in this area, the genetic response to peri-implant disease is as yet unclear and incompletely explored. This is why our study is unique, because we are exploring genetic response associated with peri-implantitis.

1.2 Hypothesis of Study:

Aa infection of dental implant abutments can induce peri-implant inflammation associated with peri-implant bone loss. Mutation of A.a virulence alters A.a mediated host inflammation and peri-implant bone loss.

1.3 Significance:

Peri-implantitis is becoming more prevalent as the growing aging population is undergoing replacement of lost dentition by dental implants to restore health and function. Despite research and development to understand this disease, there has not been a consensus within either the periodontal or the larger dental communities with regards to treatment and cure and more importantly the genetic response of this disease. This paper will create a preliminary blue print for potential biomarkers which may be explored as potential sites for host modulation and drug therapy. Additionally, this study will determine if the epithelium is a significant factor in peri-implantitis, and can potentially be a therapeutic area for peri-implantitis.

1.4 Expected Outcomes:

The expected outcome will be to observe differentially expressed genes in both the Aa and mutant group.

2. METHODOLOGY

2.1 Study Design

Aa was grown on trypticase soy agar supplemented with 0.6% yeast extract was used to grow type humidified with 5% Co2 for 3 days. Thirty-six female 5-month year old rats (Sprague-Dawley, Charles River Laboratories, Hollister, CA) were used. Two-piece titanium implants (1.2x3.0 mm) were fabricated. The abutments were attached to the external hex of all implants. The abutments were then surface treated using grit blasting aluminum oxide (50) and hydrofluoric acid. The abutments were then inoculated with either Aggregatibacter actinomycetemcomitans wild type (n=7), or Aa Double deletion mutant of leukotoxin and cytolelthal distending (DS7S-1) (n=7) and sterile sham (n=8) in vitro for 3 days. Implants were installed in maxillary bone of rats. Micro-CT scans were taken at baseline (0), weeks 3 and 8. CT scans were then overlaid to assess for bone loss. Peri-implant tissue was harvested at 8 weeks for RNA sequencing, using polymerase chain reaction was completed after to assess the presence of Aa biofilm.

2.2 PCR

Twenty two samples were used (8-controls, 7-Aa, 7-DM) and RNA isolation kit (RNeasy Mini Kit, Qiagen) was use to isolate high quality mRNA from the three groups. The samples were analyzed for quantity and quality using Bio-analyzer and spectrophotometer.

The libraries were created using (forward) QuantSeq 3’mRNA-seq library kit. 100ng of total RNA per sample was used and amplified over 17 cycles using PCR. The cDNA libraries were then pooled into equimolar concentrations. TapeStation 2200 was used to assess quality check (QC) and re-quantified using the KAPA Library Quantification Kit before loading onto the

sequencer. The sequencing was carried out on Nextseq 500, Illumina, 1x75 nt reads, high output kit, one lane.

2.3 Analysis of mRNA-sequence and QC and quantification

Raw reads were aligned to reference genome rn6 using BWA MEM.[65] Gene expression was quantified using featureCounts[66] as raw read counts. Differential expression statistics (fold-change and p-value) were computed using edgeR[67], on raw expression counts obtained from quantification, and normalized expression was reported in units of counts per million (CPM). Paired wise T-tests were completed, P-values were adjusted for multiple testing using the false discovery rate (FDR) correction of Benjamini and Hochberg.[68] Significant genes were be determined based on an FDR threshold of 5% (0.05).

2.4 Analysis

GO (Gene ontology) and pathway enrichment analysis was completed to identify upregulated and downregulated genes. GO terms combine defined terms using molecular function (MF), biological process (BP) or cellular component (CC). Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) system is used to associate large molecular datasets into genomic clustering. DAVID, was used for data mining and to analyze the gene lists by analyzing genes that were upregulated and down-regulated using $p<.05$. As well as, logFc or fold change for differential expression of genes. Zero value indicates no change, a positive value indicates upregulation and negative value indicates a downregulation of genetic expression.

3. RESULTS

Utilized pair-wise test to determine differentially expressed genes among the Aa wild type group and DM mutant group. The P values were adjusted for multiple testing using false discovery rate (FDR). There were 250 genes upregulated and 99 downregulated genes associated with DM group. The Aa group had 163 genes were upregulated and 138 genes were downregulated, p-value of 0.05. Table 8. MAP Kinase 8, MAP Kinase 4, Bpifb2 (bactericidal/permeability-increasing protein-like 2) was statistically significant in both the wild-type A.a and mutant DM group. Claudin 2 and Dynein 2 was differentially expressed in the mutant DM group exclusively.

Reviewing the DM/control group, the GO and KEGG pathways that were upregulated were mainly associated with structural, cell membrane and tight junction pathways, Claudin and Dynein. Claudin is a protein that is most important part of tight junctions and controls the flow of molecules in the intercellular space. Claudin 2, 3 and 10 were expressed under many different clusters. Claudin forms a seal to prevent water and other molecules from entering (See table 8, 9, 11, 12). It is typically not found in the oral cavity, but found in the GI tract.

Table 8: Differentially expressed genes in Wild Type Aa /control group

Gene ID	Gene Symbol	P-value
ENSRNOG00000020155	MAPK8	1.64E-06

ENSRNOG00000010119	Zmat3	1.4E-03
ENSRNOG00000012197	Bpifb2	2.1E-03
ENSRNOG00000015401	MAPK4	1.4E-03
ENSRNOG00000008510	Abtb2	3.2E-03

Table 9: Differentially expressed genes in Mutant DM/control group

Gene ID	Gene Symbol	P-value
ENSRNOG00000020155	MAPK8	2.08E-07
ENSRNOG00000012197	Bpifb2	3.31E-07
ENSRNOG00000019846	Cldn2	6.57E-05

ENSRNOG00000015401	Dynlrb2	4.9E-03
ENSRNOG00000008510	MAPK4	4.6E-03

Table 10: DM/control 206 DAVID IDs and 30 clusters

Upregulated GO TERMS	Hits	P value	Benjamini score
Tight junction: Claudin 10 (Cldn10), Claudin 2 (Cldn2), Claudin 3 (cldn3), immunoglobulin superfamily	4	1.3E-2	7.0E-1
Growth factor activity: BMP 4, fibroblast growth factor (Fgf1), Interleukin 7, myostatin, Trefoil factor 2	5	4.7E-2	9.8E-1
Cell adhesion molecules (CAMS): Claudin 10 (cldn10), Claudin 2 (cldn2), Claudin 3(cldn3), nectin cell adhesion molecule 3 (Nectin 3)	4	4.5E-2	8.6E-1
Integral component of membrane: aquaporin 5(Aqp5), ATPase sarcoplasmic/endoplasmic reticulum, Claudin 10 (cldn10), Claudin 2 (cldn2), Claudin 3(cldn3)	56	7.1E-1	1.0E0
Osteoblast Differentiation: Bone morphogenetic protein 4 (BMP4), leucine-rich repeat containing G protein coupled protein, Myocyte enhancer factor 2D (Mef2d)	3	3.1E-1	1.0E0

Negative regulation of cell proliferation: BCL2, apoptosis regulator (Bcl2), NK3 homeobox 1 (Nkx3-1), Bone morphogenetic protein 4 (BMP4), forkhead box A3 (Foxa3)	4	6.9E-1	1.0E0
Cytokine activity: bone morphogenetic protein 4 (BMP4), interleukin 19, interleukin 7, myostatin	4	1.8E-1	1.0E0

Table 11: Function of tight junction genes

Tight Junction	Function
Claudin 10 (Cldn10)	Tight junction and cellular membrane. Plays an important role in signal transduction.
Claudin 2 (Cldn2)	Found in leaky epithelium, in particular kidney, intestinal tissue
Claudin 3 (Cldn3)	Cell-cell adhesion and endothelial cells
Immunoglobulin superfamily	Glycoproteins are produced by plasma cells, producing antibodies

Table 12: Growth factor genes

Growth factor	Function
BMP4	Found in tooth development and mesodermal induction, bone formation and repair
Fibroblast growth factor	Functions in cell differentiation, proliferation and is under the control of RAS pathway. Found in tissue repair.[69]
Myostatin	Inhibits myogenesis and is involved in muscular dystrophy.[70]

Interleukin 7	Cytokine involved with B and T cell. It is found in epithelial and intestinal cells.
Trefoil factor 2	Found in intestinal tissue that functions to stabilize GI, functions to restore and maintain damaged areas in the mucosa.

Table 13: Function of cell adhesion molecules

Cell adhesion molecules	Function
Claudin 2 (cldn2), Claudin 3 (cldn3), Claudin 10 (cldn10)	See table 9 .
Nectin cell adhesion molecule 3 (Nectin3)	Regulates cell proliferation, direction, and survival.[71]

Table 14: Integral component of membrane Function

Integral component of membrane:	Function
Aquaporin 5(Aqp5)	Found in lacrimal, salivary and acinar cells. Water channel protein. It is also involved with enamel formation. Fluoride has been suggested to inhibit this protein[72]
ATPase sarcoplasmic/endoplasmic reticulum	Involved with muscular contraction and excitation through calcium release. It is found in most tissues and abundant in GI tract, spleen and lungs.
Claudin 2 (cldn2), Claudin 3 (cldn3), Claudin 10(cldn10)	See table 9. [71]

Table 15: Osteoblast differentiation Genes

Osteoblast Differentiation:	Function

Bone morphogenetic protein 4 (BMP4)	See table 10.[72]
Leucine-rich repeat containing G protein coupled protein	Functions to activate the wnt pathway, which is involved with development of organ systems: the liver, kidney, intestine, bone and eye. It is involved with bone regulation. Negatively regulates the innate immune response and acts as inhibitory agent TLR2/TLR4. Nonsense mutation of this gene is associated with low bone density.[73]
Myocyte enhancer factor 2D (Mef2d)	Functions in T cell selection, cardiac and muscle cells regulation.[71]

Table 16: Negative regulation of cell proliferation

Negative regulation of cell proliferation:	Function
BCL2, apoptosis regulator (Bcl2),	Inhibits caspase activity and apoptosis. [74]
NK3 homeobox 1 (Nkx3-1)	Found in prostate epithelium.[73]
Bone morphogenetic protein 4 (BMP4)	See table 10. [71]
Forkhead box A3 (Foxa3)	Regulates liver metabolism

Table 17: Upregulated KEGG pathways

Upregulated KEGG pathway	Hits	P value	Benjamini score
Cocaine addiction: CAMP responsive element binding protein 3-like 4 (Creb314), DOPA decarboxylase (Ddc)	3	9.5E-2	9.4E-1

Alcoholism: CAMP responsive element, calcium/calmodulin-dependent kinase kinase (Camkk2), dopa decarboxylase (ddc), histone cluster 1, Protein phosphatase 1, regulatory inhibitor subunit 1B (ppp1r1b)	5	1.4E-1	9.4E-1
Cell adhesion molecules (CAMS): Claudin 10 (cldn10), Claudin 2 (cldn2), Claudin 3(cldn3), nectin cell adhesion molecule 3 (Nectin 3) (See table 9)	6	4.5E-2	8.6E-1
Hepatitis C: Claudin 10 (Cldn10), claudin 2 (cldn 2), claudin (cldn3) (See table 9)	3	1.8E-1	9.8E-1
Tight junction: Claudin 10 (Cldn10), claudin 2 (cldn 2), claudin (cldn3) (See table 9)	4	2.2E-1	9.2E-1
Pathways in cancer: BCL2 apoptosis regulator, NK3 homeobox (Nkx3-1), RAS guanyl releasing protein 1 (Rasgrp1), Bone morphogenetic protein 4 (BMP4), Crk-like protein-like (LOC100911248), fibroblast growth factor 1 (fgf1)	6	2.9E-1	9.6E-1
MAPK signaling pathway: RAS guanyl releasing protein 1 (Rasgrp1), RAP guanine nucleotide exchange factor 5 (Rapgef5), fibroblast growth factor 1 (Fgf1), mitogen activated protein kinase 8 (Mapk8)	4	5.7E-1	9.9E-1
Ras signaling pathway: RAS guanyl releasing protein 1 (Rasgrp1), RAP guanine nucleotide exchange factor 5 (Rapgef5), fibroblast growth factor 1 (Fgf1), mitogen activated protein kinase 8 (Mapk8)	4	4.9E-1	9.8E-1

Table 18: Cocaine Addiction and Alcoholism

Cocaine Addiction and Alcoholism	Function
CAMP responsive element binding protein 3-like 4 (Creb314)	Transcription factor.[72]
DOPA decarboxylase (Ddc)	Protein that decarboxylates DOPA to dopamine [73]

Calcium/calmodulin-dependent kinase kinase (Camkk2)	Important hub for signaling pathways, can activate IP3, Toll-like receptors, which regulate cell function see (Figure 3). [75]
Histone cluster 1	Functions to compact chromatin
Protein phosphatase 1	Regulates blood glucose and insulin; it activates glycogen synthase. [76]
Regulatory inhibitor subunit 1B (ppp1r1b)	Involved with signal transduction and regulates dopamine and CaMP phosphorylation

Table 19: Pathways in cancer function

Pathways in cancer:	Function
BCL2 apoptosis regulator	Transcription factor. [72]
NK3 homeobox (Nkx3-1)	See Table 14. [73]
RAS guanyl releasing protein 1 (Rasgrp1)	Regulates B and T cell development through activation of Erk/MAP kinase cascade. [75]
Bone morphogenetic protein 4 (BMP4)	See table 10.
Crk-like protein-like (LOC100911248)	Not Applicable. [76]
fibroblast growth factor 1 (fgf1)	Protects the cell from trauma and induces cell growth, tissue repair

Table 20: MAPK signaling pathway function

MAPK signaling pathway:	Function
RAS guanyl releasing protein 1 (Rasgrp1)	See Table 17. [72]
RAP guanine nucleotide exchange factor 5 (Rapgef5)	See Table 14. [73]
fibroblast growth factor 1 (Fgf1)	See Table 17.
Mitogen activated protein kinase 8 (Mapk8)	Specific cytokine for serine/threonine and is responsible for regulating gene expression, pro-inflammatory cytokines and other processes, including cell death. [76]

There were 82 DAVID IDs and 6 clusters that were downregulated in DM/control group.

Table 21: Cluster associated with DM/Control

Downregulated GO TERMS	Hits	P Value	Benjamini score
Collagen trimer: collagen II alpha 1 chain (Col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1)	3	2.2E-2	9.2E-1
Basement membrane: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1)	3	5.0E-2	9.4E-1
Extracellular matrix: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1) (See table 20)	3	2.5E-1	1.0E0
Golgi membrane: carbohydrate sulfotransferase 15 (Chst15), cytochrome p450, family 2, subfamily	4	1.1E-1	9.9E-1
Integral component of membrane: ATP binding cassette subfamily A member 2(Abca2), Cd300 molecule-like family member F (Cd300f), cytochrome p450, family 2, subfamily e, polypeptide 1 (cyp2e1)	21	7.3E-1	1.0E0

Table 22: Collagen trimer function

Collagen trimer:	Function
Collagen II alpha 1 chain (Col2a1)	Found in cartilage formation; cartilage is able to resist compressive forces. [72]

Collagen type IV alpha 2 chain (col4a2)	Found in the basement membrane, separates the basement membrane from connective tissue "Canstatin, a cleavage product corresponding to the collagen alpha 2(IV) NC1 domain, possesses both anti-angiogenic and anti-tumor cell activity. It inhibits proliferation and migration of endothelial cells, reduces mitochondrial membrane potential, and induces apoptosis."[73]
Collagen type V alpha 1 chain (col5a1)	Member of group I collagen (fibrillar forming collagen). It binds to heparin with strong affinity.

Table 23: Golgi apparatus gene function

Golgi apparatus:	Function
Carbohydrate sulfotransferase 15 (Chst15)	Functions to transfer C-6 hydroxal group of sulfotransferase to chondroitin sulfate May function on B-cell surface. [72]
Cytochrome p450, family 2, subfamily e, polypeptide 1 (cyp2e1)	Involved with metabolism of xenobiotic material. [73]

Table 24: Integral component of membrane

Integral component of Membrane	Function
ATP binding cassette subfamily A member 2(Abca2))	Involved with sterol and lipid metabolism. There has been a link to Alzheimer's disease but role is unknown.[77]
Cd300 molecule-like family member F (Cd300f)	Inhibits myeloid and mast cells. It is involved with inhibiting osteoclast cells.[73]
Cytochrome p450, family 2, subfamily e, polypeptide 1 (cyp2e1)	Functions in liver cells and metabolizes xenobiotics.

Table 25: Downregulated KEGG Pathway

Downregulated KEGG PATHWAY	Hits	P Value	Benjamini score
Protein digesion and absorption: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1) (See table 20)	3	2.4E-2	4.9E-1
ECM receptor interaction: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1) (See table 20)	3	2.4E-2	4.9E-1
Amboebiasis: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1) (See table 20)	3	3.6E-2	5.0E-1
PI3K-Akt signaling pathway: collagen II alpha 1 chain (col2a1), collagen type IV alpha 2 chain (col4a2), collagen type V alpha 1 chain (col5a1) (See table 20)	3	2.3E-1	9.5E-1

AA/control:

The AA/control 258 David terms were upregulated and 25 clusters.

144 David IDS with 10 clusters associated with upregulated genes:

Table 26: Upregulated pathways

Upregulated pathway	Hits	P value	Benjamini score
Magnesium: ATPase phospholipid transporting 11B (Atp11b), ATPase phospholipid transporting 8B3 (Atp8b3), DNA cross-link repair 1C (Dclre1c), Epoxide hydrolase 2 (Ephx2), haloacid dehalogenase-like hydrolase domain containing 2 (Hdhd2) Serine/threonine kinase 3 (Stk3)	6	7.5E-2	8.9E-1
Epidermal growth factor: CD248 molecule (Cd248), LDL receptor related protein 5 (Lrp5), Matrilin 4 (Matn4), mucin 13, cell surface associated (Muc13)	4	1.8E-1	1.0E0
Immunoglobulin domain: IgLON family member 5(Iglon5), MER proto-oncogene, tyrosine (Mertk), Neogenin 1 (Neo1), RT1 class Ia, locus A2(RT1-A2)	6	3.3E-1	1.0E0
Protein kinase-like domain: MER proto-oncogene, tyrosine kinase (Mertk), PAN3 polyA specific ribonuclease subunit (PAN3), calcium/calmodulin-dependent protein kinase kinase 2 (Camkk2), mitogen-activated protein kinase 8 (mapk8), protein kinase domain containing, cytoplasm (Pkdcc), serine/threonine kinase 3 (stk3)	6	3.1E-1	1.0E0

Table 27: Function of Magnesium genes

Magnesium	Function of genes:
ATPase phospholipid transporting 11B (Atp11b)	N/A
ATPase phospholipid transporting 8B3 (Atp8b3)	N/A
DNA cross-link repair 1C (Dclre1c)	N/A
Epoxide hydrolase 2 (Ephx2)	plays a role in xenobiotic metabolism by degrading toxic epoxides
Haloacid dehalogenase-like hydrolase domain containing 2 (Hdhd2)	N/A
Serine/threonine kinase 3 (Stk3)	pro-apototic. It is activated during stress.

Table 28: Function of epidermal growth factor

Epidermal growth factor:	Function
CD248 molecule (Cd248),	CD28 codes for the protein called Endosialin, which is seen in angiogenesis and tumor growth and progression.
LDL receptor related protein 5 (Lrp5)	important part of the Wnt signaling pathway and is involved with osteogenesis—changes in this gene will lead to changes in bone mass. A loss of function leads to osteoporosis.
Mucin 13, cell surface associated (Muc13)	Protect the body from pathogens. Overexpression is found in breast, lung, ovarian, colon cancer. It is a marker for early detection of cancer.
Matrilin 4 (Matn4)	Involved with wound healing in pulpal dentin complex
MER proto-oncogene, Tyrosine (Mertk)	Part of protein kinase family. Involved with phagocytosis in the retina.
Neogenin 1 (Neo1)	Involved in regulation of inducing differentiation from undifferentiated cells and may function in cell adhesion.
RT1 class Ia, locus A2(RT1-A2)	Functions like MHC Class I family in foreign presentation of antigens

Table 29: Protein kinase like domain gene

Protein kinase like domain	Function
MER proto-oncogene, tyrosine kinase (Mertk)	Regulates cell proliferation, differentiation and phagocytosis
PAN3 polyA specific ribonuclease subunit (PAN3),	Protein; function unknown
Calcium/calmodulin-dependent protein kinase kinase 2 (Camkk2)	Table 16.
Mitogen-activated protein kinase 8 (mapk8)	See Table 18.
Protein kinase domain containing, cytoplasm (Pkdcc)	Seen in over 22 tissue types
Serine/threonine kinase 3 (stk3)	Activated during stress, apoptotic kinase

Table 30: 114 David IDs with 15 clusters associated with <u>downregulated genes:</u>

Downregulated pathways	Hits	P value	Benjamini score
Cytokine: C-C motif chemokine ligand 19 (ccl19), CD40 ligand (Cd40lg), bone morphogenetic protein 4 (Bmp4), colony stimulating factor 1 (Csf1)	4	2.0E-2	6.2E-1
Cytokine activity: CD40 (Cd40lg), bone morphogenetic protein (BMP4), colony stimulating factor 1 (Csf1)	3	1.8E-1	9.9E-1
Activation of MAPKK activity: bone morphogenetic protein 4 (Bmp4), eukaryotic translation initiation factor 2-alpha kinase, presenilin 1 (Psen1)	3	2.9E-2	9.9E-1
Negative regulation of apoptotic process: ADAM metallopeptidase with thrombospondin type 1 motif, 20 (Adamts20), ALMS1, centrosome and basal body associated protein (Alms1), CD40 ligand (Cd40lg), bone morphogenetic protein 4 (Bmp4), ceroid-lipofuscinosis, neuronal 3(cln3), eukaryotic translation initiation	7	4.0E-2	9.9E-1

factor 2-alpha kinase 2 (Eif2ak2), presenilin 1 (Psen1)			
Cell-cell junction: FER tyrosine kinase (Fer), cyclin dependent kinase 16 (Cdk16), leucine-rich, glioma inactivated 1 (Lgi1), Presenilin 1 (Psen1), synaptotagmin 11 (Syt11)	5	1.8E-1	9.5E-1
Lysosome: ceroid-lipofuscinosis, neuronal 3 (cln3), heat shock protein family A (Hsp70) member 8 (Hspa8), synaptotagmin 11 (syt11)	3	3.8E-1	9.7E-1
Positive cell regulation of cell proliferation : FER tyrosine kinase (Fer), bone morphogenetic protein 4 (Bmp4), colony stimulating factor 1 (Csf1)	3	7.0E-1	1.0E0
Membrane: CD40 ligand (Cd40lg), Cd300e molecule (cd300e), FER tyrosine kinase (Fer), GRAM domain containing 4(Gramd4), N-acetyltransferase 14 (Nat14), URB2 ribosome biogenesis 2 homolog (S. cerevisiae) (Urb2), adhesion G protein-coupled receptor E1 (Adgre1), aldehyde dehydrogenase 3 family, member A2 (Aldh3a2), anoctamin 2(Ano2), arylsulfatase E (Arse), cache domain containing 1 (Cachd1), ceroid-lipofuscinosis, neuronal 3 (cln3), coatomer protein complex subunit gamma 1(copg1), coenzyme Q8A(coq8a), colony stimulating factor 1 (csf1), ectotropic viral integration site 2B (Evi2b), exocyst complex component 7 (Exoc7), glycoprotein 2(Gp2), heat shock protein family A (Hsp70) member 8(Hspa8), hydroxysteroid (17-beta) dehydrogenase 6 (Hsd17b6), membrane spanning 4 domains subfamily A member 4A-like (LOC100911403), microsomal glutathione S-transferase 3 (Mgst3), mitochondrial calcium uniporter (Mcu), paroxysmal nonkinesigenic dyskinesia (pnkd), potassium voltage-gated channel subfamily D member 1(Kcnd1), presenilin 1 (Psen1), prostate androgen-regulated mucin like protein 1 (parm1), protocadherin alpha 4 (Pcdha4), purinergic receptor P2X5(p2rx5), secretogranin II (Scg3), synaptotagmin 11 (Syt11), tuberous sclerosis 1(Tsc1)	32	9.5E-1	1.0E0

Table 31: Innate immunity, innate immunity response

Innate immunity, innate immune response:	Function
Colony stimulating factor 1 (Csf1)	Involved with osteoclastogenesis and endochondral ossication. CSF1 promotes angiogenesis at chondrossseous junction. It also induces monocyte and macrophage lineage
Eukaryotic translation initiation factor 2-alpha kinase	Serine/threonine protein kinase are encoded by this gene. The activated form can inhibits protein synthesis.
RT1 Class Ia, locus(RT1-A2)	In the family of MHC-1, involved in presentation of foreign body
Adhesion G protein-coupled receptor E1 (Adgre1)	Belongs to G-protein coupled receptor 2. It is involved with cell-cell adhesion.

Table 32: Cytokine, cytokine inflammatory response

Cytokine, cytokine inflammatory response and cytokine-cytokine receptor interaction	
Bone morphogenetic protein 4 (BMP4)	Induces bone formation and cartilage
Colony stimulating factor (Csf1)	Involved with osteoclastogenesis and endochondral ossification. CSF1 promotes angiogenesis at chondrossseous junction. It also induces monocyte and macrophage lineage
C-C motif chemokine ligand 19 (Ccl19)	Induces mesenchymal bone marrow stem cell migration
CD40 ligand (Cd40lg)	Aids in B cell stimulation and IgE production
Mitogen-activated protein kinase 8 (Mapk8)	Plays a key role in T cell differentiation and apopotosis. Additionally, it is activated by TNFα and induced TNFα apoptosis.

Table 33: Positive regulation of cell proliferation

Positive regulation of cell proliferation	
FER tyrosine kinase (Fer)	Tyrosine protein kinase plays an important role in cell adhesion, cell migration and chemotaxis. It is also involved with mast cell regulation, recruiting leukocytes, promotes activation of NF-kappa-B
Colony stimulating factor (Csf1)	Involved with osteoclastogenesis and endochondral ossification. CSF1 promotes angiogenesis at chondrossseous junction. It also induces monocyte and macrophage lineage
Bone morphogenetic protein 4(Bmp4)	Induces bone formation and cartilage

Table 34: Membrane gene function

Membrane Gene function	
CD40 ligand (Cd40lg)	Aids in B cell stimulation and IgE production
Cd300e molecule (cd300e)	Expressed on myeloid cells and can activate proteins through protein tyrosine kinase binding protein
FER tyrosine kinase (Fer)	Regulates cell-cell adhesion
GRAM domain containing 4(Gramd4)	Inducing apoptosis
N-acetyltransferase 14 (Nat14)	Initiating transcription
URB2 ribosome biogenesis 2 homolog (S. cerevisiae) (Urb2),	Not available
Adhesion G protein-coupled receptor E1 (Adgre1)	Adhesion molecule
Aldehyde dehydrogenase 3 family, member A2 (Aldh3a2)	Detoxifying gene involved in lipid solubility and alcohol metabolism
Anoctamin 2(Ano2), arylsulfatase E (ARSE) [78]	Provides information for sulfatases, which are involved in cartilage and bone formation. May also play a role in Vitamin K and maintenance of bone density.
Cache domain containing 1 (Cachd1)	May regulate calcium channels
Ceroid-lipofuscinosis	N/A
Coatomer protein complex subunit gamma 1(copg1)	Lipid regulation
Coenzyme Q8A(coq8a)	Electron transport in mitochondrial matrix. It responds to tumor suppressor P53 during DNA damage, suppressing p53 apopotosis.
Colony stimulating factor 1 (csf1)	Involved with osteoclastogenesis and endochondral ossification. CSF1 promotes angiogenesis at

	chondrossseous junction. It also induces monocyte and macrophage lineage.
Ectotropic viral integration site 2B (Evi2b)	Found in lymphoid and neural cells.
Exocyst complex component 7 (Exoc7)	Targets post-golgi vesicles to plasma membrane, plays an important trafficking and secretion of proteins.
Glycoprotein 2(Gp2)	Plays an important role in innate immunity found in M cells in enteric system.
Heat shock protein family A (Hsp70) member 8(Hspa8	it helps with folding misfolded proteins
Hydroxysteroid (17-beta) dehydrogenase 6 (Hsd17b6),	N/A
Membrane spanning 4 domains subfamily A member 4A-like (LOC100911403	Expressed on B cells and B cell ontogeny
Microsomal glutathione S-transferase 3 (Mgst3),	Important mediator of inflammation and production of leukotrienes and prostaglandin
Mitochondrial calcium uniporter (Mcu)	Important for calcium uptake from the cytosol into the mitochondria
Paroxysmal nonkinesigenic dyskinesia (pnkd),	N/A
Potassium voltage-gated channel subfamily D member 1(Kcnd1	Regulation of transmembrane potassium channel activity
Presenilin 1 (Psen1)	N/A
prostate androgen-regulated mucin like protein 1 (parm1)	N/A
Protocadherin alpha 4 (Pcdha4)	Genetically similar to B and T cells receptor gene clusters Plays a role in cell-cell function in the brain
Purinergic receptor P2X5(p2rx5) [79]	Regulates osteoclasts and excessive bone loss during osteoclast inflammatory bone loss.
Secretogranin II (Scg3)	N/A
Tuberous sclerosis 1(Tsc1)	Inhibits cell growth

4. Discussion

The pathogenesis of peri-implantitis is a complex process that involves an array of bacterial species largely dependent on the susceptible host and an imbalanced shift. There is a lot of emerging data regarding this disease, which still remains elusive. Lindhe[17], found that periodontitis and peri-implantitis are diseases that are caused by bacteria. Similarly, Maruyama concluded similar findings using core-microbiota from peri-implant and periodontitis patients. [80]

Our study evaluated the gene profiling of Actinobacillus actinomycetemcomitans (Aa) wildtype and mutant Aa (DS7-1) on peri-implantitis induced model. Aa is an aggressive, gram-negative, non-motile, capnophilic pathogen, often associated with aggressive forms of periodontitis . It has the ability to produce an immune response and impair the integrity of host tissue.[81] It produces a leukotoxin, epitheliotoxin, bone resorption toxin, cytolethal toxin, and apoptoic toxin. Cytolethal toxins produce heat labile toxins.[82] Currently, the literature is limited on the induction of peri-implantitis and genetic response—many studies induce peri-implantitis via ligature wire.[83] Results of our study showed a positive association of increased inflammatory signaling pathways in response to the bacterial insult, particularly at the epithelium, resulting in peri-implantitis.

Aa LPS initiates the host response by interacting with cell surface molecules CD14 and toll like receptors mainly through TLR-4.– activating Map Kinase pathway, which stimulates the activity of various cytokines including: IL-1b, TNF-alpha, IL-6, NFkb, nitric oxide and Cox2 pathway. These cytokines were upregulated in periodontal site.[64] Additionally, they orchestrate the activation of osteoclasts through RANKL and NFkb, ultimately resulting in bone loss.

MAP kinase 4 and 8 was differentially expressed in both wild type and mutant Aa and is associated with inflammation and bone loss.[84] MAP kinase pathway has the ability to modulate cytokine, stress response, cell proliferation, and cytoskeletal organization. MAP kinase is activated through a cascade of interactions with GTPase or by phosphorylation. It can be categorized into three sub-groups. ERKs (extracellular-signal-regulated kinases), JNKs (Jun amino-terminal kinases), and P38/SAPs (Stress-activated protein kinases).[85] They are involved in the cellular response to pathogens in periodontal disease. In a cascade of two kinases MAP2K4/MKK4 and MAP2K7/MKK7 activate MAPK8/JNK1. This is initiated in response to stress, UV light or to pro-inflammatory cytokines, such as, IL-1 , EGF and TNF-a. Interestingly, "invasive process of pathogenic bacteria is frequently associated with MAP kinase signaling activity. Infection of epithelial cell lines with Listeria monocytogenes, salmonella enterica (serovar Typhimurium), or enteropathogenic Escherichia coli induces activation of ERK1/2, JNK, and P38 MAP kinase." [86]

Lipopolysaccharides can activate Map kinase 8 or the JNK pathway. Furthermore, "in all MAPK signal transduction cascades play a pivotal regulatory role in the biosynthesis of numerous cytokines, chemokines, and other inflammatory mediators that are necessary for the immune system to combat pathogenic infections."[87] Gee found that LPS-mediated pathways involved JNK for monocytes expressing CD44.[88] Porphyromonas gingivalis(Pg) (table 6) is found both in periodontitis and peri-implantitis. It activated MAPK-JNK within five minutes of exposure. This was associated with its pathogenicity and invasiveness.[86] JNKs play a role in inflammation, apoptosis and differentiation. It is associated with migration of EGF, along with cellular migration. JNK was inhibited by chemical inhibitor SP600125 and JNK1AF (knockout gene),

which impaired cellular migration.[89] In a similar knockout mice study, the researchers found that fibroblast had lower ability to close wound sites. This exhibited the critical importance of JNK to induce cellular migration in wound healing.[90] The role of MAP Kinase 8, which was upregulated in both wild type Aa and mutant DM group may fundamentally play a critical role in inducing inflammation. Although, Map kinase 4 is an "atypical MAPK" which has not been previously implicated in bacterial infections.

Cell cycle genes were upregulated in the wild type Aa group. Zmat3, also known as Wig1 or WT p43 induced gene (PAG608). This gene targets p53/p21, which are involved with regulation of cell cycle, tumor growth and mRNA stability. Wig1 binds to AU-rich elements (ARE's). "ARE's are regulatory elements, which have been implicated in post-transcriptional modification by degrading mRNA or by decreasing its efficiency."[91] Wig1 is upregulated in response to stress and was found to arrest cell cycle, rather than cell death during DNA damage.[92] In the absence of Wig1, there was increasing cell death and senescence. It is also amplified in certain tumors, particularly in cervical SCC, human papillary thyroid carcinoma, and in lung squamous cell carcinoma. Cellular senescence and carcinogenesis can occur due to cellular damage or stress through p21. Additionally, in HeLa cells, a murine B-cell hybridoma cells, Cdt from Aa was able to arrest cell cycle and B-lineage cells through P53 pathway.[92] Similarly, Cdt can induce cell cycle arrest in human fibroblast cells, suggesting the virulence factor of Aa may impair wound healing.

Bactericidal/permeability-increasing Fold Containing Family B Member 2 (Bpifl2), or innate defense peptide, is known to be expressed by oral epithelium. It was upregulated in both the

wild type Aa and mutant Aa group. The innate immune response begins with the complement pathway to combat local infections. Polymorphonuclear leukocyte (PMNs) function in a non-specific manner at an attempt to control bacterial insult. They can potentially arrest the initiation of periodontal disease via two pathways: oxidative and non-oxidative. This peptide works against gram-negative bacteria intracellularly.[93] It works by binding to lipopolysaccharides and destroying the bacterial cell wall. This antibacterial protein may play a critical role in epithelium mediated innate immune response.

The mutant Aa differentially expressed two additional proteins: Claudin-2 and Dynein. Claudin-2 is a tight junction protein that regulates water and cation movement. Claudin -2 is upregulated and internalized in response to toxins like *E.coli, H. Pylori* and *Clostridium perfingens* enterotoxins, suggesting that it plays a role in inflammatory bowel diseases (IBD).[94] This specific protein has never been explored in the oral cavity. However, Claudin-1 is influenced by P. gingivalis infection.[95] This impacts the epithelial seal making it more permeable and less resistant to bacterial insult. Moreover, altered tight junction expression is usually seen in chronic periodontitis.

Dynlrb2 or Dynein participates with an array of cellular functions, involving microtubule based movement.[96,97] In a study that evaluated light chain Dynein and concluded that it is an essential protein for mediating murine leukemia virus (MLV).[98] Similar findings related to the role of dynein and its ability to propel herpes simplex virus capsid into the nucleus.[99] The role of dynein maybe modulated by bacteria, too.

Summary of discussion:

Our study demonstrated the role of Aa in inducing the innate immune response at the epithelium, which ultimately resulted in peri-implantitis. Wild type Aa and mutant Aa induced responses that were associated with TLR4 receptors suggesting that they are signaling through conserved innate immunity mechanism. Mutant Aa appeared to strongly induce mucosal expression of Wig-1, a modulator of P53 function. Finally, the mutant Aa also induced upregulation of tight junction protein Claudin-2 and a component of Dynein, which controls microtubule function. The mutant Aa induced gene expression associated with rapid epithelial turnover and diminished epithelial structural integrity, was also seen with GO terms. This implied an impact on the structural function of the epithelium. Aa was able to induce innate immune response at the peri-implant tissues, and changes that are associated with pro-inflammatory pathophysiology. Perhaps, the epithelium can be a target area for therapy.

Limitations:

Limitations of this study that may have affected the outcome:

1) We need to replicate this study on a larger scale to ensure similar findings
2) Use of additional bacteria to assess and compare the genetic profiling
3) Animal studies can control for variables

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