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Sexual dimorphism in periodontitis in a mouse model.

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SEXUAL DIMORPHISM IN PERIODONTITIS IN A MOUSE MODEL

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DEDICATION

I would like to thank all those who have provided support and encouragement along my career path. Thank you to my loving parents for always being there. The values they instilled are ever present today. My endurance and persistence in reaching my career goals is in large thanks to them. Family, friends, mentors, and my eternal father I thank you all for being there and keeping my eyes on the finish line

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ABSTRACT

SEXUAL DIMORPHISM IN PERIODONTITIS IN MOUSE MODEL

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Background: Periodontal disease is an infection-driven chronic inflammatory disease. It occurs primarily from excessive inflammatory reactions that arise from complex exchanges between the host immune system and the tooth associated oral bacteria. It is the number one cause of tooth loss among adults[1, 2]. However, many factors confound results between males and females as to which sex is more susceptible to periodontal disease. Identifying the sex more prone to disease is integral in developing models of risk assessment and looking into the pathogenesis.

Objective: To examine in vivo and in vitro the differences between male and female mice challenged with *Porphyromonas gingivalis*.

Methods: Mice were divided into four groups consisting of male ligated, male non-ligated, female experimental ligated, and female non-ligated. All groups were given a ligature around the second molar. Experimental groups were administered *P. gingivalis* while the controls being non-ligated. After 7 days, mice were euthanized and bone losses were determined. Bone losses were measured by taking the distance from the

cementoenamel junction (CEJ) to the alveolar bone crest (ABC). Colony Forming Units (CFUs) were acquired from all groups as well. CFUs displayed bacterial clearance amongst the male and female mice. An oral gavage infection model was administered to confirm our ligature model results. Four groups were developed consisting of male experimentals, male sham infections, female experimentals, and female shams. From an in vitro perspective gingival tissue was harvested from each mouse and cytokine response levels were measured. Cytokine levels served as a method of looking into the immune system's role in periodontal disease.

Results: In comparison with male mice, female mice displayed significantly increased periodontal bone loss ($p < 0.05$) in both the ligature and oral gavage models, accompanied by elevated expression of pro-inflammatory cytokines. More oral bacteria were also detected in female mice than in males. In vitro experiments showed that macrophages from female mice respond to *P. gingivalis* with higher intensity. These findings along with the previously mentioned may contribute to more severe inflammation and bone losses in females.

Conclusion: Female mice are more likely to exhibit persistent *P. gingivalis* infection, and develop higher levels of bone loss in comparison to their male counterparts. These occurrences differ from human studies in which males are the predominant gender for this disease.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Periodontal disease is a disease of the oral cavity that is caused by bacterial infection. Periodontal disease is plaque induced, and results in gingival inflammation, tissue destruction, and alveolar bone resorption [3-6]. Alveolar bone is responsible for root support and stability of each tooth, and the resorption of underlying bone leads to eventual tooth loss [2]. Periodontal disease is the leading cause of tooth loss among adults [1, 2], and according to the University of Maryland Medical Center is estimated to afflict 75% of the American population in its various forms. The disease arises through a complex interaction between the host immune system and the oral bacteria associated within the mouth. Periodontal disease is associated with systemic diseases, including heart disease, diabetes, lung disease, and pregnancy complications [7].

Periodontal disease is a complex disease that is caused by collateral tissue damage from the pathogen-induced host immune system. The disease itself has numerous extraneous factors that are involved in disease susceptibility. These include stress, sex, age, smoking, alcohol consumption, and the above-mentioned conditions of heart disease, diabetes, and etc. The mechanisms of periodontal disease are intricate and much is still left to learn about this disease. Animal models can serve as useful tools for periodontal

disease research by eliminating extraneous variables presented in humans. Animal models serve as a means for immune system examination. Specifically targeting the immune system's affect on the host.

Gender's role in periodontal disease is controversial. There are reports revealing females to be of higher susceptibility [8, 9], and conversely there are reports pointing to males being the sex of higher susceptibility [10, 11]. These discrepancies are the manifestation of gender-related habits, such as smoking [12], alcohol consumption [13], and oral hygiene habits. Overall, in human studies males serve as the gender more susceptible to periodontal disease. There are exceptions to this with women being more susceptible to periodontal disease during pregnancy, in puberty, and in menstruation, but these are a different form of disease. Periodontitis in pregnant women causes premature and underweight deliveries [14]. Furthermore, women suffer more from autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, lupus, Hashimoto's thyroiditis, and etc.[15]. Comprehending gender's role in periodontal disease is ideal for developing methods of treatment, understanding its pathogenesis, and formulating models of risk assessment.

Periodontal disease and *Porphyromonas gingivalis*

Initiation and progression of periodontal disease requires subgingival bacteria. These bacteria are found in dental plaque, and induce the periodontal host response [16, 17]. The oral cavity is one of the most ecologically complex systems within the human body. Ample numbers of bacteria live within the oral cavity. It's estimated that over 700 bacterial species inhabit the human mouth; living on the surfaces of our teeth, cheeks, gums, and tongue [18] Surprisingly, the majority of the bacterial species within the oral cavity are common inhabitants and are not pathogens. These bacteria live in harmony

with the host, and are known as symbionts. It was initially thought for disease a sole bacterium was responsible for its cause. A one germ, one disease paradigm served as the initial theory. It's now thought that the loss of an advantageous bacterium may prove just as detrimental to the host, as acquiring a destructive bacterium in disease occurring situations.

Dental plaque is defined as "matrix enclosed bacterial populations adherent to each other and to/or surfaces or interfaces", also known as microbial biofilms [19]. These microbial biofilms cover the surface of our teeth. This occurs by microcolonies of bacteria secreting a sticky substance responsible for bacterial adherence. This sticky, glue-like substance is composed of polymers like proteins, nucleic acids, and polysaccharides.

Two types of biofilms are present within the oral cavity. Supragingival plaque inhabits the area above the gingival margin, and subgingival plaque is found below the gingival crevice (the space between the tooth and gingival tissue) [6]. Supragingival plaque is subject to more oral abrasions and saliva flow, which possess defensive components, in comparison to subgingival plaque. Subgingival plaque lives within a protected environment. An environment that is more conducive to growth, for the organisms are protected from the outside forces of saliva and oral abrasions. There are, however, components that limit the growth of subgingival plaque which include space and the innate immune system. As subgingival plaque increases, so too does its available space. The biofilm growth results in an increase in the gingival sulcus pocket and epithelial cell attachment loss [20].

To survive and grow within its niche, pathogenic bacteria need the proper nutrients and subversion methods, such as virulence factors, for eluding the host immune system [21]. Research has shown the virulent factors of *P. gingivalis* include LPS, fimbriae, and gingipains [22]. The host immune system, which resembles a surveillance system, identifies and locates foreign pathogens that are detrimental to the host. It then uses counter-attack measures to deter the invasive pathogens. Bacteria must evade defensive measures present in the oral cavity for survival. *Porphyromonas gingivalis* (*P. gingivalis*), within the gingival sulcus, interacts with the host immune system, and takes extreme measures in avoiding detection [4, 23].

According to the American Academy of Periodontology, there are various forms of periodontal disease. The different forms of periodontitis include aggressive periodontitis, chronic periodontitis. These two forms of periodontal disease are identical in many clinical aspects. Both chronic and aggressive periodontitis manifest from plaque formation lying at and below tooth surfaces of susceptible hosts. Furthermore, the oral microbes that make up the plaque biofilm within the mouth are comprised of indigenous host organisms. The physical effects of chronic and aggressive periodontal disease are a result of a host's immune and inflammatory responses [24]. Differences between chronic and aggressive periodontitis include age of onset, progression rates, patterns of destruction, inflammation characteristics, and plaque and calculus abundance within the host. Combinations of these aforementioned characteristics classify individuals into three forms of periodontal disease, which are chronic periodontitis, localized aggressive periodontitis, and generalized aggressive periodontitis [24].

The progression of chronic periodontal disease symptoms is slow and the patients tend to be older. Aggressive periodontitis consists of two forms that are considered two different diseases. Localized and generalized aggressive periodontitis are discrete from one another. The localized form tends to have minimal signs of inflammation and a thin layer of plaque formation around infected teeth. Contrarily, intensive inflammation and high levels of biofilm formation mark generalized aggressive periodontitis. These two forms of aggressive periodontitis typically affect juveniles and result in rapid levels of development [24]. Rates of progression for aggressive periodontal diseases are estimated to be 3 to 4 times that of chronic periodontal disease [25].

The host response is able to clear or control bacteria, as well as cause collateral tissue destruction [26, 27]. Immune cells such as neutrophils, macrophages, T cells are able to produce pro-inflammatory cytokines, which are a causative agent for periodontal disease. Pro-inflammatory cytokines lead to bone resorption by disrupting the balance between osteoblasts and osteoclast cell types. Equilibrium between these cell types must be achieved for healthy bone levels to occur. Infiltration of T cells, macrophages to the site of infection and, or the presence of TNF-α serve as indicator of chronic periodontal disease. The host response is extremely complex and involves both innate immunity and adaptive immunity. These immune cells promote inflammation and osteoclast production, which ultimately leads to the final stage of periodontal disease, bone loss. *Porphyromonas gingivalis* is found in dental biofilms; it is believed to one of the most important etiological pathogens causing inflammation and tissue destruction in periodontal disease. *P. gingivalis* is a non-motile, gram-negative, rod shaped, anaerobic

pathogenic bacterium. *P. gingivalis* can be found in periodontal disease lesions and is able to induce periodontal disease in animal models [28].

P. gingivalis is one of the bacteria in the "red" complex noted by Socransky et. al, 1998. The species of the "red" complex, include *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*, all showed strong detection with one another, and were discovered in periodontal locations [29]. *P. gingivalis* is also described as a keystone pathogen that serves a fundamental role in the structural maintenance of microbial community [2]. Without *P. gingivalis*, the oral community would not break into pieces, but rather the pathogenicity would be altered, as well as its ability to cause periodontal disease [30]. This bacterial pathogen is now recognized as opportunistic pathogen within the oral cavity. It is capable of host cross-talk between Toll Like Receptor 2 (TLR2) and CXCR4 (a chemokine receptor) due to its fimbriae[31]. The fimbrae bind to CXCR4, which initiates cross talk between the CXCR4 receptors and TLR 2 receptors. Ultimately, this signaling causes inhibition of pro- and anti-inflammatory cytokines. This allows for immune system's defensive measures to be repressed, and provides an environment for *P. gingivalis* to thrive. This survival strategy prolongs life for of the pathogen and provides an opportunity for growth and success, an opportunity for the pathogen to find its niche. The niche for *P. gingivalis* occurs within the periodontal pocket of the host, and is known as the gingival sulcus [22].

During progression of chronic periodontal disease, *P. gingivalis* undermines host immunity to facilitate its colonization and invasion. To establish a chronic infection, *P. gingivalis* develops strategies to evade or subvert host immunity. Its virulent factors possess the ability to manipulate the host response. Toll-like receptors (TLRs) are a

family of pattern-recognition receptors (PRRs) that recognize conserved microbial structures microbe- associated molecular patterns (MAMPs). LPS, lipopolysaccharides, from *P. gingivalis* contains atypical lipid A structures and signals through Toll-like receptor (TLR) 2 [32]. This is different from *E. coli* LPS. A study one *P. gingivalis* and *E. coli* LPs, revealed *P. gingivalis* LPS induced lower E-selectin expression on neutrophils than *E. coli* LPS [33], resulting in inefficient recruitment of neutrophils to site of infection. In macrophage, P. *gingivalis* LPS weakens TLR signaling by upregulating IRAK-M, a negative regulator of TLR signaling [34]. Virulence factors like gingipains are able to degrade TLR and cytokines [35]. Fimbriae from LPS binds to CXCR4 and instigates a crosstalk between CXCR4 and TLR2, resulting in the suppressed *P. gingivalis* clearance [36]. With the well-developed evasion strategies, *P. gingivalis* is able to survive the hostile host environment and causes chronic infection. The chronic infection with P. *gingivalis* and other associated pathogens results in initial stages of inhibited immune response, followed by persistent inflammation leading ultimately to alveolar bone loss.

Immune System Recognition

Although oral pathogens initiate periodontal disease, unregulated host immune responses cause the inflammation, resulting in collateral tissue damage and bone loss. There are two types of defense within jawed mammals. They are innate and adaptive immunity, the latter of which is also known as acquired immunity. The innate component is mediated by pattern recognition receptors (PRRs). These pattern-recognition receptors are transmembrane proteins which detect unique structures pertaining to a pathogen, known as pathogen-associated molecular patterns (PAMPs) [37, 38]. PAMPs of microorganisms include LPS, peptidoglycan, and lipoproteins [38].

Macrophages are one of the most important innate immune cells, and have a major role in host defense [39]. They have been found in periodontal diseased sites, and are associated with tissue resolution and homeostasis [40]. The functions of macrophages include eliminating invading bacteria, cell recruitment to infected sites, the removal of the surplus of neutrophils, cytokine and chemokine synthesis, and lymphocyte mediated adaptive immune response[40]. Like other immune cells, macrophages express PRRs and recognize PAMPs on pathogens [41].

Toll-like receptors (TLRs) identify friend from foe in the host system [42].Toll like receptors are key to a host's defenses. The periodontium (the tissues that surround the teeth, which are critical to tooth support) are constantly exposed to pathogens through dental plaque. Toll-like receptors are present in periodontal tissue, providing a countermeasure to the microbes attack. The TLRs serve as a defensive measure in maintaining healthy periodontal tissue [37, 38]. TLRs are typically found in the first line of cells, the defensive cells (the neutrophils, macrophages, and dendritic cells). These aforementioned cells express different TLRs, which present various forms of attack on the invasive organisms. Neutrophils for instance, are the first cells to respond. The neutrophils migrate to the infected site and initiate TLR response. Signaling through TLRs induces the production of proteins such as cytokines, chemokines, and antimicrobial peptides [39].Additionally, TLRs mediate phagocytosis and cell death, by initiating inflammation, apoptosis, and host immune responses. Signaling through TLRs enhances macrophages to protect against pathogenic microbes. However, pathogens have also developed means of deception, which deter macrophages from clearance of

microbes. These tactics allow pathogens to sneak under the radar and avoid detection by the host's macrophages [21].

Inflammation in Periodontal disease

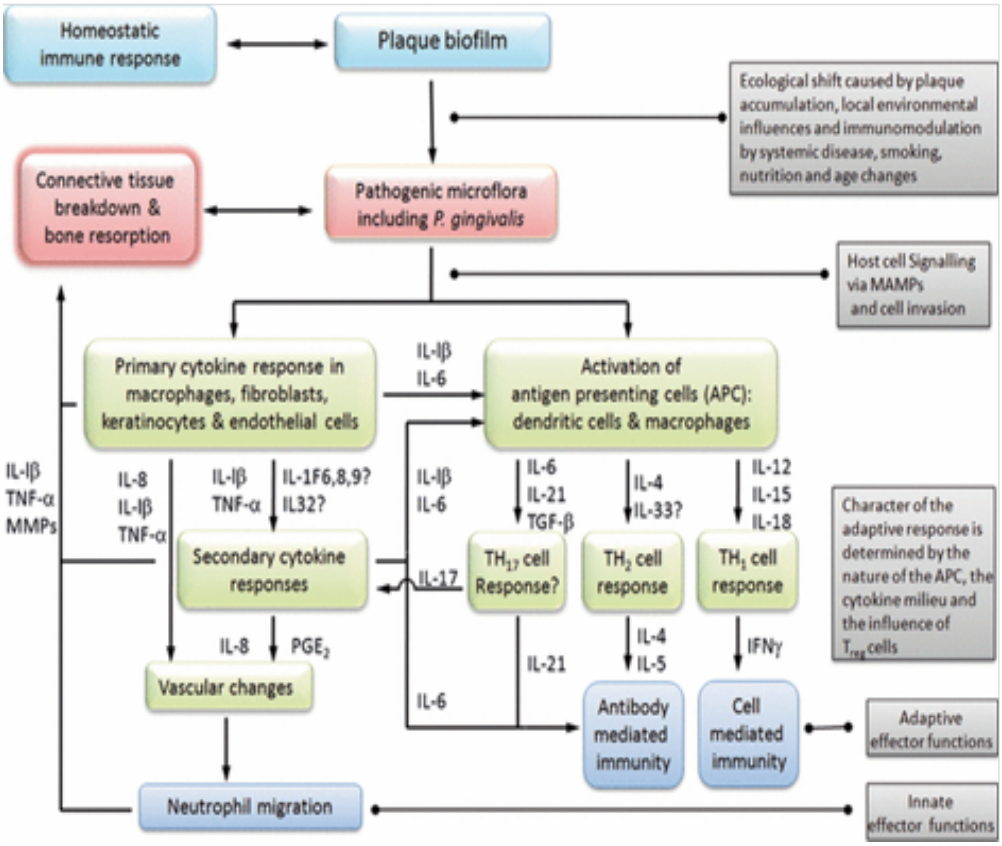
Development of periodontal disease is dependent on host's immunological response to bacterial infection and colonization. Inflammation, a form of immunological response, is a host's physical reaction to injury [1, 39]. The body's inflammatory responses can be short (acute), and, or long term (chronic). The characteristics of inflammation include redness, swelling, pain, heat, and loss of function.

There are two forms of inflammation, of mention, in the oral cavity that affect the health of the teeth and supporting structures. Both of which are marked by the acquisition of bacteria leading to cell infiltration [43]. Gingivitis is the form of inflammation that is marked by the redness of gums, and is usually resolved by brushing and proper oral hygiene. Gingivitis affects the soft tissues and epithelium. Gingivitis that's left untreated may then progresses to periodontitis. Chronic periodontal disease affects the soft tissue, the epithelium, and alveolar bone [44]. The consequences of periodontal disease ensue by bacteria buildup below the tooth surface, which leads to inflammatory response via the immune system. Leukocytes penetrate the area and inhibit the amount of bacterial invasion. Factors such as bacterial products, cytokines, and cross talk between adaptive and innate immunity all serve as signals for leukocyte recruitment [45].

A hosts' response system and its relationship to periodontal disease is complex. Evidence points to periodontal destruction occurring as a result of deficient host response; conversely, studies find periodontal destruction occurring from a strong

immune response system as well. Furthermore, a paradigm of the protective vs. destructive role of the immune system comes into effect. At what stage is the immune system playing a protective role for the host, and when does that shift from a protective role to a destructive role occur? When does the immune system do more harm for the host than good?

Cytokines are small proteins that serve as messengers between cells, and their main function is the coordination of the immune responses. Cytokines are responsible for cellular signaling cascades, which produce physical changes within the cells [46].



Periodontology, 2000 [46]

Figure 1. A diagram depiction of how cytokines regulate immune responses in the presence of a biofilm. The cytokine response to plaque biofilms results in a primary cytokine response from macrophages, endothelial cells, and others. These responding cytokines produce a cascade of signals. The signals lead to neutrophils migrating to the site of infection and mediated forms of immunity being initiated.

The interleukin-1 (IL-1) family consists of 11 members including IL-1 alpha and IL-1 beta. This family is produced in single peptides, while other interleukins typically are found as homodimers or heterodimers. IL-1 cytokine transcription occurs by way of a host of pro-inflammatory mediators such as pathogen associated molecular patterns

(PAMPs), lipopolysaccharides (LPS), and tumor necrosis factors (TNFs)[46]. IL-1 family plays an integral role in the host immune and inflammatory response systems, this interleukin family is responsible for the production of additional cell mediators, like cytokines and chemokines. Over expression of IL-1, or the inhibition of IL-1 expression, results in tissue destruction. For this reason, the IL-1 has been of interest and investigation in periodontal research, as well as its response to the bacterium *Porphyromonas gingivalis*. The IL-1 cytokine of particular interest in this study is IL-1 β. IL-1 β is identified as a fundamental player in immune response and immune-mediated diseases. IL-1 β has a unique role within periodontal disease. Rather than having a direct effect on cells and tissue, it serves as the initiator of synthesis and expression of other crucial cell mediators [46].

The tumor necrosis family (TNF) refers to two associated proteins, which are TNF- alpha (TNF-α) and TNF-beta (TNF-β) [47]. The cytokine of interest in our studies is TNF-α. TNF-α is a primary, pro-inflammatory cytokine that is produced largely by macrophages and periodontal resident cells. It is produced early on by the innate immune system in response to the bacterial invasion. This cytokine can persist and or even intensify inflammation by cellular cascade signals such as matrix metalloproteinases (MMPs) that are key signals in the matrix disruption and bone degradation [46, 48]. Furthermore, TNF-α is known to increase activity of phagocytes like neutrophils, and limits tissue repair via fibroblast apoptosis [46].

Studies using animal models have identified TNF-α to be an integral cytokine in periodontitis. A mouse study was conducted in which mice that lacked TNF-α were found to be more susceptible to infections [47]. Other studies involving mice have identified

external forms of TNF-α to support periodontitis [49]; while the inhibition [50] of TNF-α or signaling failures [51] result in destructive periodontal disease.

Bone Resorption

Alveolar bone serves as the tooth stabilizer, by holding the root in place. The alveolar bone infrastructure is dependent on the ratio between two specific cell types. These cell types are known as osteoblasts and osteoclasts [3, 52]. Osteoblasts are responsible for bone formation, and osteoclasts are responsible for resorption of bone. To maintain healthy bone levels, equilibrium must be achieved between osteoclasts and osteoblasts, or a ratio of one to one correspondingly. The imbalance of osteoclasts to osteoblasts results in periodontal disease.

The mechanism involved in osteoclast cell activity is defined as the RANK/ RANKL system. RANK (receptor activator of nuclear factor-κB), RANKL (RANK's ligand), and the soluble counterpart to RANK/RANKL is OPG (osteoprotegerin), all play valuable roles in the equilibrium between osteoblasts and osteoclasts. Here's how it works. The ligand, RANKL, binds to its receptor, RANK, which is present on the surface of pre-osteoclasts. This ligand is integral to the growth, maturation, and activation of osteoclasts. Conversely, OPG acts like a decoy receptor, by disrupting or inhibiting osteoclast formation. Ultimately, the ratio between RANKL and OPG determines the outcome for osteolytic activity and alveolar bone [2, 3]. It's believed that one of the main factors in inflammation-induced bone loss is the disruption in equilibrium between osteoblasts and osteoclasts. The two major interactions responsible for this come from 1)

toll-like receptors and inflammation induced osteoclastogenesis, and 2) and cytokines and inflammation-induced osteoclastogenesis [52].

Gender as Risk Factor in Periodontal disease

Risk factors are described as attributes or incidences linked with an increased rate of disease. They are noted for being associated with an increased rate of disease, but do not cause the disease itself. Risk factors are divided into modifiable and non-modifiable risk factors. Modifiable risk factors are behavioral and environmental factors. Non-modifiable factors are also known as determinants. They can't be modified or changed [53, 54]. There are numerous factors that influence disease in humans. These include overall health, oral hygiene, alcohol consumption, and smoking. Gender, however, has been a controversial factor in periodontal studies. Studies remain inconclusive as to the gender more prone to periodontal disease; extraneous variables confound results, and make it difficult to ascertain which gender is truly more susceptible. Sexual dimorphisms are physical differences between males and females. Identifying the sexual dimorphisms of periodontal disease is critical in risk assessment, to identify the sex that is more susceptible to disease.

Multiple factors determine periodontal disease occurrence and the gender factor is a major one in affecting disease development. It is reported that gingivitis affects 65-75% of pregnant women [55]. Moreover, women in ovulation and pre-menstruation phase are more prone to acquired periodontal diseases [56]. Ovulation induction by clomiphene citrate is able to induce gingival inflammation in women [57]. Exacerbated periodontal disease is also one of the side effects of some oral contraceptives. Surveys showed

contradictory results about the susceptibility to periodontal diseases in male and female population. Among all the periodontal office visits three quarters of those are made by women. This is despite the fact that women take better care of their teeth. On the other hand, national surveys in the USA indicated that the disease is more prevalent in men than in women. Studies have provided epidemiological evidence identifying males to be the gender more likely to develop destructive periodontal disease[58]. Despite the previous statement, men do not seem at higher risk for rapid periodontal destruction in comparison to their female counterparts[58]. Habits such as smoking [12], alcohol consumption [13], and bad oral hygiene habit, raise risks for periodontal disease occurrence. The dissimilarity in behaviors between females and males [59] interferes with the survey results and render it difficult to clarify the different biological responses to pathogen stimulation in females and males. Therefore, animal periodontal disease models are useful tools for evaluating the role that sex hormones play in periodontal disease pathogenesis.

Besides the discrepancy of disease prevalence among different sexes, periodontal disease is more rampant in pregnant women. Periodontal disease during pregnancy can cause serious problem such as preterm and underweight birth [14]. Interestingly, with enormous reports indicating higher incidences of periodontal diseases during pregnancy and its possible manifestations, as well as the cases of periodontal disease associated with puberty and menstrual cycle, studies on the working mechanisms of periodontal disease etiology are far from satisfactory [60]. During pregnancy, estrogen is elevated to reach plasma level of 6 ng/ml, which is as much as three times of the normal amount up to the end of the third trimester. The effect of estrogen on other diseases may cast light on its role in periodontal disease. Women are more susceptible to develop some autoimmune

diseases, such as multiple sclerosis, rheumatoid arthritis (RA), systemic lupus erythematosus, Hashimoto's thyroiditis, etc [61].

Animal Models for Periodontal Studies

Limitations arise from human studies. Extraneous variables such as smoking, alcohol consumption, oral hygiene, diabetes, overall health, stress, hormones, and gender are all compounding variables in studying diseases. Animal models are beneficial in eliminating factors such as these. Benefits of animal studies are that they are relatively inexpensive, they are easier to monitor and control, and they provide a definitive analysis for cause and effect relationships, which are difficult to ascertain in human clinical studies. Sadly, no single animal model mimics all aspects of human periodontal disease. Animal models, like human models, prove to be disadvantageous in experimental studies. So why use animal models? They serve as a useful model in human phenomena. They shed insight into aspects of the human body, in our case- immune response, that prove difficult from human studies. Various animal models have been used in periodontal studies ranging from invertebrates like *Drosophila melanogaster* [62], to vertebrates like non-human primates, dogs, and rodents. Animal models provide insight into the steps of periodontal disease, including 1) colonization of bacteria, 2) invasion of the bacterium from the epithelium into the connective tissue, 3) initiating the inflammatory system, 4) breakdown of the connective tissue, and 5) lastly damage control by the host and bone loss [63, 64].

Rodent models are common models in the study of periodontitis because they are easy to handle and relatively inexpensive. Mouse models are especially important due to

the large number of transgenic mice and mice with genetic depletions [64]. Different mouse models have been used to investigate etiological pathogens, host responses to pathogens, and host modification during the disease progression. An oral gavage model has widely been used as an experimental periodontitis model, in which periodontitis can been induced by orally infecting mice with oral pathogens such as *P. gingivalis*, *A. actinomycetemcomitans, Tannerella forsythia*, and etc.[65] [66].

Another animal model for periodontal disease, the ligature model, is the placement of ligatures around teeth, which initiates periodontal tissue loss. The ligature leads to greater accumulation and colonization of oral pathogens, facilitating their invasion of connective tissue. Although this model was once commonly used with non-human primates, interest in the rat and mouse ligature model has been boosted because it is simpler to work with and its cost effectiveness[66-69].

Table 1. Animal Models: Advantages and disadvantages of select animal models for studying periodontal disease development.

Animal model	Advantages	Disadvantages
Nonhuman primates	Similar dental structure, microflora, and disease to humans'. Natural or experimentally induced periodontitis.	Very expensive, with ethical and husbandry issues
Dogs	Develop natural or experimental periodontitis similar to humans	Relatively expensive, need special daily care, husbandry issues. Dentition different from humans.
Miniature pigs	Dental structure and periodontitis have some similarity to humans'. Natural or experimentally induced periodontitis.	Relatively expensive, husbandry issues; relatively few studies
Ferrets	Naturally or experimentally induced disease with similarity to humans'	Some husbandry issues
Rodents	Experimentally induced disease. Similar molar structure to humans'. Inexpensive model	Different microbiota from humans'. Small size and therefore amount of tissue for analysis. Large number of animals needed

Journal of Biomedicine and Biotechnology, 2011 [64]

CHAPTER 2

STATEMENT OF OBJECTIVES AND HYPOTHESIS

Study Objectives

The aim of this research was to investigate the role of gender in periodontal disease by using animal models. The results in human studies of periodontal disease have been confounded by many other risk factors such as smoking, alcohol consumption, stress, and disease. These factors make it difficult to determine which gender is more prone to periodontal disease. Developing an animal model is beneficial in eliminating extraneous variables that the human model present. Animal models allow us to examine the immune system's effects on a host during periodontal disease. They provide insight into this disease where ethical issues arise from human studies. Specifically, experiments can be induced into animal models to study cause and effect relationships.

The specific aims of this study were:

1. To develop an animal model for periodontal study, and determine the gender more prone to periodontal disease.
2. To examine pro and anti-inflammatory response in the presence of *Porphyromonas gingivalis*.

Study Hypothesis

Our Hypothesis is that:

1) Gender is a risk factor for periodontitis, and males are the more likely to develop disease.
2) Sexual dimorphism in periodontitis is due to differential pathogen-induced pro and anti-inflammatory response.

CHAPTER 3

MATERIALS AND METHODS

Reagents, bacteria, and mice:

P. gingivalis ATCC 33277 were grown anaerobically from frozen stocks on modified Gifu anaerobic medium-based blood agar plates for 5–6 days at 37°C, followed by anaerobic subculturing for 18–24 hours at 37°C in modified Gifu anaerobic medium broth (Nissui Pharmaceutical). This particular strain of *P. gingivalis* was used because of its known ability to induce periodontal disease. This strain has been used widely throughout periodontal research. Bacteria were harvested by centrifugation at 4000 rpm for 30 min. The cells were then washed with PBS (phosphate –buffered saline), suspended and determined for the final concentration using a spectrophotometer. The final concentration of bacteria (CFU) was standardized by using the Optical Density (OD) at 600 nm of 1.0, which is corresponding to 5 $x10^8$ CFU/ml.

Cell culture and Assays:

Thioglycollate-elicited macrophages were isolated from the peritoneal cavity of C57B/L6 mice, purchased form Jackson lab (4, 8), in compliance with established institutional policies and federal guidelines. These macrophages were used for several reasons: first, they were easy to access for harvesting; secondly, the peritoneal cavity is rich in abundance with macrophages; lastly, peritoneal macrophages are a popular choice

for macrophage studies[70]. The macrophages were cultured at 37°C and 5% CO_2 in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.05 mM 2-ME. None of the experimental treatments affected cell viability (monitored by the CellTiter-Blue assay; Promega) compared with medium-only treatments. The phenotypic characterization of peritoneal macrophages and determination of TLR2 upregulation in activated macrophages were performed by flow cytometric analysis, using the FACSCalibur and the CellQuest software (Becton–Dickinson). For these experiments, we used fluorescently labeled monoclonal antibodies to TLR2, followed by washing and flow cytometry. Our lab looked specifically at TLR2 because of its agonistic role in *P. gingivalis.*

Cytokine production (Il-6 and IL-1β) in cell culture supernatants was determined by the use of ELISA. Corning Costar 9018 ELISA plates were coated with 100 micro-liter/ well of capture antibody in Coating Buffer 4^{o}C for overnight. The wells were washed 5 times with Wash Buffer the next day. Next, the wells were then blocked with 200 micro-liter/well of 1X Assay Diluent at room temperature for 1 hour. Aspirated/ washed for a total of 5 washes. Next, 100 micro-liters/ well of sample was incubated in the appropriate wells at room temperature for 2 hours. After wash, 100 micro-liter/ well of detection antibody diluted in 1X Assay Diluent was added to the corresponding wells and incubated at room temperature for 1 hr, followed by washing and adding Avidin-HRP for 30 minutes. After thoroughly wash, substrate solution TMB were added and incubated at room temperature for 15 minutes. Thereafter, 50 micro-liters of Stop Solution was added and the plate was read at 450 nm.

In vivo mouse studies:

Ligature-induced periodontitis model:

Periodontal inflammation and bone loss in this model is induced by a 5-0 silk ligature tied around the maxillary left second molar, with the ligature placed in the gingival sulcus. *P. gingivalis* (2 x10^9 cells) was administered three separate times to the ligature sites. On the first, third, and fifth day of the experiment. The contralateral molar tooth in each mouse was left unligated (baseline control). Inflammatory bone loss was examined 7 days after placement of the ligatures, which remained in place in all mice during the experimental period. Bones were measured for the distance from cement-enamel junction to alveolar bone crest (CEJ/ABC) with the VIA-170K system.

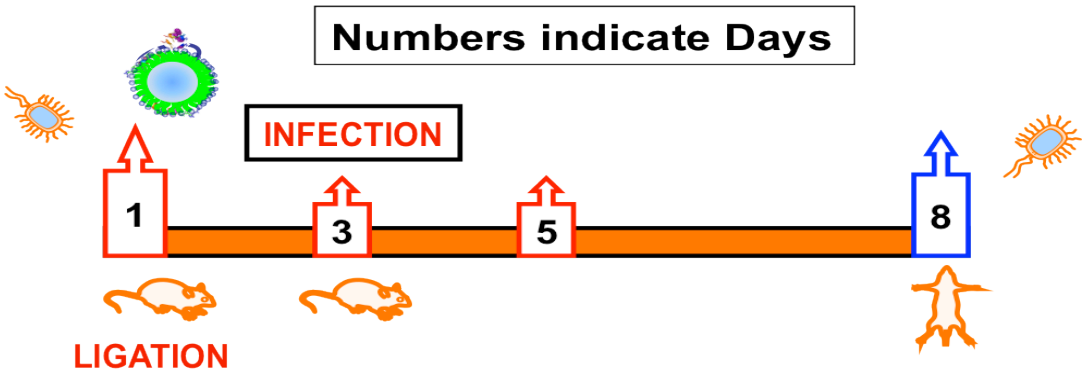


Figure 2: Schematic depiction of the protocol for periodontitis induction in ligature model.

Oral Gavage Model:

The *P. gingivalis*-induced periodontal bone loss model was used essentially as originally developed by Baker and colleagues (21) with slight modifications, as we

previously described (20). In brief, following on suppression of the normal oral flora with antibiotics (sulfamethoxazole/trimethoprim) in deionized water for 10 days, 10- to 12-wk-old mice were infected by oral gavage five times at 2-d intervals with 10^9 CFU *P. gingivalis* suspended in 2% carboxymethylcellulose. Sham-infected control animals received 2% carboxymethylcellulose alone. The mice were euthanized 6 weeks later, and assessment of periodontal bone loss in defleshed maxillae was performed under a dissecting microscope (×40) fitted with a video image marker measurement system (VIA-170K; Fryer). Specifically, the distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surfaces of the maxillary molars. The 14-site total CEJ-ABC distance for each mouse was subtracted from the mean CEJ-ABC distance of sham-infected mice to calculate bone loss. The results were expressed in millimeters, and negative values indicate bone loss relative to sham-infected control mice.

All animal protocols were approved by the Institutional Animal Care and Use Committee in compliance with established federal and state policies.

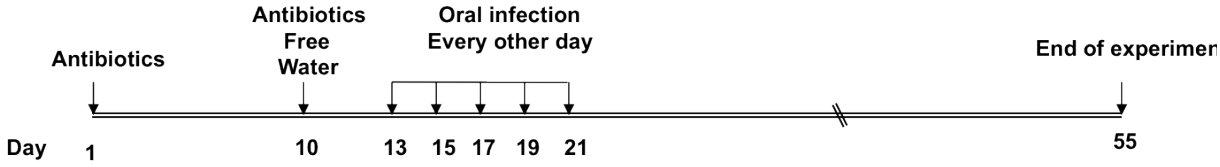


Figure 3: Schematic depiction of the protocol for periodontitis induction in oral gavage model.

Alveolar Bone Loss Reading:

The mice heads were autoclaved in water (121^{o}C for 10 minutes) and defleshed there after. The mandibular and maxillary jaws of the mice were placed in 3% hydrogen

peroxide (H_2O_2) overnight. The mice heads were pulsed in 1% bleach for one minute. The heads received a staining using 1.0% methylene blue and 0.5% eosin dyes. Following, the heads were placed in the eosin dye for 5 minutes and methylene blue for 1 minute. The heads were washed well and allowed to air dry. The bone loss measurements from the maxillary jaws were measured using a dissection microscope (x40) fitted with a video image marker measurement system (model VIA 170; Boeckeler Instruments, Inc., Tucson, Ariz.), millimeters is the standardized value. Seven sites on the buccal side were measured for the left and right molars, for a total of 14 sites. The distances were measured from the cemento enamel junction (CEJ) to the alveolar bone crest (ABC). The amount of change in the alveolar bone for each mouse was calculated by subtracting the CEJ to ABC distance for that mouse, from the average CEJ-ABC distance of the sham-infected mice.

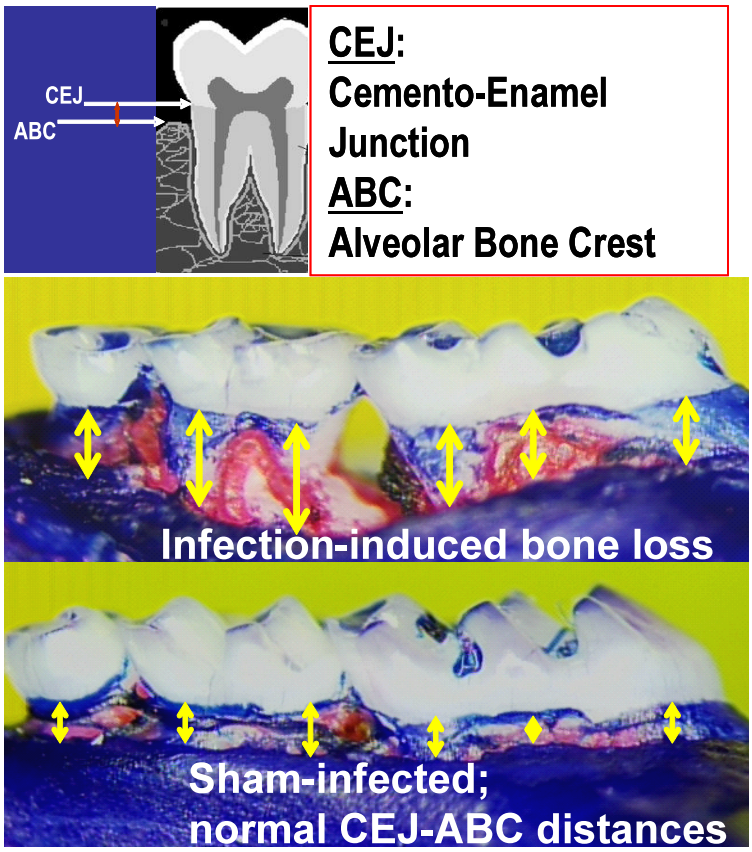


Figure 4. Depiction of how bone loss measurements were derived.

Determining the Total Number of Anaerobic Bacteria:

A sterile medium sized paper point was inserted along the gum line of the upper molars in the murine models. The paper point was held in place for approximately 30 seconds and then vortexed in 1 mL of prereduced brain heart infusion broth with the add-ins of hemin and menadione. The sample was vortexed for approximately 30 seconds. Next 50 micro-liters of broth was acquired and plated on blood agar. The plates were given two weeks to culture in an anaerobic chamber before being counted. The colonies were counted based on the number of black-pigmented colonies.

Gingival Inflammation Determination:

The gingival tissue was harvested from the upper jaw around the molars. A small segment of tissue was gathered after euthanasia from each mouse. A segment of tissue was obtained from both the left and right sides. The left side corresponded to the infected site, and the right corresponded to the uninfected location. The gingival tissues were placed in RNAlater (Ambion Cat #AM7020) and kept at -20 ^{o}C until used for RNA isolation. RNA was isolated from the tissue using an RNeasy mini kit (Qiagen Cat#74104). Each gingival tissue was homogenized in 600 µL solution of RLT buffer and β-mercaptoethanol. The lysates were centrifuged for 3 minutes at full speed (14,000 RPM) at 4 ^{o}C. The supernatant was pipetted into a new tube. Six hundred microlitters of 70% ethanol were added to the lysates and mixed by pipetting to ensure homogeneity. 700 µL of sample were transferred to an RNeasy spin column placed in a 2 mL collection tube. The samples were centrifuged for 1 minute at 10,000 RPM. The flow through was

discarded. If the samples exceed 700 µL, then the step was repeated. 350 µL of RW1 buffer was placed into each spin column for washing. The samples were then centrifuged for 1 minute. Next a solution of DNase and RDD buffer was added to each sample. A ratio of 70 µL to 10 µL or RDD buffer to DNase respectively, per sample. The DNase I incubation mix (DNase and RDD buffer mixture) was placed into each spin column and allowed to sit for 15 minutes. Another 350 µL of RW1 buffer was placed into each column and centrifuged for 1 minute. Next, 500 µL of RPE buffer was added to the spin column. RPE is supplied as a concentrate and therefore ethanol must be added to the buffer before using. The samples were centrifuged for 1 minute following the RPE buffer. Another 500 µL of RPE buffer was placed into each spin column and this time centrifuged for 2 minutes. Each sample was placed into a new 2 mL collection tube and the old tubes were discarded. The samples were then centrifuged for 1 minute. The samples in the spin columns were placed into a new 1.5 mL tube and 50 µL of RNase-free water were added to each. The samples were centrifuged for 1 minute. Based on concentrations of RNA determined by NanoDrop, samples were converted into cDNA via reverse transcription. The High Capacity cDNA Reverse Transcription Kit from Applied Biosystems was used. The following reagent mixture was used.

Reagents	Amount (µL)- per sample
10x Buffer	10
25xdNTP	4
10x Random Primers	10
RT	5
RNase Inhibitor	5

DEPC H_2O	16
Total	50

Table 2. Reagent mixture required for Reverse Transcriptase.

40ng of RNA in 50 µL of DEPC H2O was transferred into each tube while remaining on ice. Next, 50 µL of reagent mixture was transferred into each tube. The samples were then centrifuged. The PCR machine was placed on the rt2 setting. The protocol was then run at the desired volume of 100 µL.

Real Time PCR:

The cDNA samples were taken from the freezer and allowed to thaw on ice. A mixture of 100 µL sample, 980 µL Ultra Pure Water and 1080 µL Taqman Master Mix were placed used for a total volume of 2,160 µL. The tubules were capped and vortexed to mix the samples completely. The samples were then centrifuged. The qPCR plate was prepared for the experiment by removing the plate cover, and centrifuging the plate at 10,000 RPM for 1 minute. Next the cover of the plate was removed and 20 µL of mix (cDNA plus master mix) was placed into each well. The plate was then centrifuged again. The plate was then run using the qPCR machine and 7500 software. A plate template was developed for the experiment.

Statistical analysis:

Data were evaluated by analysis of variance and the Tukey–Kramer Multiple Comparisons Test using the InStat program (GraphPad Software, San Diego, CA). Where appropriate (comparison of two groups only), two-tailed t tests were performed. $P < 0.05$ was taken as the level of significance.

CHAPTER 4

RESULTS

More severe periodontitis in females in the Ligature Experiment:

To identify the role of gender differences in periodontitis with the exclusion of other behavior-related risk factors, we investigated the periodontal phenotype of female and male mice in a ligature-induced periodontitis model, which was mentioned in materials and methods. Female and male C57BL/ 6 mice were ligated around 2nd molars and infected with live *P. gingivalis*. The mice were infected with *P.gingivalis* every other day for a total of three infections. The controls were sham-ligated and sham-infected mice (non-ligated). Eight days later, mouse jaw bones were obtained and measured for the distance between the cemento-enamel junction and alveolar bone crest of each molar (3 molars on both sides) in the mouse cavity. We found that both female and male ligated mice showed significantly greater CEJ-ABC readings than the non-ligated controls, while female ligated mice showed higher levels of CEJ-ABC reading in comparison to the male ligated group (Figure 5). Both the female and male non-ligated groups, the control groups, were of equal values in bone loss, which served as an indication to a true control group being established. Our results indicated that female mice are more susceptible to bone loss in the *P. gingivalis* ligature model.

Inflammation is able to induce alveolar bone loss and is also indication of periodontitis. In order to determine the severity of periodontitis, we tested gingival inflammation in female and male mice. IL-1, IL-6, and TNF have been well known for

their presence in periodontitis patients and their detrimental function in periodontal disease and bone loss [71]. Analysis of the periodontal inflammatory response by real-time quantitative PCR showed differences in certain periodontitis-related pro-inflammatory cytokines between female and male mice. We observed significant higher upregulation in the expression of IL-1 and IL-6 in females, which are critical in periodontitis progression (Fig. 6). Although not significant, TNF expression was also upregulated in female in higher level. IL-17 is an important pro-inflammatory cytokine, which is paid more attention due to its tight link to newly found Th17 cells. Our recent publication showed that IL-17 caused more severe periodontitis[72]. In Fig. 6, ligated female mice expressed higher IL-17 compared to their male counterparts. On the other hand, the expression of anti-inflammatory cytokines IL-10 and TGF did not show difference in female and male gingival tissues (Fig 6), implying that more gingival inflammation in females caused more severe periodontitis.

Because oral pathogens are responsible for initiating and progressing periodontitis, anaerobic colony forming units (CFUs) within the oral cavities of male or female mice were examined (Figure 7). Before the ligation, oral bacteria of the males and females were determined for the baseline. The oral bacteria of pre-ligation mice are essentially equal in colony forming units. Eight days after ligation, we found that female mice had a much greater bacterial burden than that of their counterpart male mice, which suggested the involvement of bacteria in this bone-loss model (Fig. 5). More bacterial burden in female mice may be due to lower clearance ability of female mice. Another possibility is that higher inflammation in female mice provides more nutrients and optimal environment for the survival of oral bacteria. In summary, female mice

showed higher alveolar bone loss, gingival inflammation, and a greater bacterial burden within the oral cavity, which indicated female mice were more prone to more severe periodontitis, at least in ligature-induced periodontitis animal model.

Bone loss in female and male mice in an oral gavage model:

To determine whether our observations of female mice developing more severe periodontitis was specific to the ligature model, we used another model, oral gavage model to test bone loss in female and male mice. Female and male mice were orally infected with 10^9 p. gingivalis for 5 times as described in materials and methods. Sham-infected mice were used as controls. At the extermination of the experiment, we tested the CEJ-ABC readings of the female and male mice (Fig 8A). To calculate the bone loss value, CEJ-ABC readings of the controls were subtracted from those of experimental mice (Fig 8B). The results showed that females developed more bone losses than male mice in oral gavage models.

Female macrophages up-regulated more TLR2 expression responding to *P. gingivalis* infection

The results in both the ligature and oral gavage models imply that females were prone to more severe periodontitis. In order to determine the mechanism and involved signaling pathway, we looked into the TLR response of female and male macrophages induced by *P. gingivalis* stimulation. TLRs are important PRRs that recognize PAMP from pathogenic bacteria. Fimbriae and LPS from *P. gingivalis* are important virulent factors and act through TLR2 signaling pathway [73]. TLR2 is a major receptor for *P.*

gingivalis infection. TLR2 signaling is able to lead to NF-κB activation and induction of pro-inflammatory cytokines such as TNFα and IL-6. The signaling through TLR2 not only induces innate immunity, but also promotes adaptive immunity [74]. Investigation on the TLR2 stimulation in female and male macrophage cells might help us understand the differences in inflammation and bone losses. Macrophage cells are one of the most important immune cells and are widely investigated for its capability of phagocytosis, antigen presentation, and cytokine production. TLR2 expression on macrophage cell surface was tested to determine the stimulation through TLRs. After stimulation *with P. gingivalis*, the macrophages expressed higher level of TLR2, implying the stimulation of TLR (Fig 9). Furthermore, macrophages from female mice up-regulated more TLR2 than males, showing that macrophages from females respond better to *P. gingivalis* stimulation and lead to stronger TLR2 signaling than males (Fig 9).

Female macrophages produce more inflammatory cytokines than male macrophages when responding to *P. gingivalis* infection

To understand whether discrepancies in TLR2 response led to different levels of inflammation in female and male mice upon *P. gingivalis* stimulation, we examined pro-inflammatory cytokine production. Specifically, examining the ability of peritoneal macrophage cells *in vitro* when responding to *P. gingivalis* stimulation. Peritoneal macrophages were isolated from female and male mice, and then stimulated with *P. gingivalis,* and pro-inflammatory cytokines were measured in the supernatants. IL-1β and IL-6 were tested for their significant role in periodontitis. As shown in Fig. 10, macrophages from both female and male mice all responded well to *P. gingivalis*

stimulation and produced IL-1β and IL-6. Interestingly, female macrophages produced significantly higher levels of IL-1β and IL-6 than male macrophages (Fig. 10). IL-1β and IL-6 are well known for their detrimental function in PD and bone loss [71]. The in vitro results match well with gingival cytokine expression in ligature models. This strongly implies that by producing more pro-inflammatory cytokines, females might be more prone to more severe bone loss and periodontitis.

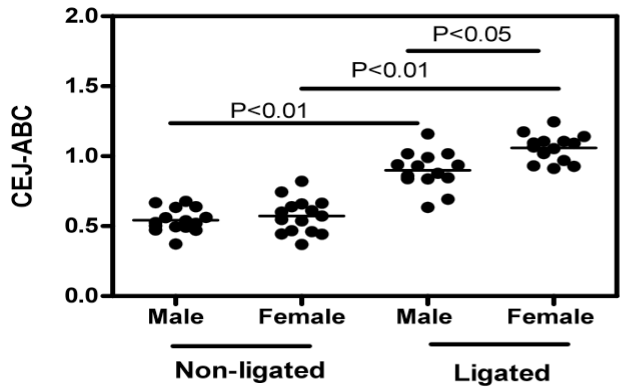


Figure 5. Ligation Model Experiment: Periodontal bone loss in ligated female and male mice. Mice of 8–10 wk of age were used in the experiment. A ligature was placed around the second molar on the left side and infected with *P. gingivalis*, in three doses. The non-ligated right side served as the control. The distance (in mm) from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 predetermined maxillary buccal sites, and the readings were totaled for each mouse. Total bone losses

measured amongst control and experimental groups. P value was labeled to show the significance.

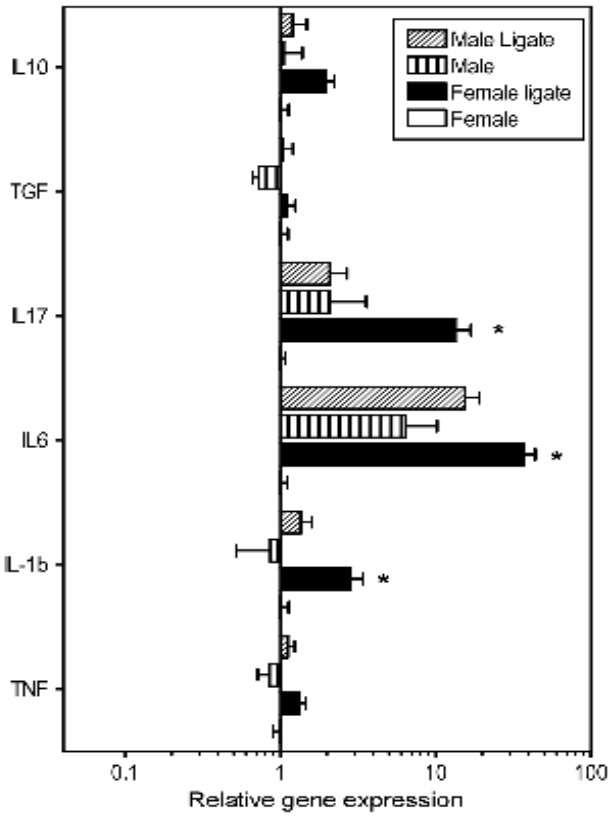


Figure 6. Relative expression of cytokines in the gingival tissues. Quantitative real-time PCR (qPCR) was used to determine gingival mRNA expression levels for the indicated receptors (normalized against GAPDH mRNA levels). The gingivae used were excised from either female or male mice (sham-ligated or ligated with *P. gingivalis*). Results are shown as fold induction relative to female sham-ligated mice. Each data point represents the mean ± SD of 5 separate expression values, corresponding to qPCR analysis of total gingival RNA from individual mice. A minimum of 2-fold difference was a requirement for further testing of statistical significance.

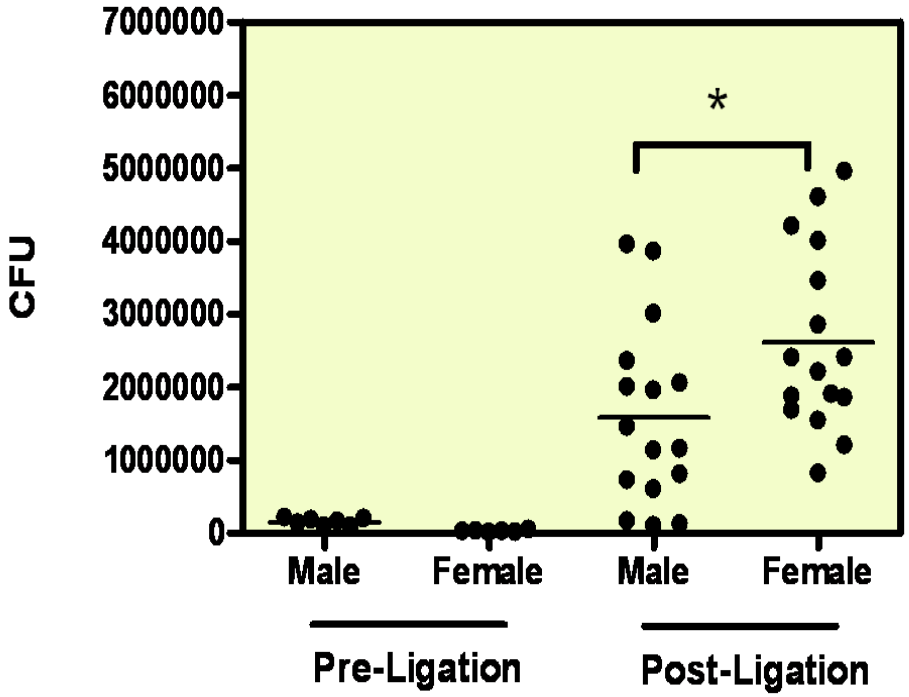


Figure 7. Assessment of the number of retrieved anaerobic bacteria from oral cavity of female and male mice pre and post-ligation. The swabs were retrieved from oral cavities from either female or male mice (pre-ligated or 8 days after ligation with 3 times infections of *P. gingivalis*). Each data point represents the reading from individual mice. Asterisks indicate statistically significant differences ($p < 0.05$) between ligated females and males.

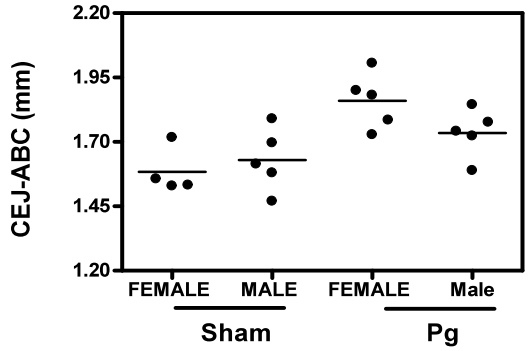


Figure 8A: Oral Gavage Measurements (previous page)

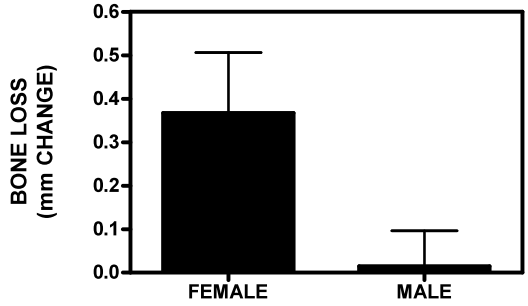


Figure 8B: Difference Between Experimental and Control Groups in Oral Gavage (above)

Figure 8A and 8B. Bone losses in female and male mice after oral *P.gingivalis* infection, the oral gavage model. Upper jaw was harvested and both left (L) and right (R) sides were measured for CEJ/ABC distance. The first diagram (8A) provides a mouse-by-mouse depiction of bone loss readings. Male and female mice that received the bacterium infection displayed higher levels of bone loss in comparison to the control (sham) infected mice. The second diagram (8B) displays female and male mice vs. bone loss (mm change). This was conducted by subtracting the bone loss of the experimental group

mice with the sham-infected mice. The female experimental reading was subtracted from the female control. Data represented the mean ± SD from 5 mice. (B) Total alveolar bone losses in female mice were more than male mice following *P.gingivalis* infection.

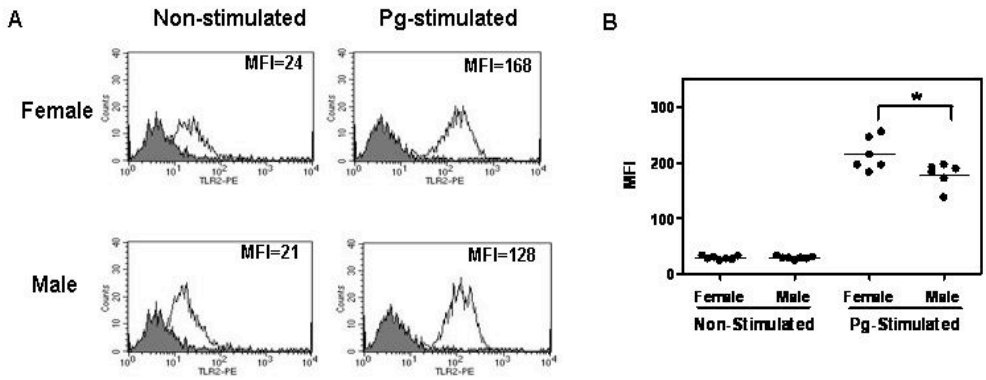


Figure 9. MFI (Median Fluorescent Intensity) in vitro Study: Female and male macrophages expressed up-regulated TLR 2 at different levels post-infection. Peritoneal macrophages were harvested from female or male mice. After incubated with *P.gingivalis* with MOI=10, macrophages were stained with TLR2-PE and being analyzed by FACS using a FACS-Caliber (BD bioscience). (A) The histograms shown are from one of six independent sets of experiments that yielded similar findings. Numbers in the histograms are mean fluorescent intensity values. (B) Each data point represents the median fluorescence intensity (MFI) of macrophage TLRs from individual mice. Asterisks indicate statistically significant ($p < 0.05$) differences between macrophages from female and male mice (n=6).

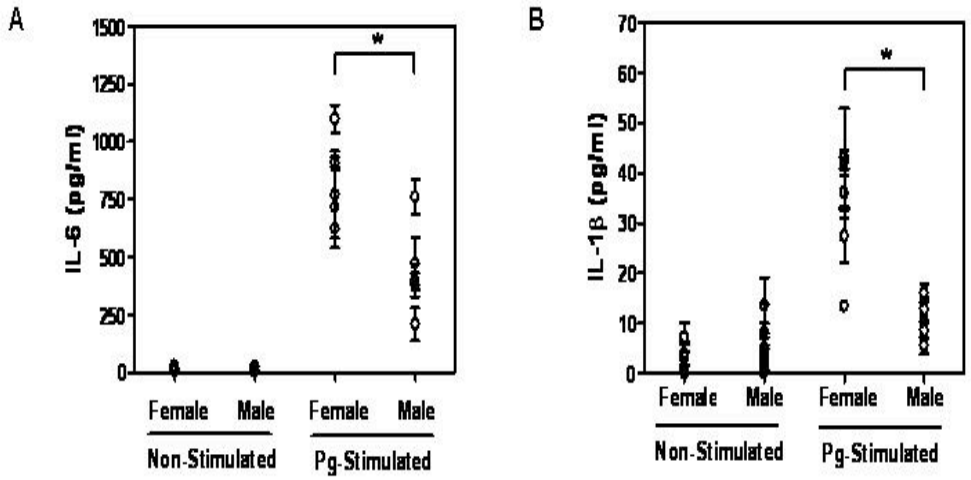


Figure 10. IL-6 and IL-1β cytokine expression following *P. gingivalis* infection: Peritoneal macrophages were harvested from female or male mice. After overnight incubation with *P.gingivalis* with a MOI=10, supernatants were harvested and tested for (A) IL-6 and (B) IL-1β secretion by ELISA. Each data point represents the mean value (with SD) of cytokine production from individual mice (n= 5). Asterisks indicate statistically significant ($p < 0.05$) differences between female and male macrophages.

CHAPTER 5

DISCUSSION

Gender's role in periodontal disease is controversial due to many extraneous variables confounding results[75-78]. However, studies for the majority show males to be the sex predisposed to periodontal disease. Animal models exclude human behaviors and variables such as smoking, [12], alcohol consumption [13], and oral hygiene habits, which provides a means for examining gender's true role in this disease. The differences amongst females and male mice shed light into the pathogenesis of periodontal disease, and are useful in developing models of risk of assessment and economical alternatives for future periodontal studies.

The findings illustrated in our ligature-induced periodontitis model suggested a higher degree of periodontitis in female mice, in contrast to their male counterparts. These findings were consistent with our cytokine analysis, which revealed the gingival tissue of female mice to have significantly elevated expression for pro-inflammatory cytokines; $p < 0.05$; (Fig. 7). The fact that increased CEJ–ABC distances in female mice was accompanied by elevated expression of pro-inflammatory cytokines, strongly supported periodontal disease among infected female mice. We next looked into anaerobic recovery of bacteria. Bacterial colonies were higher amongst female mice.

The higher levels of colonies developing within the female mice elucidated to the fact that female mice have a better environment for bacterial growth. The rich nutrient supply provided by female mice resulted in higher levels of anaerobic biofilms and ultimately periodontal disease. The colonies exacerbated the immune system, which in turn stimulated the inflammatory response leading to tissue degradation and bone resorption. In summary, our findings indicated that the periodontal tissues of female mice showed clear signs of increased inflammation and elevated alveolar bone loss compared with male mice.

A follow up experiment, the oral gavage, was conducted for determining the validity of the ligature mouse model. This animal model confirmed our results from the ligature model. The oral gavage model produced similar results when compared to the ligature model. Our results revealed that female mice are also more susceptible to *P. gingivalis*-induced bone loss in the oral gavage model. (Fig 8)

To better understand the causes of sexual-dimorphism in periodontitis in animal models, an in vitro analysis was carried out to test host responsiveness to *P. gingivalis*. Possible differences in TLR2 expression were investigated in peritoneal macrophages from female and male mice. Stimulation of macrophages with *P. gingivalis* from both female and male macrophages displayed significant ($p < 0.05$) upregulation of Toll-Like Receptor 2. This implied that macrophages from females are more responsive to *P.gingivalis* stimulation. Hyper-responsiveness of female cells to pathogens induced higher production of pro-inflammatory cytokines, which led to more inflammation and bone loss. Indeed, our test on the production of cytokines IL-1[79] and IL-6[80], which are

important mediators of periodontitis, showed that the macrophages from female mice produced higher amounts of these pro-inflammatory cytokines (Fig. 10).

Males are found to be the gender prone to human periodontal studies [81], which is contradictory to what we examined in our mouse model. This inconsistency might be due to the fact that periodontitis is a complex disease, which is influenced by many factors. There is no single animal model that can represent all the aspects and answer all the questions for this disease. However, animal models are still the best tools for mechanism studies. These models avoid ethical complications in human research. Various models of study can be used, mimicking different aspects of the disease. In this case, the ligature model and oral gavage model might not be suitable for research on sexual dimorphism in chronic periodontitis, or it may only reflect certain aspect in chronic periodontitis in different sexes.

It's worth noting that Baer [25] eluded to the fact that females are 3 times more likely to develop aggressive periodontitis than males. Another report [82] concluded that aggressive periodontitis, or at least localized juvenile periodontitis, affects females more often than males with a ratio of 2.5:1. We believe our findings, pertaining to the in vivo and in vitro studies, can prove useful as an animal model for studying aggressive periodontitis amongst males and females. This is a different form disease from chronic periodontitis, but serves as a possible direction for our animal model.

Another direction involves looking into hormones, specifically estrogen. Estrogen was reported to increase inflammation levels and cause higher incidences of some autoimmue diseases in females [83]. Sex hormones play a major role in bone loss, a key attribute to periodontal diseases. In periodontal disease, estrogen might cause destruction

in several ways. The direct effect of estrogen on bone metabolism is of specific interest, and currently under intense investigation. Millions of women develop osteoporosis after menopause. Hormone loss after menopause in women is associated with significant bone loss, and hormone applications have been used for aged women with osteoporosis. This proves hormones are protective for bone loss. The loss of estrogen is reported to induce bone loss [84]. Reports showed that estrogen is able to directly cause the suppression on osteoclast differentiation [85], as well as the apoptosis in osteoclasts [86]. Contrarily, the protective function of estrogen in bone loss is still not clear. For instance, ovariectomy (OVX) before puberty actually promotes bone formation in a mouse model [87]. This suggests the removal of estrogen is beneficial for bone formation. The function of sex hormones in bone loss remains controversial, and obviously isn't fully understood. These discrepancies may be due to the different tissues involved and models of study. Estrogen, furthermore, affects the development of immune cells and changes the overall environment in host. Estrogen is able to modulate T cells and osteoblasts to synthesize cytokines, which are important in bone metabolism. For example, TNF and IL-1 are potent osteoclastogenic factors and induce receptor activator for nuclear factor (NF)-κB ligand (RANKL) on bone marrow stromal cells and oseteoblasts [88]. The essential role of RANKL/RANK signaling in osteoclast differentiation is well established, as mentioned in the above section on bone resorption. Loss of estrogen also leads to IL-6 upregulation and osteoclast development [89]. Estrogen inhibits TNF production by human peripheral blood mononuclear cells (PBMCs) in response to LPS stimulation, while enhanceed TNF induction without stimulation [90].

Estrogen's function in immunity has never been clarified, because estrogen's function could be dependent on: dosage, the type of immune cells, and the type of stimulation. For example, reports have revealed estrogen to enhance NF-κB in human T cells [91], however, observations from other reports have elucidated to the fact the estrogen down-regulates NF-κB activation and inflammation in rat astroglial cultures and vascular smooth muscle [92, 93]. Our future goal is to determine the role of estrogen in sexual dimorphism in periodontitis.

In closing, future directions for experimental work include the role of estrogen in periodontal disease. Secondly, Examining this animal model and its correlation to aggressive periodontitis. This includes looking at the bacterium *Aggregatibacter actinomycetemcomitans,* a known bacterial component in periodontal disease[94].

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CURRICULUM VITAE

Name:	Gleason, Robert Cleveland		
Position:	Masters Student in Oral Biology		
Education:			
Kentucky Wesleyan College	B.S.	05/2010	Chemistry
University of Louisville	M.S.	05/2013	Oral Biology
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A) Personal Statement:

The purpose of this proposed research is to develop an animal model for periodontitis. In particular, we aim to examine the differences between males and females when infected with *Porphyromonas gingivalis*. Periodontitis is an infection-driven chronic inflammatory disease, which results primarily from excessive inflammatory reactions arising from complex interactions between the host immune system and the tooth-associated oral bacteria. I attended Kentucky Wesleyan College for my undergraduate studies. While at KWC I majored in Chemistry and minored in Biology. I'm Masters Candidate in Oral Biology. I was new to research when I came to U of L in the fall of 2011. The only lab experience I possessed was from the required courses of my undergraduate career. I have gained experience and a strong foundation under the mentorship of Dr. Liang. My techniques include isolation of RNA from mouse gingivae, PCR and real-time PCR, bone loss measurements and work with cell cultures. I'm thankful for learning experience, and knowledge and experiences gained in the vast field of Oral Biology. I'm diligent and persistent individual. I'm committed to the oral health field, and hope to better it through research and eventually work in dentistry. Thank you to the University of Louisville and the Masters of Oral Biology program for this opportunity.

B) Positions and Employment:

August 2011- May 2013
University of Louisville: Oral Biology Masters Student

September 2011- August 2012
Sam's Club: Cashier

August 2008- May 2010
Kentucky Wesleyan College: General Chemistry Lab Proctor
Kentucky Wesleyan College: General Chemistry and Organic Chemistry Tutor

C) Recent Work
March 2013
University of Louisville: Graduate School Symposium Participant
I presented data on my current research pertaining to the sexual dimorphism of periodontal disease in a mouse model.

May-August 2012
University of Louisville Summer Research Program
Conducted experiments pertaining to the study of sexual dimorphism of periodontal disease in a mouse model and presented our findings at Research Louisville that fall.