Targeting mTOR to Reverse Age-Associated Periodontal Disease

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Abstract

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Old age is the single greatest risk factor for morbidity and mortality in developed nations. Successful interventions that delay the biological aging process or increase healthy longevity in people could have profound benefits for quality of life, productivity, and reduced healthcare costs (*1, 2*). The FDA approved mTOR inhibitor, rapamycin, represents an outstanding candidate for such an intervention. The field of Geroscience, which seeks to understand how aging causes disease and develop preventative and therapeutic approaches, has shown that this geroprotective intervention impacts major age-related diseases such as heart disease and Alzheimer's disease. In contrast to major age-associated diseases, the relationship between biological aging and oral health is often neglected among broader dental, biomedical, and Geroscience research communities. We present in Chapter 1 that implementing Geroscience within oral health research is imperative to our geriatric population, who experience age-related

diseases also in the oral cavity, such as periodontal disease and oral cancer. In Chapter 2, we explore if the geroprotective compound rapamycin impacts the oral cavity by investigating its effect on periodontal bone loss, a hallmark of an age-associated oral disease known as periodontal disease. Chapter 3 then addresses the groundwork for properly designing Geroscience studies using animal models to understand oral health and disease. Lastly, in Chapter 4 we take the framework we have addressed from Chapter 3 and our observations in Chapter 2 to uncover the novel finding that rapamycin reverses age-associated periodontal disease. This work is transformative as it capitalizes on advances in Geroscience to enhance our understanding of the mechanistic and molecular basis for age-associated changes in the periodontium leading to disease. We go beyond just addressing knowledge gaps to extend oral healthspan but uncover possible interventions that target aging hallmarks in the oral cavity - ultimately leading to clinical strategies to treat periodontal disease in elderly patients.

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DEDICATION

For UWSOD Class of 2015

Chapter 1. TOWARDS INTEGRATING GEROSCIENCE AND ORAL HEALTH SCIENCE

1.1 INTRODUCTION

Geroscience is a multidisciplinary field seeking to understand the biology aging leading to age-related diseases and disorders (*3-5*). The United States Census Bureau expects by the year 2035 for the first time the population over the age of 65 will outnumber the children under 18 years old. Such change will lead to nearly a quarter of Americans being 65+ years old, and triple the number of 85+ (*6*). This demographic shift will have drastic economic, social, and health-related challenges (*1, 2*). Thus, integrating Geroscience within all biomedical research is critically important for our society as age is the single greatest risk factor for most diseases, conditions limiting healthspan, and mortality in developed nations (*7*). By understanding the biology of aging, we may ultimately translate interventions that extend lifespan and healthspan in model organisms to humans.

1.2 DENTISTRY AND GEROSCIENCE

Despite incorporating Geroscience into the various areas of biomedical research, inquiries still are primarily focused on major age-related disease, such as cancer, heart disease, or Alzheimer's disease. For example, within the National Institute on Aging (NIA) more than one third of the 2014 budget was allocated to study of Alzheimer's Disease (*2*). In contrast to these major age-associated diseases, the relationship between biological aging and oral health is often neglected among broader biomedical, geroscience, and even dental research communities. Intriguingly, a branch of medicine that has continuously observed the demographic shift of the aging population along with chronic diseases is dentistry.

With the population aging, the elderly is a growing patient demographic in dental practices. Consider the fact that majority of the prescribed medications encountered by oral health care providers in the dental office are those related to patients with one or more chronic, age-related conditions, including heart disease or diabetes (*8, 9*). A dentist is expected to understand these medications and its interactions with oral disease and treatments. Along with major age-related disease, an elderly patient walking into a dental office is also at increased risk for age-associated oral diseases, including dental caries, periodontal disease, salivary dysfunction, and oral cancer (*10*).

From an economic standpoint, the cost of dental care ranks the fourth highest, with periodontal disease accounting for 10% of the total healthcare burden and other possible links to chronic illnesses accounting for 75% (*11*). As one of the few professions visited by the general population at least once a year, dentists and other oral health care providers serve a unique opportunity to diagnose, assess, monitor, prevent, or even reverse age-related declines starting in the patient's mouth. Such clinical intervention is one mode to address the consequence of economic and health-related challenges approaching with the aging demographic shift.

1.3 CONCLUSION

One goal of the Geroscience Initiative is to raise awareness of the importance of understanding the contribution of aging biology to age-related diseases, and ultimately develop multi-disease preventative and therapeutic approaches (*3, 4*). By understanding the mechanisms of aging oral biology, it may be possible to manipulate the process of aging to delay many age-related declines and chronic disease in the mouths of our elderly patients.

Generally, the direction in oral health research has been on the pathogenesis and treatment of individual diseases, or methods to replace existing oral biology such as dental implants or restorative materials. Although disease-specific, replacement-focus questions has

led to significant advancement in management of oral diseases, we have been unsuccessful in delaying, ameliorating, and even reversing the onset and progression of age-related oral deterioration. As a consequence, a majority of treatments in the elderly are completed after the disease progresses, such as removing dental caries *after* the decay has already destroyed the tooth structure or attempts to prevent more periodontal bone loss only *after* the onset of periodontal disease. Thus, implementing Geroscience Initiatives within oral health research will aid in translating findings and therapeutics more reliably to prevent or reverse age-associated oral dysfunction and disease in people.

For the overall health of an organism, *all* organ systems within the body must maintain optimal functionality (*3*), thus studies on how aging affects the oral cavity is critical. In the next chapter, we attempt to integrate the field of Geroscience into oral health research by analyzing the effects of a geroprotective intervention on age-associated changes in the murine mouth.

Chapter 2. LINKING GEROSCIENCE WITH AN AGE-ASSOCIATED ORAL DECLINE

2.1 ABSTRACT

This chapter is adapted and compiled from (12)

Interventions that target biological mechanisms of aging have potential to enhance quality of life by delaying morbidity and mortality. The FDA approved drug rapamycin represents a candidate to increase lifespan and delay age-related dysfunction in rodents and humans. Transient treatment with rapamycin or a rapamycin derivative has also been shown to rejuvenate immune function in aged mice (*13*) and elderly people (*14*) and alter bone metabolism (*15*). Periodontal disease is an important age-related oral disease involving altered immune function, bone homeostasis, and changes to the oral microbiome. Periodontal disease is clinically defined by loss of periodontal bone and by connective tissue degeneration. We have discovered that normative aging causes significant periodontal bone loss in two different mouse strain backgrounds and that rapamycin treatment attenuated age-associated periodontal bone loss in mice. This represents the first intervention shown to substantially attenuate age-associated periodontal bone loss.

2.2 INTRODUCTION

Age is the single greatest risk factor for many diseases including Alzheimer's disease, heart disease, diabetes, and periodontal disease (*7, 16, 17*). By delaying the biological aging process, it may be possible to reduce the impact of age-related diseases, which could have profound benefits for quality of life and reduced healthcare costs. In dentistry, age is risk factor for many oral diseases including periodontal disease, salivary dysfunction, and oral cancer (*7, 10, 16-18*).

The FDA-approved drug rapamycin, a specific inhibitor of mTOR, is a promising candidate to increase healthspan in humans. Treatment with rapamycin has been shown to increase lifespan in numerous species (*19*) and to delay or reverse multiple age-associated phenotypes including cancers (*20*), immune senescence (*13*), declining muscle function (*21, 22*) in mice, and cardiac dysfunction in both middle-aged mice (*23-25*) and companion dogs (*26*). Moreover, treatment with rapamycin derivative RAD001 was reported to rejuvenate and improve immune function in healthy elderly people (*14, 27*).

Periodontitis is an age-associated, bacterial-induced inflammatory disease of the oral cavity, which results in alveolar bone loss and tissue degradation (*28, 29*). Recent epidemiologic data in the U.S. population suggest that more than 60% of adults aged 65 years and older have periodontitis (*16, 17*). Thus, periodontal disease represents an important health concern among elderly people, for which there is not currently any effective treatment to reverse the condition.

As rapamycin is the most effective pharmacological agent for improving healthspan in mice (*30*), with known anti-inflammatory (*31*), and microbiome-modulatory effects (*21*), we assessed the effects of rapamycin on periodontal bone levels in aged mice.

2.3 RESULTS

2.3.1 Establishing Periodontal Bone Loss Assay

As periodontal bone loss is a hallmark for periodontal disease, we first evaluated periodontal bone levels by high-resolution μCT imaging of the maxilla. We compared young (2-3 month) versus old (24 month) C57BL/6JNia female mice and found a significant loss of periodontal bone. This was quantified by the distance between the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) at 14 predetermined maxillary sites, bilaterally (Figure 1.1 and 1.2).

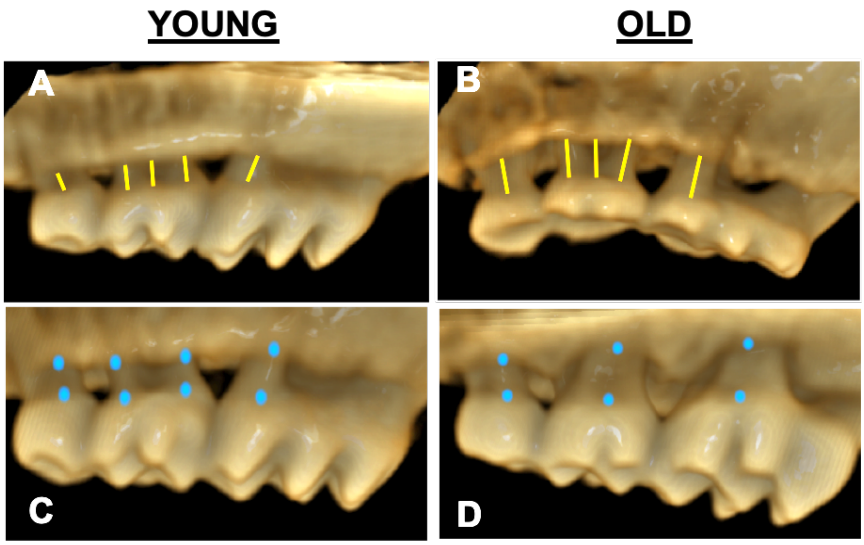

Figure 1.1. Normative aging causes periodontal bone loss in mice.
Representative μCT scan showing significant periodontal bone loss in old mice vs. young mice. A) Buccal aspect is shown for young mouse and a (B) old mouse. Yellow lines represent distance between the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) as landmarked by an observer who was blinded to the identity of each animal. The larger distance in panel B compared to panel A shows periodontal bone loss in the aged animal compared to the young animal. Distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured at 14 predetermined maxillary sites, C) 8 predetermined maxillary buccal sites and D) 6 predetermined maxillary palatal sites, bilaterally. The readings were totaled for each mouse.

2.3.2 Short-Term Rapamycin Treatment Attenuates Periodontal Bone Loss

Implementing the periodontal bone loss assay, we analyzed periodontal bone loss in C57BL/6JNia mice treated with either vehicle control (Eudragit) or 14ppm encapsulated rapamycin (eRAPA) in the diet for 8-weeks beginning at 22 or 24 months of age. In both males and females, rapamycin-treated animals had significantly greater periodontal bone levels after 8-weeks of treatment compared to the controls (Figure 1.2, $p<0.005$).

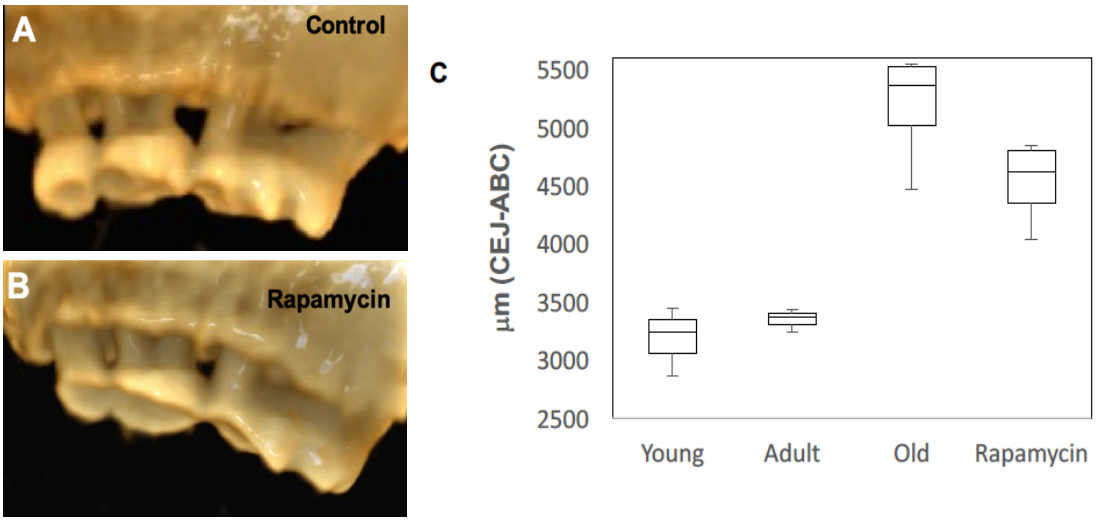

Figure 1.2. A single, short-term 8-week treatment with rapamycin attenuates periodontal bone loss in aged mice.
Female 24-26-month old C57BL/6 treated were treated with either a control diet or 42 ppm eRAPA diet for 8-weeks. µCT image analysis indicated less periodontal bone loss in the rapamycin treated mice at the end of the treatment period compared to control animals. Representative images of (A) control and (B) rapamycin-treated mice after 8-weeks. C) Boxplot showing total distance measured from CEJ-ABC buccal and palatal aspect only for 26-28-month-old female C57BL/6 mice treated with control or 42 ppm rapamycin beginning at 24-26 months of age. Rapamycin-treated animals had significantly greater periodontal bone levels compared to controls ($p<0.005$) each mouse.

2.3.3 Chronic Rapamycin Treatment Attenuates Periodontal Bone Loss

The National Institute on Aging Interventions Testing Program (ITP) has published several studies showing that dietary eRAPA can increase lifespan in the genetically heterogeneous UMHET3 mouse strain at doses ranging from 4.6 ppm to 42 ppm (*32-35*). We obtained the cranioskeleton from female UMHET3 mice treated with Eudragit, vehicle chow or with chow with 42 ppm eRAPA from 9 months of age until 35-37 months old and examined their periodontal bone levels by high-resolution µCT imaging of the maxilla. Aged UMHET3 mice were about a year older than previously shown aged C57BL/6JNia mice and had severe tooth wear on the palatal aspect (Figure 1.3), which caused the palatal landmarks to be difficult to identify. As a result, only the buccal aspect was evaluated bilaterally for quantitation of periodontal bone levels. As in transiently-treated C57BL/6JNia mice, UMHET3 mice treated with

rapamycin throughout life showed significantly greater periodontal bone levels compared to age-matched controls (Figure 1.3, $p<0.005$)

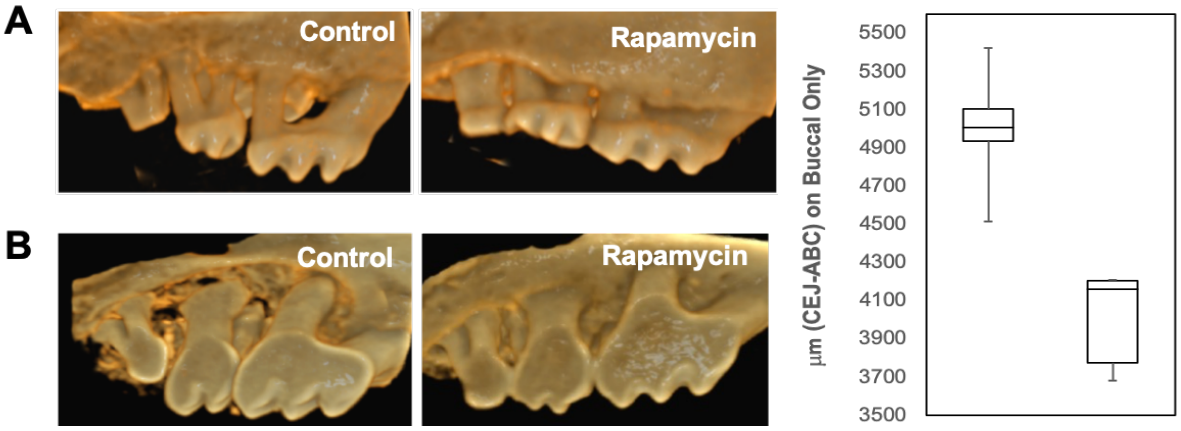

Figure 1.3. Chronic treatment with rapamycin attenuates periodontal bone levels in UMHET3 mice. A) Representative scans of buccal aspect from 32-36-month UMHET3 mice treated either Eudragit vehicle control or 42 ppm eRAPA rapamycin beginning at 9 months of age. B) Representative palatal aspect scans. C) Boxplot showing distance measure from CEJ-ABC buccal aspect only for 32-36 month old UMHET3 treated with control or 42 ppm rapamycin beginning at 9 months of age. Rapamycin treated animals had significantly greater periodontal bone levels compared to controls ($p<0.005$).

2.4 DISCUSSION

Periodontal disease is a major health concern among the elderly and may contribute to comorbidities including diabetes, cancers, cardiovascular disease. Currently, there is no treatment for age-related periodontal disease beyond preventative dental care, periodontal surgeries, and tooth removal, and approaches to prevention are imperfect. In this chapter, we demonstrate that the geroprotective compound rapamycin attenuates periodontal bone loss in aging mice. Mice treated with rapamycin for 8-weeks beginning around 22 months of age (roughly the mouse equivalent of 70 years of age in people) show about half the age-related periodontal bone loss (relative to 2-3-month-old mice) as age-matched controls. However, as the samples retrieved for this study was considered incidental tissues, we were only capable of completing a general observational study.

To further investigate how rapamycin attenuates age-associated periodontal bone loss, a study examining the changes in the periodontium during the normative aging process was

designed. However, unlike traditional models and studies that attempt to artificially replicate oral disease, we approached our study from a strictly Geroscience perspective in the subsequent chapters.

2.5 MATERIALS AND METHODS

Animal Care

Skulls utilized in this study came from previous completed studies. All animal procedures were previously approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Washington and the University of Michigan, in compliance with established Federal and State policies. The carcasses were stored in 10% neutral buffered formalin and then transferred in 1X PBS or 70% ethanol. The cranioskeleton were then retrieved for periodontal bone analysis. IACUC approval was not needed for scanning as samples were considered incidental tissue. Detailed information on the studies where the animals were collected is stated below:

C57BL/6JNia Mice

C57BL/6JNia mice in this study were obtained from the National Institute on Aging Aged Rodent Colony and housed as previously described (*23*). Female mice arrived at 17 months old, and were aged in house to 22 months, at which point they were given either rapamycin-containing diet (14 ppm rapamycin encapsulated in Eudragit, “eRapa”, in LabDiet 5053 chow) or control diet containing the encapsulation alone. Male mice arrived at 20 months old, were aged to 24 months old at start of treatment, and were treated with either control diet or eRapa diet at 42 ppm for 8-weeks. All animals were monitored daily. After 8-weeks, the animals were sacrificed by cervical dislocation.

UM-HET3 Mice

UM-HET3 mice were produced at the University of Michigan using the protocols of the National Institute on Aging Interventions Testing Program (ITP). In brief, genetically heterogeneous UM-HET3 mice were produced by a cross between CByB6F1/J mothers (JAX #100009) and C3D2F1/J fathers (JAX #100004). They were housed at four female mice per cage from weaning and at 9 months of age were given a diet containing encapsulated rapamycin (LC Labs) at 42 ppm (mg of drug per kg of food). Control mice received Purina 5LG6 food without added drug. Mice that died were not replaced, so that cage density declined at older ages. Cages were inspected daily. Date of death was noted for mice found dead, and mice found to be so ill that they were expected to die within the next 24–48 h were euthanized, with the date of euthanasia taken as the date of death for life table calculations. Mice chosen for this study were all females, and were selected because they had died at ages between 1054 and 1132 days. Mean age at death for the selected Control mice (1070 days) did not differ from that of the selected Rapa mice (1096 days), to minimize age at death as a possible confounding variable.

Microcomputed (μCT) Tomography

Cranioskeletons were scanned in a SkyScan 1076 microcomputed tomography (microCT) system at the Small Animal Tomographic Analysis Facility (SANTA) at Seattle Children's Research Institute. Resolutions were at 18 or 35-μm with following settings: 55 kV, 179μA, 360-ms exposure, 0.5 Al filter, 0.7° rotation step, and 3-frame averaging. Raw scan data were reconstructed with NRecon 1.6.9, and three-dimensional (3D) renderings were generated with Drishti 2.4 (Limaye 2012).

Periodontal Bone Loss Assay and Landmark

3D rendered images were randomized and landmarked by five-independent observers. For the the C57BL/6JNia mice, n=8 control, and n=8 rapamycin were analyzed, while for the UMHET3

mice n=6 control and n=6 rapamycin were analyzed. Periodontal bone loss was measured as distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) on 8 predetermined landmarks on the buccal surfaces of the maxillary molars and 6 predetermined landmarks on the palatal surfaces of the maxillary molars. The CEJ-ABC distances were totaled for each mouse through the Drishti software. Landmarks were completed by five-independent observers and means were calculated. A two-tailed *t* test was performed. $p < 0.05$ was taken as the level of significance.

Chapter 3. GEROSCIENCE PERSPECTIVE FOR GERIATRIC ORAL HEALTH RESEARCH

3.1 ABSTRACT

This chapter is adapted and compiled from (36)

While age is the greatest risk factor for oral health decline and diseases, a majority of preclinical oral health research has not adequately considered the importance of aging in research aimed at the mechanistic understanding of the aging oral cavity. We have attempted to provide insights from animal studies in the Geroscience field and apply them in the context of oral health research. In this chapter, we discuss a framework for defining and utilizing age-appropriate rodents and present experiment design considerations, including genetic background and site specificity.

3.2 INTRODUCTION

Current research on age-associated oral disease is largely limited to clinical studies seeking to characterize changes in the oral cavity during disease and to preclinical studies using animal models. A limitation of most preclinical studies is that they exclusively utilize artificial disease models in young rodents, thereby losing the contribution of the aged local and systemic environment. We suggest that the failure to utilize properly aged animals for preclinical, geriatric studies of oral disease limits the clinical relevance of such studies. Oral health research could potentially benefit from a greater consideration of the importance of aging biology to oral diseases.

3.3 Animals Models in Oral Health Research

While no single animal model species can replicate all aspects of human oral structure and function, studies using non-human primates, dogs, and rodents have provided important insights into mechanisms impacting oral health. Among these species, rodent models have been used extensively as the periodontal anatomy of rodent molars is similar to humans (*37, 38*). For instance, relationships between immune function, microbial changes within the oral cavity, and inflammation have been established through studies performed in rodent models (*39, 40*).

Common techniques used in rodent models of oral disease include oral gavage of periodontal pathogens or placement of ligatures in gingival sulcus. Both techniques ultimately result in the clinical phenotype of periodontal disease, such as periodontal inflammation and alveolar bone loss. For example, oral infection with *Porphyromonas gingivalis* and *Fusobacterium nucleatum* in a 4-week old rodent model revealed more alveolar bone loss and inflammatory response when both bacteria were combined (*41*). Another method to induce the clinical disease phenotype of periodontitis is the usage of placing silk sutures around rodent molars, a technique called the ligature model. The ligature model allows biofilm accumulation, disrupts the gingival epithelium, and enhances bone loss (*39*). For example, utilizing this method revealed that *P. gingivalis* exacerbates RANKL-dependent alveolar bone resorption via TLR2/TLR4 signaling in 8-10 week old mice (*42*).

However, many of these studies, including ones discussed above, reflect the biology of disease modeled in adult or still-developing, juvenile mice that may not reflect the biology of naturally developing human oral disease, which most often occurs in an aged individual who may already be experiencing declines in health. Despite many important discoveries, we believe that progress in understanding the mechanisms of age-associated oral diseases, such as

periodontal disease or oral cancer, have been hindered by a lack of appropriately designed preclinical research in the context of normative aging.

3.3.1 Defining an "Old" Mouse

The National Institute on Aging (NIA) and The Mouse in Biomedical Research (*43*) suggest that "old" are mice 18-24 months of age, middle-age is 10-14 months, and young is 3-6 months. In fact, most Geroscience studies would not consider a mouse to be old until 18-24 months of age. In order to model human geriatric dental health in mice, the animals used for these studies should be roughly similar in biological age to older people.

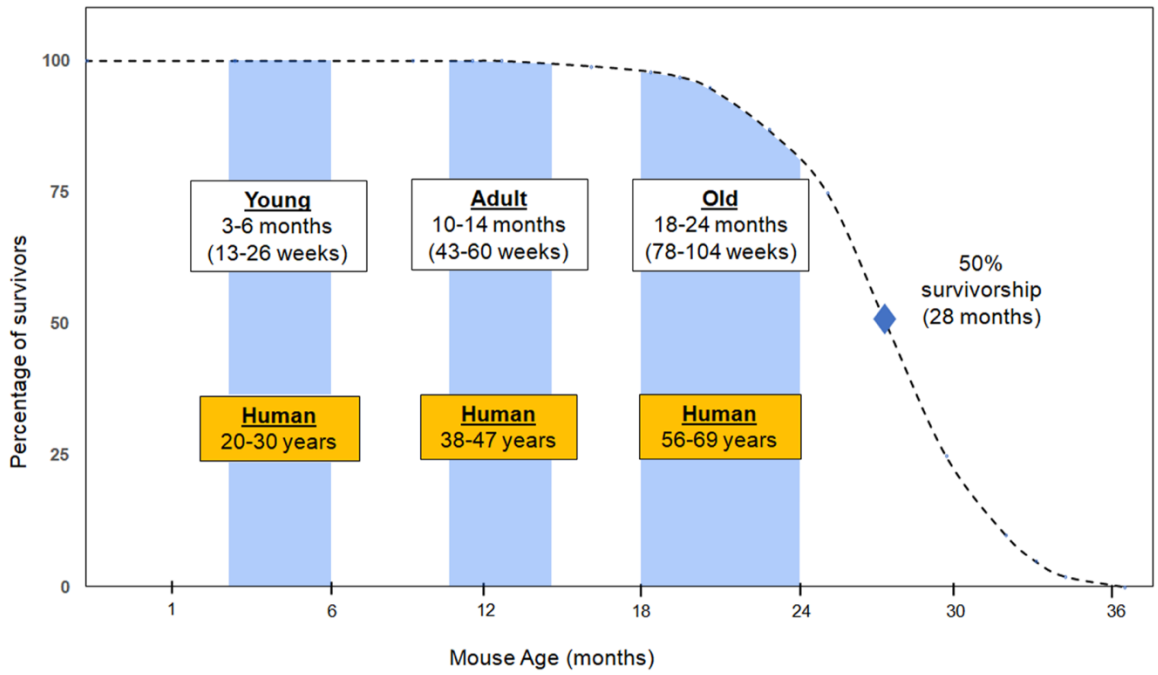

Figure 2.1. Mouse age is an important factor when modeling human age-associated oral diseases. A survival curve for C57BL/6JNia mice maintained in the laboratory *Adapted from (43).* A rough comparison of human age ranges is shown for different mouse ages. In order to recapitulate the contributions of physiological changes that can only occur in an aged model, a properly aged, old mice need to be used.

For example, a 3-month-old young mouse is roughly equivalent to 20-year-old human, while an 18-month old mouse will be approximately equivalent to a 56-60-year-old human. To understand the mechanisms of oral disease that are particularly important in the elderly, even

older mice (perhaps up to 30 months of age) should be used; however, it is important to consider the differences between pathological changes due to disease progression versus changes associated with normative aging, particularly when utilizing animals at advanced ages. Thus, it is suggested that mice at least 18 months of age, and preferably 20-24 months of age, are appropriate for most rodent studies of age-related oral health (Figure 2.1).

3.3.2 Strain Background and Site Location

While there is no "right" choice of strain background, it is important to keep in mind that different mouse strains do differ somewhat in lifespan and disease burden. Some strains, for example are particularly prone to certain forms of cancer. For example, Balb/C mice have a 44% incidence rate of lymphoma at 13 months of age, while SJL/J mice have a 91% incidence rate of reticulum cell sarcoma at the same age (*44*). With respect to longevity, there are wide ranges among mouse strains for both median and maximum lifespan and interesting sexual dimorphisms that can go both directions. For example, BALB/cBY median lifespan for males has been reported as 707 days and 757 days for females, while C57BL/6J median lifespan has been reported as 901 days for males and 866 days for females, but these values also vary from study to study (*44, 45*). In another study of 31 inbred mouse strains, median lifespan ranged from 251 days to 964 days across the different strains, with correspondingly high variance in a variety of age-associated phenotypic measures (*46*). The Jackson Laboratory Mouse Phenome Database contains a large number of age-related measures for different mouse strains and can be a useful resource when designing studies of aging biology in mice (*47*).

Along with strain variances, facility differences may impact Geroscience studies investigating oral health and diseases. From vendor and genetic backgrounds to subtle differences in housing conditions, such as bedding, cage, and diet, can change the biology of the mice. A consequence and confounding factor in oral health research is the alteration of the oral microbiome due to environmental impacts. Studies have shown that subtle housing

differences significantly alters the gut microbiota (*48-50*), thus such variation may also reshape the oral microbiome. For example, it has been reported that mice genotype and housing environment are important factors in the susceptibility of bacterial-induced periodontitis models (*51-53*). The differences in the baseline oral microbiome due to environmental or genetic factors may potentially explain the variation to periodontal disease susceptibility. Overall, in addition to considering strain variances, utilizing different facilities to potentially rule out confounding effects of the environment should also be taken into consideration.

3.4 CONCLUSION

Recently, the National Institute of Dental and Craniofacial Research (NIDCR) has also begun to promote collaborations to improve the oral health of older adults living the United States by “addressing knowledge gaps in the etiology and management of dental, oral and craniofacial diseases associated with aging”. This represents an important opportunity to better understand the role of aging biology in oral health and disease. Here we have attempted to provide some insights from the Geroscience field to facilitate oral health studies in rodent models. In the next chapter, we have implemented a majority of these considerations into our study design. We reveal in the subsequent chapter that by properly integrating Geroscience perspectives to understand oral health and disease has brought us closer to the possibility of translating these findings in our elderly patients.

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Chapter 4. RAPAMYCIN REVERSES AGE-ASSOCIATED PERIODONTAL DISEASE

4.1 INTRODUCTION

Age is associated with failure to maintain homeostasis resulting in degradation of the cellular maintenance and repair processes (*54*). Such deterioration is the single greatest risk factor for many human diseases including cardiovascular disorders, neurodegenerative diseases, diabetes, and cancer. Interventions that target specific aging hallmarks have shown delay or prevent age-related disorders and extend lifespan. The specific mTOR inhibitor rapamycin is one such intervention that extends lifespan in mammals and impacts aging phenotypes.

Rapamycin is an FDA approved drug which directly inhibits the mechanistic target of rapamycin complex I (mTORC1). Transient or life-long treatment with rapamycin extends lifespan and healthspan in multiple organisms, ranging from nematodes to primates (*55*). Rapamycin extends lifespan of mouse in both sexes, even administered at 9 or 20 months of age in genetically heterogenous UMHET3 mice (*32, 35*), or late in life 20-21 months of age (*56*), and the extension is both dose- and sex-dependent. In addition to extension of lifespan, rapamycin has been shown to rejuvenate the immune function in both old murine models (*57*) and health elderly humans (*14, 27*), alter bone metabolism (*15, 58*), and remodel the microbiome (*56*).

The periodontium, the specialized tissue that surround and support the tooth consisting of the gingiva, periodontal bone, cementum, and periodontal ligament, is one site in the entire body where immune function, bone remodeling, and microbiome exist in a homeostatic environment. A disruption in this balance leads to a chronic inflammatory disease known as periodontitis. By definition of the American Academy of Periodontology (AAP), periodontitis

[periodontal disease] is inflammation of the periodontal tissue [periodontium] resulting in clinical attachment loss, alveolar bone loss, and periodontal pocketing, and associated with variable microbial pattern. Most recent epidemiologic data in the U.S. population suggests that more than 60% of adults aged 65 years and older have periodontitis (*16, 17*), while being associated with other age-related conditions such as heart disease, diabetes, and Alzheimer's disease (*10, 18, 59*). Thus, periodontal disease represents a potential cause for diminished healthspan (*60*) and a major cause of lower oral health-related quality of life (OHRQol) in the elderly (*61, 62*) for which there is currently no effective treatment.

We have shown in a previous chapter that both short-term and chronic rapamycin treatment was able to attenuate periodontal bone loss, a clinical defining phenotype of periodontal disease (See Chapter 2), albeit the analysis was completed with incidental tissues where the actual studies were completed at least a few years prior. In this study, we have included appropriate study design considerations by implementing Geroscience into oral health research (Chapter 3) and analyzed age-associated changes in the periodontium during the normative aging process in C57BL/6 mice in two separate laboratory sites. Here, for the first time, we show that short-term rapamycin treatment diminishes inflammation in the gingiva and periodontal bone, regenerates periodontal bone, and remodels the oral microbiome, ultimately reversing age-associated periodontal disease.

4.2 RESULTS

4.2.1 Multi-Institution Study Design

To examine the impact of rapamycin on the periodontium during normative aging, we designed a multi-institutional study between the University of Washington (UW) and the Jackson Laboratory (Jax) (Figure 3.1). The UW cohorts of C57BL6/JNia (hereafter termed NIA-UW Colony) were received directly from the National Institute on Aging (NIA) and acclimated within

the UW facilities in Seattle, Washington. The Jax cohorts of C57BL/6J (hereafter termed JAX Colony) were born and raised within the Jax facilities in Bar Harbor, Maine. We then treated mice at both sites with encapsulated rapamycin (eRAPA) in the diet at 42ppm, which has shown to significantly improve survival in the NIA Interventions Testing Program (ITP), or control food (eudragit) (*35*) (Figure 3.2). Most importantly, all mice were properly aged Geroscience standards to where young animals were 6-month old, adult animals were 13-month old, and old animals were 22-month old (See Chapter 2).

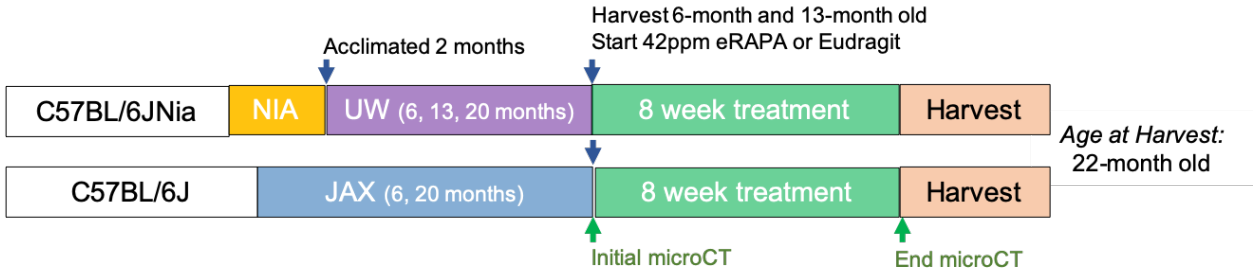

Figure 3.1. Mouse age is an important factor when modeling human age-associated oral diseases.
All mice were of C57BL/6 background. The NIA-UW colonies were received directly from the NIA Aged Rodent Colony at 4, 11, and 18-months, then acclimated for two months within the UW facilities (ARCF) until they reached 6 (Young), 13 (Adult), and 20-months (Old). The Young and Adult cohorts were harvested for oral tissues and microbiome. The Old cohorts were randomized and either given Eudragit or 42ppm eRAPA within the food for 8-weeks. For the JAX colonies, an initial μCT image was taken prior to the 8 week treatment and then a final μCT before harvest. All animals were harvested at the end of 8-weeks, ~22-months old.

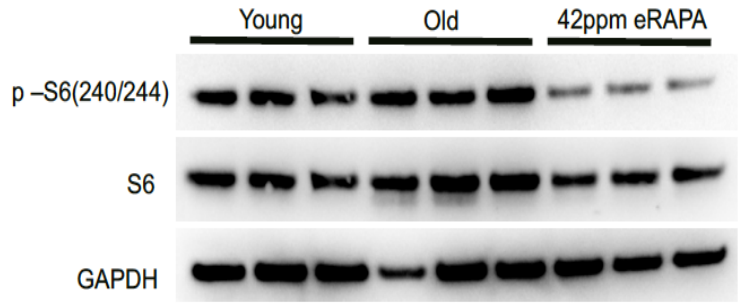

Figure 3.2. 42ppm eRAPA decreases mTOR signaling in murine oral cavity.
Western blotting verifying that rapamycin inhibits mTOR signaling. We show a decrease in phosphorylation of ribosomal protein S6, a downstream substrate of mTOR. Individual periodontal bone samples per lane is shown as representative of periodontium.

4.2.2 Rapamycin Decreases Inflammation in the Aging Periodontium

One result of the failure to maintain homeostasis during the aging process is the senescence of the immune system and associated increase in inflammation (*63-66*), a collective termed known as inflammaging. The nuclear factor-κB (NF-κB) is the hub of immune and inflammatory response that has been shown to be activated during periodontal disease (*67, 68*) as well as during the aging process (*69-71*). Deregulation of the NF-κB signaling can lead to overproduction of pro-inflammatory cytokines and development of chronic inflammatory disorders, such as rheumatoid arthritis and Crohn's disease (*72-74*). We first evaluated the NF-κB hub by analyzing both NF-κB p65 and IκBα expression levels in the gingiva and periodontal bone. Amongst the various structures supporting the tooth and oral cavity, we have initially focused on the gingiva and periodontal bone as inflammation and bone loss are one of the first clinical hallmarks of periodontal disease.

The gingival epithelium is a critical interface between the external and internal environment, while serving as a first line of defense to the multitude of microbial species in the oral cavity. While initially thought as only a protective layer, the gingiva is a major immunosurveillance organ in the oral cavity which tailors immune mechanism in response to the diverse microbiome, continuous barrier mechanical damage from hygiene regimens and mastication, and airborne pathogens via antimicrobial peptides and inflammatory mediators (*75-77*). Along with the gingiva, the immune mechanism is also critical to periodontal bone homeostasis.

Bone is a dynamic and active tissue, undergoing constant renewal in response of hormonal, mechanical, and nutritional influences. The periodontal bone is in constant interplay with bone resorbing osteoclasts and bone forming osteoblasts and maintaining homeostasis in the midst of the continuous remodeling cycle. As stated above, dysregulated NF-κB signaling

causes an overproduction of inflammatory mediators, and one consequence such increase in the periodontium is periodontal bone resorption, a hallmark of periodontal disease (*28, 78*). Thus, analysis of NF-κB in both the gingiva and periodontal bone will uncover if this major inflammatory-mediating hub is altered during the normative aging process.

We discovered that in both the gingival tissue and periodontal bone, there was an increase in NF-κB p65 expression with corresponding alteration of IκBα levels, showing that there was age-associated increase in NF-κB signaling in the periodontium. Further, 8-weeks of rapamycin treatment was sufficient to ameliorate this increase in expression (Figure 3.3).

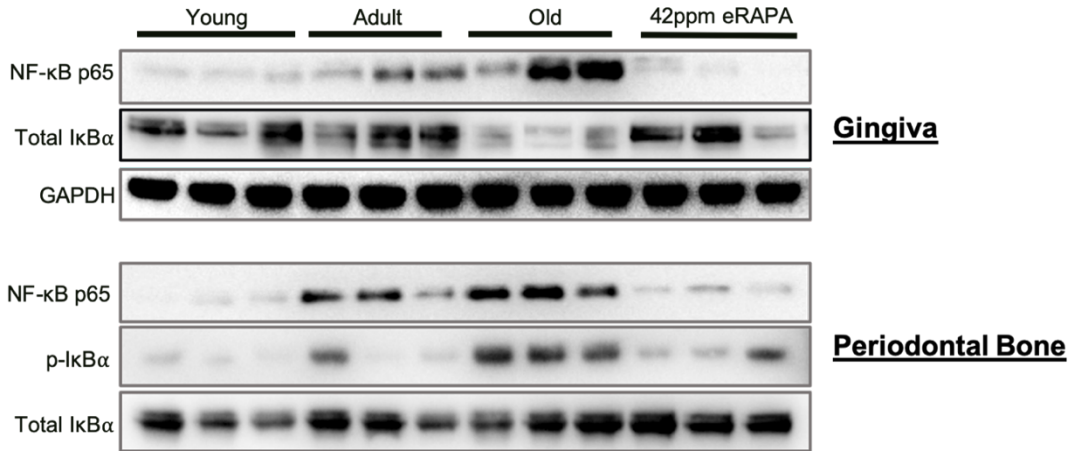

Figure 3.3. Rapamycin alters increased NF- κB expression in periodontium
NF- κB p65 and IκBα expression was determined by Western blot analysis of total lysates from the gingiva and periodontal bone of properly aged animals (Young, Adult, and Old) and Old animals treated for 8-weeks with rapamycin (42ppm eRAPA). GAPDH was used a loading control. In both the aging gingiva and periodontal bone there is an overall increase in expression of NF- κB p65 with corresponding alteration of IκBα. 8-weeks of 42ppm eRAPA treatment attenuated the altered changes seen with the increased NF- κB p65 expression. For the gingiva, each lane represents pooled gingiva from animals co-housed (n=1-2), and each lane for the periodontal bone western blot represents individual samples.

As NF-κB modulates transcription of various inflammatory cytokines and chemokines during chronic inflammation, we extended our examination and evaluated the inflammatory cytokines and chemokines downstream. Old gingival tissues showed increased levels of known markers associated with periodontal disease such as TNF-α, IL-1β, and CXCL5 (Figure 3.4.A). In the old periodontal bone samples, there was also increase in inflammatory markers such as

TNF-α, IL-1α, IL-1β, IL-6, and MMP9 (Figure 3.4.B). 8-weeks of rapamycin treatment altered the expression of age-associated chemokine and cytokine changes in both the gingival tissue and periodontal bone (Figure 3.4). Together, along with NF-κB signaling increase with age, our data indicate that 8-weeks of 42ppm eRAPA is sufficient to attenuate the age-associated increase in inflammation in both the gingiva and periodontal bone.

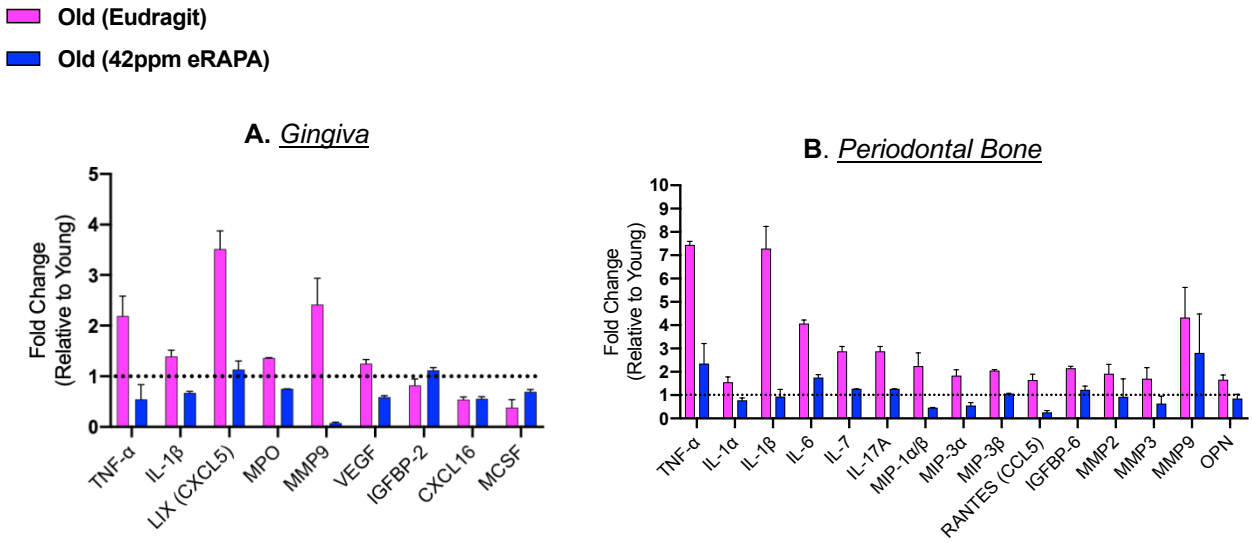

Figure 3.4. Aging alters inflammatory cytokine and chemokine profiles, and rapamycin modifies the expression profiles.
Protein expression levels of mouse cytokines and chemokines was determined by a spotted nitrocellulose membrane assay (Proteome Profiler Mouse, R&D Systems) by loading pooled samples from gingiva (A) and periodontal bone (B) of Young and Old (Control, Eudragit), and Old animals treated for 8-weeks with rapamycin (42ppm eRAPA). Data is shown per manufacture's protocol, with fold-change relative to young animals (set to 1), expressed as mean ± SEM. Statistical significance set to $p < 0.05$.

4.2.3 Rapamycin Reverses Age-Associated Periodontal Bone Loss

The periodontal bone is constantly remodeled, and the disruption of bone homeostasis occurs during periodontal disease ultimately leading to periodontal bone loss. As we have seen an increase in inflammation within the aging periodontium, we extended our observation by evaluating periodontal bone homeostasis. To assess the periodontal bone homeostasis during normative aging, we evaluated RANKL expression, which is critical mediator in osteoclast formation where over-expression leads to bone resorption (*28, 79*), then completed histological

analysis to evaluate TRAP+ cells which indicate osteoclast numbers (*78*), and finally μCT scanned the NIA-UW and JAX colonies to measure periodontal bone levels. We discovered a significant increase in RANKL expression (Figure 3.5.A) and $TRAP^+$ cells (Figure 3.5.B) in the periodontal bone, as well as periodontal bone loss with age (Figures 3.6 & 3.7).

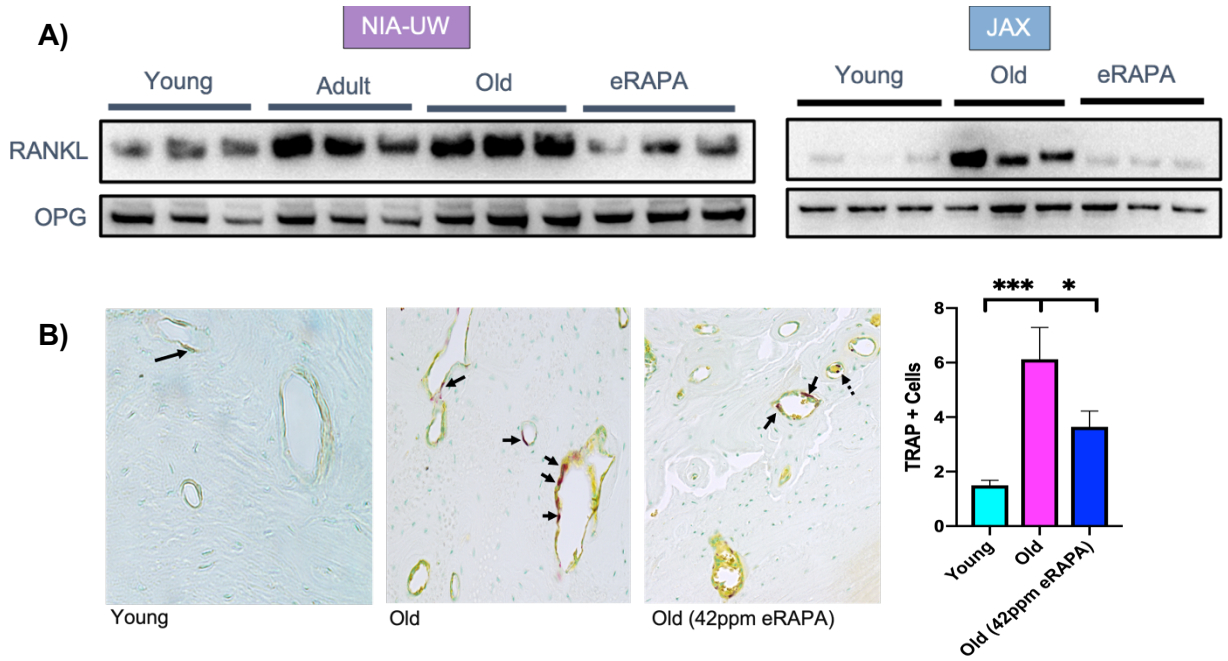

Figure 3.5 Rapamycin attenuates age-associated increase in RANKL expression and TRAP+ cells in periodontal bone
(A) RANKL and OPG expression was determined by Western blot analysis of total lysates from the periodontal bone of aged animals (Young, Adult, and Old) and Old animals treated for 8-weeks with 42ppm rapamycin (eRAPA). The periodontal bone within both the NIA-UW and Jax Colonies showed an increased expression of RANKL while 8-weeks of rapamycin treatment ameliorated the increased RANKL expression. Each lane represents individual periodontal bone samples. (B) Representative histological sections at 200x magnification. Sections have undergone TRAP azo-dye staining with FastGreen counterstain. Enumeration of $TRAP^+$ cells within the periodontal bone from two-independent observers reveals an increase number of $TRAP^+$ cells with age, and diminishes with rapamycin treatment. Statistical analysis completed with unpaired t-test, with significance set to $p<0.05$. *** $p<0.005$, * $p<0.05$

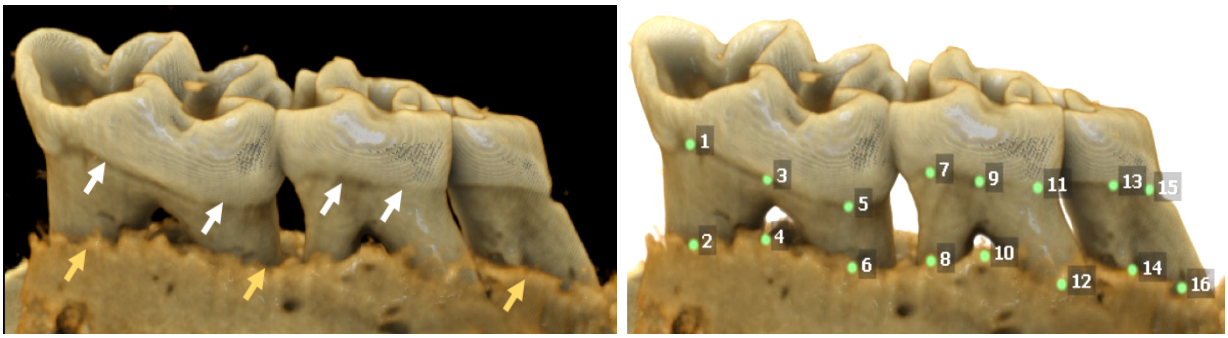

Figure 3.6. Assay for Measuring Periodontal Bone Loss
Representative image of a mandible is shown. Periodontal bone loss was measured as distance from the cementoenamel junction (CEJ, white arrows) to alveolar bone crest (ABC, orange arrows) on 16 predetermined landmarks on the buccal aspect of maxillary and mandibular periodontium. The CEJ-ABC distances were totaled for each mouse.

As rapamycin has been shown to affect osteoclast survival (*15*) and periodontal bone (*12*), we assessed periodontal bone levels in the old animals treated with rapamycin. Molecular changes in the aging periodontal bone, including increased RANKL expression and TRAP+ cell numbers, were attenuated with rapamycin treatment (Figure 3.5). Also, consistent with our previous report, age-associated periodontal bone loss was attenuated with just 8-weeks of rapamycin treatment in the old animals (Figure 3.7).

Utilizing our multi-institution collaboration, we then asked whether the attenuation in periodontal bone loss in the rapamycin-treated animals was prevention or reversal. To answer this question, we imaged the same old animal before and after 8-weeks of rapamycin or control-fed diet. In the control-fed old animals, 8-weeks did not significantly alter periodontal bone loss levels. Surprisingly, old animals treated with rapamycin for 8-weeks had a significant gain in periodontal bone, indicating a reversal of periodontal bone loss (Figure 3.8). Taken together, 8-weeks of 42ppm eRAPA reversed the age-associated periodontal bone inflammation and loss in old animals.

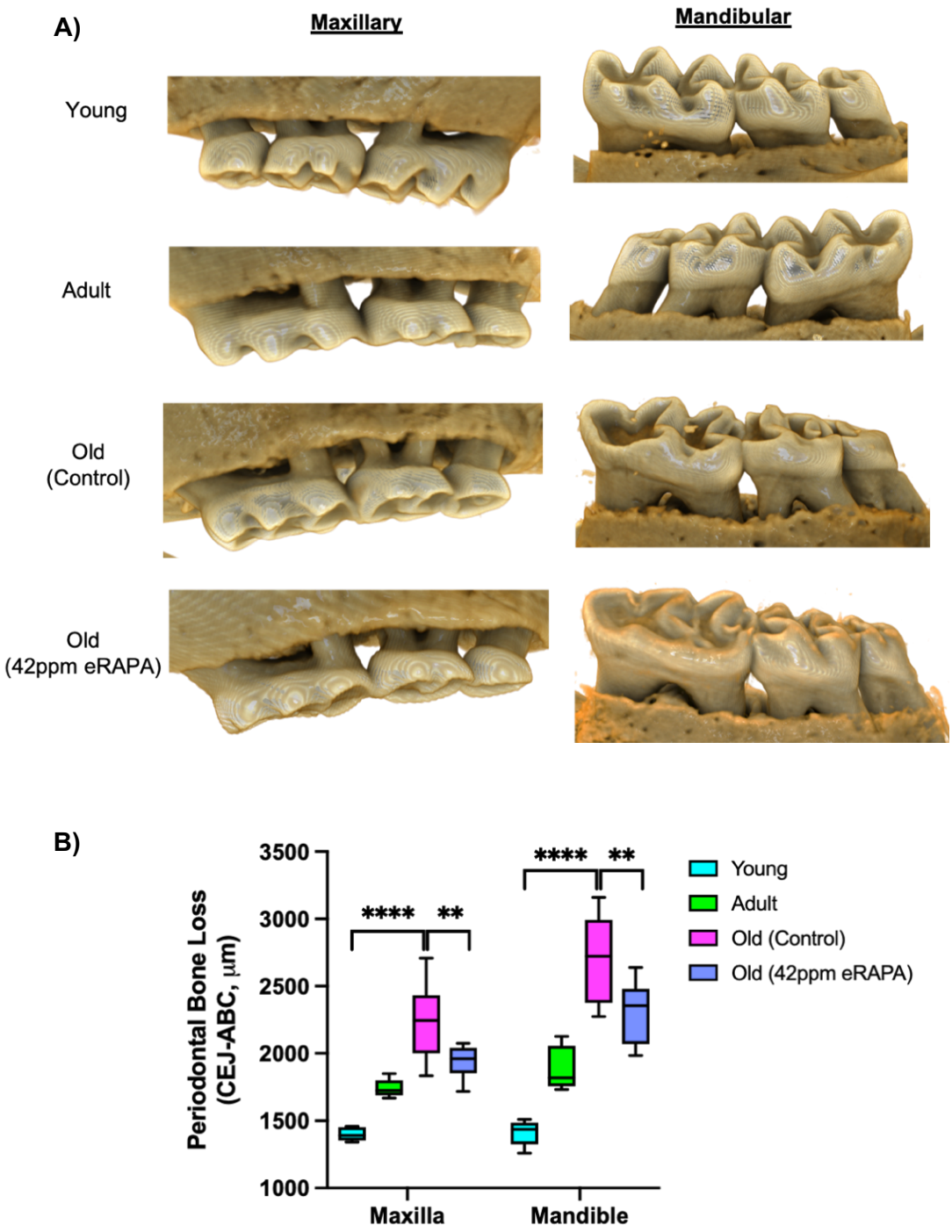

Figure 3.7. Rapamycin attenuates age-associated periodontal bone loss (NIA-UW)
A) Representative images of maxillary and mandibular teeth of Young, Adult, Old, and Old treated with 42ppm eRAPA revealing age-associated periodontal bone loss with age. 8-weeks of 42ppm eRAPA attenuated periodontal bone loss. B) Box-and-whiskers plots shows median, 25th and 75th percentile with whiskers at the 5th and 95th percentile. Statistical analysis was completed using unpaired t-test, with $p< 0.05$ were considered statistically significant. ** $p<0.01$,****$p<0.001$

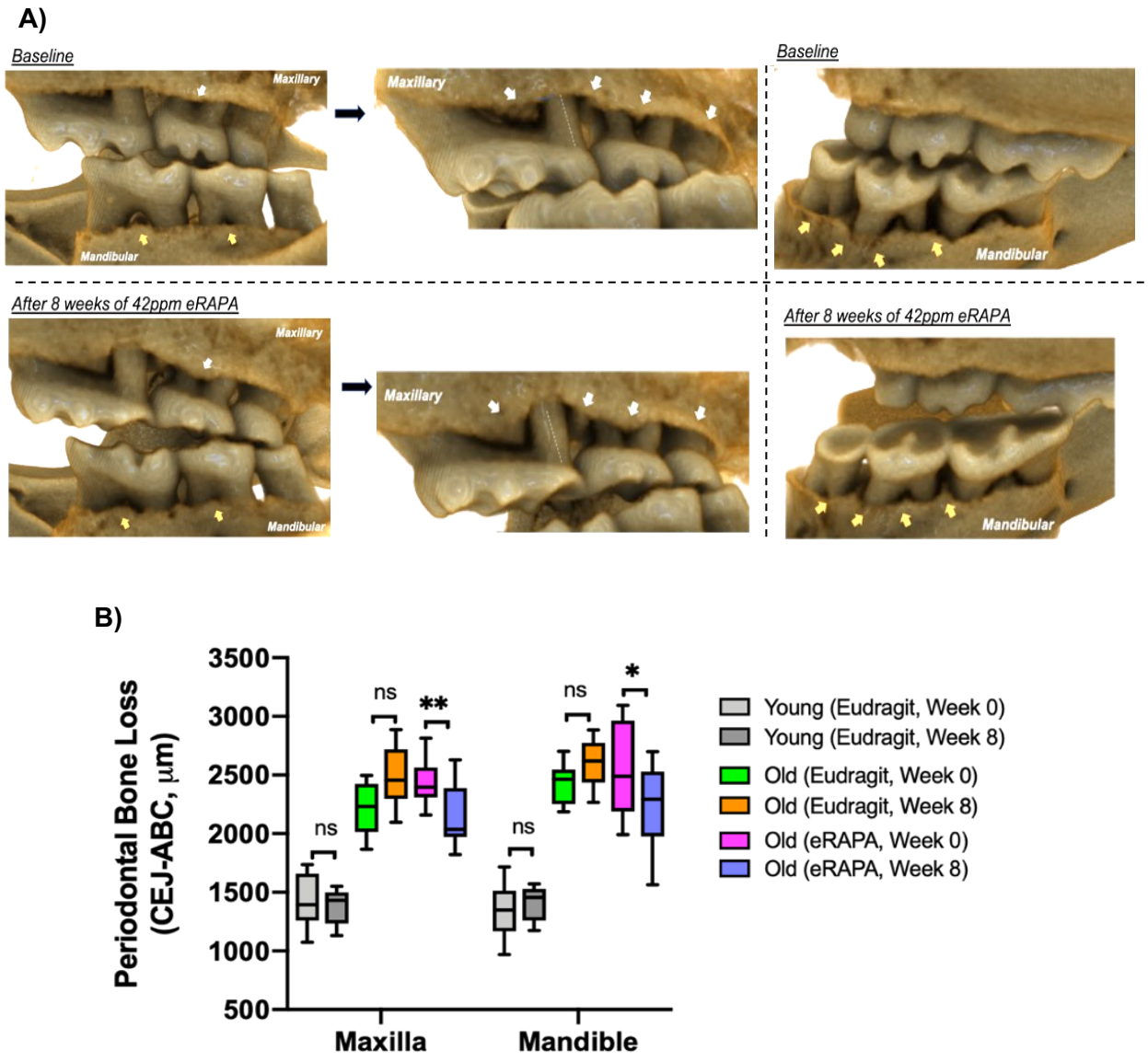

Figure 3.8. Rapamycin reverses age-associated periodontal bone loss (Jax)
A) Representative images of maxillary and mandibular teeth of the same Old animal at baseline and then 8-weeks with 42ppm eRAPA. White arrows indicate maxillary alveolar bone crest, and orange arrows indicate mandibular alveolar bone crest. On both the maxilla and mandible, there is periodontal bone loss around and in-between the molars, but after 8-weeks of 42ppm eRAPA the bone loss is reversed. B) Representative image of the maxillary and mandibular teeth of a different old animal. Orange arrows indicate alveolar bone crest on mandible. After 8-weeks of 42ppm eRAPA treatment, there is reversal of periodontal bone around the mandibular molars. C) Box-and-whiskers plots shows median, 25th and 75th percentile with whiskers at the 5th and 95th percentile. Longitudinal comparison was completed with the same animal at baseline or after 8-weeks with either eudragit (control) or 42ppm eRAPA. Statistical analysis was completed using paired t-test, with p-values < 0.05 were considered statistically significant. **p<0.01, *p<0.05

4.2.4 Rapamycin Remodels the Oral Microbiome

An estimated 40 trillion bacteria reside in the human body with a ratio of 10 bacterial cells to every 1 human cell (*80*). Such contrast in number emphasizes the important relationship between the microbiota to its host to maintain health. Of all the external and internal surfaces of the human body, including skin, gastrointestinal, or conjunctiva, the oral cavity is a unique, heterogenous environment for bacterial colonization.

The oral cavity is composed of distinct locations, such as the tongue, palate, cheek, gingiva, and tooth, where environmental conditions and location-specific biological properties selects for distinct microbial communities (*81*). One major biological change that can affect the bacterial species balance is aging. For example, the gut microbiota profile is altered in elder people compared to adults (*82-85*). While the different microbiome profiles can be attributed to age-related declines, such as weakened immune response, dietary changes, or use of medications, generally is there an overall reduction in gut commensals, such as lactobacilli, while increase in opportunistic microbiota, like as enterobacteria, in the elderly (*84, 86, 87*). However, in contrast to the gut microbiota, little is known about the development of the oral microbiota during the aging process and its role in maintaining periodontal health and disease.

The shift in the microbial communities in the oral cavity in healthy versus disease sites is a potential indicator and predictor of periodontal health, as changes within the microbiome community are associated with changes in the clinical status of the periodontal tissues (*28*). The oral cavity is unique in that controlled subclinical levels of inflammation, which is induced by oral commensal bacteria, is part of an active process leading to oral homeostasis (*53, 88*). During periodontal disease, the underlying biofilm triggers an overactive inflammatory response leading to periodontium degradation and tooth loss. In humans, the growth of periopathogens such as *Poprhyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia* has been shown in periodontal disease (*29, 89-91*) .

Advancement in culture-independent high-throughput technologies, such as proteomics or genomics, has led to more in-depth analysis of the oral microbiome. The most common genomics approach in investigating the oral microbiome community is based on the 16S ribosomal RNA (16s rRNA) gene. The 16S rRNA gene is comprised of approximately 50 functional domains and encodes for the 30S small ribosomal subunit that is evolutionarily conserved in all prokaryotes, making it a universal target for bacterial identification.

By implementing 16S rRNA genomic sequencing, we discovered that beta diversity, which measure the diversity between samples, is significantly different with age in both the UW and Jax animals. Visualization of the beta diversity via principal coordinate analysis (PCoA) shows a clear separate segregation and clustering of control, old animals versus old animals treated with rapamycin for 8-weeks, in both sites (Figure 3.9).

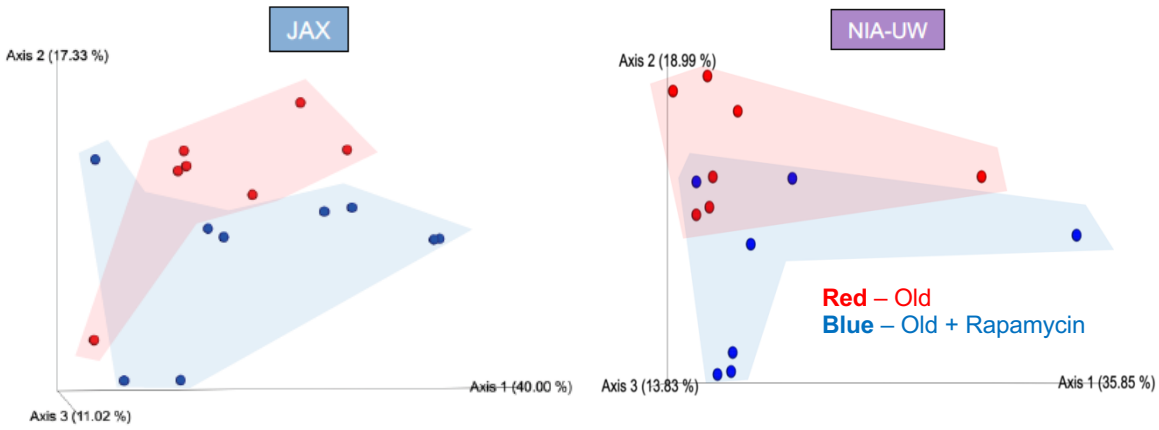

Figure 3.9. Beta diversity differences in microbial compositions in old animal treated with rapamycin
Beta diversity patterns using Principal Coordinate Analysis (PCoA) of weighted UniFrac distances. PCoA of weighted Unifrac distances reveal clustering within each group and spatial segregation between old animals and old animals treated with rapamycin in both sites, Jackson Laboratory (Jax) and University of Washington (NIA-UW). Red dots indicate Old animals. Blue dots indicate Old animals treated with 8-weeks, 42ppm eRAPA.

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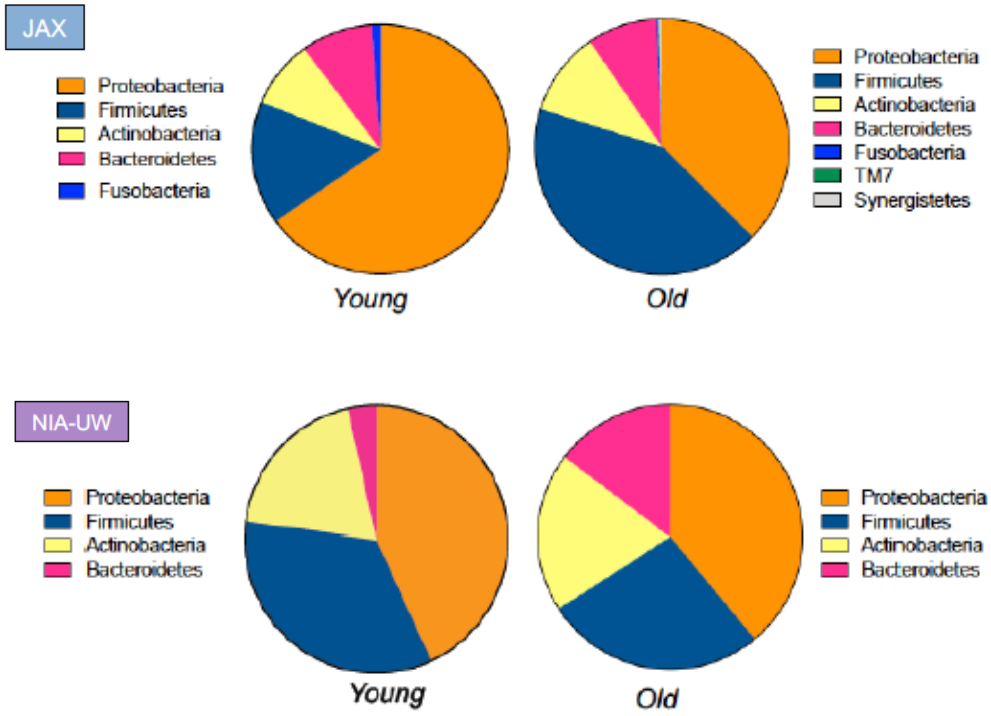

Figure 3.10. Oral microbiome is altered with normative aging.
Relative abundance plots of the oral microbiome at the phylum level. The average relative abundance of major microbial phyla is shown. Young and Old is shown for animals raised at the Jackson Laboratory (Jax, Bar Harbor, ME) and animals raised at the UW ARCF (NIA-UW, Seattle, WA). Site-dependent difference at the phyla level is seen with normative aging.

We then examined the oral microbial composition at the phylum level. With normative aging there is a relative difference in the phylum abundance in the periodontium across both UW and JAX (Figure 3.10). For example, there is an increase relative abundance of Firmicutes, Bacteroidetes, Actinobacteria, TM7, and Synergistetes, while decrease of Proteobacteria and Fusobacteria within the JAX animals with age. There were also facility-specific phyla detected. For example, TM7 and Synergistetes were only detected in the old animals in the Jax facility (Figure 3.10). In the old, UW-NIA animals, there was also a decrease in Proteobacteria and increase in Bacteroidetes and Firmicutes. Results from both locations are in line with previous reports showing in chronic periodontitis there is abundance of Synergistetes and Bacteroidetes, and decreased abundance of Proteobacteria (*92, 93*). With 8-weeks of rapamycin treatment, old

animals in both facilities showed increase in the relative abundance of Proteobacteria and decrease of Actinobacteria and Bacteroidetes (Figure 3.11).

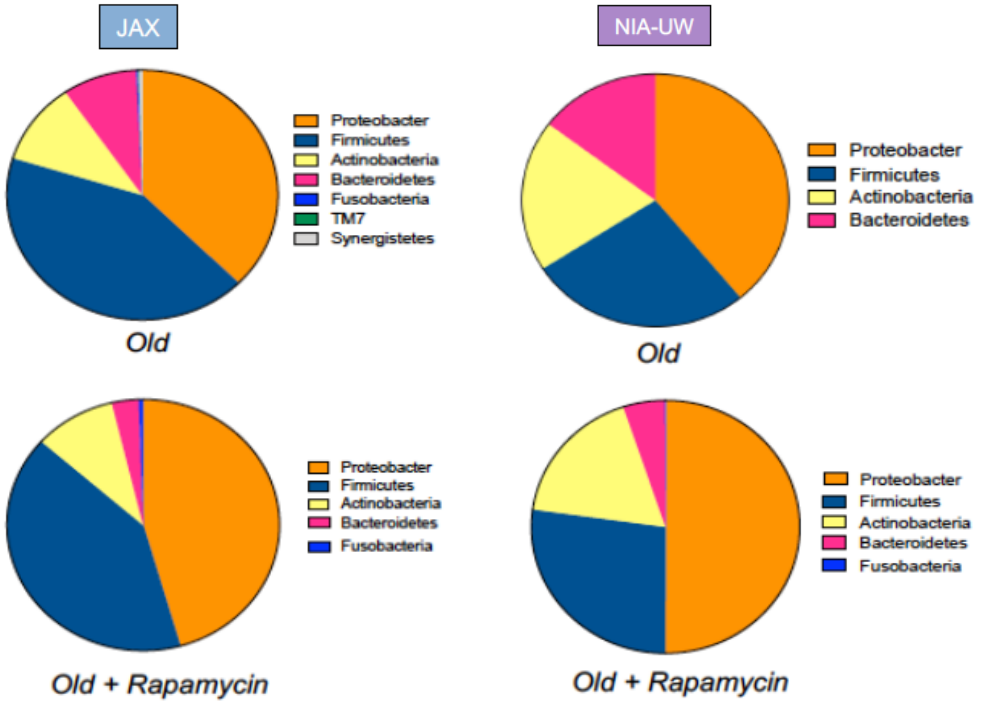

Figure 3.11. Oral microbiome is altered with rapamycin treatment.
Relative abundance plots of the oral microbiome at the phylum level. The average relative abundance of major microbial phyla is shown. A) Relative abundance in Old and Old + Rapamycin is shown for animals raised at the Jackson Laboratory (Jax, Bar Harbor, ME) and for animals raised at the UW ARCF (NIA-UW, Seattle, WA).

The 16s rRNA analysis revealed that there is an age-associated change in the oral microbiome in both facilities, while both young and old animals have a distinct oral microbiome composition that is facility-dependent. Moreover, regardless of the facility-dependent differences in the oral microbiome of the old animals, 8-weeks of rapamycin shifts the oral microbiome in old animals, specifically altering levels of Proteobacteria, Actinobacteria, and Bacteriodetes phylum.

4.3 DISCUSSION

Periodontal disease is a major health concern among the elderly and may contribute to other age-related comorbidities, including diabetes, cardiovascular disease, cancers, and dementia. Currently, there is no effective treatment for age-related periodontal disease beyond preventative dental care, periodontal surgeries, and tooth removal, and approaches to prevention are imperfect. In this report, we demonstrate that the geroprotective compound rapamycin reversed age-associated periodontal disease by attenuating both gingival and periodontal bone inflammation with age, regenerating periodontal bone loss, and altering the oral microbiome in only 8-weeks in old mice (roughly the mouse equivalent of 70 years of age in people).

Clinical treatment and attempts have been made to target hallmarks of periodontitis to attenuate or reverse the disease, such as gingival inflammation, bone regeneration, or altering the microbiome. However, these possible interventions are site and tissue specific with limited success, and majority of studies proposing potential therapies have modeled age-associated periodontal disease in young adult, or still-developing, juvenile mice. This is a potential problem as such studies may not reflect the biologically of natural developing human oral diseases, which most often occurs in an aged individual who may already be experiencing declines in immune function and other physiological systems. Here we show that with just normative aging in the murine model, the hallmarks indicating periodontal disease, including both gingival and periodontal bone inflammation, periodontal bone loss, and variable microbiome, occurs.

In the aging gingiva and periodontal bone (hereafter collectively termed periodontium), NF- κB with altered IκBα expression was upregulated which indicate an increase in the proinflammatory signaling pathway (*94*). The NF- κB pathway has roles in chronic inflammatory diseases, such as in rheumatoid arthritis, by regulating expression of various pro-inflammatory mediators (*73*) and mediates osteoclastogenesis (*68*). In the periodontium we discovered an

increase in inflammatory cytokines and chemokines, such as TNF-α, IL-1α, IL-1β, IL-6, and RANTES, as well as adhesion molecules, such as MMPs, with age. Within the periodontal bone, we also detected an increased expression of RANKL, which is a major mediator in bone resorption, and increase in a histochemical marker for osteoclasts, TRAP. The molecular data is complemented with our μCT analysis showing periodontal bone loss in the aging mouse at two different housing sites. In addition, our 16s rRNA sequencing data reveals an age-associated change in the oral microbial composition in two separate facilities. Based upon these collective results, we conclude that with age mice develop periodontal disease, a bacterial-induced chronic inflammatory disease of the supporting tissues of the tooth (periodontium) leading to periodontal bone destruction.

With known effects on immune rejuvenation (*14, 57*), bone homeostasis (*15, 95, 96*), and microbiome shifts (*56*), we gave old mice an 8-week treatment with the geroprotective compound rapamycin. This short-term treatment with 42ppm rapamycin-containing diet was sufficient to mitigate chronic inflammation in the periodontium and reverse periodontal bone loss. Further, we show a facility-dependent change in the oral microbiome with age, but a facility-independent alteration of the oral microbiome with rapamycin treatment, specifically a decrease in Bacteriodetes and increase Proteobacteria phylum, in both sites. Interestingly, we see the Bacteriodetes phylum consistently increased with age, and is decreased with rapamycin treatment in both facility sites. The Bacteriodetes is a major phylum revealed to play a role in periodontal disease, while bacteria such as *Porphyromonas gingivalis* and *Treponema denticola,* two red-complex periododontal pathogens, are part of this lineage (*97, 98*). Attenuation of overall periodontium inflammation, reversal in periodontal bone loss, and shift in the oral microbiome after 8-weeks of rapamycin treatment all indicate a reversal of age-associated periodontal disease.

While the role of the microbiome is thought to play a critical etiology of periodontal disease (*99-103*), we believe the changes to the oral microbiome are a result of systemic response by rapamycin rather than a direct effect on the microbiome. Our current belief is such that rapamycin alters the aging periodontium environment, which then alters the oral microbiome. This rationale is based upon the fact that the microencapsulated rapamycin in the chow is targeted to deliver the active ingredient in the lower intestines (*104, 105*), and bacteria do not have the mTOR gene (*106*). Moreover, we are currently still investigating whether the oral microbiome composition in old animals treated with rapamycin is similar to young oral microbiome or a completely new composition. Although this is the first demonstration of a geroprotective agent that can enhance oral health during aging, we predict that other geroprotective interventions may have similar effects on periodontal disease. Consistent with this hypothesis, metformin has shown to extend lifespan and healthspan in mice (*107, 108*), and there is limited evidence that metformin may help preserve periodontal bone in diabetics (*109*).

Currently, the exact hallmark(s) of aging that rapamycin targets in the periodontium to reverse disease is still unknown. We speculate that the aging hallmark of cellular senescence to play a role in the aging periodontium and disease pathogenesis. Cellular senescence is a cell-cycle arrest mechanism that prevents the proliferation of damaged or dysfunctional cells. Senescent cells are found primarily in renewable tissues, accumulate over the lifespan of rodents, nonhuman primates, and humans, and is metabolically active developing the senescence-associated secretory phenotype, or SASP (*110-113*). Within our study, we noted that majority of the factors that were altered in the aging periodontium were not only inflammatory cytokines and chemokines, but also SASP factors (Figure 3.4). As the gingiva and periodontal bone are both renewable tissues, constantly being re-epithelialized and remodeled throughout life, it is possible that during the aging process there is an increase in cellular senescence burden and rapamycin is reducing this burden. Of interest, it is worth noting that rapamycin has previously been shown to attenuate the pro-inflammatory SASP factors (*114,*

115), which could underlie some of the effects seen in this study. To test the possibility that cellular senescence in part regulates the changes observed in the aging periodontium, we probed via western blotting the cellular senescence biomarker $p16^{Ink4a}$, which has been shown to be upregulated during the aging process in multiple tissue (*113*). Promisingly, we discovered that even in the aging periodontium there was an increase in p16 expression (Data not shown). In light of this preliminary discovery, we are currently examining the role of $p16^{Ink4}$ in the aging periodontium through utilizing a recently established system to remove $p16^{Ink4}$ cells in old animals (*116, 117*).

4.4 CONCLUSION

Rapamycin is an intervention that extends lifespan and healthspan in species ranging from invertebrates to mammals, has shown to rejuvenate the immune function in both old mice models and healthy elderly humans, impact bone metabolism, and reshape the microbiome. All such impacts during the aging process also occurs in the periodontium, where a disruption in either immune function, bone homeostasis and/or microbiome leads to chronic inflammatory disease called periodontal disease. With age, the risk and prevalence of periodontal disease increases significantly. Past and recent studies have attempted to model age-associated periodontal disease by utilizing artificial mechanisms in young animals and have lacked conclusions that translate more reliably to age-associated periodontal disease in humans.

We have designed our study with a Geroscience emphasis to investigate the impact of normative aging on the periodontium, and discovered periodontal disease, per definition by American Academy of Periodontology, occurring in aged animals. We further show that rapamycin is able to reverse this age-associated oral disease with just 8-weeks of treatment by targeting various hallmarks of periodontal disease. This is the first intervention shown to enhance periodontal health during aging by reversing the multiple age-related changes in the periodontium.

Rapamycin have been used clinically for many years, safety and dosing is established and are compatible with treatment in otherwise healthy people (*14, 27*). We propose that rapamycin could easily be tested in a clinical population to assess whether it can attenuate and even reverse periodontal disease. If successful, such a demonstration may represent a paradigm shift in treatment of this significant age-associated oral health problem.

4.5 Materials and Methods

Mouse Studies

Seattle, WA

Experiments were performed on 4, 11, and 18-month old C57BL/6JNia obtained from the National Institute on Aging Aged Rodent colony and acclimated within the Animal Research and Care Facility (ARCF) (Seattle, WA, USA). A separate experiment with a cohort of C57BL/6J at 6 and 18-month old was completed at the Jackson Laboratory (See below, Bar Harbor, ME, USA). For all the experiments in Seattle, animals were housed in individually in Allentown NexGen Caging (Allentown, Allentown, NJ) containing corncob bedding and nestlest. Mice were fed irradiated Picolab Rodent Diet 20 #5053 (Lab Diet, St. Louse, MO). Animals were maintained in specific pathogen free facility within a *Helicobacter spp.*-free room. Mice were housed in groups and inspected daily. Mice were euthanized according to the following criteria (modified from the Intervention Testing Program protocol [Harrison et al., 2009]) when they showed one of these symptoms: (1) inability to eat or drink, (2) severe lethargy, as indicated by a lack of response such as a reluctance to move when gently prodded, (3) severe respiratory difficulty while at rest, indicated by a regular pattern of deep abdominal excursions or gasping, or showing any combination of the following features: (a) severe balance and gait disturbance, (b) an ulcerated or bleeding tumor, visible to the naked eye and breaking through the skin of the animal, or rapid weight gain associated with visible or palpable masses, or (c) Body Condition Score equal to 1 or loss of 20% of body weight in the course of seven days.

Bar Harbor, ME

A cohort of mice are transferred into the Jax Center for Biometric Analysis and brought into the imaging suite in groups of 10 mice per scan group. The mouse specimen scan order was determined by a distributed manifest from the Shock Center for the U of W group. The mice are identified and weighed. Anesthesia is induced at 2-3% isoflurane of O2 gas. Under anesthesia, the mouse is placed in a prone position in the CT scanner and maintained with an isoflurane level of 1.2-1.5% for the duration of the scan. An X-ray fluoroscopic radiograph is used to position the mouse for a whole head scan with bone mineral density phantoms included on the specimen positioning bed. After the CT scan, the mouse is placed in a warmed isolation cage and allowed to fully recover from the anesthesia. At the end of the imaging session, the cohort is returned to animal housing facility.

Encapsulated Rapamycin Feeding Model

For experiments, encapsulated rapamycin was obtained from Rapamycin Holdings, Inc. Irradiated PicoLab Diet 20 5053 pellets were ground and mixed with encapsulated rapamycin at 42ppm. 300ml of 1% agar melted in steril water, and 200ml of sterile distilled water were added per kilogram of powdered chow in order to make pellets. Pellets were stored at -20 °C until use. Control food contained the same concentration of agar and encapsulated material (eudragit) without rapamycin at the concentration that matched the rapamycin chow. 20 mice (10 on eudragit, 10 on rapamycin) received assigned diet treatments at 20 months of age, lasting for 8-weeks.

Micro-computed Tomography (μCT) Analysis

Samples were scanned in a Skyscan 1076 and 1173 microCT system at the Small Animal Tomographic Analysis Facility (SANTA) at Seattle Children's Research Institute and Friday

Harbor Laboratories at the University of Washington. Resolutions were 8-18 μm with following settings: 5 kV, 179μA, 360-ms exposure, 0.5 Al filter, 0.7° rotation step, and 3-frame averaging. Raw scan data were reconstructed with NRecon 1.6.9, and three-dimensional (3D) renderings were generated with Drishti 2.7 (Limaye 2012). For periodontal bone loss, 3D rendered images were randomized and landmarked by independent observers. Periodontal bone loss was measured as distance from the cementoenamel junction (CEJ) to alveolar bone crest (ABC) on 16 predetermined landmarks on the buccal aspect of maxillary and mandibular periodontium. The CEJ-ABC distances were totaled for each mouse through the Drishti software, and means calculated.

For bone volume analysis, DICOM images were uploaded to Analyzepro 1.0 (AnalyzeDirect, Overland Park, KS, USA) and analyzed for average volume as a group and individually. Fixed hemimandibles and maxilla were scanned in 70% ethanol.

Jackson Laboratory (JAX) Imaging Parameter and High-Resolution Secondary Reconstructions

The mouse is scanned in a Perkin-Elmer Quantum GX in vivo Micro-CT tomograph. The accelerating voltage is 55 kV with a source current of 145 micro-amps. The duration of the regional CT scan is 4 minutes over 360 degrees rotation. The reconstruction volume is 25 mm FOV for a resolution of 50 microns which encompasses the entire skull and mandible with bone mineral density phantoms in the field of view. The calculated dose for these settings is 521 milliGray for the scan (*118*). Two secondary reconstructions are performed from the acquired data. The first is a 12.8 mm FOV at 25 microns resolution which encompasses the upper and lower jaw and BMD phantoms. The second is a 7.8 mm FOV at 17 microns resolution to match the imaging parameters by the previous UW work. The FOV is 7.8 mm is sufficient to encompass the teeth at the back of the upper and lower jaw.

JAX Image Processing

The native Perkin-Elmer Viewer VOX image files are converted to Drishti Volume Exploration and Presentation Tool NetCDF format volumes using custom code specific for this study (*119*). Drishti conversion was performed with no loss in data fidelity or information. Perkin-Elmer native XML files are included for information purposes in identifying image and acquisition parameters. Each CT scan includes all 3 resolution images (50, 25, and 17 microns) as part of the data package for the mouse at the particular time point.

Western Blot and Proteome Profile Analysis

For protein analysis by Western Blot, gingival tissue and alveolar bone was dissected and immediately placed in liquid nitrogen. Total cellular proteins were extracted using bead-based, cryogrinding in RIPA Lysis and Extraction Buffer (Thermo Scientific, MA, USA) and EDTA-free Halt™ protease inhibitor cocktail included to prevent protein degradation during extraction process. Gingival tissue was pooled from co-housed animals. Protein concentration was determined by Bradford colormetric assay (Thermo Scientific). 10-20 μg of total protein was separated by SDS-PAGE on 10% or 12% (w/v) polyacrylamide gel, then transferred to PVDF membrane using Trans-Blot Turbo Transfer System (Bio-Rad, CA, USA). Dependent upon strength and signal of antibody, membranes were stripped with Restore™ Plus Western Blot Stripping Buffer, and reprobed for total antibody, or duplicate gels were ran and separate blots were probed. Antibodies to NF-κB p65 (D14E12) XP® (8242, Cell Signaling Technology), phospho-IκB (B-9, Santa Cruz), IκB (32518, Abcam), GAPDH (D16H11) XP® (5174, Cell Signaling Technology) were probed.

Detection of cytokine proteome was completed using Mouse XL Cytokine Array Kit (R&D Systems, Bio-Techne Corporation, MN, USA). Gingiva and alveolar bone samples were individually pooled, protein concentration determined by Branford colormetric assay to load 200μg of protein lysate. Data analysis was completed per manufacture's protocol.

Detection and imaging were performed using ChemiDoc™ XRS+ (Biorad, USA) and Image Lab Software (Biorad, USA).

Histology

Tissues were fixed in Bouin's solution, and demineralized in AFS (acetic acid, formaldehyde, sodium chloride). Mandibles were processed and embedded for paraffin serial sectioning to collect coronal (buccal-lingual) of 5 μm in thickness. Sections were stained for tartrate-resistant acid phosphatase (TRAP) to examine osteoclast activity and numbers (Sigma-Aldrich Kit, St. Louis, MO, USA), and Fast Green counterstaning and examined with Nikon Eclipse 90i Advanced Research Scope. Representative images (40x) for alveolar bone.

Microbiome Analysis

Whole mandible samples were homogenized using a FastPrep24 (M.P. Biomedicals) with bead-beating tubes and ceramic beads (Percellys). Microbial DNA was extracted using QIAmp® DNA microbiome kit (Qiagen, Valencia, CA, USA) and DNA was further purified using DNA Clean & Concentrator kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. The V3-V4 variable region of the 16s ribosomal RNA gene was amplified using gene-specific primers with Illumina adapter overhang sequences (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). Each reaction mixture contained 2.5 μl of genomic DNA, 5 μl of each 1 μM primer, and 12.5 μl of KAPA HiFi HotStart ReadyMix. Amplicon PCR was carried out as follows: denaturation at 95°C for 3 minutes, 35-40 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, followed by a final extension step at 72°C for 5 min. PCR products were verified using gel electrophoresis (1% agarose gel) and cleaned with AMPure XP beads (Agencourt, Beckman

Coutler Inc., Pasadena, CA, USA). Amplicons were then indexed using the Nextera XT Index Kit V2 set A and set D (Illumina) and purified again with AMPure XP beads to remove low molecular weight primers and primer-dimer sequences. Samples were pooled into a single library which was analyzed using the TapeStation 4200 High Sensitivity D1000 assay (Agilent Technologies, Waldbronn, Germany) and Qubit High Sensitivity dsDNA assay (Thermo Fischer Scientific) to assess DNA quality and quantity. The final pooled library was then loaded on to an Illumina MiSeq sequencer with 10% PhiX, which served as an internal control to balance for possible low diversity and base bias present in the 16S amplicon samples, and was run for 480 cycles and generated a total of 4.1 million paired-end reads (2x240 bp).

Bioinformatics:

Raw paired-end sequences were imported in to Qiime2 and were trimmed by 8 nt from the 5' end and truncated to 239 nt for the forward and reverse reads. Only forward reads were used in further analysis due to poor reverse read quality. Trimmed forward reads were then demultiplexed and denoised using the DADA2 package (*120*). Taxonomy was then assigned using the feature-classifier suite and the Human Oral Microbiome Database (HOMD) (*121*). Samples were then filtered for singletons, taxonomic contaminants, and excluded samples with less than 3000 reads. Alpha and Beta diversity using Permanova and 999 iterations was also completed in Qiime2. Further analysis was done in the R suite (*122*) using the Phyloseq package (*123*).

Statistical Analysis

Results for μCT analysis, quantitative histology, proteome analysis are expressed as mean ± standard error of mean (SEM). Data were analyzed where appropriate using Student's *t*-test or paired t-test (comparing two groups only), or one-way analysis of variance (ANOVA) with post-hoc Tukey test for multiple comparisons, where p-values < 0.05 were considered statistically

significant. Statistical analysis was completed using GraphPad Prism 8.00 (Graphpad, Software, La Jolla, CA, USA).

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VITA

Jonathan was born in Tacoma, Washington, and moved to Puyallup, Washington where he lived before attending the University of Washington, Seattle. Jon was making pretzels or making coffee as a barista before becoming a research scientist in the Department of Oral Biology (now Oral Health Sciences) joining the lab of Sun Oh Chung. He earned is Doctor of Dental Surgery (DDS) at the University of Washington School of Dentistry in 2015 and joined the lab of Matt Kaeberlein in the Department of Pathology, School of Medicine in 2016. In 2019, Jon earned a Doctor of Philosophy at the University of Washington in the discipline of Oral Health Sciences.