The impact of CE proteins on periodontal disease and the oral microbiome in white spot lesions patients

by

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ABSTRACT

Background: Periodontal disease is a pathological condition that affects the soft and hard tissues that surround teeth. Although its overall etiology is well- studied, there remains less clarity as to the etiology of the severe forms of this disease (SvP). The epithelial barrier is one of the greatest shields of our body and, at the same time, the portal of entry for many aggressors. To understand more about the etiology of SvP, we have focused on the cornified epithelium (CE), which is the outermost layer of the skin and oral mucosa. The insoluble protein, loricrin, comprises most of the protein mass and supports the barrier between environments. Loricrin downregulation has been linked to inflammatory skin disorders, which suggests that this protein may play a key role in the barrier function of the CE. Signal Transducer and Activator of Transcription 6 VT (Stat6VT) transgenic mice are a model of CE protein downregulation used to study skin disorders. These mice overexpress the transcription factor Stat6, increasing levels of T Helper Cell Type 2 cytokines, which compete for a mutual co-factor (p300/CBP) and consequently decrease expression of loricrin and filaggrin, two CE proteins. Porphyromonas gingivalis (Pg), a major periodontal disease pathogen, can also affect CE protein expression through chronic activation of Stat6VT. Our study aims to determine the impact of deficiency/mislocalization of several CE proteins on periodontal health in the context of infection by Pg and investigate the localization and expression of CE proteins in human gingival samples of patients that presented with SvP compared to healthy controls. Hypothesis: There are two hypotheses: 1. SvP patients will show downregulation/mislocalization of CE proteins accompanied by changes in epithelial differentiation and increased signs of inflammation in histological sections of uninvolved gingival tissues compared to healthy controls. 2. Stat6VT mice will develop an increased oral immune response and more significant alveolar bone loss compared to littermate controls in response to a pathogen challenge. Methods: Fifteen patients were recruited from the periodontology residency program at the University of Alberta. Patients with Stage III or IV and Grade C periodontitis (SvP group) or included as healthy controls. Discarded healthy tissue samples containing keratinized gingiva were collected during various periodontal procedures. The human samples were assessed for loricrin, filaggrin, cytokeratins 1, and 14 expressions. For the animal study, plasma was then carefully collected and used to analyze Th2 cytokines and systemic inflammatory status. Alveolar bone loss was assessed using microcomputed tomography, and tissue morphology of the soft tissues was qualitatively and quantitatively assessed by histological examination for several proteins. Results: Loricrin expression was visibly downregulated in all SvP patients when compared to controls. Filaggrin, on the other hand, showed a similar signal to controls in Stage III C patients, and a decreased signal in Stage IV C patients. CK1

presentation was more widespread but had lower expression (qualitatively) in all SvP patients. CK14 expression was also more widespread over several layers, instead of being contained to a single layer as in controls, and a decrease in expression was also observed in all SvP patients. Pathogen-infected Stat6VT mice presented increased alveolar bone loss compared to controls in 9 out of 16 examined sites. This increase in bone loss was accompanied with increased tissue inflammation, based on CD45+ cell count (35.30 ± 1.539 vs. 22.86 ± 1.067; $p<0.0001$). CK1 expression was dramatically decreased in Stat6VT mice (6.556 ± 0.76 vs. 19.94 ± 2.868; $p<0.0001$). CK14 expression was more broadly expressed in Stat6VT mice, with positive cells extending up into the granular layer, exceeding confinement to the basal layer, as observed in controls. Ki67 positive cells were decreased in Stat6VT mice (38.54 ± 2.637 vs. 53.97 ± 3.870; p:0.0013) and scattered in the distribution. Conclusions: Our study found decrease and difference in localization of important proteins of the oral epithelium, such as loricrin, filaggrin, CK1 and CK14. These changes in epithelial differentiation that may exacerbate the effects of Porphyromonas gingivalis infection in Stat6VT mice. There were similarities between the mouse model and the most severe forms of human periodontitis, suggesting similar etiologies. The hypothesis that barrier integrity may contribute to SvP needs further study. The identification of Stat6VT mice as a model of SvP may significantly impact mechanistic study.

PREFACE

This thesis is an original work by Raisa Queiroz Catunda. All procedures were approved by the Animal Care and Use Committee (ACUC) of the University of Alberta (AUP 00002935), and both projects involving human subjects were approved by the Health Research Ethics Board (The role of a Loricrin in aggressive periodontal disease: Pro00062112; Oral microbiome and Stephan curve kinetics in white spot lesion development among orthodontic patients: Pro00099341). The study design, planning, and experiment development were done in collaboration with Dr. Febbraio. Animal experiments were performed with the collaboration of Karen Ho. The experiments of the human study were performed in collaboration with Dr. Bryant Roy. Sample collection of human gingiva was performed with the collaboration of the periodontology residents. Dr. Maria Alexiou and Dr. Pranidhi Baddam scanned all the mice heads for the microCT analysis, and Shrushti Patel assisted in analyzing these results. Sara Moradipoor and Ramesh Mahdavifar assisted in CD45 and Ki67 cell count analysis. Dr. Mark H. Kaplan generously donated the Stat6VT transgenic as sperm for rederivation. The cytokine array experiment was performed by Sara Moradipoor and analyzed by Dr. Febbraio. Dr. Khaled Altabtbaei was the main collaborator in the white spot lesion project's design, development, and analysis. Several images were designed with the collaboration of Milton Neto. References are presented at the end of each chapter. Results chapters consist of two papers that were submitted for publication and chapters 3-6 consist of already published papers.

Chapter 3 of this thesis has been published and the authors are Catunda RQ, Ho KK, Patel S, Febbraio M tittled as “A 2-plane micro-computed tomographic alveolar bone measurement approach in mice” on Imaging Sci Dent. 2021 Dec;51(4):389-398.

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Catunda, RQ is the first author of all published chapters of this thesis and she was involved with concept, literature review, data analysis and manuscript composition.

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CHAPTER 1

INTRODUCTION

CHAPTER 1: INTRODUCTION

1.1 Oral health

The World Health Organization (WHO) has defined health as "*a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity*" [1]. In the same sense, WHO also defined oral health as the absence of any type of oral disease, chronic orofacial pain, oral and pharyngeal cancer, oral infection, soft tissue injuries surrounding teeth, periodontal disease, tooth decay, tooth loss, malformations, congenital or other diseases and disorders that affect and limit functional capacities, such as biting, chewing, smiling and talking, as well as the psychosocial well-being of an individual [2]. Thus, oral health must be considered as an integral part of general health, and is essential for an individuals' quality of life, since oral diseases cause pain and discomfort that affect general health. Furthermore, oral diseases also affect populations through the burden on the healthcare system and the costs associated with prevention and therapies [1, 3]. Oral diseases thus prove to be a major public health problem, not only because of their multidimensional character, complexity, functional and visual importance, but because, even though infrequently deadly, they still result in a plethora of negative social, economic and psychological impacts in an individual's life [4].

1.2 Periodontitis

The periodontium is the structural part of the oral cavity that functions in anchorage and protection of teeth in the mandible and maxilla. It is constituted by the gingiva, periodontal ligament (PDL), cementum, and alveolar bone. Clinically, healthy gingiva presents a pale pink color (lighter than buccal mucosa) with a stippled surface (orange peel-like appearance) and firm consistency [5] (Figure 1).

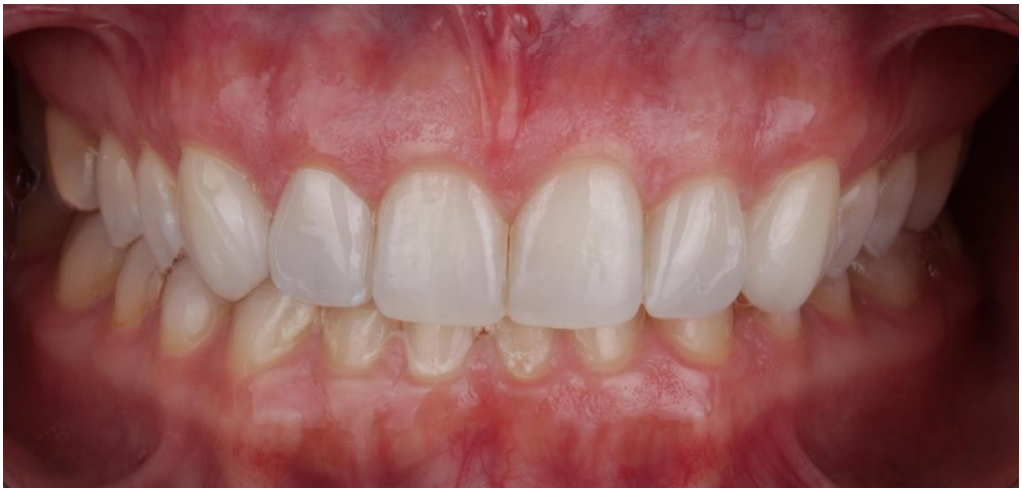

Figure 1– Healthy gingival appearance of a 34-year-old female patient. Note the pale pink color, the stippled gingival surface, knife edge margins and the tight adaptation.

Periodontitis is an infectious disease that has as major etiological factors specific microorganisms present in the oral biofilm which affect teeth supporting structures leading to loss of attachment, and with increasing progression, bone, and if left untreated, eventually loss of the dental element [6-9]. Periodontal disease definition and classification have undergone several revisions during the last decades, in the face of advances in the fields of epidemiology, microbiology, molecular biology, as well as changes in methodological and therapeutic approaches [10-14]. In this way, information has been obtained that contributes to conceptualization of the pathophysiology of this infection and its determinants, and by extension, as a public health problem oral disease.

The current classification of periodontal diseases, established at the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions in 2017, includes a multidimensional grading and staging system [14]. Grades are from A-C, based on the risk of disease progression, adding components such as health status and smoking. Staging takes into account probing depth, percentage of bone loss, clinical attachment loss (CAL), extent and existence of bony defects, mobility of teeth, furcation involvement, and any tooth loss related to periodontitis [14]. Prevalence studies since the publication of the 2017 classification showed a decrease in the total prevalence of periodontitis compared to the older classification [15-18]. However, this is probably due to “non-periodontal causes" (traumatic gingival recession, CAL in the distal aspect of a second/third molar, root/cervical dental caries, amongst others) being

removed from periodontitis diagnosis. Regardless of the differences in prevalence between the old and new classification systems, periodontitis still comprises a high percentage of oral disease all over the globe, with recently published ranges varying from 28.7-43.7% of the affected population (from low to high-income countries, respectively). It is the leading cause of tooth loss [19, 20].

1.2.2 Severe forms of periodontitis

The former 1999 Classification of Periodontal Diseases and Conditions presented a condition known as aggressive periodontitis [9]. Aggressive periodontitis was considered a distinct entity from other forms, as it showed an early age of onset, rapid rate of progression and familial history [10]. In addition, in this form of periodontitis, it was typical to observe very minimal plaque and calculus accumulation associated with a fast rate of periodontal damage [9]. The 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions recognizes periodontitis as a spectrum disorder. This was meant to recognize a wide variation in symptomology and severity. Therefore, aggressive periodontitis is now obsolete nomenclature, and forms of periodontitis that present with a rapid rate of destruction are now classified as Grade C, Stage III or IV periodontitis [21]. This thesis uses the term "severe forms of periodontitis" (SvP) for these forms of periodontitis, for simplicity. Please note, when discussing literature prior to 2017, the old nomenclature will be used, since not all patients in those past studies would be classified as Grade C, Stage III or IV.

1.2.3 Etiology

Establishment of a highly pathogenic biofilm initiates the host immune/inflammatory response that may lead to the destruction of periodontal tissues. However, this is inconsistent in all individuals [13, 22]. The inflammatory reaction is microscopically and clinically visible in the affected periodontium and represents the host response to the plaque microbiota and its products. Therefore, these processes act in the gingival tissues to protect against microbial attack and prevent

microorganisms from spreading or invading tissues. In some cases, these host defense reactions can be harmful, because neighboring connective tissue, cells and structures are also susceptible to damage [10, 11, 23]. Furthermore, the inflammatory and immunological reactions may extend and reach deeper tissue levels, including the connective tissue and the sulcus, and may also involve the alveolar bone in this destructive process. Thus, such defensive processes may, paradoxically, account for the most tissue damage observed in gingivitis and periodontitis [24-26]. While much is known, there remain gaps in our understanding, and given the prevalence of this disorder worldwide, a better understanding of its etiology is necessary in order to address adequately.

Since the change in the classification, exploring the etiology, and how it fits with this new stratification remains ongoing [27-30]. Previous studies suggested that genetic factors [10, 31, 32] and red-complex bacteria had the greatest contribution to disease [33-35]. Socransky and collaborators in 1998 described six microbial complexes that compose the subgingival biofilm of adults [8]. The yellow, blue, green, and violet complexes form the base of the biofilm pyramid: they are the initial colonizers of the dental surface and are not related to periodontal disease [8]. These basal complexes provide receptors and create ecological conditions for the growth and propagation of bacteria of the orange complex, implicated in the pathogenesis of periodontal diseases[8]. Among bacteria in the orange complex, *Prevotella intermedia* is noteworthy as it is highly prevalent and has several important virulence factors (adhesins, fimbriae, proteases, lipopolysaccharide, extracellular polysaccharides, among others) [36]. The orange complex precedes and creates conditions for the establishment of the red complex, composed of the species *Porphyromonas gingivalis* (Pg), *Treponema denticola*, and *Tannerella forsythia*. These bacteria are critical contributors to the pathogenesis of periodontitis, and are related to increased pocket depth and the presence of bleeding on probing [8]. Genera and species related to periodontal disease have broadened past the red complex, now also comprising *Filifactor alocis*, *Synergistetes*, and *Peptostreptococcaceae* [37, 38], as well as *Actinomyces actinomycetemcomitans,* which had been previously associated with aggressive periodontitis [39, 40]. A recent study, however, pointed out that the microbiota associated with different periodontitis presentations is insufficient to explain clinical variability, implicating other factors as significant[40, 41].

There is a great focus on barrier dysfunction in the etiology of diseases in dermatology and gastroenterology [42, 43]. For example, a theory to explain the manifestations of atopy (an allergic

reaction), known as the "outside-in" hypothesis, suggests dysfunction of the skin/gut barrier leads to sensitization of the immune system and a hyperactive state of the organism and, consequently, the manifestations of atopy [44]. We have explored the same concept as an underlying etiology in the most severe forms of periodontitis, in which individuals with seemingly little plaque and calculus manifest an increased immune reaction to pathogenic bacteria [45-48].

1.2.3.1 Epithelial layers and the cornified envelope

Keratinocytes, the most prevalent cell type present in the epithelium, can produce keratins which, together with several structural proteins, create a tight barrier. Through a highly organized differentiation process, keratinized epithelium forms a genuine shield against the environment and any foreign aggressors, while it is preventing dehydration [49-51]. Epithelial layers can be identified by morphological features and by the expression of specific cytokeratins (Figure 2). Cytokeratins 1 (CK1) and 10 (CK10) are generally found in the supra-basal layers of oral keratinized epithelium, whereas cytokeratins 14 (CK14) and 5 (CK5) are typically expressed in the basal layer of both keratinized and non-keratinized epithelium [52-54]. The process of differentiation is tightly regulated but found to be disrupted in certain disease states and inflammatory processes [51, 55]. CK5 and CK14 assemble into keratin intermediate filaments; these proteins extend from desmosomes in the direction of the nuclear lamina, and along with other proteins, create the cytoskeleton of epithelial cells [49]. As basal keratinocytes migrate to the spinous layer, they lose their mitotic potential and a different set of proteins is synthetized [49]. CK1 and CK10, which replace CK5 and CK15, are the first proteins expressed during differentiation that leads to cornification/keratinization [49, 56].

The term keratin derives from Greek "*keratos*" which was used to denominate horn, as that nomenclature was historically used to classify proteins extracted from horns, claws and hooves [57]. There are a great variety of keratins and they are classified according to their biomechanical properties, molecular weight, distribution and preferential synthesis [56]. Keratins in nails and hair are cysteine-rich, and considered the "hard keratins while the "soft" or cytokeratins are present in the forms of filaments in epithelial cells [55, 58]. The main functions of keratins are to provide

mechanical support in preservation of cellular architecture, in intracellular cargo transportation, junction development, during mitotic activity, in regulation of protein synthesis and cell growth, and in protection [56].

According to Rao *et al.* (2014), the classification of keratins is as follows:

a. Biomechanical properties:
   Type I: acidic (CK9, CK10, CK12, CK28, CK31, CK40) Type II: basic (CK1, CK8, CK71, CK86)
b. Molecular weight:
   Low: present in the granular and simple types of epitheliums Intermediate: present in the stratified epithelium
   High: present in the keratinized stratified epithelium
c. Distribution:
   Soft: present in the mucosa and skin Hard: present in hair/nails
d. Preferential synthesis: Primary: produced constantly
   Secondary: produced in addition to or in the case of an absence of primary keratins

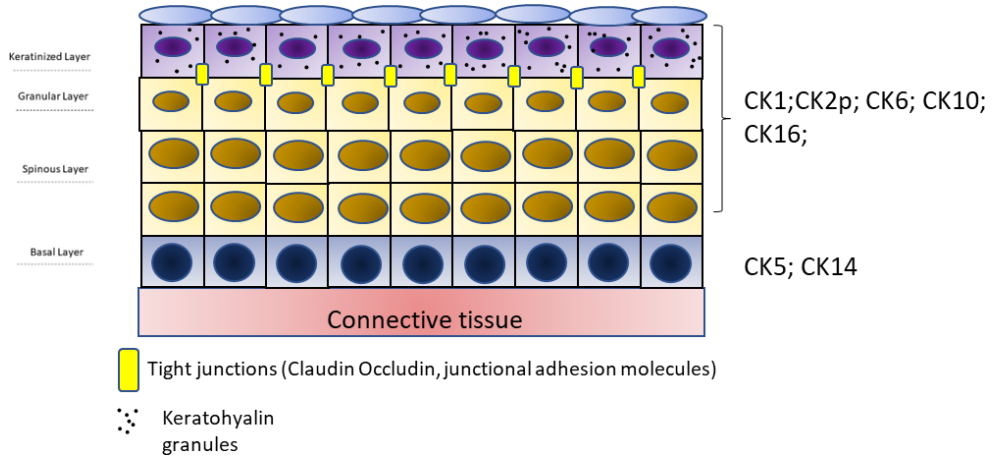

Figure 2 – Illustration of the keratinized stratified epithelium: connective tissue is shown in light red at the very bottom, followed by the basal layer, spinous layer, granular layer and keratinized layer. Some cytokeratins are also illustrated in this image CK1, CK5, CK10, and CK14. In yellow is also possible to observe the tight junctions,

mostly formed by occludin, claudins, and junctional adhesion molecules and the keratohyalin granules are represented by dark dots in the upper layers.

The oral cavity is formed as a stratified squamous epithelium containing keratinized, non-keratinized and specialized mucosa [59]. The epithelium provides great mechanical strength to the structures formed. Four layers can be identified within the epithelium:

a. Basal or germinal layer: consists of prismatic or cuboid cells, which rest on the basal lamina, which separates the connective tissue from the epithelium. There is intense mitotic activity in this region, to provide constant epithelium renewal.
b. Spinous layer: presents polygonal or cuboid cells, with a central nucleus and expanded cytoplasm. These cells are joined with desmosomes, a type of tight junction between cells, which gives this layer a spiny appearance.
c. Granular layer: composed of flat cells with a central nucleus, which contain numerous keratohyaline granules.
d. Depending on the oral cavity location, the external layer can be keratinized or non- keratinized. Keratinized Layer: this layer is composed of flattened dead cells without a nucleus (or containing small nuclei) and without organelles. They have reinforced cell membranes and keratin-filled cytoplasms. [56].

In the oral cavity, there are two types of keratinization:

1) Orthokeratinized: This is the most common type of epithelium found in the masticatory mucosa. It also composes the specialized mucosa of the dorsal surface of the lingual papilla.
2) Parakeratinized: This type of epithelium is associated with the attached gingiva and is part of the masticatory mucosa. In contrast to orthokeratinized epithelium, in parakeratinized, it is hard to distinguish between the granular and spinous layers; pyknotic nuclei are retained in the outermost layer and some filaments can still be observed in the cytoplasm [59, 60].

In the skin, the outermost layer is known as the stratum corneum or cornified layer [51]. The differentiation process culminates in cell bodies without organelles, comprised of keratin intermediate filaments and lipids. Within this layer are flattened, protein-rich cells, known as

corneocytes [61]. The cornified envelope (CE) is an approximately 15nm thick structure that is formed on the internal side of the corneocyte membrane via cross-linking of keratin filament associated proteins, such as involucrin, loricrin, filaggrin and others, by transglutaminases [51, 56]. During the differentiation process, the lamellar granules in the keratinocytes fuse with the plasma membrane and release their contents into the extracellular space of the granular and spinous layers. The secreted lipids are arranged into the extracellular lipid lamellae of the cornified layer [62]. Then there is formation of a lipid envelope, created by ceramides, that bind in a covalent manner to the CE along with the lipids that are extracellular and play an important role in the impermeability of this structure (Figure 3a, 3b) [51].

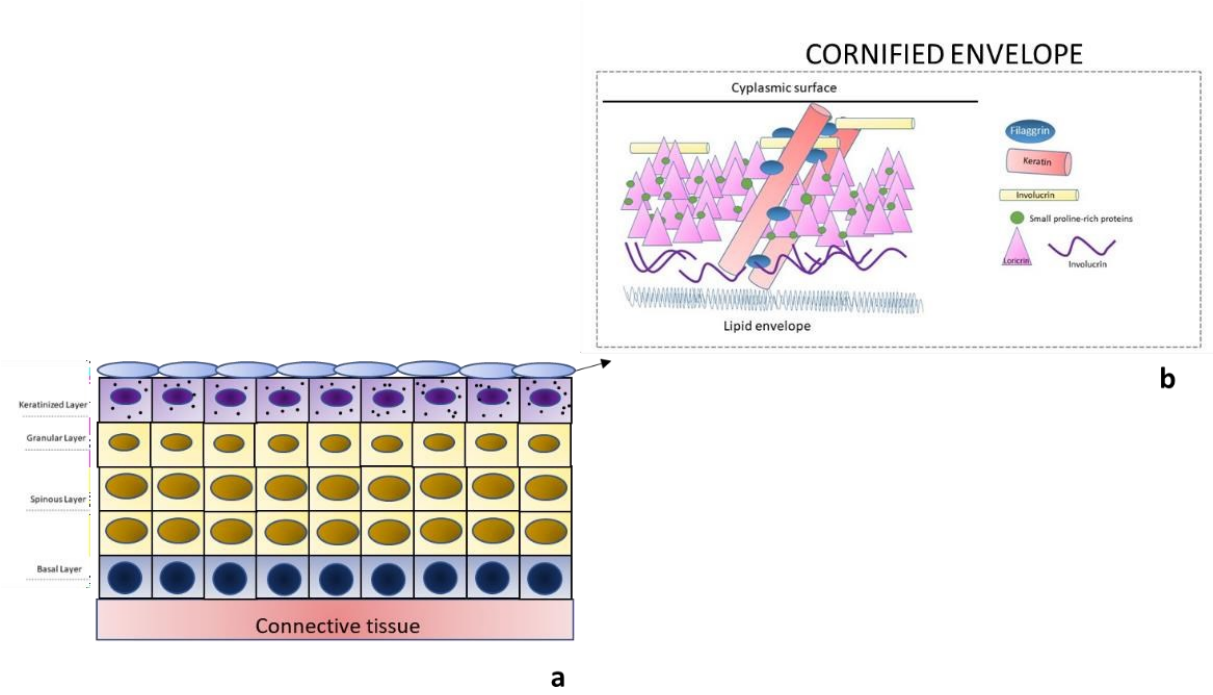

Figure 3 – The cornified envelope. **a**. Stratified keratinized epithelium; **b**. Enlarged image of the cornified envelope, illustrating some key proteins present and how they closely interact.

1.2.3.2 Loricrin and the epithelial barrier

The structures that bind the corneocytes tightly together are the corneodesmosomes; these are "modified" desmosome-like structures, which eventually are degraded in the upper layers to allow desquamation to occur [63]. Transglutaminases (1, 3 and 5) in the CE have an important role, as

they are responsible for the formation of stable isopeptide bonds [49]. Loricrin is the most abundant protein in the keratinized layer (~70-80%). It is a 26 kilodalton insoluble protein that has the highest level of expression in humid tissues, such as the oral mucosa, and promotes elasticity and defense against mechanical stress [64-68]. Transglutaminase crosslinking sites in loricrin result in a tight meshwork. As such, it creates a robust barrier between the external and internal environment [69-72]. The keratinized epithelium of the oral cavity recapitulates many of these features with similar result.

Different types of inflammatory responses are elicited when the periodontium is under duress. In periodontal disease patients, the involvement of T cells is multifaceted, and data support a role for T helper cells in both protection and pathogenesis [73-76]. Pg infection is characterized by the presence of both T-helper cells type 1 (Th1) and type 2 (Th2) [73-77]. The differentiation of precursor CD4+ T cells into Th1 or Th2 cells is determined in the priming phase of these cells and is defined by a diversity of extracellular factors (cytokine environment, dose of antigen, and the source of co-stimulation) [78]. Th1 cells normally secrete interferon-gamma and tumor necrosis factor alpha, are linked to the initial inflammatory response, and promote cell-mediated immune responses. On the other hand, Th2 cells secrete IL-4, IL-5, IL-6, and IL-13, which increase B cell proliferation and differentiation, and are associated with humoral-type immune responses [77-80]. As a result, high levels of pathogen-specific immunoglobulins are released to overcome the foreign body threat [78]. IL-4 has been shown to be an important mediator of periodontitis- induced inflammatory responses, and also predominates in certain skin diseases [81-86].

Vohwinkel syndrome and loricrin keratoderma are inherited skin disorders that have been associated with loricrin mutations [87-90]. A common mutation is the insertion of an extra guanine in a stretch of guanines (codons 230-231) that results in a frameshift and incorrect termination [87-92]. For this specific mutation, one study found loricrin expression and localization to be comparable to controls and one study showed that loricrin was observed inside the nucleus [87-90]. Loricrin mislocalization may interfere with normal CE formation, as it potentially causes late or discontinuous differentiation, which can result in a parakeratotic epithelium [93].

Clinically, studies point to loricrin mutation/mislocalization/downregulation as a possible etiology for skin barrier dysfunction, leading to the aforementioned inherited disorders as well as psoriasis, atopic dermatitis and aggressive periodontitis. These conditions suggest that issues that

arise during the process of keratinization or transglutamination can lead to changes in barrier function and the manifestation of disease [42, 50, 84, 94]. Two unbiased studies comparing the transcriptomes between healthy and aggressive periodontal disease patients, or aggressive and chronic periodontal disease patients, showed profound down-regulation of loricrin and filaggrin, implicating the integrity of the keratinized epithelium in the pathogenesis. More recently, the connection to IL-4 in aggressive periodontitis patients has been made, and also implicates barrier dysfunction [85, 86, 89-91].

As mentioned above, IL-4 has been shown to be an important mediator of periodontitis-induced inflammatory responses. An important signaling pathway activated by IL-4 is the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-Stat) pathway. IL-4 attaches to its cell-surface receptor leading to dimerization and recruitment of JAK2 [81, 95]. JAK2 then phosphorylates tyrosine residues, allowing Stat6 proteins to bind. Stat6 proteins are also phosphorylated, leading to dimerization and translocation into the cell nucleus, where they bind DNA, resulting in transcription of target genes (Figure 4) [81, 95]. In the progression of periodontal disease, Th2 cells have a greater contribution, given the predominately increased number of B lymphocytes observed (Figure 5) [96-99]. Mechanistically, Stat6 activation downstream of IL-4 signaling is key to understanding potential barrier function disruption, through downregulation of keratinized epithelial proteins, including loricrin and filaggrin [95, 100, 101]. In keratinocytes, loricrin and filaggrin transcription requires a co-factor, p300/Creb-binding protein. The same co-factor is used by Stat6. Chronic IL-4 secretion leads to sequestration of this co-factor by Stat6, resulting in decreased transcription of these keratinized epithelial proteins (Figures 6) [81].

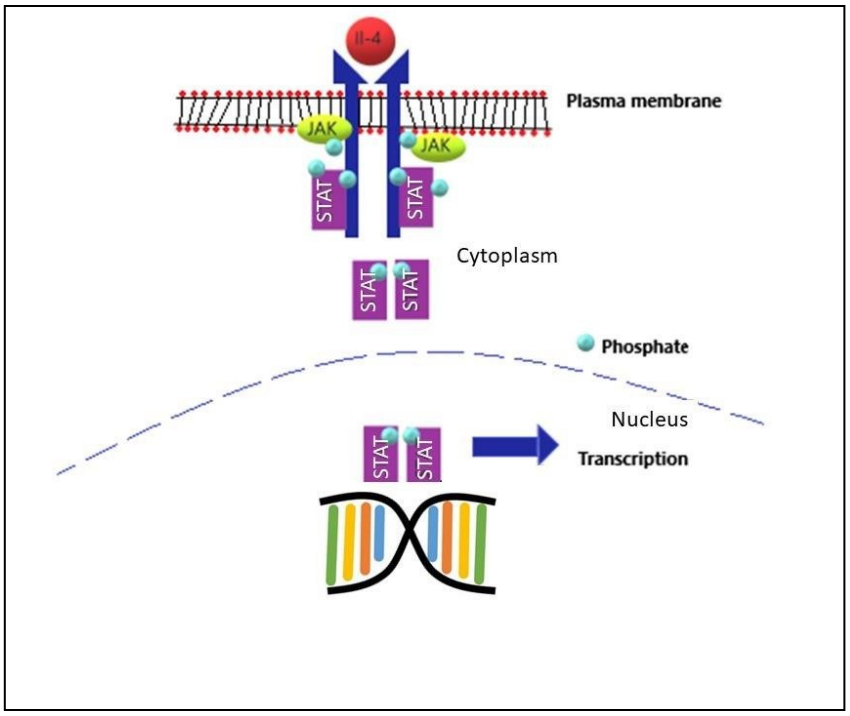

Figure 4 – JAK-Stat pathway. IL-4 binding to its cell-surface receptor leading to dimerization and recruitment of JAK, which results in phosphorylation (addition of phosphates, light blue circles) of tyrosine residues. This allows two Stat6 proteins to bind to the now phosphorylated domain. Stat6 is also phosphorylated, leading to its dimerization. The Stat6 dimer then translocates into the nucleus and binds to the promoter regions of DNA containing Stat6 binding sites, resulting in the transcription of specific target genes.

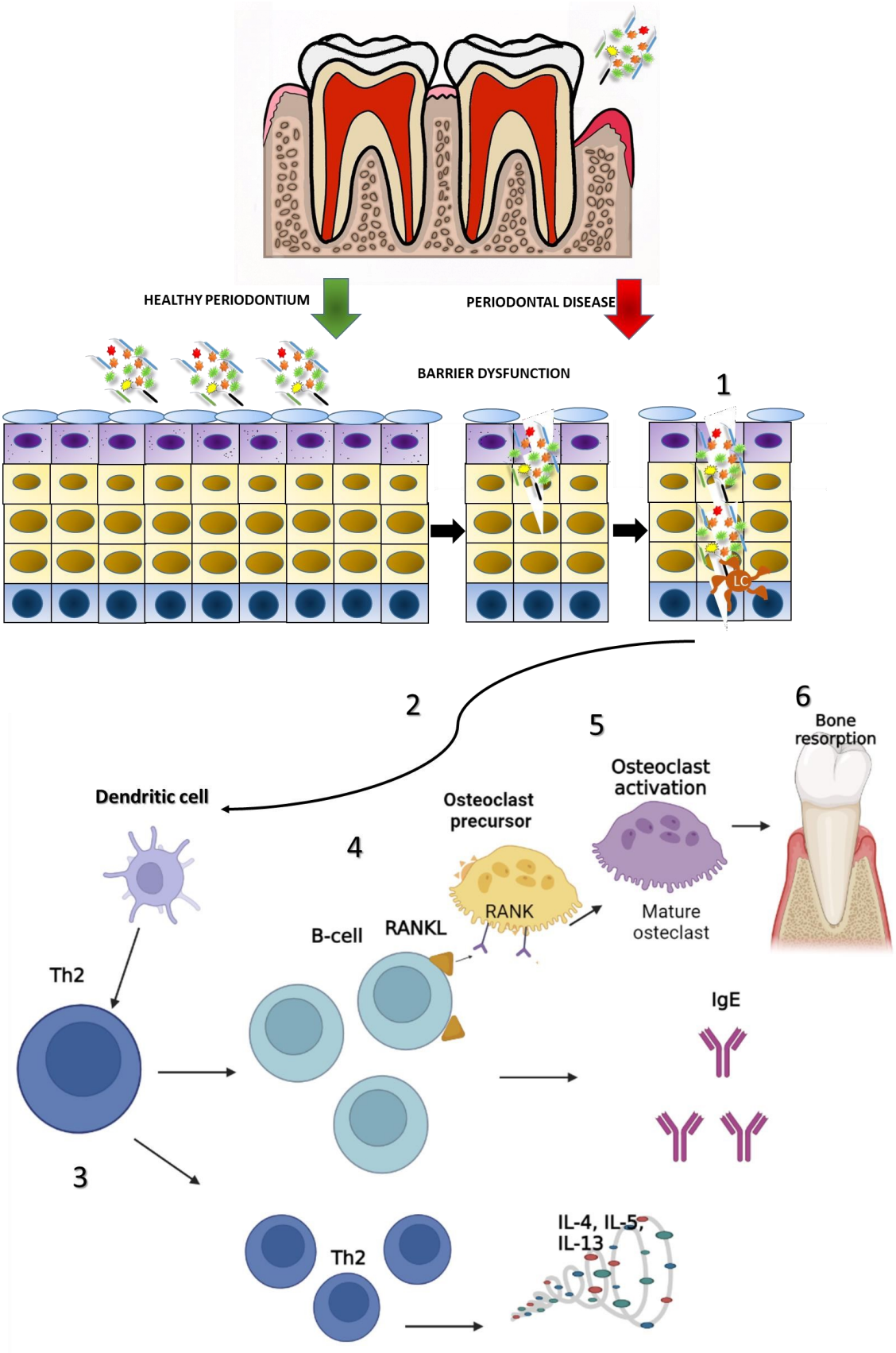
HEALTHY PERIODONTIUM
PERIODONTAL DISEASE
BARRIER DYSFUNCTION
1
LC
2
Dendritic cell
Th2
3
4
B-cell
RANKL
Osteoclast precursor
RANK
5
Osteoclast activation
Mature osteclast
6
Bone resorption
IgE
Th2
IL-4, IL-5, IL-13

Figure 5 – Periodontitis initiation and immune response in the context of a dysfunctional barrier: 1: In the context of a dysfunctional barrier, bacteria and bacterial lipopolysaccharide are detected by immune cells. 2: Multiple cytokines are released that stimulate macrophages and dendritic cells to secrete IL-4; 3: This induces a Th2 response. Activated T-cells express RANKL on their surface; 4: B-cells are activated by the TH2 response to also express RANKL; 5: Osteoblasts, express the receptor for RANKL, RANK on their membrane and interact with B- and T-cells. This leads to differentiation into osteoclasts; 6: Osteoclasts resorb alveolar bone. Due to the reduced integrity of the epithelial barrier, the amount of plaque bacteria needed to activate the immune response is much reduced, explaining the paradoxical clinical presentation of some patients with SvP.

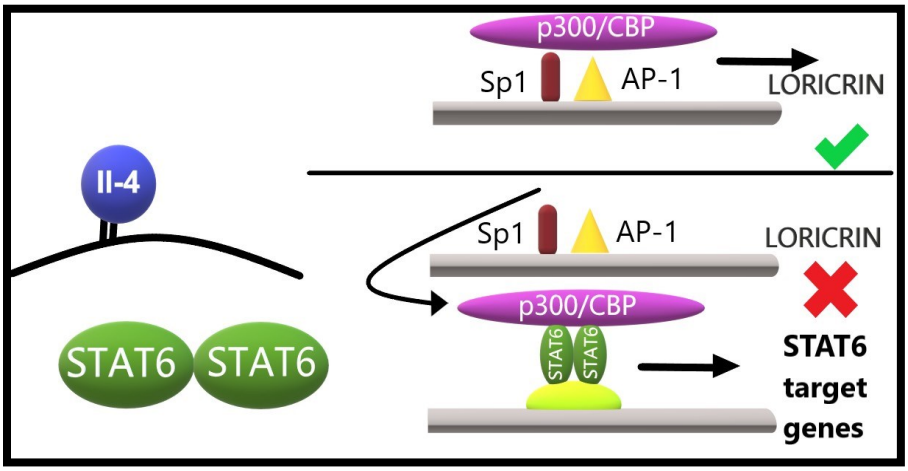

Figure 6 - Chronic IL-4 signaling leads to downregulation of loricrin by sequestration of a common co-factor, p300/creb-binding factor (CBP). In keratinocytes, p300/CBP binds to the transcription factors Sp1 (specificity protein one-- transcription factor) and AP-1 (Activator protein 1 - transcription factor) in the loricrin gene promoter, stimulating transcription of loricrin. In the circumstance of chronic Stat6 activation, due to IL-4 signaling, p300/CBP instead forms a complex with Stat6, resulting in less loricrin gene transcription.

1.2.3.3 Stat6VT mouse model

In periodontitis research, several mouse models have been used, however, none of them to investigate the role of barrier dysfunction [102-107]. In dermatology research, the concept of a dysfunctional barrier is well-established and multiple mouse models have been created for mechanistic studies [50, 108, 109]. As loricrin is the most abundant protein in the CE, to explore a defect in epithelial barrier function, we considered the use of a loricrin knockout mouse [110, 111]. This mouse was created more than 20 years ago, but interestingly, presented only with a

transient phenotype ~4-5 days after their birth, due to the compensatory upregulation of other cell envelope proteins. Subsequently, other animal models to mimic skin conditions have been generated, including a transgenic that overexpresses IL-4, and a constitutively active Stat6 transgenic (Stat6VT) [81, 95, 112, 113]. Both these latter mice have clinical similarities and are established atopic dermatitis models, showing lesions at similar time points, increased expression of IL-4-stimulated genes and peripheral B cells, and decreased loricrin expression [100, 112-117]. We were able to establish a collaboration with Mark Kaplan from the University of Illinois School of Medicine, who generated the Stat6VT model, and provided us with sperm to derive the mice used in our studies.

Stat6VT transgenic mice have been used for approximately two decades to mimic IL-4-stimulated responses in keratinocytes [95, 100, 117, 118]. The "VT" refers to the valine and threonine residues in the Stat6 protein, which were each replaced with alanine, resulting in constitutive activation of the Stat6 protein in the absence of IL-4 receptor binding. Therefore, this mutant form can activate downstream gene transcription without a stimulus. The mutant Stat6 transcription factor is expressed in T and B cells via the CD2 locus control region promoter. In T lymphocytes, Stat6VT expression drives T cell differentiation and activation in the direction of the Th2 phenotype [100]. This transgenic model has ~2x less loricrin gene expression when compared to wildtype mice; and decreased loricrin protein that varies from 95% in regions with skin lesions to 75% in uninvolved regions [95]. Interestingly, no one had examined the oral cavity of these mice.

1.2.1 Objective

The objective of this study is to investigate the impact of CE protein deficiency/mislocalization on periodontal health in the context of Pg infection and to determine the localization and expression of CE proteins in human gingival samples of patients that presented with SvP compared to healthy controls.

1.2.1 Hypothesis

a. We hypothesize that Stat6VT mice will develop an increased oral immune response and more significant alveolar bone loss compared to littermate controls in response to a pathogen challenge.
b. We hypothesize that SvP patients will show downregulation/mislocalization of CE proteins accompanied by changes in epithelial differentiation and increased signs of inflammation in histological sections of uninvolved gingival tissues compared to healthy controls.

1.3 White Spot Lesions

Defects in enamel development, presenting as enamel hypoplasia or opacity, are caused by damage or changes in the enamel organ due to inherited and acquired conditions [119]. In contrast, white spot lesions (WSL) are considered one of the early clinical manifestations of dental caries [120]. If not arrested, these milky white opacities, as a result of enamel demineralization, can progress to frank caries [121]. The impact of caries on quality of life, including pain and negative consequences on mastication, phonation, aesthetics and social interaction is well-established [122]. Enamel color changes are one of the main reasons patients seek aesthetic treatments. Such changes occur because of hypoplasia, fluorosis, drug pigmentation, and demineralization [123]. As there is a small tooth surface between the gingival tissue and the bracket on maxillary lateral incisors, these teeth seem to be the most affected. This fact results in an aesthetic compromise due to stains and changes in dental morphology, leading to dissatisfaction with the smile's appearance [119, 124].

WSLs can be associated with the use of fixed orthodontic appliances (Figure 7), as braces make oral hygiene more difficult for the patient, especially in areas around the brackets, and favors the creation of new habitats for biofilm accumulation [123, 125]. The incidence and prevalence of WSL development in orthodontic patients can vary dramatically (23.4-72.9% and 30-75.6%, respectively); this great variation may depend on the specified parameters (age, diagnosis criteria, initial caries assessment). Incidence is generally assessed throughout orthodontic treatment (18-24 months on average), and for most studies that included prevalence, patients were at least 12 years old [126-137] In a study by Hadler-Olsen *et al*. (2012), the mean increase in the WSL index for patients wearing fixed appliances was 1.9. In contrast, the increase in the WSL index in healthy patients not wearing any appliance was 0.4 [138].

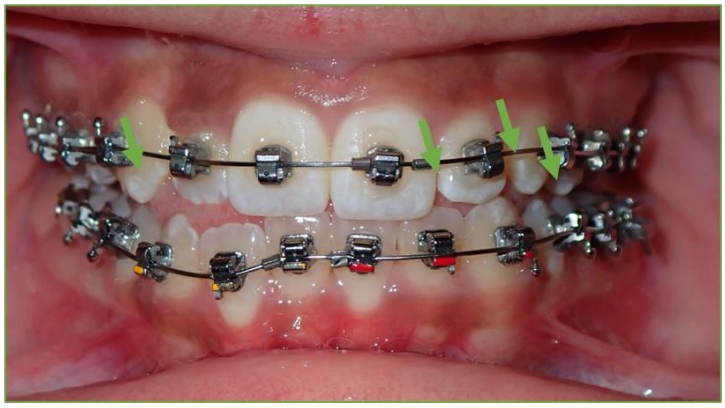

Figure 7 – 13-year-old orthodontic male patient presenting with WSL. Note the generalized WSL around the brackets (light green arrows).

An intriguing finding about WSL occurrence in orthodontic patients is that while we would anticipate that those who have good oral hygiene would not develop these lesions, studies have shown that even when oral hygiene compliance was excellent or moderate, 15 and 42% of such patients, respectively, still got lesions [127]. In one study, patients with good or moderate oral hygiene had a prevalence of WSLs ranging from 23-68% [138]. This study found that, on average, there was the development of 1 new WSL for patients with good oral hygiene, 1.4 for patients with moderate oral hygiene, and 3.3 for patients with poor oral hygiene [138]. These studies suggest that other yet-to-be-identified factors may also contribute to WSL development in the context of orthodontic appliances. Interestingly, not all orthodontic devices/approaches have the same predisposition to affect oral hygiene. A systematic review found that compared to clear aligners, orthodontic brackets tended to lead to poorer periodontal health in patients, however, both promote dysbiosis of the oral microbiome compared to patients who were not undergoing orthodontic treatment [139, 140].

The assessment of WSLs is generally done according to the modified WSL index by Gorelick *et al*. (1982), which is based on the evaluation of the buccal surfaces of individual teeth for their presence or absence [129]. The severity of individual WSLs is scored as follows (Figure 8): 0. no white spot formation; 1. slight white spot formation (thin rim); 2. excessive white spot formation (thicker bands); 3. white spot formation with cavitation.

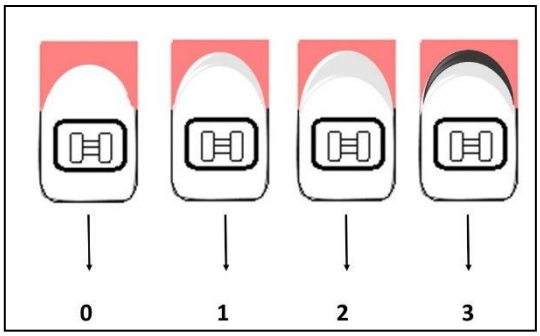

Figure 8 - White spot lesion scoring system. 0: no visible white spot or surface disruption (no demineralization); 1: visible WSL that covers less than one-third of the surface, without surface disruption (mild demineralization); 2: visible WSL that covers more than one-third of the surface, with a roughened surface but not requiring restoration (moderate demineralization); and 3: visible cavitation, requiring restoration (severe demineralization).

1.3.1 Microbiome

As caries is a multifactorial disease, there are several potential contributors associated with the occurrence of WSLs [141]. The oral cavity is an anatomical site with a great diversity of microorganisms, comprising fungi, viruses, and bacteria. The oral microbiome forms a large community that consists predominantly of commensals and beneficial microorganisms [142-144]. The concept goes beyond microbiota and includes the relationship between microbial cells and human cells through their genomes, transcriptomes, proteomes, and metabolomes (Figure 9) [145]. In the past, one way to study and identify the microorganisms in a specific microbiome was by culturing them individually in the laboratory. However, the main limitation in single culturing microorganisms is that many cannot grow in an isolated manner, because they depend on specific conditions/microorganisms that are not replicable in a laboratory environment. Technological advances have led to the development of molecular techniques, such as 16S ribosomal RNA (rRNA) sequencing, which makes it possible to identify species in a biological sample without the need for culturing. This is the key advantage of the metagenomics approach (study of the microbiome) [146].

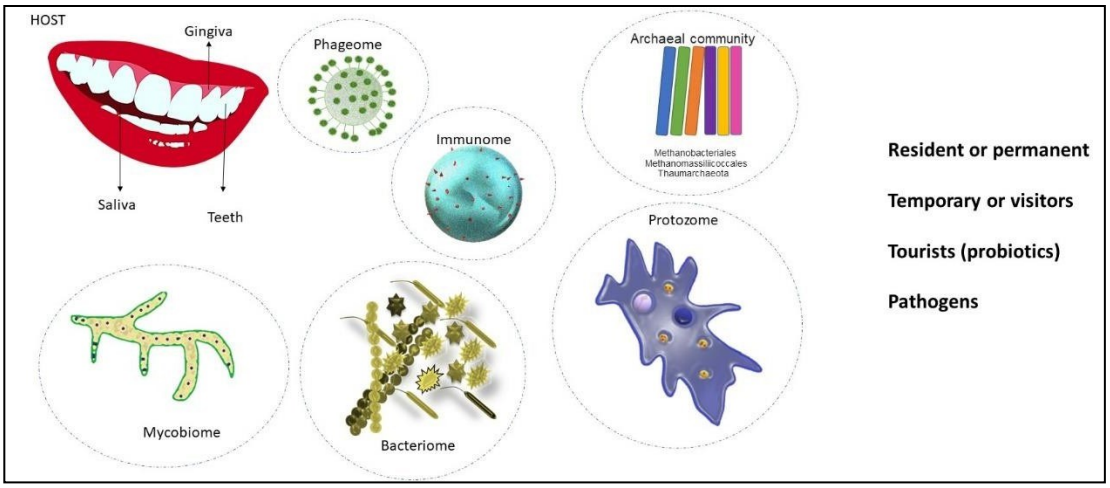

Figure 9 – The oralome constituents in dashed circles and the dynamics of the core microbiota on the right side.

Metagenomics allows samples to be collected from their natural environment, within the complex communities where they usually reside, and permits researchers to comprehend biology as a whole. It transcends the individual organism, and it focuses on the community--how organisms might influence each other's activity and serve a joint function [146, 147]. Our oral microbiome reflects, to a large extent, the composition of the microbiomes of those with whom we live, our diet, oral hygiene, oral and systemic diseases, medications, and interactions with our immune system [148]. Microbiome studies have revealed that every human being carries potentially pathogenic microorganisms, however, in healthy individuals, they can coexist with the host and the remaining microbiome without causing any imbalance [145]. Consequently, the shift to a pathological state may reflect the introduction of some disruptive stimulus that promotes the takeover by pathogens.

Given this innovative technology's current availability, recent studies offer a broadening viewpoint on the role of bacteria in WSL development, suggesting that *Streptococcus mutans* in the initiation of dental caries may not be as central as was previously assumed [123, 141, 149, 150]. It is known that the caries initiation process results from the activity of aciduric and acidogenic microorganisms, known in the literature as streptococci mutans and the non-mutans streptococci (*Streptococcus sanguinis, Streptococcus oralis, Streptococcus gordonii*), together with *Actinomyces sp.* [151, 152]. The number of *Lactobacilli* were frequently used as a diagnostic

test to predict caries activity, however, they are virtually absent at the WSL stage, in support of the conclusion that they are probably not involved in caries initiation [152, 153].

The colonization of enamel surfaces that have been professionally cleaned starts with *Streptococcus sanguinis, Streptococcus oralis*, and *Streptococcus mitis*, which belong to the non-mutans streptococci group. They comprise ~95% of all *Streptococci* in dental plaque and make up 56% of the total early colonizers [154]. As the biofilm matures, colonization by *Streptococcus mutans* increases, promoting imbalance with commensals. This shift in the microbiota promotes a decreased pH and a cariogenic ecosystem. A recent study of the microbiome in different carious lesions, from WSLs to dentin caries, showed that WSLs had a very restrictive niche compared to open dentin cavities. *Streptococci, Rothia, Leptotrichia, and Veillonella* were at higher levels in carious enamel lesions, whereas *Lactobacillus, Shlegelella, Pseudoramibacter,* and *Atopobium* appeared to be associated with dentin lesions (Figure 9). When it comes to *Streptococcus mutans*, surprisingly, a low proportion were found in WSLs (0.73%). This demonstrates the importance of studying other bacteria and their roles as etiologic contributors to tooth decay [153, 155, 156].

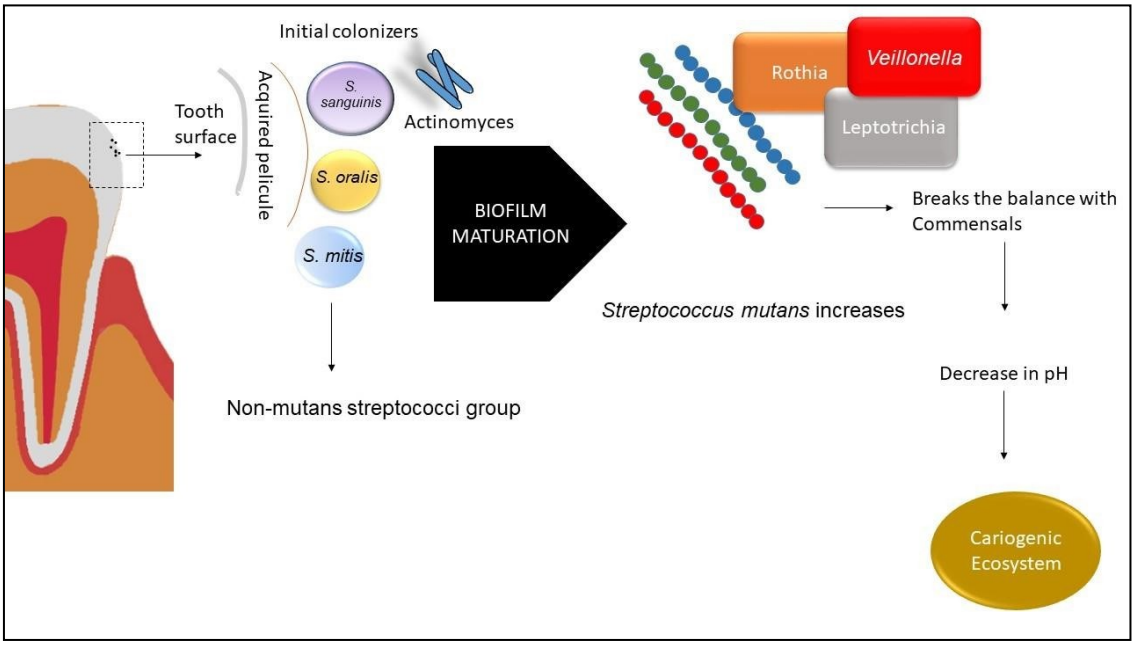

Figure 10 – Microbiome progression in WSL initiation. Tooth structure, on the far left, illustrating the initial colonizers, the biofilm maturation in sequence, the dysbiosis in the plaque leading to a decrease the plaque pH and the creation of a cariogenic ecosystem.

1.3.1.2 Stephan curve kinetics and saliva

Most studies regarding the impact of fixed orthodontic appliances in developing WSLs have focused on specific bacteria species rather than characteristics and features of the microbiome, and often did not consider the dynamic protective effects of saliva [126, 129, 133-136, 157, 158]. Saliva is a complex fluid mixture that humidifies and lubricates the oral mucosal surface, allowing swallowing, phonation, and digestion of the food bolus by enzymatic action. It is also a neutralizer of acids that can help prevent tooth decay and other oral diseases [159]. Salivary buffering capacity plays a vital role in determining the constituency of the microbiome, varies between individuals, and may be determined by multiple factors, including genetics, saliva flow, oral or systemic diseases, medications, and oral microbiome constituents. Decreased saliva buffering capacity contributes to pathological biofilm development, enamel demineralization, and predisposition to dental caries [123, 160, 161]. Some protective effects of saliva can be delineated by the Stephan Curve, which measures salivary pH over time following an oral sucrose challenge [162].

The essential association between pH and dental caries was determined in pioneering work in 1944 by Stephan [163]. In his classic clinical trial, Stephan evaluated the pH generated in dental plaque after patients rinsed their mouths with a sucrose rinse. The three phases of the Stephan curve are 1. a rapid drop in pH, due to fermentation of sucrose by acid-producing bacteria; 2. if the pH drop is below 5.5, the critical pH, demineralization of enamel; 3. gradual increase back to baseline pH, which occurs within 30 to 60 minutes in normal, healthy subjects (Figure 10). In Stephan's study, patients could be classified into caries-free to high caries activity groups based on the association between pH drop and development of lesions over 24 months [163]. After establishing the association of caries development with the kinetics of the Stephan curve, another study found a cause-and-effect relationship between carbohydrate substrate availability and the scale and duration of the plaque pH fall [164]. Besides damaging the enamel, prolonged low pH in plaque and saliva effectively selects for acidogenic and aciduric bacteria [165].

The same pattern of acidification and neutralization occurs in saliva. If the neutralization effect of saliva is diminished, this may lead to a higher prevalence of WSLs. Saliva buffering capacity is independent of oral hygiene and most likely genetically determined in healthy

individuals. In patients that are orally (no caries activity) and systemically healthy and not taking any medications, differences in saliva Stephan curve kinetics may underlie alterations in the oral microbiome that are more predisposing to WSL development in the context of fixed brackets, and that are likely due to a microbiome more predisposing to the habitation of acid-producing bacteria.

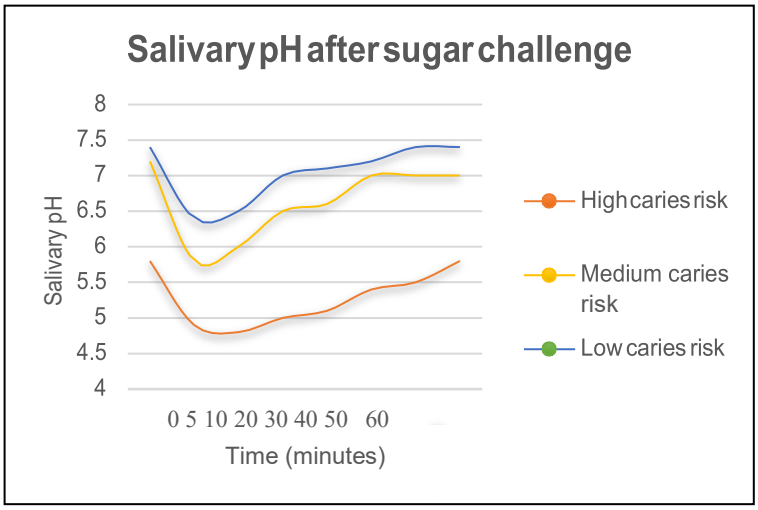

Figure 11 - Stephan curves for different caries activity groups. Green group = caries free; yellow group = slight caries activity; red group = extreme caries activity. Adapted from Stephan R.M., 1944 [163].

1.3.1.2 Objective

The objective of this study is to determine if baseline differences in Stephan Curve kinetics and oral microbiome features correlate with WSL development in orthodontic patients with fixed appliances.

1.3.1.3 Hypothesis

We hypothesize that Stephan Curve kinetics, combined with predisposing microbiome features, can serve as markers to determine which participants with fixed appliances are more likely to develop WSLs.

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CHAPTER 2
METHODOLOGY

ANIMAL AND HUMAN STUDY

CHAPTER 2: METHODS

2.1 Ethical approval

All procedures were approved by the Animal Care and Use Committee (ACUC) of the University of Alberta (AUP 00002935). Both projects involving human subjects were approved by the Health Research Ethics Board (The role of a Loricrin in aggressive periodontal disease: Pro00062112; Oral microbiome and Stephan curve kinetics in white spot lesion development among orthodontic patients: Pro00099341).

2.2 Chemicals and Supplies

If not identified, chemicals, plasticware, and supplies were from Fisher Scientific.

2.3 Animal study

2.3.1 Animal model

Dr. Mark H. Kaplan (Indiana School of Medicine) kindly donated Stat6VT transgenic sperm for rederivation by the Jackson Laboratory (Bar Harbor, ME). Both sexes of Stat6VT hemizygous mice were bred to C57BL/6j (wild-type) mice in the viral antigen-free facility at the University of Alberta and then relocated into conventional or biocontainment facilities (experiment-dependent). Mice were fed a standard chow diet (4% total fat) and provided water *ad libitum*. After genotyping, mice were separated into sex-matched Stat6VT positive or littermate control groups. All Stat6VT mice used in experiments were hemizygous.

2.3.2 Genotyping

Ear notches were used for identification and genotyping. Each sample was digested in 200µl of 0.1M ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate, 0.1M NaCl, 0.05M Tris, pH 7.5 containing 100ng/ml of proteinase K in a 55°C water bath, overnight. Nucleic acids were separated from protein, and other tissue constituents by serial extraction with equal volume Tris-saturated phenol and chloroform. DNA was precipitated with two volumes of 100% ethanol. The pellet was air dried and resuspended in 100ul Tris-EDTA (0.01M Tris, pH 8, 0.001M EDTA) containing 20ng/ml of RNAse A. In general, 1:100 dilutions of the DNA were used for PCR.

Our collaborator, Dr. Mark H. Kaplan, shared with us his laboratory genotyping protocol for polymerase chain reaction (PCR). We acquired the DNA oligo primers from Integrated DNA Technologies. Primers HAA9 (5'- GCC TAC CAT GGT GCC TTC TTA TG – 3') and VT FLAG (5'- TAT GCT TGT CAT CGT CCT TGT AGT CA – 3') targeted the novel FLAG tag incorporated in the Stat6VT construct to differentiate transgenic from wild-type mice. Ready-to-use PCR master mix (DreamTaq Green, K1082, Thermo Fisher Scientific) containing DreamTaq DNA polymerase, 2X DreamTaq green buffer, deoxyribose nucleotide triphosphates and 0.004M magnesium chloride was used for PCR. The final master mix consisted of 1X PCR master mix, 1µm of each primer and 2ul of DNA in a total reaction volume of 10µl. The samples were processed according to the PCR thermocycling protocol described in Table 1.

The next step was to resolve the PCR products on a 1.5% agarose gel. Tris-borate-EDTA (TBE) was used as the running buffer (0.089M Tris, 0.089M boric acid, 0.002M EDTA). A ChemiDoc Imaging system (model XRS+, Bio-Rad) was used to detect and document the results (ChemiDoc MP Imaging System, version 4.1, Bio-Rad).

Table 1 – PCR thermocycler protocol *

Temperature (Celsius)	Procedure	Time (min)
94º	Initial denaturation of DNA template	2
94º	Denaturation	0.5
54º	Primer annealing	0.5
72º	Extension	1
Repeat	Cycles	34x
4º	Hold	∞

* Model used: T100 Thermal Cycler (Bio-Rad)

2.3.3 Mice sample size calculations

We performed a power calculation to determine the number of animals per group. We considered that 25% bone loss would be significant, based on similar studies in the literature [1-3]. Using the website: https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html, we determined our sample size to be 9 (at 80% power, 0.05 alpha value). We included extra animals (at least two) per group in case of malocclusion or unexpected morbidities/mortalities (especially associated with skin lesions at later time points).

2.3.4 Animal Study design

Transgenic Stat6VT mice have been used widely for studies in the realm of skin and inflammatory disorders, however, no previous research has been published investigating the oral cavity [4-7]. Results from previous work in our lab showed that Stat6VT male mice consistently developed skin lesions at ~10-12 weeks of age; females had earlier and more variable disease onset [8]. Faxitron analysis of eleven femurs each from male and female Stat6VT and control mice showed no differences in bone mineral content and bone mineral density for males at this age; females showed significant differences [8]. We chose 10–12-week-old males for our pathogen

challenge model because skin disease onset and progression were more uniform, and bone mineral density and content did not differ from controls (Figure 12a, 12b). We reasoned that skin lesions indicated active disease that could manifest in the oral cavity if challenged.

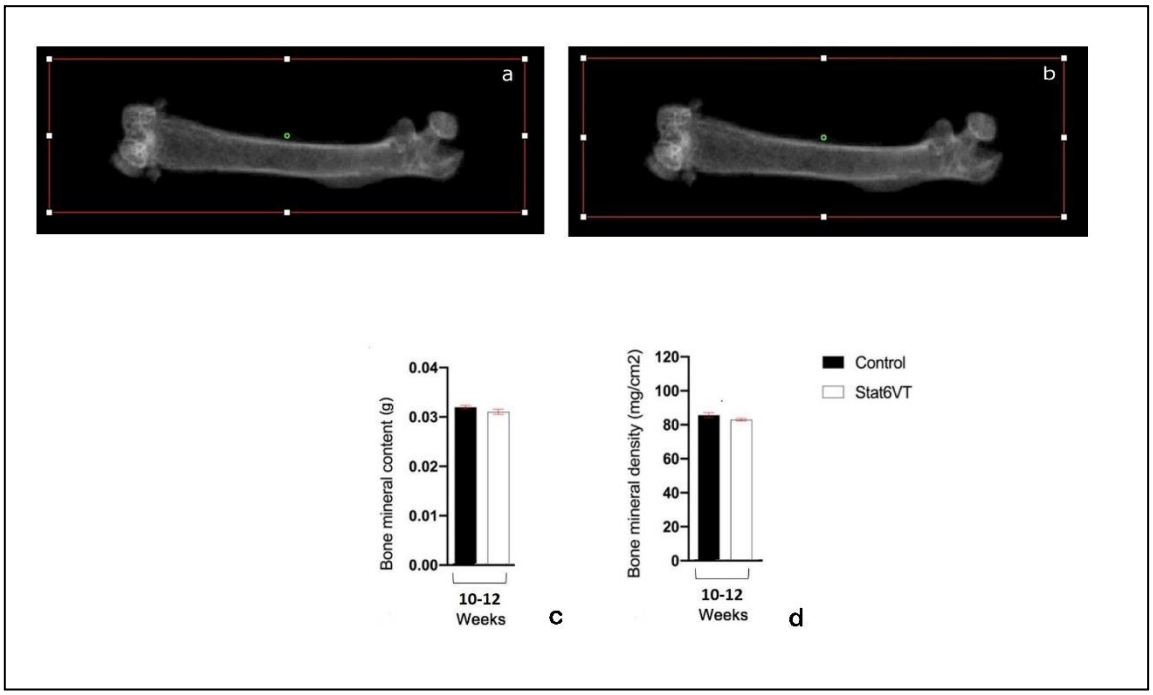

Figure 12 – Bone mineral content and bone mineral density. Representative images of femurs from **a.** Control male mouse (BMC: 0.03230g; BMD: 80.945 mg/cm2); **b.** Stat6VT male mouse (BMC: 0.03064g; BMD: 80.848 mg/cm2);

c. Bone mineral content of 12-week-old male control and Stat6VT mice (n=11/group); **d.** Bone mineral density of 12-week-old male control and Stat6VT mice (n=11/group).

To determine potential differences in alveolar bone levels and inflammation at baseline, we assessed alveolar bone loss by a standardized 2-plane landmark microCT protocol prior to Pg-infection in 12-week old control and Stat6VT male mice [9]. The details of the protocol are in chapter 3, briefly, there were 8 measurements each for the 1st and 2nd mandibular molars: 2 on the sagittal plane and 6 on the coronal plane. The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured and found to be significantly greater at four sites on the coronal plane and one site on the sagittal plane in Stat6VT mice compared with controls (Figure 13a-f, n = 11/group). H&E staining showed an increase in rete pegs and clear cells that could be indicative of inflammation in Stat6VT mice. We then confirmed this finding by staining masticatory mucosal tissues with a pan leukocyte marker, CD45: Stat6VT mice had a mean of 19.08 ± 0.7432 CD45 positive cells/40x field, compared to control mice, which had a mean of

11.08 ± 1.654 CD45 positive cells/40x field (n = 5/group, p = 0.0012). There was also significantly more proliferation in the epithelium of Stat6VT mice compared to controls, as measured by the number of Ki67 positive cells/40x field (control: 29.57 ± 3.416; Stat6VT: 43.86 ± 3.420, n = 5/group, p = 0.0039). CK1 and CK14 expression were largely similar between the groups (Figure 14a-d). Loricrin expression was very similar in intensity, however it appeared in lower layers in Stat6VT mice (Figure 14e, Zf).

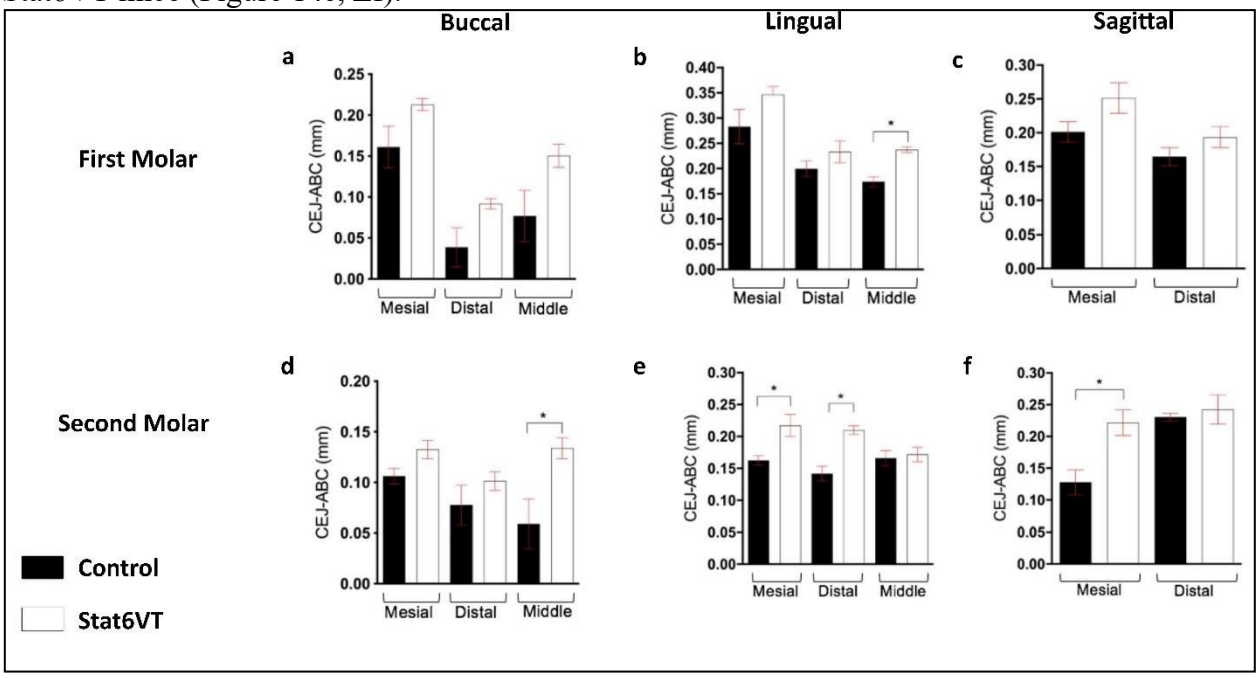

Figure 13 - MicroCT measurements of the distance from the CEJ to the ABC in 12-week old male Stat6VT and control mice prior to Pg infection (n =5/group) **a.** First molar buccal aspect, **b.** First molar lingual aspect, **c.** First molar sagittal aspect, **d.** Second molar buccal aspect, **e.** Second molar lingual aspect, **f.** Second molar sagittal aspect. *p<0.05

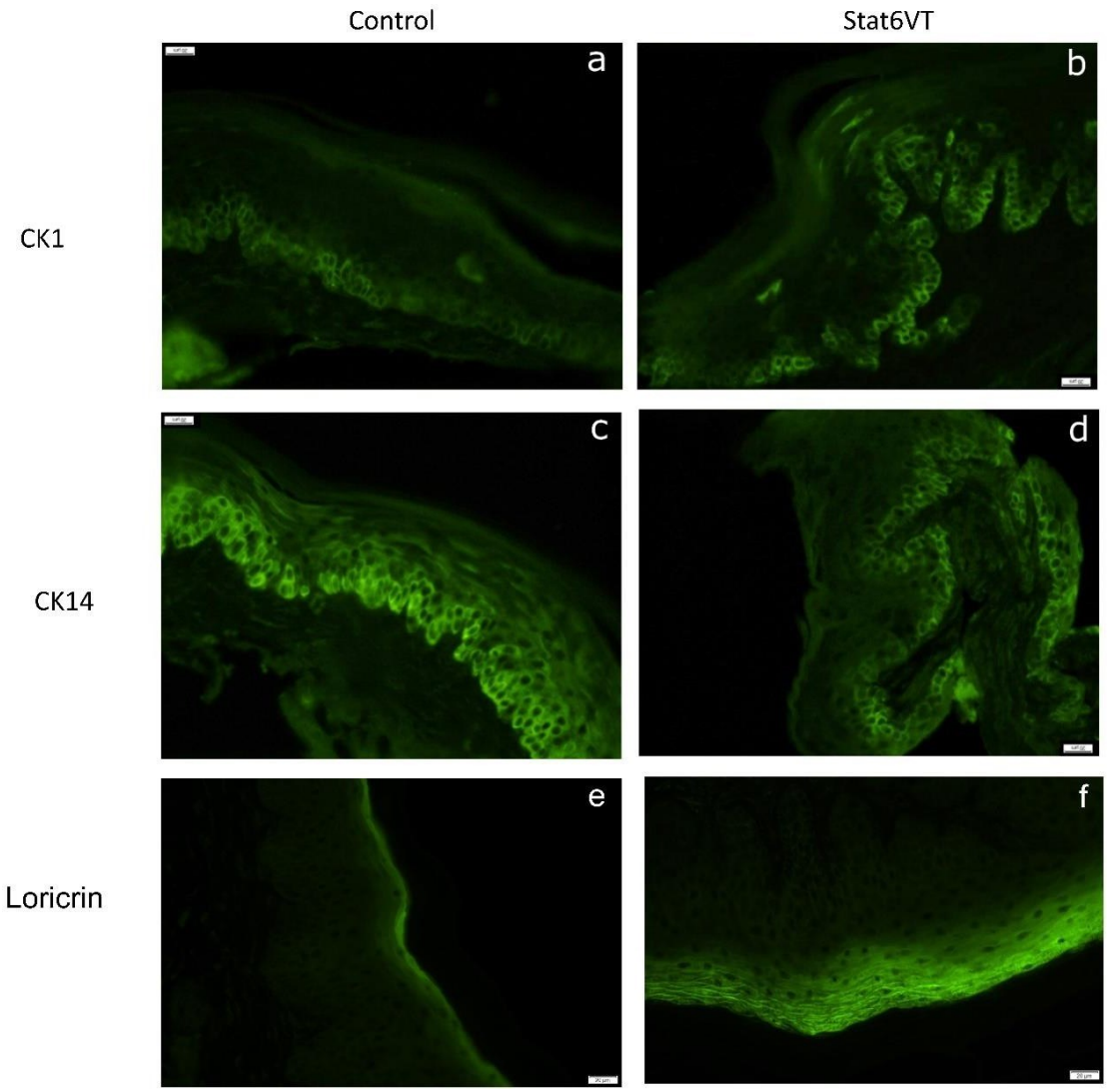

Figure 14 - CK1 positive cells in the masticatory mucosa prior to Pg infection of 12-week old male mice (n =5/group) **a.** Control, **b.** Stat6VT mice. CK14 positive cells in the masticatory mucosa prior to Pg infection of 12-week old male mice (n =5/group); **c.** Control, **d.** Stat6VT mice. Scale bar = 20µm. Loricrin staining in the masticatory mucosa prior to Pg infection of 12-week old male mice (n =5/group) **e.** Control and **f.** Stat6VT mice. Scale bar = 20µm.

2.3.5 Pg Infection

Porphyromonas gingivalis (American Type Culture Collection strain #33277) bacteria were grown under anaerobic conditions in Schaedler's broth containing vitamin K and hemin (BBL Schaedler Broth with Vitamin K1, L007496, Becton Dickinson). The components of Schaedler's broth (Becton Dickinson) are shown in Table 2.

Table 2 - Schaedler's Broth with Vitamin K1 and Hemin*

Reagents	Amount
Casein	8.1g
Peptic digest of animal tissue	2.5g
Papaic digest of soybean meal	1.0g
Dextrose	5.82g
Yeast extract	5.0g
Sodium chloride	1.7g
Dipotassium phosphate	0.82g
Hemin	0.01g
Vitamin K1	0.01g
L-cystine	0.4g
TRIS aminomethane	3.0g
Milli-Q water	Up to 1000ml

*Autoclaved for 15 minutes at 121°C, pH 7.6 ± 0.2 at 25◦C

Pg bacteria were grown to saturation in a 2.5L anaerobe jar (AnaeroPack, R685025, Thermo Fisher Scientific) for 24-48 hours using anaerobe packs (BD BBL™ GasPak™) to produce the correct atmosphere [10]. Cultures were centrifuged at 20,000 x g for 5 minutes. Bacteria were resuspended at a concentration of ~10^9/ml in sterile phosphate-buffered saline (PBS) containing 2% carboxymethylcellulose (9004324, Sigma-Aldrich), which is a thickener used to promote bacterial adherence to structures in the oral cavity [10]. Ten to twelve-week-old male Stat6VT and littermate control mice were weighed to determine the proper dose of ketamine/xylazine for anesthesia, administered via intraperitoneal injection (100mg/kg ketamine and 10mg/kg xylazine in a volume of 100ul). For the mice to be infected with oral lavage (utilizing a microbrush), we had them reach stage 3 plane 2 anesthesia every time.This was repeated every other day for two weeks in the Bio-Containment facility at the University of Alberta. Control mice

received vehicle alone. This is a modification of the classic technique used by Lalla *et al*. [11, 12]. Mice were allowed to recover on a water-jacketed heat pad prior to return to their cages.

2.3.6 Sample collection and preservation

Mice were euthanized by intraperitoneal pentobarbital injection (200 mg/kg, Euthanyl, BiMeda-MTC Animal Health Inc, 00141704). For plasma analyses (cytokine array), about 1mL of blood was collected from the heart via cardiac puncture, utilizing an EDTA-coated 1cc syringe containing EDTA to a final concentration of 5mM. In order to separate erythrocytes and leukocytes from plasma, whole blood was centrifuged for 5 minutes at 3783 x *g*. Plasma was then carefully collected, aliquoted (200µl/tube), and stored at -20°C until cytokine analysis could be performed. Soft tissues were collected from the gingiva, palate, and mandibles using sterile instruments and a dissecting microscope (model DM2000, Leica Microsystems) to achieve optimal accuracy. Small incisions were made at the commissures of the mouth with a scalpel and the palate was peeled back, starting from the incisive papilla to the soft palate (Figure 14). Then keratinized gingival tissues from the buccal left molar region were collected. Tissue samples intended for histology were fixed in 10% formaldehyde (28908, Thermo Fisher Scientific) at 4°C for 24-48 hours (depending on sample size) on a rotating platform. Samples were rinsed with PBS three times and immersed in PBS at 4°C until microCT scanning (mandibles) or further processing was performed. Mandibles were decalcified for histological preparation.

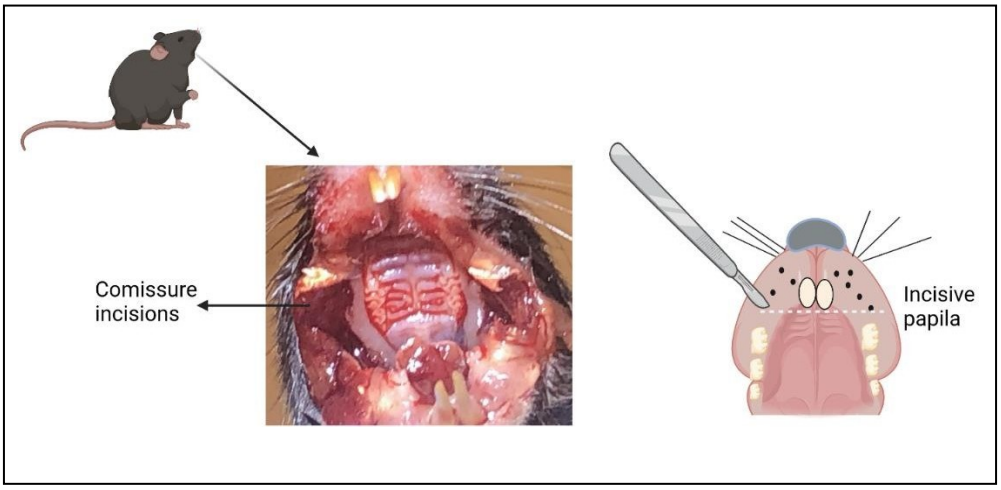

Figure 15 – Mouse palate showing commissure incisions. Dotted white line indicates where the incisive papilla was dissected.

2.3.7 Decalcification

Mandibles were immersed in 0.5M EDTA, pH 7.4. Samples were incubated in 10ml of EDTA solution on a rotating platform in a 15ml conical tube, and the solution was changed every other day for 8 weeks [13]. The samples were then soft enough to be processed and embedded in paraffin [13]. Mandibles were cut in half; therefore, embedded samples consisted of two hemi-mandibles (left and right side) (Figure 16a, 16b).

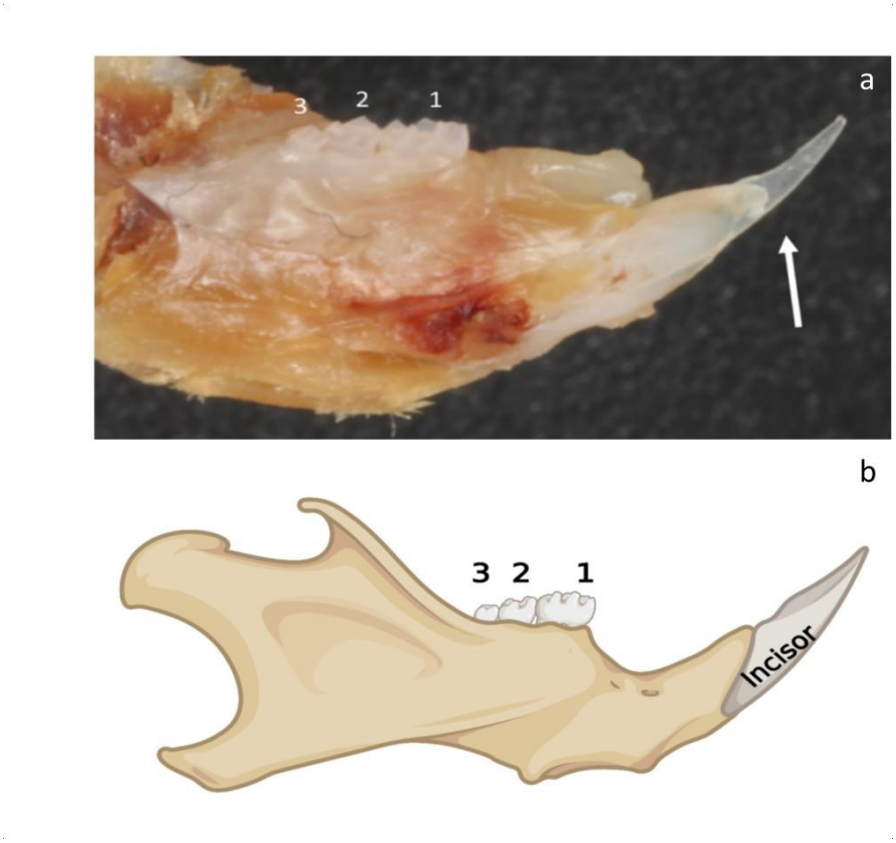

Figure 16 – **a.** Photograph of a mouse left hemi-mandible. Arrow indicates translucent incisor (one indicator of tissue softness). 1: first molar; 2: second molar; 3: third molar; **b.** Cartoon rendition of a mouse left hemi-mandible. Figure created with Biorender software.

2.3.8 Tissue processing

2-5mm thick tissue samples were positioned in histologic cassettes and immersed in 50% ethanol for 2 hours before using the automated processing cycle. The cassettes are resistant to most histological solvents, and their slots offer maximum flow-through for good fluid exchange and proper drainage. Each cassette was labeled individually in pencil to avoid chemical erasure of the unique label (pen ink is easily dissolved by xylene) (Figure 17). Next, the samples were wrapped in lens paper (Fisherbrand, 11-996). Then the labeled cassettes were placed inside the processor metal baskets and set into the automatic tissue processor machine (Leica Biosystems, TP1020). The processing cycle utilized is shown in Table 3. Each step of the processing requires the diffusion of a solution into the tissue after the the dispersion of the prior solution in the series.

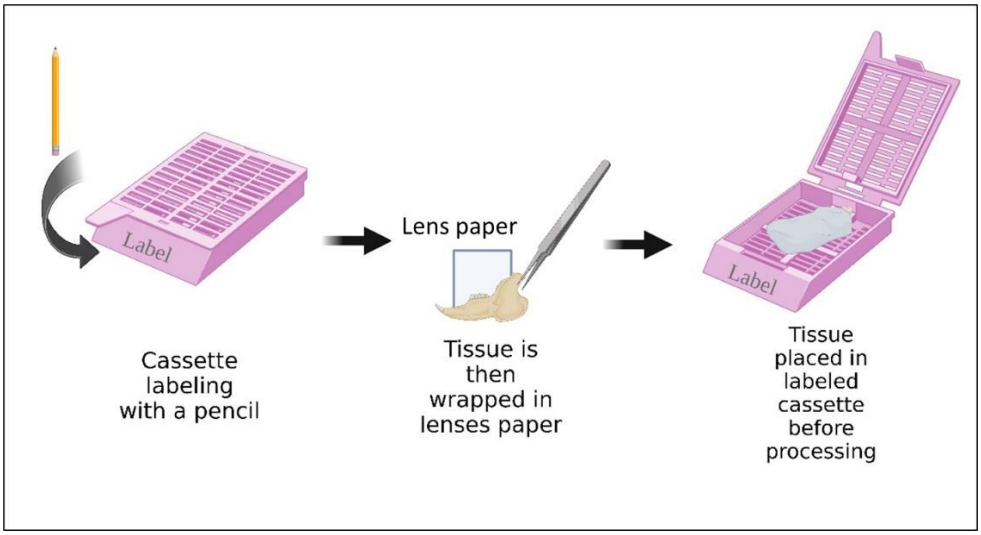

Figure 17 – Schematic of cassette labeling and tissue placement. Figure created with Biorender software.

Table 3 – Tissue processing cycle.

Solution	Change (s)	Time (h)
70% Ethanol	2	1
96% Ethanol	1	1
100% Ethanol	1	1

100% Ethanol	1	1
100% Ethanol	3	1.5
Xylene	3	1.5
Paraffin wax (58-60°C)	2	2

2.3.9 Tissue embedding

Once the embedding stage was reached, the histological cassette lid was snapped off, such that the cassette forms a base for the paraffin wax block (Figure 18). Tissues were embedded in paraffin (Histoplast Paraffin Wax, 22900700, Thermo Fisher Scientific), which is similar in density to the tissue. The embedding was performed using a Leica Biosystems Embedding Center (EG1160). While multiple tissues can be processed together, at the embedding stage, each hemi-mandible with surrounding tissue was placed individually into the bottom portion of the histology metal mold and sealed with the cassette. For consistency in orientation, the buccal side of each specimen was placed at the bottom, and the lingual/palatal side was placed facing up. In addition, occlusal surfaces were placed facing the up. To ensure sectioning was performed, as much as possible, parallel to the long axis of the roots, the occlusal surfaces of the first molar were orientated 90 degrees perpendicular to the floor of the embedding mold. Incisors were kept on all mandibular samples to enhance orientation and landmarking. Once samples were embedded, they were left on a 4°C cooling platform for at least 1 hour or until the paraffin had completely hardened.

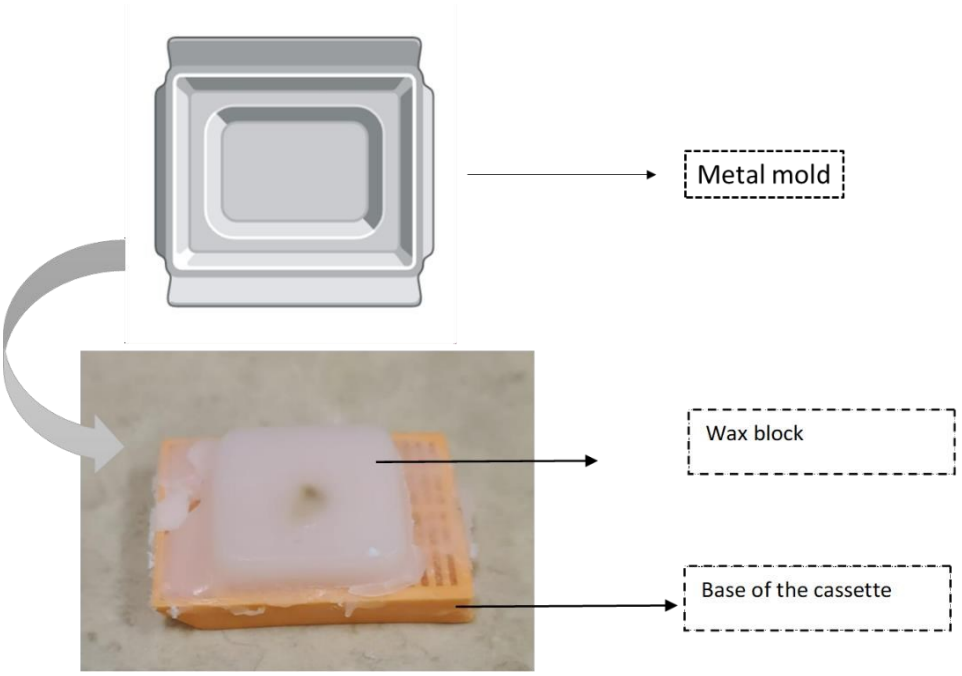

Figure 18 – Metal mold and resulting wax block with embedded tissue (dark spot) on a tissue cassette.

2.3.10 Microtome sectioning

After embedding, tissues were sectioned using a Leica microtome (model Histocore AUTOCUT, 14051956472, Leica Biosystems) and sections collected onto Superfrost Plus microscope slides (22037246, Thermo Fisher Scientific). Sections were 5-7µm in thickness. After sectioning, samples were placed in a slide rack and allowed to dry overnight. Orientation for sectioning is illustrated in Figure 19.

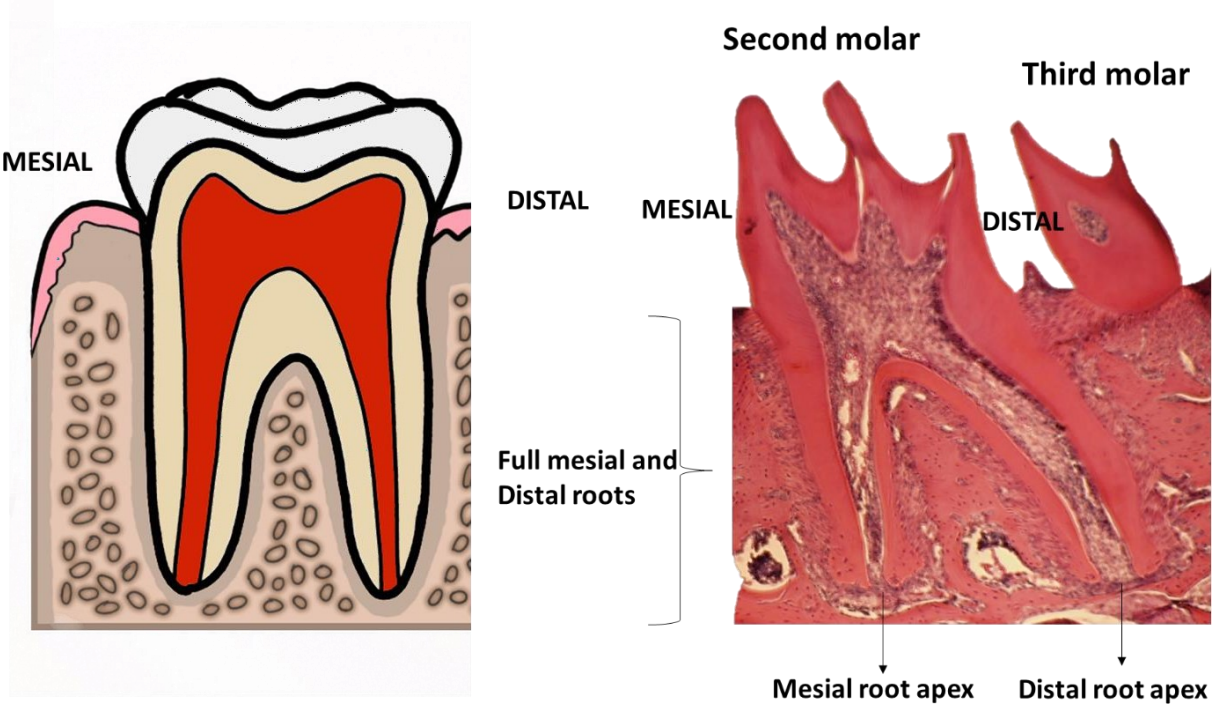

Figure 19 – Cartoon representation of desired orientation of selected slides on the left side and actual H&E histological section on the right side.

2.3.11 Hematoxylin and eosin (H&E) staining

Using landmarks for selection of best sections and orientation, samples were chosen for staining. Landmarks for hard tissue were: the crown of the first or second molar, mesial and distal roots present, as shown in Figure 15. Our lab used the modified protocol of Slaoui *et al*. to perform H&E staining [14]. Initially, slides were placed on a metal slide tray and heated to 65°C in a vacuum oven (ThermoFIsher Scientific Hi-Temp Vacuum Oven, 3625A) for 10 minutes to initiate de-paraffinization. Then slides were placed in a glass slide staining rack and the tissues went through a xylene immersion and then graded ethanol washes (100%, 95%, and 70%). Next, the

tissues were re-hydrated in a bath of Milli-Q water. Tissues were stained with undiluted hematoxylin (Protocol, 245656) for about 4 minutes. Following this, slides were carefully washed with Milli-Q water and incubated for 30 seconds in undiluted eosin (Protocol, 245658). Lastly, the tissues were dehydrated by immersion in graded ethanol solutions (95% and 100%), followed by a xylene immersion for 6 minutes. Slides were then placed on a paper towel to remove excess xylene. For mounting, a very thin layer of Permount (Fisher Chemical, SP15100, Thermo Fisher Scientific) was applied on a glass cover slip (22mm x 60mm). Stained slides were allowed to dry overnight on a slide rack and imaged under a light microscope (model DM2000, Leica Microsystems). Images were captured using cellSens Standard software (Version 4.1, Olympus).

2.3.12 Immunofluorescence

We adapted the immunofluorescence protocol from the study by Christodoulou *et al*. [15]. Slides were de-paraffinized for 10 minutes at 65°C in a vacuum oven. Tissues were re-hydrated according to the protocol shown in Table 4. Antigen retrieval was achieved using a solution consisting of 0.006M citric acid, 0.01M trisodium citrate buffer, pH 6.0. The slides were placed in this solution inside a polypropylene slide mailer (HS15983G, Heathrow Scientific) and then heated in a microwave for 1 minute at 90°C and left immersed for 40 minutes. Three washes were performed using sterile PBS on a shaking platform (multi-purpose rotator, Thermo Scientific) for 5 minutes each. Then the tissue samples on the slide were circled for isolation with an ImmunoPen (ImmEdge Hydrophobic Barrier PAP Pen, H4000, Vector Laboratories).

Table 4 – Deparaffinization protocol for immunofluorescence.

Solution	Time (min)
Xylene	10
Xylene	10
100% ethanol	10
100% ethanol	10
95% ethanol	5
70% ethanol	5
Milli-Q water	5

2.3.13 Blocking

Blocking was based on the host of the secondary antibody, in our case, goat. The blocking solution contained 10% goat serum (100µl stock solution diluted in 900µl Tris Buffered Saline – (0.05M Tris, pH 7.6; 0.015M sodium chloride)). Tissues were blocked for 45 minutes in a humid chamber. The chamber consisted of a raised plastic stand (e.g. pipette tip rack) above layers of paper towels saturated with Milli-Q water in a plastic container that had a lid (Figure 20).

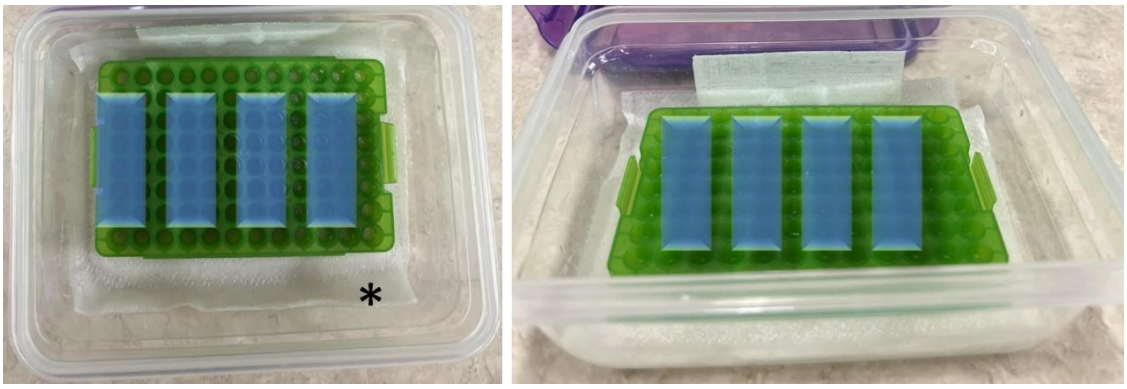

Figure 20 – Humid chamber: plastic container, a pipette tip rack (green), and paper towels soaked in Milli-Q water (*); blue rectangles represent where slides were placed. This was closed with a lid during incubations.

2.3.14 Primary antibodies

After the blocking step, the designated primary antibody at the specific dilution (Table 5) in 1% BSA was placed onto the tissue sample, incubated at room temperature for 1-2 hours in a closed humid chamber, and then placed at 4°C overnight. Table 5 describes information relevant to the primary antibodies used.

Table 5 – Details of the primary antibodies used.

Primary antibody	Host	Type	Dilution in 1% BSA	Brand (cat#)
Loricrin	Rabbit	Polyclonal	1:100	Abcam (85679)
Ki67	Rat	Monoclonal	1:300	Invitrogen (SolA15)
CD45	Rat	Monoclonal	1:400	Santa Cruz (53665)
CK1	Rabbit	Monoclonal	1:500	Abcam (185628)
CK14	Rabbit	Monoclonal	1:500	Abcam (119695)

2.3.15 Secondary antibodies

The following day, the primary antibody was removed by tapping the slides horizontally, and the slides were washed 3x in a solution of PBS + 1% Tween-20 for 5 minutes each on a shaking platform. Secondary antibody, at specific dilution as shown in Table 6, in TBS (50 mM Tris-Cl, pH 7.6; 150 mM NaCl) and BSA 1%, was then applied The details for the secondary antibodies used are shown in Table 6.

Table 6 – Details of secondary antibodies used.

Secondary Antibody	Dilution	Brand (cat #)	Markers
DyLight 488 conjugated goat anti-rabbit	1:500	Thermo Fisher Scientific (A32731)	Loricrin, CK1.CK14
Alexa Fluor 647 conjugated goat anti-rat	1:500	Thermo Fisher Scientific (A21247)	CD45, Ki67

After incubation, three washes in PBS for 5 minutes each were performed in a darkened room and then tissues were mounted utilizing SlowFade Gold Antifade Mountant 4',6-diamidino- 2-phenylindole (S36938, Thermo Fisher Scientific). Slide images were captured with an immunofluorescent microscope (Olympus, model IX73) equipped with imaging analysis software (cellSens Dimension, version 1.15, Olympus). Image capture settings were kept constant to allow for comparison between slides/groups.

2.3.16 MicroCT

Prior to histology, fixed mandibles were scanned using a 3-dimensional microCT (model U-SPECTII/ CT, Milabs) at 25μm voxel size resolution with an exposure of 75ms, voltage of 50kV, and current of 0.24mA. Reconstruction of the images was done utilizing MILabs software (version 2.38). During the reconstruction phase sagittal, coronal, and axial planes were aligned. Because mandibles were still attached to the craniofacial complex, the nasal septum and occlusal plane of the first molars were used as references to place the samples inside the machine. 3D reconstructions were made using Avizo software (version 9.1, Thermo Fisher Scientific). For alveolar bone measurements, we used a protocol developed and published by our laboratory previously [9]. The steps of the protocol are described in Chapter 3.

2.3.17 Cytokine array

Blood was collected as described in Section 2.3.6. The cytokine array was performed on plasma from male Pg-infected Stat6VT and control mice, and uninfected Stat6VT and control mice. For the Pg-infected Stat6VT and control plasma analysis, a mouse cytokine antibody array - membrane was used to perform cytokine profiling (AB193659, Abcam). For the uninfected Stat6VT and control analysis, a 16-well tray cytokine array kit was used (QAM-INF-1, RayBiotech) and performed entirely by Sara Moradipoor (former Research Assistant in Dr. Febbraio's lab) according to the manufacturer's directions.

For the cytokine array of Pg-infected Stat6VT and control mice, 2 membranes embedded

with cytokine targets were provided by the manufacturer. These were initially blocked in blocking buffer at room temperature for 30 minutes. Then the blocking buffer was aspirated and the membrane was incubated with 1ml of undiluted plasma pooled from 5 mice per group for 2 hours at room temperature on a shaking platform. The membranes were washed with kit included wash buffer for 5 minutes on a shaking platform 3x. Next the membranes were washed with a second kit contained wash buffer twice for 5 minutes on a shaking platform . A total of 2ml of biotinylated antibody cocktail (provided by the manufacturer) was loaded onto the membranes (1ml per membrane) and incubated for 2 hours at room temperature on a shaking platform. After aspiration, membranes were washed sequentially with the two wash buffers, as previously described. The membranes were then placed onto Whatman chromatography paper and any excess wash buffer was carefully removed by blotting the membranes' edges with the chromatography paper. The membranes were next transferred onto a plastic sheet and saturated with 500µl of chemiluminescence detection buffer. Another plastic sheet was placed directly on top of the membranes and transferred to the ChemiDoc (model XRS+, Bio-Rad) for imaging, using a capture mode that took images at 1 to 10-second intervals over a total time period of 3 minutes.

For data analysis of the Pg-infected mice cytokine array, data normalization was performed according to the manufacturer's instructions. The quantity of biotinylated antibodies for each positive control spot is constant from array to array. Therefore, the positive control signals can be used to normalize signal responses for comparison of results across multiple arrays. We used the following formula for normalization, as suggested by the manufacturer (User Manual, AB193659, Abcam, Version 3):

"X(Ny)=X(y)*P1/P(y)"

Where:

P1 = mean signal density of Positive Control spots on reference array P(y) = mean signal density of Positive Control spots on Array "y" X(y) = mean signal density for spot "X" on Array for sample "y" X(Ny) = normalized signal intensity for spot "X" on Array "y" "

After the normalization step, we then compared relative expression levels between the 2 groups. The data are presented as the relative expression, or fold change, of a certain cytokine (Stat6VT *vs* controls). Fold change was calculated as the average signal intensity in the control group subtracted from the average signal intensity in the Stat6VT group, divided by the signal intensity in the control group.

For data analysis of the uninfected Stat6VT and control mice analysis, briefly the manufacturer provides an array specific cytokine standards that allow for the creation of a standard curve for each cytokine and this facilitates cytokine quantification. The slides were then analyzed via Quantibody® Q-Analyzer (an array specific, Excel-like software) which allows for for intra- and inter-slide normalization for large numbers of samples.

2.4 Human tissue samples from patients with severe forms of periodontitis

2.4.1 Human tissue sample size calculation

In a pilot study, our lab investigated if there was a difference in protein expression in gingival samples of 12 SvP and 11 healthy patients, from the University of Alberta dental clinic and Universidade de Guarulhos (São Paulo, Brazil) [16]. This was accomplished using a commercially available validated ELISA (SEC568Mu – Cloud-Clone Corp.). Similarly to the pilot, we had a 0.86 effect size, alpha = 0.05 and at 0.8 power, the sample size was calculated to be 20/group [16]. The mix of patients from Canada and Brazil were only used for the pilot.

As previously described in Chapter 1, SvP refers to stage III and IV grade C periodontitis patients. Patients that presented with any systemic comorbidities or smokers were excluded from our study. Healthy and SvP patients were screened and diagnosed by periodontology residents and

confirmed by a periodontology instructor. After a careful explanation of the research and tissue collection, patients proceeded to read and sign the informed consent form (Appended at the end of this chapter - A). Tissues were collected during routine surgeries (gingivectomies, crown lengthening procedures, or any procedures that would allow us to collect keratinized oral tissue) by periodontology residents and stored by Dr. Christopher Bryant Roy (periodontology resident and contributor to this project). An illustration of one of the surgical procedures is shown in Figure 21a, 21b. Tissues were placed in 4% formalin, incubated on a rotating platform at 4ºC for at least 24 hours, then washed three times in sterile PBS and finally stored in PBS until they were processed.

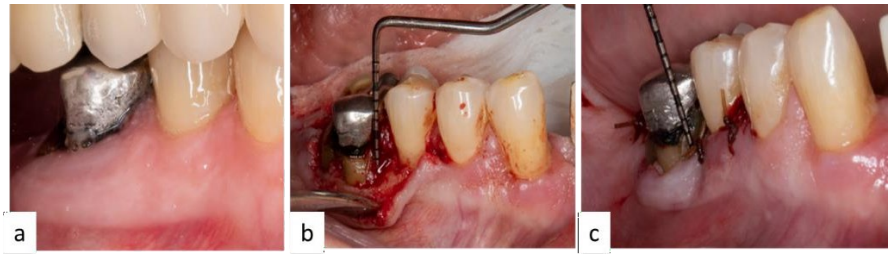

Figure 21 – **a.** Crown lengthening procedure of tooth #46. **b.** Measurement of the distance from the CEJ to the alveolar bone with a periodontal probe. **c.** Surgery finalized, sutures in place. (Pictures courtesy of Dr. Christopher Bryant Roy).

2.4.1 Histology

The procedures for processing, embedding, H&E and immunofluorescence staining utilized the same methods as described in Sections 2.3.8 to 2.3.15, with slight differences. For the human samples, there was no hard tissue involved, thus no decalcification was needed. For human tissue orientation, samples were positioned in the histological cassettes such that both epithelium and connective tissue were perpendicular to the metal molding cassette (meaning also

perpendicular to the microtome blade). Orientation was easily accomplished given the difference in color between connective and epithelial tissues after fixation (Figure 22a, 22b).

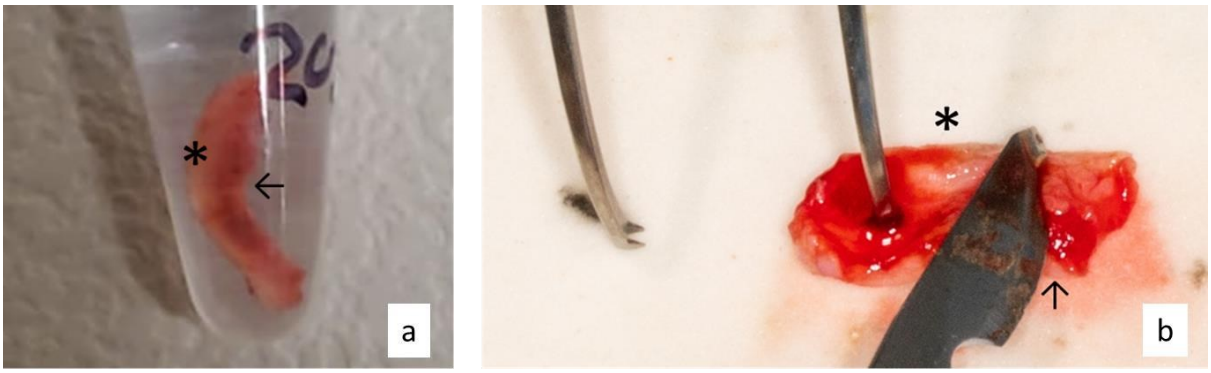

Figure 22 – **a**. Gingival tissue fixed in 10% formaldehyde. * denotes epithelium; arrow denotes connective tissue. **b.** A closer image of the tissue, once again illustrating the difference in color between connective and epithelial layers (images generously provided by Dr. Christopher Bryant Roy).

2.5 Statistical analyses

Results are presented as mean ± standard error (S.E.). Kolmogorov-Smirnov normality and equality of variances tests were applied to the results. If they were normally distributed and had equal variances, they were assessed by a non-paired, two-tailed t-test or one-way ANOVA. If normality wasn't met, the non-parametric Mann-Witney or Kruskal-Wallis test was used. The statistical significance was set at $p<0.05$. GraphPad Prism 9 and IBM SPSS version 28 software were used for statistical analyses.

2.6 Stephan curve kinetics, white spot lesions and the microbiome

2.6.1 Sample size calculation

The sample size for control and WSL patients (cases) was calculated at 80% power with a confidence level of 95% (precision of at least 5% is recommended when expected prevalence

ranges between 10-90%) [17-21]. Previous experience in the orthodontic clinic at the University of Alberta showed that 60% of patients developed at least some signs of a WSL (Flores-Mir, C., unpublished observations). As a result, the number of patients needed was 16 (8 per group). Due to the longitudinal study design, to allow for possible drop-out of 20%, we set a recruitment target of 10 patients per group, for a total of 20 patients.

2.6.2 Inclusion and exclusion criteria

Patients were assessed for good oral health prior to appliance placement based on the simplified oral hygiene index (OHI-S). Briefly, six teeth were scored for their debris and calculus indexes (Fédération Dentaire Internationale notation: #16: upper right 1st molar, #11: right upper central incisor, #26: upper left 1st molar, #36: lower left 1st molar, #31: left lower central incisor, #46: lower right 1st molar) (Figure 23). Debris was scored 0-3 as follows: 0: no debris, 1: soft debris < ⅓ of tooth surface, 2: soft debris >⅓ and <⅔ of tooth surface, and 3: debris covering >⅔ of tooth surfaces (Figure 24). A similar scoring was done for calculus based on crown coverage. The sum for each index was divided by the number of teeth examined (6) and then added together for the total OHI-S score. The scores related to oral health as follows: 0.1-1.2: good; 1.3-3.4: fair; 3.1-6.0:poor [22]. The oral hygiene was followed at 3, 6 and 12 months by the orthodontic residents (as they have notes for oral hygiene on their orthodontic chats) and double checked by a second resident.

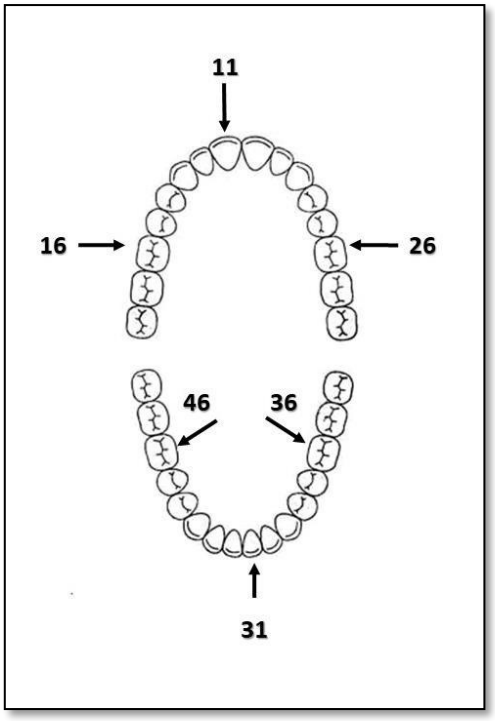

Figure 23 – Sites chosen for the OHI-S.

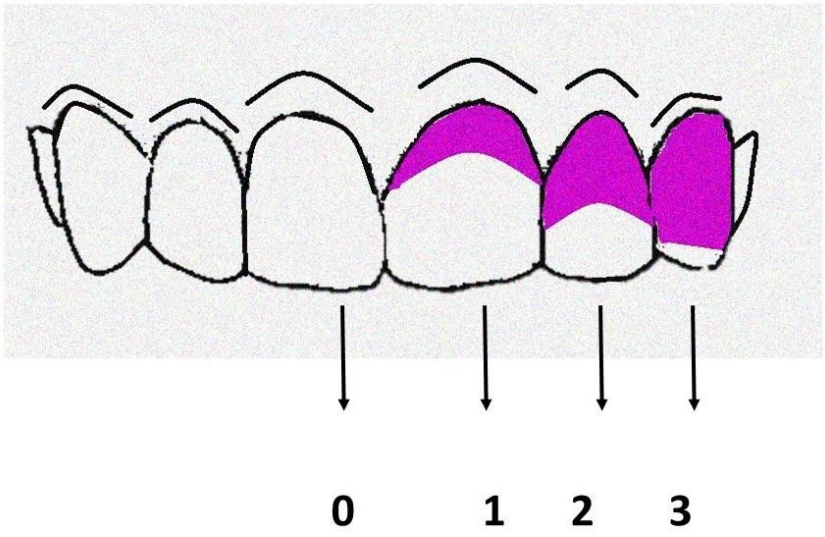

Figure 24 – Simplified Oral Hygiene Index (OHI-S).

Patients with OHI-S scores between 0.1-1.2 (good) that had fully erupted second molars and a treatment plan for fixed, self-ligating orthodontic appliances (at least from 1st molars to 1st

molars) for a minimum of 12 months duration, were recruited into this study from the Graduate Orthodontic Clinic at the University of Alberta. Written informed consent was obtained prior to participation (appended at the end of this chapter - B). Patients with systemic disease, xerostomia, clefts, generalized dental problems, on a daily supplemental fluoride regimen, or ongoing medication for a chronic disease and those who smoked were not included in our study. Patients who already had WSLs, hypoplastic or fluorotic enamel before the start of orthodontic treatment were also not included. Patients had hygiene appointments within three months prior to bracket placement.

2.6.3 White spot lesion detection

WSLs were assessed according to the modified WSL index by Gorelick *et al*. (1982), based on evaluating the buccal surface of individual teeth for their presence or absence [23]. The severity of each WSL was scored as follows (Figure 25): 1. no white spot formation; 2. slight white spot formation (thin rim); 3. excessive white spot formation (thicker bands); 4. white spot formation with cavitation. The scoring was performed under direct illumination using a dental chair light after drying the teeth with compressed air for 5 seconds. Assessments for WSLs by direct clinical examination and photographs occurred at 4 time points: baseline, 3 months, 6 months and 12 months. All buccal surfaces were visualized

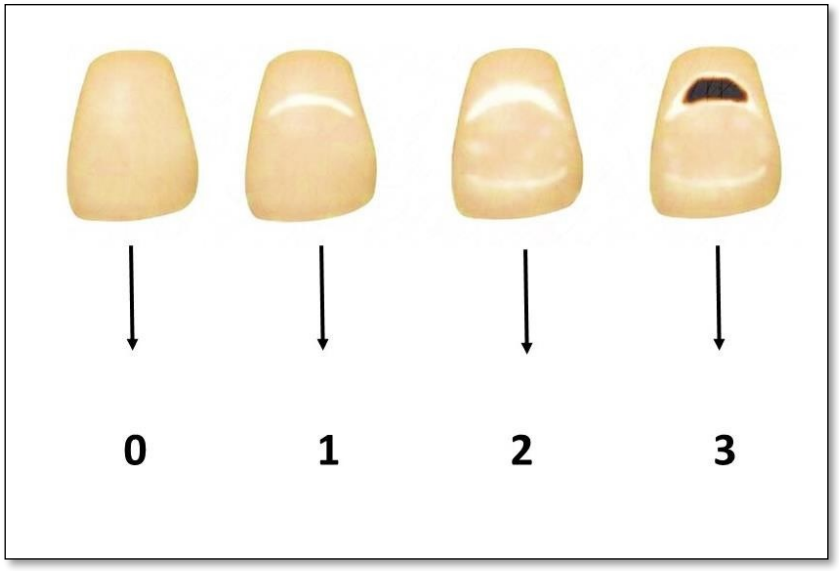

Figure 25 – White spot lesion scoring system. 0: no visible white spot or surface disruption (no demineralization); 1: visible WSL that covers less than one-third of the surface, without surface disruption (mild demineralization); 2: visible WSL that covers more than one-third of the surface, with a roughened surface but not requiring restoration (moderate demineralization); and 3: visible cavitation, requiring restoration (severe demineralization).

2.6.4 Saliva collection and Stephan curve

Stephan curve kinetics were only determined before the placement of fixed appliances. Patients were asked to refrain from eating, drinking or chewing gum for 30 minutes. Patients rinsed with water for 30 seconds and an initial ~1 ml sample of saliva was obtained for pH analysis and ~2 ml for microbiome analysis. Next, patients rinsed with a 10% sucrose solution for 30 seconds and saliva samples (~0.5 ml) were collected after 5, 15, 30 and 45 minutes. pH was determined using a microelectrode (Cole Parmer pH meter PH6+, Quebec, QC).

2.6.5 DNA isolation

Immediately after obtaining saliva samples the steps recommended by the kit manufacturer used were followed to preserve and isolate the DNA (Microbiome DNA Isolation Kit, Norgen Biotek Corp, Thorold, ON). Briefly samples were heated at 55ºC for one hour, then mixed with lysis buffer and lysis additive, followed by incubation at 65ºC for 5 minutes. The mixture was vortexed and centrifuged at 20,000 × g. The supernatant was then transferred to a new tube and Binding Buffer I was added, mixed and incubated on ice for 10 minutes. The lysate was centrifuged for 2 minutes at 20,000 × g. The supernatant was carefully transferred to a collection tube and an equal volume of 70% ethanol was added and vortexed. Then the spin column was assembled with one of the collection tubes and the clarified lysate containing ethanol was added onto the column and centrifuged for 1 minute at 10,000 × g. The column was washed twice with wash solution and centrifuged for 1 minute at 10,000 × g. Finally, the column was placed into a new elution tube, elution buffer was added and the column centrifuged at 200 × g, followed by 1 minute at 20,000 × g. The purified genomic DNA was stored at -20°C.

Genomic DNA concentration was determined by fluorometric analysis using the Qubit® 4 Fluorometer system (Invitrogen by Thermo Fisher Scientific - Q33238) in conjunction with the Qubit™ 1x dsDNA High-Sensitivity Assay Kit (Invitrogen by Thermo Fisher Scientific – Q33231) generously shared with our laboratory by Dr. Khaled Altabtbaei. Briefly, standards were diluted as follows: 10µL of the standard were added to 190µL of Qubit working solution containing a fluorescent nucleic acid binding reagent selective for dsDNA (Qubit working solution) in Axygen PCR-05-C tubes (VWR – 10011-830). After the standards were assessed, 1µL of sample was added to 199µL of the Qubit working solution. Samples were vortexed and incubated at room temperature for 3 minutes prior to reading. All Qubit®-based nucleic acid quantifications were performed utilizing Qubit® 4 Fluorometer system. The minimum DNA concentration required by Génome Québec for sequencing was 10ng in a volume of at least 10uL. All collected saliva samples met the minimum criteria as shown in Table 7.

Table 7 – Average readings from Qubit.

Sample	Read 1 (ng/uL)	Read 2 (ng/uL)	Average (ng/uL)
1	13.5	13.9	13.7
2	20.2	20	20.1
3	8.59	8.61	8.6
4	10.1	9.99	10.045
5	48.7	48.3	48.5
6	1.55	1.53	1.54
7	4.1	4.08	4.09
8	31.1	30.9	31
9	7.27	7.16	7.215
10	14.6	14.4	14.5
11	7.39	7.33	7.36
12	44.5	44.3	44.4
13	12.9	12.8	12.85
14	4.65	4.62	4.635
15	1.45	1.44	1.445
16	43.5	43.3	43.4
17	41.2	40.9	41.05
18	17.9	17.8	17.85
19	14.1	14	14.05
20	8.82	8.75	8.785
21	7.26	7.23	7.245

The next step was to assess for DNA quality, purity and integrity, as required by Génome Québec. Based on the results from the Quibit analysis, approximately 200ng of DNA were added to sterile water, and DNA agarose gel loading dye (ThermoFisher Scientific – R0611). The DNA was electrophoresed through a 0.8% agarose gel containing a final concentration of 0.85µM ethidium bromide (ThermoFisher Scientific – BP1302 – 10). The running buffer was TBE and the DNA was electrophoresed at constant amperage (15 mA) for 25 minutes. Images of the gel were captured utilizing the ChemiDoc™ MP Imaging System (model XRS+, Bio-Rad) and Image Lab

5.0 software (ChemiDoc MP Imaging System, version 4.1, Bio-Rad). DNA was deemed of good quality for sequencing if free of protein and RNA contamination (no high or low molecular weight bands), and if there was no visible smearing (indicative of degradation). All saliva samples were of good quality.

2.6.6 16s RNA Sequencing

Samples were placed in a labeled and sterile 96-well plate (Eppendorf twin.tec, Full Skirt, 951020401) and covered with a clear adhesive film (Microseal 'B' seal, Biorad, MSB1001) and shipped to Génome Québec on dry ice.

The Ilumina MiSeq PE 300bp sequencing platform was used, and as shown in Figure 26, all samples reached adequate coverage. Two sets of primers were used, as one or the other may, in some cases, more effectively identify genera. This allowed for the retrieval of a broader microbiome spectrum than what would be achievable with one primer set. To avoid overestimation, primer averaging was done on operational taxonomic units (OTU) [24]. Adaptors were removed using CutAdapt [25]. Parameters for trimming and overlap needed for merging were determined with Figaro [26]. Merged sequences were algorithmically corrected to produce Amplicon Sequencing Variants (ASVs) using opensource software DADA2 [27]. DADA2 was used to bin the nucleotide-corrected ASVs to their identifying taxa using the naive Bayesian classifier against a SILVA rRNA database (v138.1, provided by DADA developer here: https://zenodo.org/record/4587955#.Ykc0By971jc) [27].

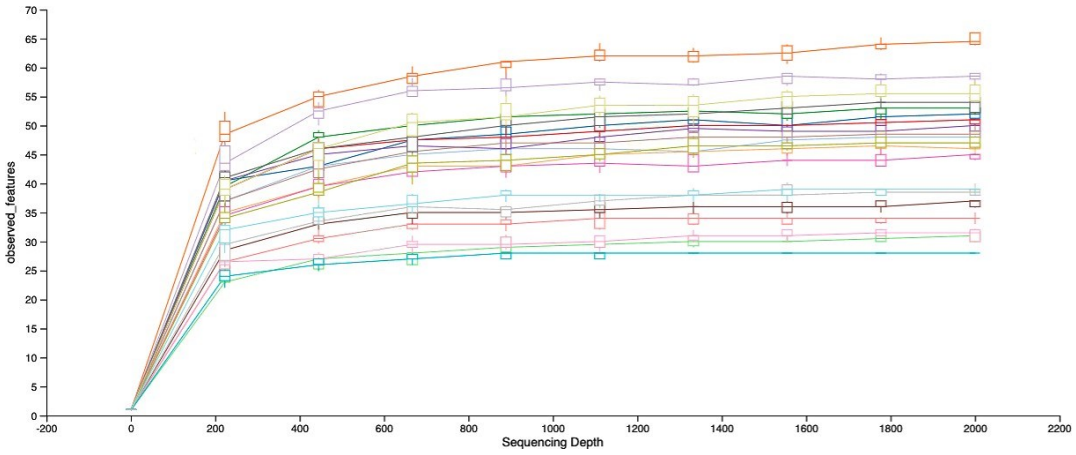

Figure 26 - Alpha rarefaction curves (observed features) demonstrating that enough coverage has been achieved.

Statistical analysis

Data were entered into Excel (version 2208, Microsoft), and statistical calculations were done in IBM Statistical Package for Social Sciences (SPSS, version 28). For Stephan curve analysis, the mean and standard error of the mean (mean ± SEM) of pH were calculated for each assessment time and were stratified between the case and control groups. Three variables were included (a) time, (b) pH at baseline (c) number of teeth with WSL. Data normality according to the occurrence of WSLs was verified by the Kolmogorov-Smirnov test. Two-way repeated-measures ANOVA was performed for the occurrence of WSLs and evaluation time, with Bonferroni correction for multiple comparisons. For microbiome analysis, alpha and beta-diversity were interrogated using an automated pipeline (Forays into Automating Laborious Analysis of Phylogeny (FALAPhyl): https://github.com /khalidtab/FALAPhyl). Feature-abundance testing was examined via Linear discriminant analysis (LDA) Effect Size (LefSe) [28]. Since it was unknown if the effect of pH on bacteria would follow a linear or unimodal function, Spearman

correlation and canonical correspondence analysis (using Vegan in RStudio) were performed to interrogate the two relationships, respectively [29, 30].

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APPENDIX A – INFORMED CONSENT

Faculty of Medicine and Dentistry

Division of Periodontology

PARTICIPANT CONSENT FORM

Title of Study: The role of a Loricrin in aggressive periodontal disease **Principal**

Investigator: Dr. Liran Levin (780-407-5562) **Research/Study Coordinator:** Dr. Raisa

Catunda

Why am I being asked to take part in this research study?

You are being asked to be in this study because we are trying to learn about one of the important causes of aggressive periodontal (gum) disease. During your procedure, gum tissue is normally discarded. Instead, we would like to collect this tissue that is normally disposed of for our study. The study will not change anything that the Dentist/Hygienist would do normally, it would only involve saving the tissue that is normally discarded for our study.

We would also like to collect a very small amount of the liquid that surrounds your teeth.

Before you make a decision one of the researchers will go over this form with you. You are encouraged to ask questions if you feel anything needs to be made clearer. You will be given a copy of this form for your records.

What is the reason for doing the study?

We are studying Aggressive Periodontal Disease or AP. AP is a very bad form of gum disease that occurs in young people in their 20s and 30s. It is so bad that they can lose all of their teeth in a very short time. You can imagine that it is very traumatic for the young person. Not a lot is known about why AP occurs. We think it may be caused by a decrease in a component of the gums, called loricrin. We would like to use the tissue that is normally thrown away during your procedure to measure the amount of loricrin in people with or without AP.

Our gums are composed of many cells that form a tight seal against the millions of bacteria found in our mouth. The tight seal is created by a protein

called loricrin holding the cells together. Some bacteria that are found in the mouths of people with AP can trick the gum cells into producing less loricrin. We think this results in a weakening of the tight seal, allowing bacteria to invade. The body then tries to kill the bacteria, but in the process, can also destroy the bone that holds teeth in place. The body's response to bacteria is called "inflammation". It is similar to what happens when you have a cut and it gets infected: it becomes red and swollen with fluid. The fluid around your gums contains elements of inflammation that we can measure. We think those elements will be increased in the gum fluid of people with less loricrin because they will be fighting the invading bacteria.

What will happen in the study?

For participants who have a healthy mouth and are undergoing a crown lengthening procedure, we ask that you allow us to save the tissue that is normally discarded as a result of the procedure. Collection of this tissue will not change the procedure you undergo in any way.

We then ask that you also allow us to collect a small amount of the gum fluid around your teeth. To do this, we will place the tip of a small square of paper against your gums to absorb the fluid. We will do this at several places in your mouth.

You will not feel any more discomfort than what occurs during the normal crown lengthening procedure, and the collection of the fluid will take less than 2 minutes.

For participants with Aggressive Periodontal Disease, when you come to the clinic for periodontal surgery or scaling and root planing ("deep cleaning") as part of your normal treatment plan, we already occasionally remove tissue that has inflammation. Normally, this tissue is discarded.
We ask that instead, you allow us to save this tissue for our study. Collection of this tissue will not change the procedure you undergo in any way.

For the collection of fluid, we will place the tip of a small square of paper against your gums to absorb the fluid. We will do this at several places in your mouth.

You will not feel any more discomfort than what normally occurs during your regular treatment, and the collection of the fluid will take less than 2 minutes.

So basically, you will have the same exact procedure that you would have had, but instead of throwing away your tissue, we will collect it for our study. We will also collect a small amount of fluid from around your teeth.

What are the risks and discomforts?
Since you are undergoing the procedure anyway, there are no changes in risks or discomfort as a result of collecting the tissue compared with throwing it away. Placing the paper against your gums to absorb the liquid causes no discomfort and has no risks.

What are the benefits to me?
There are no specific benefits to you. However, by participating, you are contributing to our knowledge of how AP causes such bad gum disease and tooth loss, and you may consider that this may help us treat AP patients better in the future.

What will I need to do while I am in the study?
Basically, there is no change in what you need to do. You will undergo the same exact procedure, except we will save instead of discard your tissue. It will take us less than 2 minutes to collect the gum fluid.

Do I have to take part in the study?
Participation in this study is entirely voluntary. If you decide to be in the study, you can change your mind and stop being in the study at any time during the procedure, and it will in no way affect the care or treatment that you are entitled to.

Are there other choices to being in this research study?
If you would like to participate, we will need to save the gum tissue instead of throwing it away as well as collect the fluid sample from your gum.
If you choose not to participate, we will just discard the tissue as done usually.

What will it cost me to participate?
There is no additional cost to participate in our study.

Will my information be kept private?
During the study we will be collecting health data about you. We will do everything we can to make sure that this data is kept private. No data relating to this study that includes your name will be released outside of the study doctor's office or published by the researchers. When we collect your tissue and fluid, we will give it a random number. This random number will not be in your file, so no one will know that the sample is yours. We will also not document in your file that you were a participant in the study. The only

other information that the lab researchers will have is your age (not your date of birth) and your gender. The researchers will also be told whether you are an AP patient or a healthy patient.

By signing this consent form, you are giving permission for the study doctor/staff to collect, use and disclose the information about you from your personal health records as described above (your age and your gender, and whether you are an AP patient or a healthy patient). After the study is done, we will still need to securely store your health data that was collected as part of the study. At the University of Alberta, we keep data stored for 5 years after the end of the study.
If you decide not to participate in the study, we will not collect your health information or the samples.

What if I have questions?
If you have any questions about the research now or later, please contact Raisa Catunda at our office (780-407-5562).
If you have any questions regarding your rights as a research participant, you may contact the Health Research Ethics Board at 780-492-2615. This office is independent of the study investigators.
This study is being conducted/sponsored by the University Hospital Foundation. The Institution and study doctor are getting money from the study sponsor to cover the costs of doing this study. You are entitled to request any details concerning this compensation from the Principal Investigator (Dr. Levin).

CONSENT

Title of Study: The role of a Loricrin in aggressive periodontal disease

Principal Investigator(s): Dr. Liran Levin **Phone Number(s):** 780-407-5562
Study Coordinator: Dr. Raisa Catunda **Phone Number(s):** 780-407-5562

Yes No Do you understand that you have been asked to be in a research study? ☐ ☐ Have you read and received a copy of the attached Information Sheet? ☐ ☐ Do you understand the benefits and risks involved in taking part in this research study? ☐ ☐ Have you had an opportunity to ask questions and discuss this study? ☐ ☐

Do you understand that you are free to leave the study at any time, ☐ ☐ without having to give a reason and without affecting your future dental care?

Has the issue of confidentiality been explained to you? ☐ ☐

Do you understand who will have access to your records, including ☐ ☐ personally identifiable health information?

Do you want the investigator(s) to inform your dentistthat you are ☐ ☐ participating in this research study? If so, give his/her name _____

Who explained this study to you?

_____

I agree to take part in this study: Signature of Research Participant

_____(Printed Name)

_____

Date:_____

Signature of Witness

_____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee_____Date

_____

THE INFORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A SIGNED COPY GIVEN TO THE RESEARCH PARTICIPANT

APPENDIX B – INFORMED CONSENT

PARTICIPANT CONSENT FORM

Title of Study: Oral microbiome and Stephan curve kinetics in white spot lesion development among orthodontic patients wearing fixed appliances

Principal Investigator: Carlos Flores-Mir (780.492.7409)

Research/Study Coordinator: Raisa Catunda (780.407.5562)

Why am I being asked to take part in this research study?
White spot lesions are early signs of tooth decay and a frequent occurrence during orthodontic treatment. It is the first time a study of this kind is being performed in participants wearing orthodontic appliances. You are being asked to be in this study because we are trying to learn about one of the important causes of tooth decay (cavities).

Before you make a decision one of the researchers will go over this form with you. You are encouraged to ask questions if you feel anything needs to be made clearer. You will be given a copy of this form for your records.

What is the reason for doing the study?

This project examines saliva, as well as aspects of the oral microbes with the objective of identifying participants that are more susceptible to white spot lesions to allow for early intervention and for the development of preventative strategies. There will be no changes to your orthodontic treatment if you decide to participate in this study.

What will happen in the study?

During your orthodontic treatment, pictures are taken to follow-up the progress of the treatment, we will be using these pictures to assess any signs of early decay development. You will also be asked to provide some saliva prior to appliance placement in order for us to investigate acidity in your saliva and microbes that can be important in the cavity development.

- The very first visit we will ask you to arrive 1 hour prior to your orthodontic appointment in order to do the salivary and plaque collection. Since the salivary collection occurs in a sequential manner, we would be collecting saliva every 10 minutes (collection itself should not take more than 30 seconds);

- Your orthodontic treatment will not change if you accept to participate in this research project;
- You will be asked to rinse your mouth with water for about 30 seconds and open your mouth for us to collect a small amount of the plaque around your teeth. To do this, we will rub a small brush against your teeth. We will do this at several places in your mouth. This will be done only at your first visit before appliance placement.
- After plaque collection, you will be asked to spit in a tube for us to collect the baseline saliva sample. We will ask you to rinse your mouth with a 10% sucrose solution for 30 seconds and then we will ask you to spit in the tube again after 5, 10, 15, 30 and 45 minutes. This will be done only at your first visit before appliance placement.

What are the risks and discomforts?

There are no risks in participating in this study. The only discomfort that could arise is that you will have to remain with your mouth opened for plaque collection.

What are the benefits to me?

You are not expected to get any benefit from being in this research study. This study may help identify participants who are more susceptible to cavity development during orthodontic treatment. This would allow clinicians to create new preventive measures in order to decrease prevalence of cavities during orthodontic treatment.

What will I be asked to do while I am in the study?

There is no change in what you need to do. Regardless of participation in our study, all participants will receive thorough oral hygiene instructions, including the advice to brush and floss teeth at least three times a day with typical, commercially available fluoride toothpaste. All participants will also be advised to reduce their consumption of carbonated soft drinks, acidic juices, sugar-containing candies, and snacks during orthodontic treatment at every visit.

Do I have to take part in the study?

Being in this study is your choice. If you decide to be in the study, you can change your mind and stop being in the study at any time, and it will in no way affect the care or treatment that you are entitled to.

Are there other choices to being in this research study?

You do not have to join the study in order to receive treatment. The orthodontic resident will explain all treatment options available to you.

What will it cost me to participate?

There is no additional cost to participate in our study.

Will I be paid to be in the research?

A one-time parking payment cost of $20.00 will be provided after the first data collection stage is completed (saliva and plaque collection) as an additional time over the regularly scheduled appointment is needed. No payments will be provided for the other appointments as they will be part of the regularly scheduled orthodontic appointments.

Privacy and Confidentiality

During the study we will be collecting health data about you. We will use the data to help answer research questions and we will disclose your information with others such as other researchers involved.

Below we describe in more detail how your data will be collected, stored, used and disclosed.

What data will we be collecting?

During this study we will be collecting data about you. Examples of the types of data we may collect your age, your health conditions, your health history, your medications and digital intra-oral images. We will only look for and collect the information that we need do the research. We will get this information by asking you questions and collecting the samples outlined in this form. We will also look at your medical chart (paper or electronic) held by the study doctor or other doctors you have seen (such as your family doctor).

How will the study data be stored?

The study data we collect which will include your name will be securely stored by the study doctor during and after the study. We will also put a copy of this consent form in your clinical record, so that doctors you see in the future will know you were in the study. In Canada, the law says we have to keep the study data stored for at least 25 years after the end of the study. The study doctor will not release your name to anyone unless the law says that they have to.

How will the study data be used?

We will do everything we can to make sure that this data is kept private. No data relating to this study that includes your name will be released outside of the study doctor’s office or published by the researchers. When we collect your plaque and saliva, we will give it

a random number. This random number will not be in your file, so no one will know that the sample is yours. This coded study data will be kept by the researchers in a secure manner and will be used now and in the future to

- learn more about how white spot lesions develop during orthodontic treatment
- learn more about how to treat white spot lesions
- identify susceptible individuals that wear orthodontic appliances at an early stage of white spot lesions.

This coded study data may also be shared with people who work with the researcher and with regulatory authorities. The researcher and/or the people they work with may be located outside of Canada, in countries that do not have the same privacy laws as in Canada. However, because nothing will contain your name, no one who uses this information in the future will be able to know it came from you. The risk to your privacy, then, should be very small.

When the study is done, the study coordinator may place your coded study data into a secure database. The coded data may then be used to answer other research questions in the future. Only researchers who have the training and experience to do the research (also known as “qualified researchers”) will be allowed to use the data. The data will be de-identified and kept at the University of Alberta for at least 25 years.

Who will be able to look at my health data?

During research studies it is important that the data we get is accurate. Therefore, your study data and original medical records may also be looked at by people from: the study sponsor, the University of Alberta auditors and members of the Research Ethics Board and/or other foreign regulatory authorities.

By signing this consent form you are saying it is ok for the study doctor/staff to collect, use and disclose information from your medical records and your study data as described above.

If you would like to see the study data collected about you, please ask the study doctor. You will be able to look at the study data about you and you can ask for any mistakes to be corrected. The study doctor may not be able to show you your study data right away and you may have to wait until the study is completed or another time in the future before you can see your study data.

If you leave the study, we will not collect new health information about you, but we will need to keep the data that we have already collected.

What if I have questions?

If you have any questions about the research now or later, please contact Raisa Catunda at our office (780-407-5562).

If you have any questions regarding your rights as a research participant, you may contact the Health Research Ethics Board at 780-492-2615. This office is independent of the study investigators.
This study is being conducted/sponsored by the American Association of Orthodontists Foundation. The Institution and study doctor are getting money from the study sponsor to cover the costs of doing this study. You are entitled to request any details concerning this compensation from the Principal Investigator (Dr. Carlos Flores-Mir).

CONSENT

Title of Study: Oral microbiome and Stephan curve kinetics in white spot lesion development among orthodontic patients wearing fixed appliances

Principal Investigator(s): Carlos Flores-Mir **Phone Number(s):** 780.492.7409
Study Coordinator: Raisa Catunda **Phone Number(s):** 780.407.5562

	Yes	No
Do you understand that you have been asked to be in a research study?	☐	☐
Have you read and received a copy of the attached Information Sheet?	☐	☐
Do you understand the benefits and risks involved in taking part in this research study? ☐ ☐		
Have you had an opportunity to ask questions and discuss this study?	☐	☐
Do you understand that you are free to leave the study at any time, without having to give a reason and without affecting your future medical care? Has	☐	☐
the issue of confidentiality been explained to you?	☐	☐
Do you understand who will have access to your records, including personally identifiable health information?	☐	☐
Do you want the investigator(s) to inform your family doctor that you are participating in this research study? If so, give his/her name _____	☐	☐

Who explained this study to you? _____

I agree to take part in this study:

Signature of Research Participant _____

(Printed Name)

_____
(Signature)

Date:_____

Signature of Witness _

_____

I believe that the person signing this form understands what is involved in the study and voluntarily agrees to participate.

Signature of Investigator or Designee_____Date _____

ORMATION SHEET MUST BE ATTACHED TO THIS CONSENT FORM AND A SIGNED COPY GIVEN TO THE RESEARCH PARTICIPANT

CHAPTER 3
METHODOLOGY

A TWO-PLANE MICROCT ALVEOLAR BONE MEASUREMENT APPROACH IN MICE

A 2-plane micro-computed tomographic alveolar bone measurement approach in mice

Raisa Queiroz Catunda[1], Karen Ka-Yan Ho[1], Srushti Patel[1], Maria Febbraio[1,*]

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ABSTRACT

Purpose: This study introduces a standardized 2-plane approach using 8 landmarks to assess alveolar bone levels in mice using micro-computed tomography.

Materials and Methods: Bone level differences were described as distance from the cemento-enamel junction (CEJ) to alveolar bone crest (ABC) and as percentages of vertical bone height and vertical bone loss, comparing mice infected with *Porphyromonas gingivalis* (Pg) to controls. Eight measurements were obtained per tooth: 2 in the sagittal plane (mesial and distal) and 6 in the coronal plane (mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual).

Results: Significant differences in the CEJ-to-ABC distance between Pg-infected mice and controls were found in the coronal plane (middle-lingual, mesiobuccal, and distolingual for the first molar; and mesiobuccal, middle-buccal, and distolingual for the second molar). In the sagittal plane, the distal measurement of the second molar was different. The middle-buccal, mesiobuccal, and distolingual sites of the first and second molars showed vertical bone loss relative to controls; the second molar middle-lingual site was also different. In the sagittal plane, the mesial sites of the first and second molars and the distal site of the second molar showed loss. Significantly different vertical bone height percentages were found for the mesial and distal sites of the second molar (sagittal plane) and the middle-lingual and distolingual sites of the first molar (coronal plane).

Conclusion: A reliable, standardized technique for linear periodontal assessments in mice is described. Alveolar bone loss occurred mostly on the lingual surface of the coronal plane, which is often omitted in studies. ***(Imaging Sci Dent 2021; 51: 389-98)***

KEY WORDS: X-Ray Microtomography; Alveolar Bone Loss; Periodontitis; Porphyromonas Gingivalis

Introduction

Periodontitis is a multifactorial inflammatory disease that can lead to an irreversible destruction of the soft and hard tissue structures in the oral cavity, ending in tooth loss. It occurs as a result of complex interactions between the dental plaque biofilm, which contains bacteria, and a susceptible host's efforts to fight the infection, with contributions from environmental and epigenetic factors.[1] Although the subgingival environment is characterized by a large variety of bacteria, with more than 300 species identified from different individuals and ~40 at a single site, only a few species are associated with periodontitis, and they are mainly Gram-negative and anaerobic. One key pathogenic bacterium in periodontitis is *Porphyromonas gingivalis* (Pg).[2]

Studies of the etiology and progression of periodontal disease, as well as pharmacological and surgical interventions, have been widely explored in animal models.[3-8] There remain, however, unanswered questions, given the ethical barriers associated with non-intervention in humans. The benefit of animal models is that they enable a longitudinal assessment of the disease, with similar onset and progression characteristics in several animals, in addition to allowing analysis of the cellular and molecular

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composition of the tissues involved.[4,9,10] The selection of an experimental model is determined by the objective of the scientific research. Mice are a good model for studying periodontitis because they show susceptibility to experimental induction of periodontal disease, are relatively low-cost and easy to handle, have been extensively characterized in terms of their inflammatory and immunological processes, and can be readily manipulated genetically.[11]

Clinical attachment level and, consequently, alveolar bone level, are factors accounted for in the most recent classification of periodontal diseases in humans.[1] With the help of a periodontal probe, the measurements are assessed at 6 standardized sites per tooth: mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual. In mice, the alveolar bone level is a tangible 2-dimensional (2D) measurement that has been used over the years to assess periodontal disease, various treatment modalities, and pathology progression.[3,7,12,13] However, there is a clear lack of standardization of linear measurements in mice, even after the introduction of micro-computed tomography (micro-CT). Regardless of the technology used for assessment, in some studies, sites are randomly assigned, while in others, the sites are established, but standardization of the measurement remains unclear. The most commonly used landmarks comprise the interproximal region (mesial and distal), either within the sagittal plane or in the coronal plane.[3,7,12,13]

There are already multiple variables that can make it difficult to compare data collected from animals from study to study, such as strain, method of periodontal disease induction, age, environmental factors, and diet.[9,10,14] Therefore, a standardized method for alveolar bone level assessment is essential for reproducibility and in order to compare results among studies. The goal of this paper is to introduce a standardized 2-plane approach with 8 linear landmarks for the assessment of alveolar bone levels in mice utilizing micro-CT. Furthermore, the utility of this method is demonstrated in a study of mice orally infected with Pg.

Materials and Methods

Animals

In this study, 10- to 12-week-old C57Bl/6j male mice were used. The animals were maintained and bred in a specific pathogen-free facility at the University of Alberta. The mice received water and were fed a standard rodent chow diet (4% total fat) *ad libitum*. They were kept in the same environmental conditions concerning temperature, humidity, and light/dark cycles. Ethics approval from the University of Alberta Animal Care and Use Committee was granted for this research project prior to study commencement (AUP 2935).

Bacteria

Pg bacteria (ATCC strain 33277) were grown for 72 hours on a blood agar plate under anaerobic conditions and then inoculated into Schaedler broth containing vitamin K and hemin for 24-48 hours, until the optical density of the culture reached 1.3 at 660 nm (approximately 10^9 colony-forming units (CFU)/mL). Cultured Pg were resuspended in saline containing 2% carboxymethyl cellulose (as a thickener to promote adherence) before oral inoculation of the mice.

Experimental design

A periodontal disease induction protocol was used as an experimental model to determine whether this standardized measurement method would adequately and reliably discriminate bone loss. In order to determine the sample size, a power calculation was performed. A 3% difference in bone loss relative to control and a 15-20% difference between groups was considered to be statistically and clinically significant, as per the current literature.[7,12,15] This yielded 20 teeth/group (left and right first molars and left and right second molars (=4 teeth × 5 animals/group). Since significant variability in measurements of the third molar was noted in previous studies (unpublished data), only the first and second molars were included in the present study.

Pg infection

Ten mice were divided into 2 groups, Pg-infected and control. Infection was carried out by oral lavage with 200 µL of carboxymethyl cellulose containing ~10^{10} CFU/mL Pg, using a micro-brush to apply the suspension at the gingival margin throughout the mouth on alternate days for 2 weeks (7 applications total). The mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylene (intraperitoneal injection) for the inoculation. This procedure, a modification of the classical technique of Lalla et al.,[16] was performed in a level 2 biocontainment facility. Two weeks after the last inoculation, the mice were euthanized by pentobarbital overdose (200 mg/kg, intraperitoneal injection) followed by bilateral thoracotomy, according to Canadian Council on Animal Care guidelines.[17]

The heads were collected and fixed in 4% formaldehyde for 48 hours.

Histological assessment of inflammation

The micro-CT findings were compared to the histological assessment of inflammation for validation. Following micro-CT scanning, the jaws were decalcified, embedded in paraffin, and sectioned (7-µm thickness). After de-paraffinization, the slides were stained with hematoxylin and eosin and with a pan-leukocyte marker (CD45) to identify inflammatory cells (catalog #53665, Santa Cruz Biotechnology Inc., Dallas, TX, USA), according to the supplier's instructions. CD45 positive cell counts were performed by 3 independent examiners and the results were calculated as the ratio of the difference between the Pg-infected group divided by the control group (fold change formula). A total of 50 images (5 per animal) of the masticatory gingiva were analyzed.

Scanning of the mandibles

The mouse heads were 3-dimensonally (3D) micro-CT scanned at a 25-µm voxel size resolution using the following settings: 360°; exposure, 75 ms; voltage, 50 kV; current, 0.24 mA (MILabs U-CT, Utrecht, Netherlands). Each head was placed separately and scanned and saved as a Neuroimaging Informatics Technology Initiative (NIftI) file, a format commonly used in imaging informatics. In this format, the first 3 dimensions define the 3 dimensions in space (x, y, and z). The scanned image was then reconstructed using MILabs Software (MILabs, Utrecht, Netherlands) aligning the sagittal, coronal, and axial planes, using the nasal septum and the occlusal plane of the first molars as references. The reconstructed scan was saved as a "parcel" file (a tool that bundles all the parts of the files into a way they can be compacted and exported).

Quantification of alveolar bone level

Three-dimensional reconstructions were performed using Avizo software (version 2019.1, Berlin, Germany). To assess the alveolar bone levels of mice, a measurement protocol with 3 landmarks was defined: the cemento-enamel junction (CEJ), alveolar bone crest (ABC), and root apex. The distance from the CEJ to ABC was measured in millimeters (up to 2 decimal points). These measurements were completed for the first and second molars of the mandible on both sides, since mandibular molars are most often used for measurements in the literature and are birradicular (anatomical consistency).[3,13,18-20]

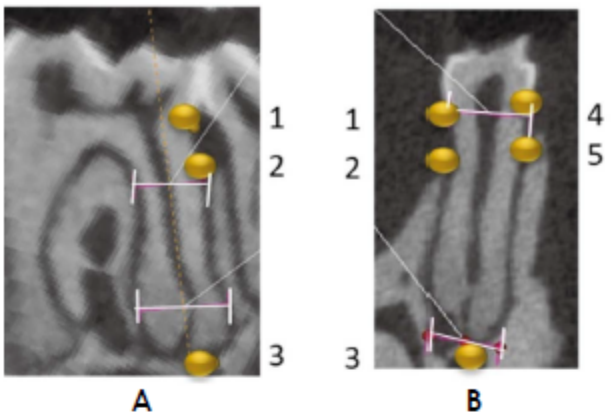

Fig. 1. A. Sagittal landmarks (dots). 1. cemento-enamel junction. 2. alveolar bone crest. 3. root apex. B. Landmarks of the coronal plane (the line defines the ruler used to define the middle of the tooth). 1. cemento-enamel junction (lingual). 2. alveolar bone crest (lingual). 3. root apex. 4. cemento-enamel junction (buccal). 2. alveolar bone crest (buccal).

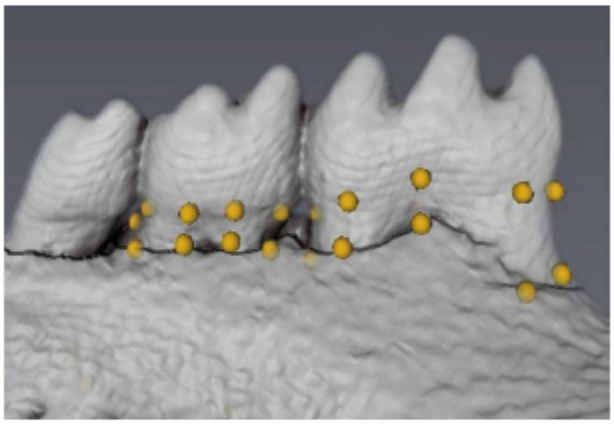

Fig. 2. Three-dimensional lingual view of the landmarks (dots) used for linear measurements.

Similar to human examinations, a total of 8 measurements were obtained: 2 in the sagittal plane (mesial and distal, Fig. 1A) and 6 in the coronal plane (mesiobuccal, middle-buccal, distobuccal, mesiolingual, middle-lingual, and distolingual, Fig. 1B), totaling 32 measurements per animal (first and second mandibular molars). Figure 2 shows a 3D view of measurements from the buccal perspective.

Landmark standardization according to planes

In order to achieve standardized sagittal measurements, the coronal plane of the first molar was initially oriented. Sagittal plane measurements were taken, using the soft-

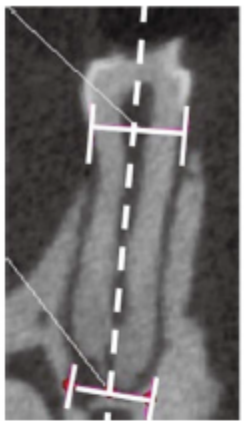

Fig. 3. Definition of the sagittal plane in the coronal plane (the same for both roots). In white: ruler tool; white line + dashed red line represents the middle point.

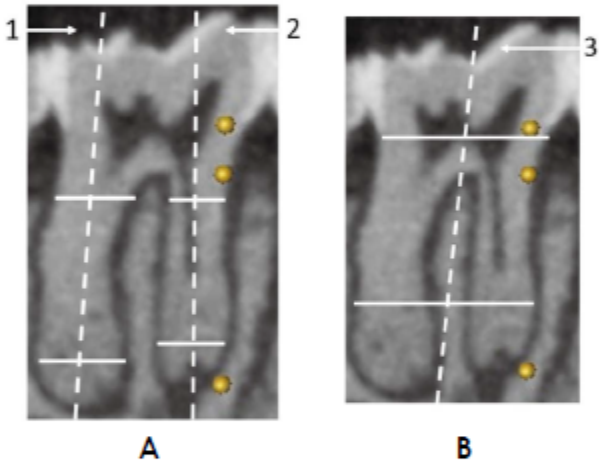

Fig. 4. A and B. Definition of the coronal plane in the sagittal plane for mesial, distal, and middle measurements. 1. Orientation plane for lingual and buccal coronal distal measurements. 2. Orientation plane for lingual and buccal coronal mesial measurements. 3. Orientation plane for lingual and buccal coronal middle measurements (dots demarcate landmarks; dashed lines represent the mid-points of the root/pulp chamber).

ware's measurement line and ruler tools to precisely ensure the center of the middle pulp chamber and root apex (Fig. 3). This can be achieved by drawing a line that passes through the middle of the CEJ, ABC, and root apex.

For coronal measurements, the sagittal plane was used for orientation of the buccal and lingual sides (Fig. 4). The coronal plane measurement line was precisely aimed at the middle of the floor of the pulp chamber in the designated root and the middle of the last third of the root. Again, the ruler tool with millimeter markings was used to ensure that the center of the root was established (the root was divided in thirds and the ruler was used from each root external wall–buccal and lingual–to establish the middle point).

To obtain the middle-buccal and middle-lingual measurements, the plane was defined as the center of the pulp chamber and the middle distance between the last third of the 2 roots (Fig. 4C). After the planes were established, measurements could then be performed.

Results were presented as the distance in 3 different ways: 1) millimeters from the CEJ to the ABC; 2) as a percentage of vertical bone height (utilizing the formula CEJ-ABC / total root length [TRL: distance from the root apex to the CEJ] × 100); in humans, vertical bone height is classified as the remaining vertical bone height and the vertical bone height that has been lost, whereas in mice, there is no standardization regarding what would be "clinically" significant; therefore, the calculation of how much vertical height was lost within the same animal was called "vertical bone height" to avoid any confusion with actual bone loss, which is described in #3 below; and 3) bone loss relative to the animals that were not exposed to Pg, calculated using the formula below:

$$\text{Vertical bone height } \% = \frac{(\text{CEJ to ABC})}{\text{TRL}} \times 100$$

Bone loss (%) = (Vertical bone height of PG-infected group) − (Vertical bone height of control group)

All alveolar bone measurements were performed by calibrated and blinded examiners. Using the Euclidean distance formula below (calculated from the Cartesian coordinates of the points using the Pythagorean theorem), data were collected for statistical interpretation.

$$d(p,q) = \sqrt{\sum_{i=1}^{n} (q_i - p_i)^2}$$

p, q = 2 points in Euclidean n-space, q_i, p_I = Euclidean vectors, n = n-space

Statistical analysis

Prism software was employed to perform statistical analyses (GraphPad, La Jolla, CA, USA). Data were analyzed for normal distribution using the Shapiro-Wilk test. If normally distributed, data were subjected to the parametric t-test; if not, the non-parametric Mann Whitney-U test was used. The mean and standard error of the mean (SEM) were calculated for both groups. Statistical signifi-

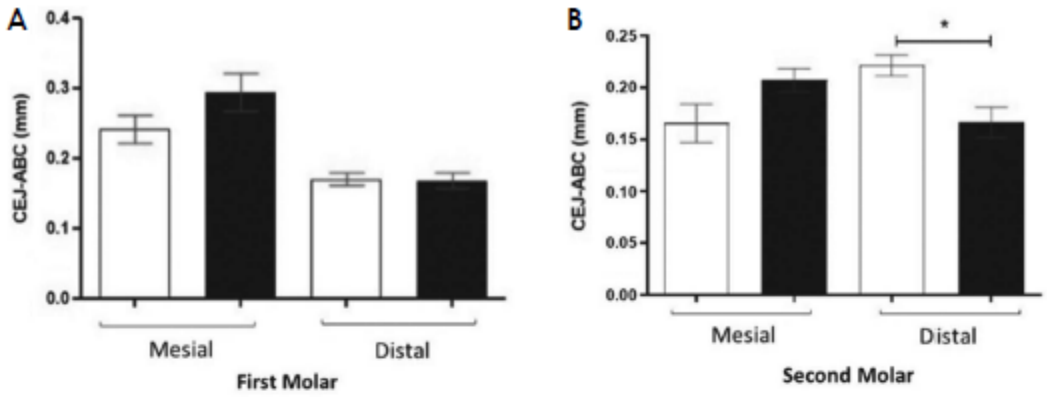

Fig. 5. Distance measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) in millimeters for each group (Pg-infected versus control) in the sagittal plane for mesial and distal sites. A. First molar. B. Second molar. *$P<0.05$, n = 10 first and 10 second molars/group. Pg: *Porphyromonas gingivalis*.

cance was set at $P<0.05$.

To calculate the error of the method, 3 blinded examiners were calibrated, and inter- and intra-examiner correlations were evaluated using the Cohen kappa for all measurements. Five animals were randomly selected, and a total of 160 measurements were compared (first and second molar, right and left side of the mandible; 4 teeth/mouse for a total of 20 teeth). The analysis was performed by each examiner with a 1-week interval between each measurement. The average error accepted was $\leq 3\%$ or 0.02 mm.

Results

The inter-examiner Cohen kappa scores for measurements were determined to be 0.80, 0.85, and 0.90 for each measurement cycle, and the intra-examiner Cohen kappa was 0.61, 0.70, and 0.80 for each examiner (all showing "substantial" to "strong" agreement).

The Pg-infected group presented some degree of alveolar bone loss in both planes, as expected. However, the differences in the percentage of vertical bone loss and the ABC-to-CEJ (mm) distance depended upon which planes were analyzed.

In the sagittal plane (Fig. 5), only 1 site (the distal site of the second molar) showed a statistically significant difference in the CEJ-to-ABC distance ($P<0.05$) (Fig. 5B). In the control group, the distance varied from 0.16 mm to 0.24 mm; in the Pg-infected group, the distance varied from 0.16 mm to 0.29 mm. The average ± SEM values for the control group versus the Pg-infected group per site were as follows: mesial first molar: 0.24 ± 0.02 mm versus 0.29 ± 0.02 mm; distal first molar: 0.16 ± 0.008 mm versus 0.16 ± 0.01 mm; mesial second molar: 0.16 ± 0.01 mm versus 0.20 ± 0.01 mm; distal second molar: 0.16 ± 0.01 mm versus 0.22 ± 0.01 mm.

The average values of the combined coronal measurements of the CEJ-to-ABC distance (control versus Pg-infected group) were as follows: 0.15 ± 0.01 mm versus 0.19 ± 0.01 mm for the first molar ($P<0.05$) and 0.12 ± 0.006 mm versus 0.15 ± 0.005 mm for the second molar ($P<0.05$) (Figs. 6A and B). When analyzing all CEJ-to-ABC distance measurements in the coronal plane separately, the distolingual and middle-lingual measurements of the first molar and the distolingual measurement of the second molar showed statistically significant differences compared to the control group ($P<0.05$). On the buccal surface, the mesial measurement of the first molar and second molar, as well as the middle-buccal measurement for the second molar showed differences that were statistically significant ($P<0.05$) (Figs. 6C-F).

In terms of vertical bone loss, of the 6 locations in the coronal plane for each molar, 4 sites showed loss relative to control on the buccal side: the middle and mesial sites of the first molar (1.71% and 1.14%, respectively) and the middle and mesial sites of the second molar (1.05% and 3.32%). On the lingual side, 3 locations showed loss: the distal site of the first molar (2.08%) and the middle and distal sites of the second molar (4.43% and 1.15%) (Figs. 7A and B). In the sagittal plane, the sites that showed loss were the mesial sites of the first molar and second molar (3.11% and 4.82%, respectively) and the distal site of the second molar (5.18%) (Fig. 7C).

In terms of percentage of vertical bone height, for the sagittal plane, the control group showed variation from 12.50% to 16.19%, whereas the Pg-infected group ranged

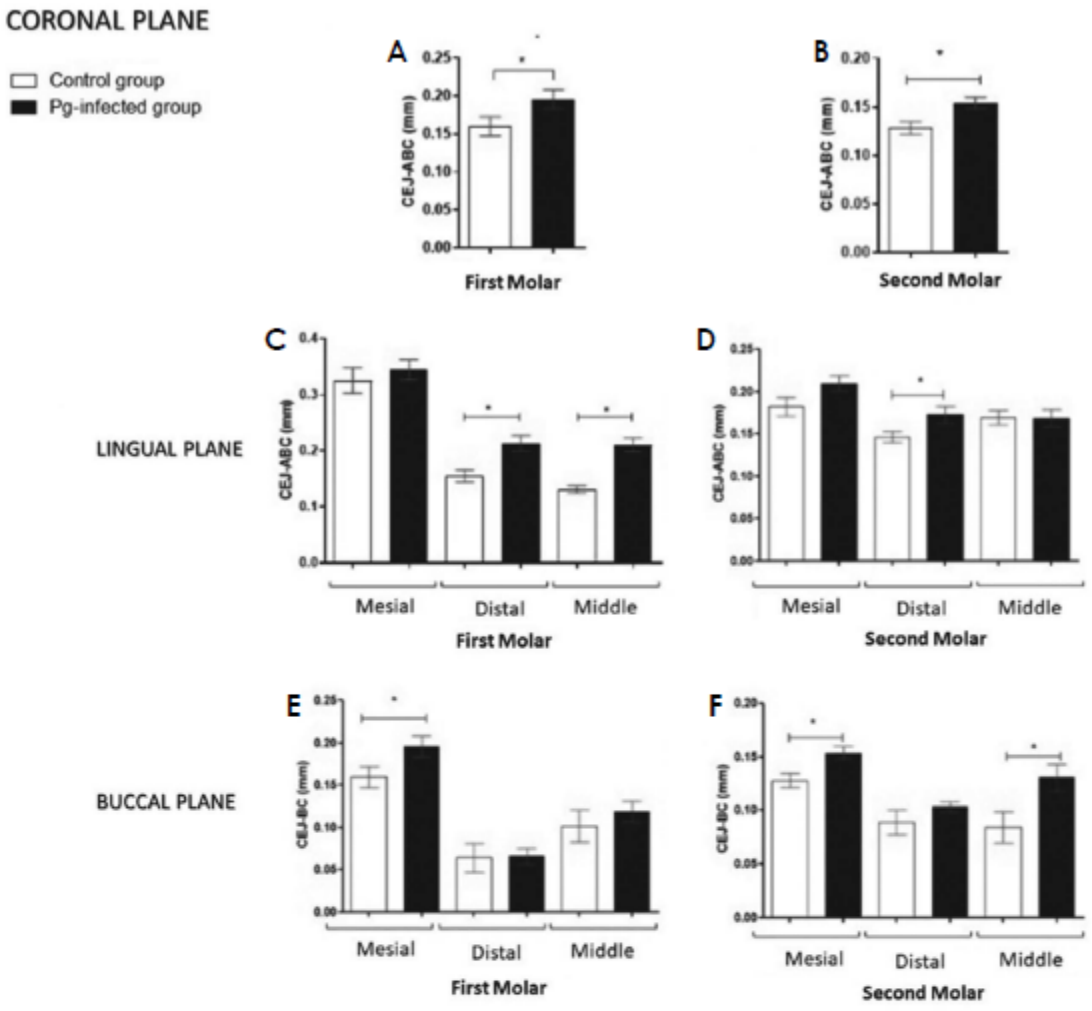

Fig. 6. Distance measured from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) in millimeters for each group (Pg-infected versus control) combined in the coronal plane. A. First molar. B. Second molar; Individual measurements of the lingual surface. C. First molar. D. Second molar; Individual measurements of the buccal surface. E. First molar. F. Second molar. $*P<0.05$, n = 10 first and 10 second molars/group. Pg: *Porphyromonas gingivalis*.

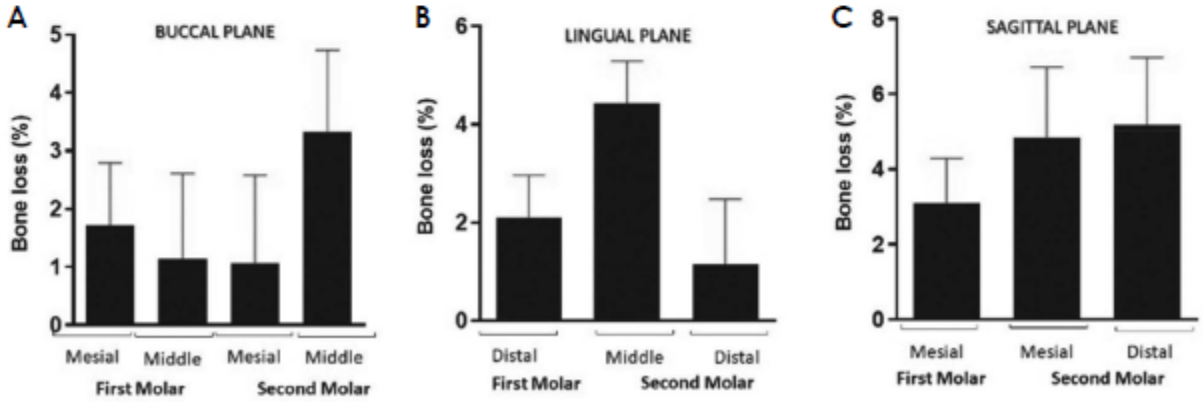

Fig. 7. Percentage of bone loss in the Pg-infected group relative to control. A. Buccal surface. B. Lingual surface. C. Sagittal plane. Only sites that presented bone loss are shown. Pg: *Porphyromonas gingivalis*.

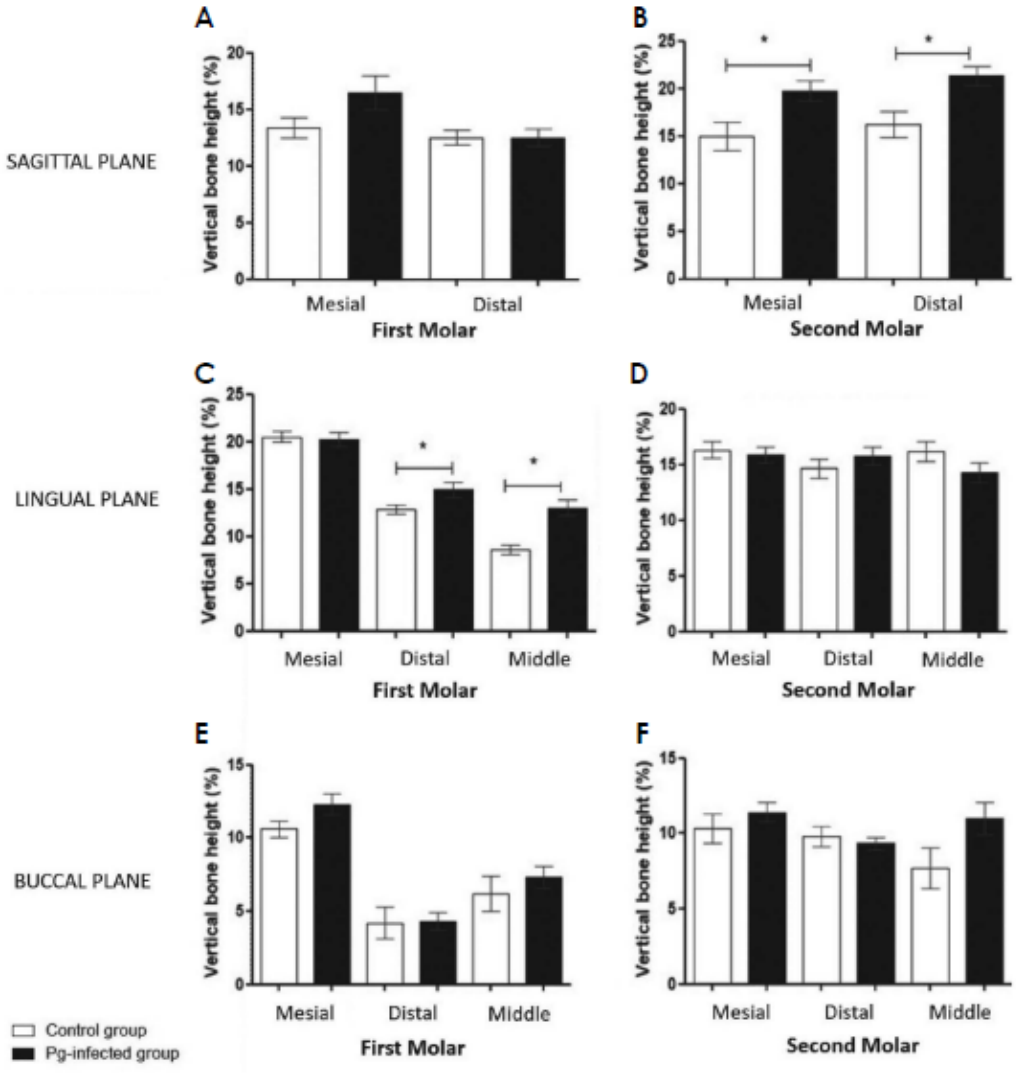

Fig. 8. Alveolar bone height shown as the percentage of total root length for Pg-infected and control groups. Combined measurements in the sagittal plane. A. First molar. B. Second molar; individual measurements of the lingual surface. C. First molar. D. Second molar; individual measurements of the buccal surface. E. First molar. F. Second molar. *$P<0.05$. Pg: *Porphyromonas gingivalis*.

from 12.49% to 21.37% (Figs. 8A and B). Statistically significant differences were observed at the mesial and distal sites of the second molar ($P<0.05$). In the coronal plane, the percentage of vertical bone height varied from 4.21% to 20.5% in the control group and from 4.34% to 20.15% in the Pg-infected group, with the lowest percentages observed on the buccal surface and the highest percentages on the lingual surface (Figs. 8C-F). Out of the 6 locations in the coronal plane, 2 sites showed a significant difference relative to control: the middle-lingual and distolingual sites of the first molar ($P<0.05$).

A classic method of assessing oral inflammation is via histology; hematoxylin and eosin staining and immunocytochemistry with a specific inflammation marker (CD45) were utilized to show that oral infection with Pg induced inflammation concomitant with bone loss (Fig. 9). The Pg-infected group presented more CD45-positive cells than the control group (2.53-fold increase; $P<0.05$).

Discussion

This manuscript describes a standardized micro-CT approach to perform linear periodontal measurements of 8 different sites per mandibular molar in a murine model.

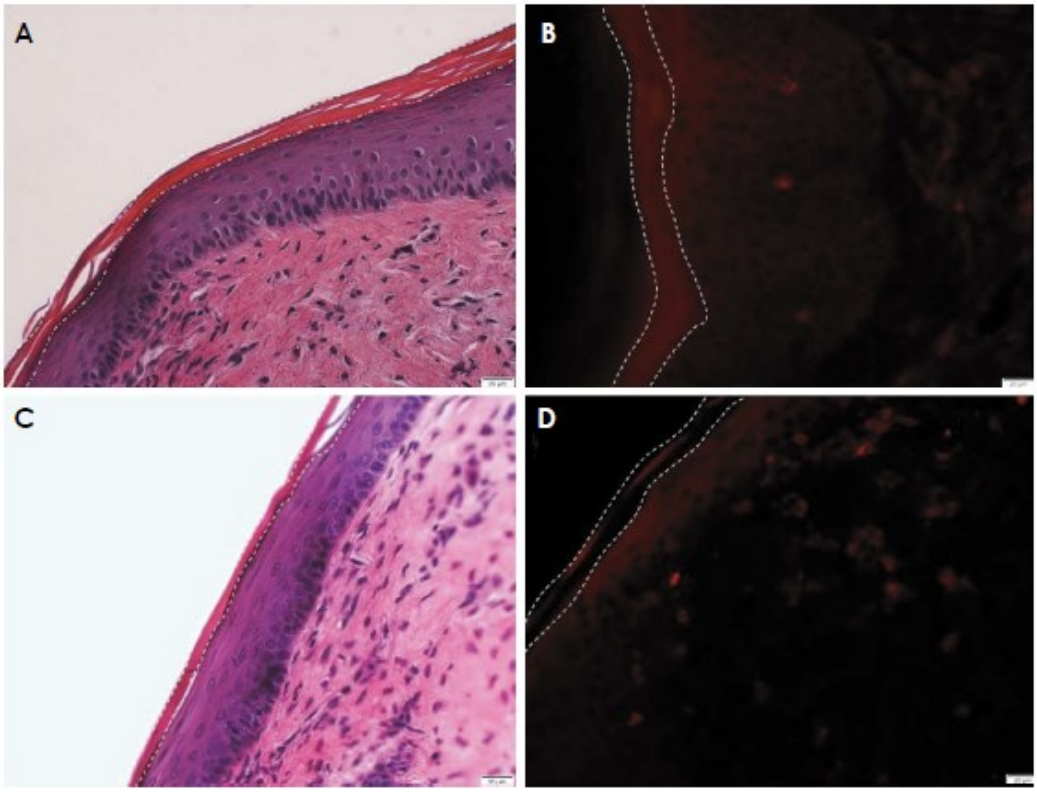

Fig. 9. Hematoxylin and eosin-stained oral cavity tissue from masticatory gingiva. A. Control and C. Pg-infected mice; CD45-positive cells in the oral cavity tissue from masticatory gingiva. B. Control and D. Pg-infected mice. Dashed lines show the keratinized oral tissue border for localization purposes; CD45-positive cells were visualized by red fluorescence (magnification: ×400). Pg: *Porphyromonas gingivalis*.

There is a great variety of linear measurements described in the literature,[3,7,13,18,20,21] but the lack of consensus regarding the sites chosen and the variability in the assessed teeth and single-plane measurements remain concerns. In Figure 10, alveolar bone loss is shown in a 3D view of the lingual sites (middle and mesiobuccal). The results clearly show that if only the sagittal or buccal surfaces, for example, were measured, there could be an underestimation of the disease extent.

When performing research using an animal model of periodontal disease, many factors play a role in the degree and severity of the disease. Some of these factors include the species of animal, the method utilized to promote bone resorption (e.g., bacteria or ligation), the specific bacteria used for infection, the period of study following infection or ligation, and the methods associated with disease evaluation. The methodology used to perform measurements should not be a source of bias introduced into the evaluation.[5,8-10,14,15,21,22] The importance of a consistent set of sites and landmarks reflects the fact that linear measurements are technique-sensitive and minor changes in angulations/positions of the sample might impact the results.[7]

The most commonly used measurement to determine alveolar bone loss in mice is the distance from the CEJ to the ABC.[3,6,7,18,21,23-26] The results of the present study showed that alveolar bone loss occurred mostly on the lingual surface of the coronal plane, a surface not often included in studies, and in the sagittal plane.[7,18,21] Studies found in the literature that did include the coronal plane (buccal and lingual/palatal), combined their results as an average; therefore, it is not possible to confirm whether those findings are similar to the findings of the present study.[3,6,15,23-27] The studies that analyzed bone loss utilizing a percentage method had findings that varied considerably, with reported values of 6-8%,[12] 10-30%,[13] 15-29%,[20] and 5-28%.[28] Those studies included vertical bone loss, bone loss compared to control, or the bone volume fraction. There was substantial variability (5-30%) due to the sites measured or methodology applied; howev-

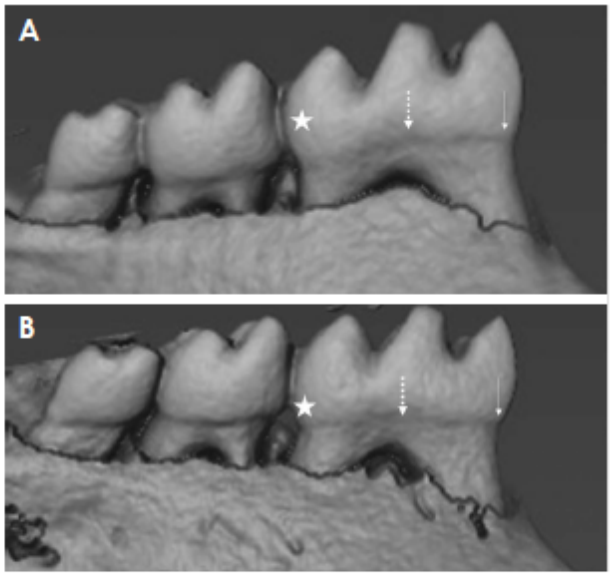

Fig. 10. Lingual view of 3 mandibular molars. A. Control mouse. B. Pg-infected mouse. Stars: distolingual, dashed arrows: middle-lingual, arrows: mesiolingual.

er, some of the reported values are broadly similar to the variability found in the present study (4.34-21.37%).

It is fundamental to note that the goals of an individual study often dictate the method of infection and the type of bacteria used for infection (or a combination), and that different methods will generate different patterns of bone resorption. Oral lavage with Pg was used in this study as a test method of infection. It is paramount to have a measurement strategy that examines the tooth completely, similarly to what is done in human periodontal probing to assess bone loss. This ensures that no instances of isolated bone loss will be excluded from the analysis.[1,29-32]

Vertical bone height characterizes the length of the part of a tooth that appears above the ABC (measured based on the CEJ) versus the part that lies below (CEJ up to the root apex). This number can also be expressed as a ratio or a percentage.[33] After Pg infection, all planes presented some degree of bone loss. When assessing alveolar bone loss, the vertical bone height percentage was compared by subtracting the value found in the Pg-infected group from that found in the control group to assess how much loss occurred at each site. This is important because animals and their root length may vary in size. Expressing the measurement in this manner eliminates any root size bias; therefore, this method is often used in the literature to assess both bone loss and bone gain.[18] The present study showed that lingual sites had a higher percentage than buccal sites, but similar percentages when compared to sagittal, mesial, and distal sites. In the literature, the studies that included vertical bone height, unfortunately, only included buccal sites.[12,34] This could be a very significant omission, given that lingual sites showed percentages of vertical bone height of up to 20% and buccal sites had a maximum of 12% in the present study; the latter value is similar to what has been published in the literature.[12,34] When determining any type of loss, it is important not to underestimate or overestimate the disease. Since lingual sites showed double the percentage of vertical bone height of buccal sites, this dramatic difference should be considered when conducting assessments.

Some of the limitations of the present study are that only 1 type of software was used (Avizo); therefore, the landmarks and planes might not be applicable to other types of software that do not allow the user to move the sample freely. The linear measurements accounted for isolated bone loss but not intra-bone loss; therefore, volume and density would need to be measured to assess alveolar bone internally. This method was utilized to measure alveolar bone in C57Bl/6j male mice molars; therefore, the measurements may be applicable to animals with similar dento-alveolar anatomy such as hamsters and rats, but not larger mammals, given the variability across species in the number and shape of teeth.

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Conflicts of Interest: None

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CHAPTER 4
LITERATURE REVIEW

DIAGNOSIS OF AGGRESSIVE PERIODONTITIS: A DILEMMA?

QUINTESSENCE INTERNATIONAL
PERIODONTOLOGY

Raisa Queiroz Catunda

Diagnosis of aggressive periodontitis: A dilemma?

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Periodontitis is one of the leading causes of tooth loss in the adult population. This disease can be classified into various categories, and one of the most destructive amongst them is aggressive periodontitis (AgP). The incidence of AgP is lower than other types of periodontitis. However, it affects young individuals and can cause severe destruction of tooth-supporting structures including tooth loss if left untreated. The current classification for diagnosing periodontal disease was established by the American Academy of Periodontology (AAP) in 1999. This classification provided strict guidelines to aid in AgP diagnosis. These include three main factors: systemically healthy individual, rapid loss of clinical attachment, and familial aggregation. In spite of these specific guidelines, AgP diagnosis is often missed clinically due to various reasons. There is still a vast variation in the diagnostic criteria for identifying AgP and not all practitioners utilize the AAP guidelines for their diagnosis. Furthermore, the definition of the disease might be changing in the future to better represent the current understanding of the disease. Since early diagnosis and prompt treatment is key in treating these patients, it is important to have calibration in the diagnosis process. This review aims to identify sources of variation and ambiguity in diagnosing AgP among dental practitioners. For this purpose, we have conducted an extensive literature search and outlined the various diagnostic aids for AgP patients reported in the literature. Understanding and correcting these variations can simplify the diagnostic process leading to faster treatment of patients affected with AgP. This review also emphasizes the importance of minimizing the bias in identifying patients with AgP and highlights the best tools for this purpose.
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Key words: bone loss, epidemiology, oral hygiene, periodontal pocket, plaque, prevalence

Aggressive periodontitis (AgP) is a relatively rare and rapidly progressive disease affecting mostly adolescents and young adults who are otherwise considered healthy. AgP is characterized by a rapid attachment loss, increased probing depths, and vertical loss of the supporting alveolar bone resulting in the appearance of infrabony defects, which if left untreated, can lead to tooth loss (Figs 1 and 2).[1,2]

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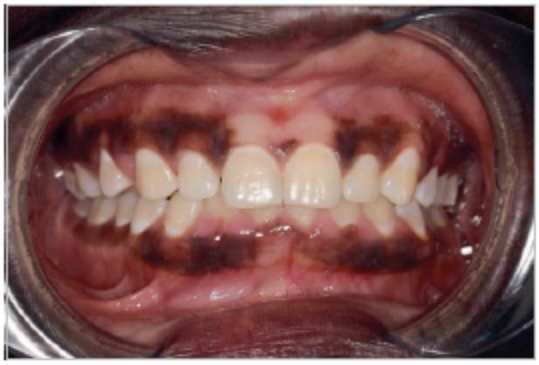
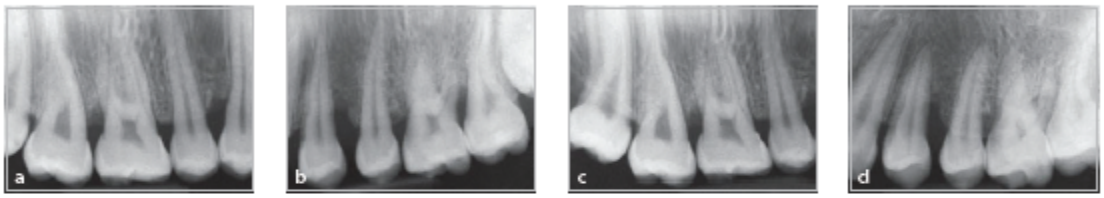


Fig 1 Frontal view of a patient with aggressive periodontitis: a 17-year-old African-American male. Note the absence of local factors intraorally.



Figs 2a to 2d Periapical radiographs of affected sites in the same patient as Fig 1 (left and right maxillary premolars and molars). Note the vertical bone loss around the premolars and molars with furcation arrows noted in the first molar on both left and right maxillary quadrants.

The rapid periodontal destruction present in AgP is characterized by a highly virulent microbiota and/or a high level of susceptibility of the individual.[3] There is a significant familial pattern noted in this disease.[1,4] The risk of tooth loss and impairment of function and esthetics, especially in young patients, most commonly affected by AgP, emphasizes the need for prompt diagnosis and efficient treatment of the disease.[4,5]

Depending on the extent and severity of AgP progression, the treatment can consist of a nonsurgical phase with antibiotic therapy, a surgical phase with regenerative options, or in some cases extraction of the affected teeth.[2,5-7] Thus, it is vital to diagnose this disease early to control the progression and extent of surgical needs in an AgP patient. Correct and early diagnosis of these patients results in prompt treatment and stringent maintenance, increasing the chance of success with appropriate treatment.[8]

With the current nomenclature and the diversity in periodontitis diagnosis patterns, this review aims to examine the history that led to the factors that define AgP, explore the variation in diagnosis and the importance of minimizing the bias in the diagnosis of AgP, as well as highlight the best available diagnostic tools.

DATA SOURCES AND RESOURCES SELECTION

This review included studies with human subjects only. There was no sample size, age, sex, year, language, or type of study restriction. A search was performed utilizing the following databases: Medline, Embase, PubMed, and Google Scholar. The databases, medical subject headings (MeSH), search strategy, and total findings are shown in Table 1. After downloading all articles from the databases, duplicates were excluded. All the remaining downloaded articles were searched for duplication using RefWorks software (ProQuest).

Table 1	Medical subject headings used across the databases and search strategy	
Database	**Search strategy**	**Results**
Medline	1. exp aggressive periodontitis/di [Diagnosis] (148)	82
	2. exp diagnosis/ or exp early diagnosis/ (7861428)	
	3. exp questionnaire/ (872108)	
	4. 2 or 3 (8349041)	
	5. 1 and 4 (82)	
PubMed	(("aggressive periodontitis"[All Fields] OR "juvenile periodontitis"[All Fields]) OR "early onset periodontitis" [All Fields]) OR "prepubertal periodontitis"[All Fields] AND (("1999/01/01"[PDAT] : "2017/12/31"[PDAT]) AND "humans"[MeSH Terms] AND (("infant"[MeSH Terms] OR "questionnaire"[MeSH Terms]) OR "early diagnosis" [MeSH Terms] OR "diagnosis"[MeSH Terms]))	456
Embase	1. exp aggressive periodontitis/di [Diagnosis] (55)	28
	2. exp diagnosis/ or exp early diagnosis/ (5809153)	
	3. exp questionnaire/ (552025)	
	4. 2 or 3 (6267562)	
	5. 1 and 4 (28)	
Google Scholar	With all of the words: Aggressive periodontitis	100
	With at least one of the words: Aggressive periodontitis or juvenile periodontitis or early onset periodontitis or prepubertal periodontitis	
	Where my words occur: anywhere in the article	
	Return articles dated between: 1999–2017	
	Chosen by relevance (first 10 pages)	
Total		666

REVIEW

Study selection

In total, 666 articles were identified from three databases, and 100 more were identified from gray literature (Google Scholar) as shown in Table 1. A total of 130 full text articles were downloaded and reviewed, of which 74 were subsequently excluded. Overall, 51 publications were included in this narrative review (Fig 3).

DISCUSSION

History of aggressive periodontitis classification

Prior to the term "aggressive periodontitis" (AgP), several descriptions were used, some of which are still in use today, causing confusion among the dental community. Initially, Butler introduced the term "juvenile periodontitis" in 1969.[9] This term was further strengthened by Baer in 1971, when he divided the disease into two categories: the localized form that affected the first molars and incisors, and the generalized form that affected most of the dentition.[10] Later, nomenclature such as "pre-pubertal periodontitis" was introduced and referred to periodontal disease occurring during or shortly after eruption of permanent teeth. It was described as a rare condition and, for this reason, there were no reports of clinical attachment loss (CAL) and the number of teeth involved.[11] In contrast, juvenile periodontitis was defined as a disease that occurs in systemically healthy children and adolescents, characterized by rapid destruction of the alveolar bone.[11]

Newer terminology was introduced in 1989 when the term "early onset periodontitis" (EOS) was used by the American Academy of Periodontology (AAP) and in

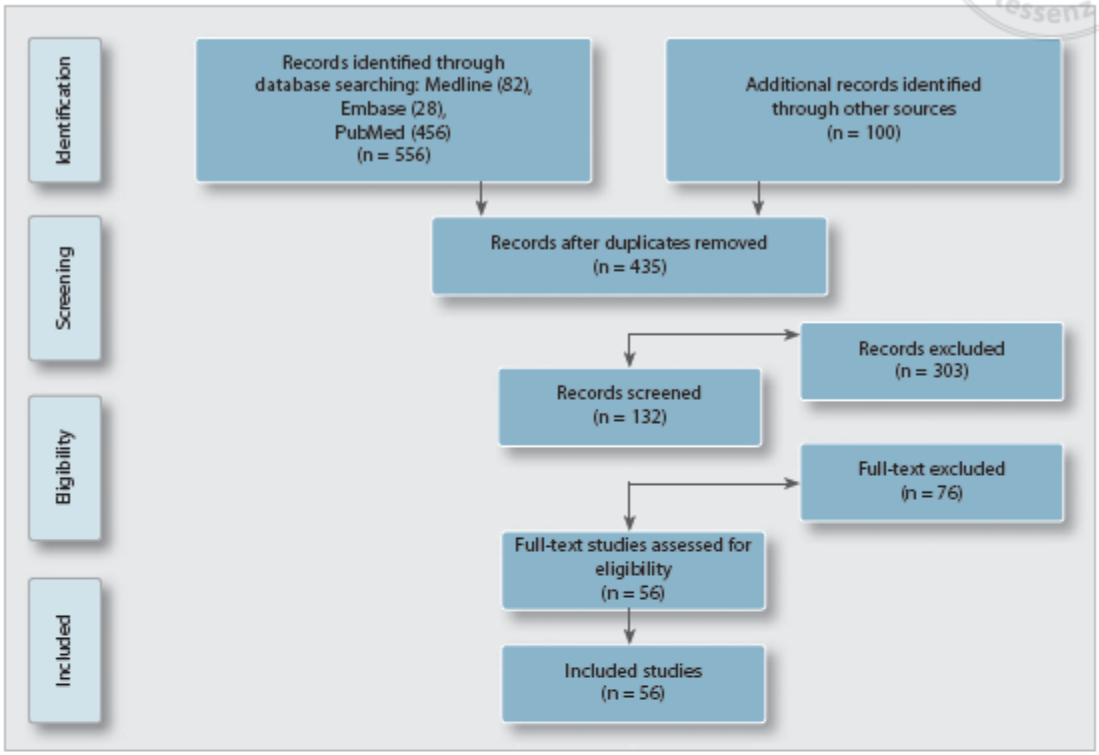


Fig 3 Flow diagram of literature search process.

the European classifications of 1993 as a designation for a group of destructive periodontal diseases affecting young individuals (prepubertal, juvenile, and progressive periodontitis).[12] However, the term "early onset" implies that it occurs in young individuals with knowledge of the time of onset of disease. These data are not always available in the clinical setting. Also, in most situations, there is uncertainty about setting an arbitrarily upper age limit for patients with early-onset periodontitis.[13] Several examples of this uncertainty are outlined in the 1999 AAP classification.[13]

Current classification

Due to all the previous classifications, it appeared that there was no agreement on how to narrow down specific characteristics that were unique to these patients. Therefore, based on the knowledge of the shortcomings of the 1989 classification system, a new classification was proposed in 1999 by the AAP.[13] The term "pre-pubertal periodontitis" was removed from use, since most of the generalized cases of this condition were related to systemic diseases. Cases not related to systemic disorders were re-allocated in the groups of "chronic" (CP) or "aggressive" periodontitis, depending on the characteristics of each case, since age ceased to be a primary factor for classification.[13] In this classification, EOS was called "aggressive periodontitis," which could be either localized or generalized.[14]

According to Armitage,[13] classification of localized (LAgP) or generalized (GAgP) is made considering the affected sites. LAgP is selected when there is circumpubertal onset with localized presentation of loss of

attachment in the first molars and/or incisors with interproximal loss of at least two permanent teeth, one of which is a first molar, and involving no more than two teeth other than first molars and incisors.[13] GAgP is considered in patients under 30 years of age, when there is generalized interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors.[13,14]

To further clarify these diagnostic criteria, the AAP also introduced primary and secondary features that can aid in identifying AgP patients. The primary factors are that patients must be systemically healthy, and there should be rapid loss of attachment, and family aggregation. Secondary factors that are usually present (but not necessarily in all cases) are: amounts of microbial deposits inconsistent with the severity of periodontal destruction, high proportions of *Actinobacillus actinomycetemcomitans* (and in some populations *Porphyromonas gingivalis* may be present), phagocytic abnormalities, hyperresponsive macrophages, including elevated levels of cytokines, progression of attachment loss, and bone loss may be self-limiting.[14] The classification recognized that not all features could be present in a patient, and therefore the diagnosis should be based primarily on the clinical and radiographic data and patient history.[13]

The definition of the disease may change in the near future to better represent the current understanding of the disease.

Prevalence of AgP

As mentioned above, familial aggregation was a significant factor in patients with AgP. It was important to report if any subgroup of the population was more prone to this disease than others. Knowing the prevalence of a disease worldwide is of fundamental importance in the establishment of new treatment approaches and health care need planning.[15] Reports in the literature show that prevalence studies (before and after the new classification) have been performed in the following countries: Iran, United States of America, Sudan, Brazil, Uganda, Chile, Turkey, Nigeria, Japan, United Kingdom, Finland, Norway, Switzerland, and Israel.[6,16-31] The prevalence varied between 0.02%[32] and 6.1%,[6] regardless of the ethnicity, sex, severity, or localization.

In studies that considered ethnicity, there was a higher prevalence in black people than in Caucasians,[24,26,32] varying from 6.9%[33] to 7.3%[34] in specific African ethnicities. Some studies even pointed to black individuals having 40- to 43-times higher prevalence than white.[32,35,36] Regarding the prevalence by sex, women seemed to be affected at earlier ages than men,[2,5,6,14] but few studies have found significant differences between males and females.[23] When the studies divide the classification into LAgP and GAgP, the prevalence varies from 0.01%[37] to 4.3%[33] and 0.02%[38] to 4.3%,[34] respectively.

Where does the dilemma start? Controversy in diagnosis

Even though the 1999 AAP classification is almost 20 years old, the current issue in studies that have tried to diagnose AgP lies in the lack of standardization; the cross-sectional studies after 1999 do not always follow all the criteria suggested by Armitage.[6,13,16-31] A wide variety of criteria has been used by different authors to determine indices of periodontal disease. These criteria differ in standards of the measurement of probing depth (PD) and CAL, periapical and interproximal radiographs, as well as the selection of sample size of the studied population.[30]

With the great variability in diagnosis reported globally, there is a need to understand why there is a lack of homogeneity when adhering to the classification. The controversy resides in the lack of complete comprehension of the guidelines and misinterpretation. The results from the present literature search showed that some authors included patients with systemic diseases and classified them as AgP patients, and others included patients with modifying factors[20,33] or only used radiographs as criteria,[29,31] which can also have an influence in the initial assessment, leading to incorrect/incomplete diagnosis.[40]

Another factor mentioned by researchers when investigating risk factors, is the great difficulty in distin-

guishing true risk factors from associated cofactors. According to Garcia et al,[41] when only risk factors are considered, without previous knowledge of their effect with time, false or an exaggerated periodontal diagnosis can occur.

Although AgP shows a strong trend for familial aggregation, it is often neglected while evaluating the patient's medical/dental and family history. In periodontal studies after the AAP new criteria were introduced, authors did not seem to investigate previous family dental history.[6,28,42-44] Some clarified the medical history of patients and family members, but there has been little research investigating whether close relatives of the affected patient had any previous periodontal history.[32,45-47]

Another source of misdiagnosis lies in the protocol used for clinical examination, such as differences in the extent (full-mouth vs half-mouth), the number of periodontal sites assessed in each tooth, and the diversity in periodontal probing devices available (Fig 4).[48]

In order to minimize the variation among practitioners, researchers tried to add more features to differentiate between the different types of periodontal diseases. According to Ranney,[49] the diagnostic criteria should not have any overlaps to avoid confusion between different types of diseases, and he suggested to set fixed age limits for CP and AgP patients.

Additionally, many authors have written well-documented reports in which they have suggested additional features that can be used in the diagnosis process. These further add to the variation presented to dentists. For example, when defining AgP, Stawinska et al[50] suggested that the following exclusion criteria should be considered: patients over 60 years of age, previous periodontal treatment (last 3 years), pregnancy and lactation, systemic disorder that could affect the periodontal tissue, smoking, and nonsteroidal anti-inflammatory medication or antibiotic therapy in the last 6 months. They also suggested some useful tools towards the characterization of AgP: probing pocket depth (PPD), bleeding on probing (BOP), evaluation of furcation areas, the extent of gingival inflammation using the Papillary Bleeding Index (PBI), and the accumulation of dental plaque assessed using the Approximal Plaque Index (API). Other authors suggested the addition of Gingival Index (GI), Calculus Index (Calculus Surface Severity Index, CSSI), and CAL.[42,51]

Thus, it is evident that the diagnostic criteria for AgP have relied not only upon the AAP classification criteria but also on clinician/researcher's experience, personal preference, and the methodologies deemed reliable. While this may work for certain individuals, these different techniques and criteria for disease diagnosis are not easily calibrated among dentists, and this causes further discrepancy.

How can AgP be correctly diagnosed?

It is clear that there is no "perfect" tool for AgP diagnosis. It is a multifactorial disease and a challenge to diagnose. However, a good "diagnosis tool" should include the currently used classification, such as the AAP classification, so that variation amongst studies and practitioners can be minimized. It is also important to have a clear view of the differential diagnoses; as AgP is a relatively rare disease, some clinicians end up misdiagnosing it as either CP or gingival diseases modified by systemic factors.[51] Additionally, clinicians can use other tools for diagnosis that they find helpful, but it is important to ensure the AAP criteria is fulfilled first in order to facilitate standardization.

A task force report was recently published by the AAP (2015), aiming to further clarify the current classification of attachment loss, differentiation between CP and AgP, and localized versus generalized periodontitis.[14] Apart from emphasizing the previous guidelines (1999), the task force debated the accuracy of CAL measurements, which are challenging to perform, especially when the margin of the gingiva is coronal to the cementoenamel junction (CEJ), and there can be confusion if the gingival margin is considered to be at the same level as the CEJ.[14] They mentioned three factors, in general, to classify an individual (not under any current periodontal treatment) as having periodontitis: one or more sites having inflammation (BOP), radiographic bone loss, and increased CAL.[15]

There was emphasis by the task force on the differential diagnosis with CP. Often, the dental practitioner or the periodontist sees a patient at a certain point and, depending on the accuracy (or even the existence) of dental records, they can track the progression of the disease. One of the emphasized criteria was the age-dependent limits; age should not the single criterion for differentiation, but patients younger than 25 years at the time of the onset of the disease can be used along with other criteria to differentiate AgP from CP. Other criteria, such as low levels of biofilm and calculus, should always be taken into consideration to support a broader diagnosis of AgP.[14]

Regarding the extent of the disease, the classification as generalized or localized is also a point of misinterpretation. The lack of standardization of even the extension of measurements (full-mouth vs half-mouth examination protocols) can have a huge impact in the categorization in LAgP or GAgP. The task force suggested the usage of percentage of affected teeth rather than affected sites, to minimize the chance of diagnostic errors regarding extension.[14]

CONCLUSION

AgP is a devastating disease and has a negative impact on the quality of life of affected patients.[52] This disease is still not clearly understood and several mechanisms are involved in the disease process.[53] It is, therefore, crucial to establish a standardized diagnostic method to correctly detect cases in the early stages. There are many different sources of misdiagnosis of AgP, as reported by various studies. These include clinical examination, clinical history (risk factors), progression of the disease, and familial aggregation investigation. Currently, there are no flawless diagnostic tools, but the probable solution is to follow the current definition of the disease by the AAP, to perform complete periodontal examination with a full-mouth protocol, include complete history (medical and dental) of the patient, and assess the rate of disease progression. The definition of the disease might change in the future to better represent the current understanding of the disease. Accuracy of diagnosis is of great importance for the prompt treatment and orientation of patients, improving their masticatory functions, esthetics, and quality of life.

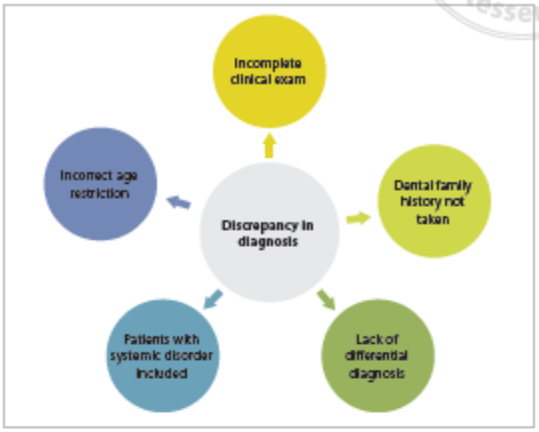


Fig 4 Potential sources of diagnosis discrepancies

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CHAPTER 5
LITERATURE REVIEW

PREVALENCE OF PERIODONTITIS IN
YOUNG POPULATIONS: A SYSTEMATIC REVIEW

Prevalence of Periodontitis in Young Populations: A Systematic Review

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Purpose: To assess the prevalence of periodontitis in young populations (previously termed aggressive periodontitis – AgP) and report on the earliest known occurrence of this disease.

Materials and Methods: A search was performed covering the last 18 years utilising the following databases: Medline (Ovid), PubMed and Embase. Four reviewers evaluated each study. Review findings were summarised using the PRISMA Statement for reporting and Joanna Briggs Institute (JBI) Critical Appraisal tools for quality assessment, respectively. Twenty-two articles were included in this systematic review, consisting of 6 prevalence studies and 16 case reports. Only prevalence studies were considered for prevalence estimates.

Results: The average reported prevalence of periodontitis in young populations was 1.7% (ranging from 0.66% in Argentina to 5.9% in Israel). The prevalence was higher for the localised form of this disease. Permanent teeth were the most common dentition affected (114 out of 115 affected patients). In terms of age, the prevalence was 0.6%, 0.8% and 1.6% for the age groups 2–12, 20–25 and 13–20, respectively. There was no significant difference noted between males and females. The earliest age diagnosed with periodontitis was 3 years 7 months.

Conclusion: The prevalence of periodontitis in young patients is ranges widely, which could indicate populational predispositions, underdiagnosing or lack of standardisation in diagnosis.

Key words: aggressive periodontitis, demographics, epidemiology, misdiagnosis

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Periodontitis is a multifactorial disease in a susceptible host caused by bacterial insult over prolonged periods of time. The predisposing factors include strain and concentration of bacteria, quality of oral hygiene, host related factors, and many other elements.[37]

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Periodontitis primarily affecting young people (previously termed aggressive periodontitis; AgP) is a rapidly progressing disease of complex aetiology.[5] This disease is caused by a multitude of environmental and hereditary factors that alter cellular interactions and host response.[53] Identification and classification of this periodontal disease has been a source of contention resulting in ambiguity in diagnosis.[10] The latest classification of periodontal and peri-implant diseases published in 2018 aims to remove this ambiguity and make diagnosis of this disease easier.[9] Since this classification has been recently established, for the purpose of this study, we limited our search from 1999 to 2017, during which period the previous classification was employed.[5]

The 1999 classification characterised periodontitis in young populations (AgP) by an early onset and aggressive destruction. This disease most frequently occurs in individuals younger than 25 years of age.[34] It shows rapid progression, without a corresponding amount of plaque or calculus.[31] It also shows familial aggregation and, surprisingly, the affected individuals are otherwise healthy.[21]

Previously classified 'aggressive periodontitis' had two forms of the disease based on its extent and severity: localised and generalised. The localised form was defined as ≥ 2 permanent teeth (no more than two teeth other than

Table 1 Search strategy used across the databases

Database	Search Strategy	Results
Medline	1 exp Aggressive Periodontitis/ (1973) 2 prepubertal periodontitis.mp. (89) 3 EOS periodontitis.mp. (0) 4 juvenile periodontitis.mp. (816) 5 1 or 2 or 3 or 4 (2252) 6 exp Young Adult/ (606853) 7 exp Child, Preschool/ or exp Child/ (1759324) 8 exp Adolescent/ (1847479) 9 exp Adolescent/ or exp Students/ or schoolchild.mp. (1926200) 10 exp Infant/ or kindergarden.mp. (1058884) 11 exp Pediatrics/ (53234) 12 6 or 7 or 8 or 9 or 10 or 11 (3618567) 13 limit 12 to humans (3594828) 14 12 and 13 (3594828) 15 5 and 14 (1171) 16 limit 15 to (yr ="1999 -Current" and ("all infant (birth to 23 months)" or "all child (0 to 18 years)" or "all adult (19 plus years)" or "newborn infant (birth to 1 month)" or "infant (1 to 23 months)" or "preschool child (2 to 5 years)" or "child (6 to 12 years)" or "adolescent (13 to 18 years)" or "young adult (19 to 24 years)")) (442)	442
Pubmed	(("aggressive periodontitis"[All Fields] OR "juvenile periodontitis"[All Fields]) OR "early onset periodontitis"[All Fields]) OR "prepubertal periodontitis"[All Fields] AND (("1999/01/01"[PDAT] : "2017/12/31"[PDAT]) AND "humans"[MeSH Terms] AND (("infant"[MeSH Terms] OR "child"[MeSH Terms] OR "adolescent"[MeSH Terms]) OR "infant"[MeSH Terms] OR "adult"[MeSH Terms]))	1085
Embase	1 exp Aggressive Periodontitis/ (759) 2 prepubertal periodontitis.mp. (89) 3 EOS periodontitis.mp. (0) 4 juvenile periodontitis.mp. (798) 5 1 or 2 or 3 or 4 (1609) 6 exp Young Adult/ (177277) 7 exp Child, Preschool/ or exp Child/ (2409910) 8 exp Adolescent/ (1414052) 9 exp Adolescent/ or exp Students/ or schoolchild.mp. (1577444) 10 exp Infant/ or kindergarden.mp. (952784) 11 exp Pediatrics/ (93250) 12 6 or 7 or 8 or 9 or 10 or 11 (3399940) 13 limit 12 to humans (3027625) 14 12 and 13 (3027625) 15 5 and 14 (674) 16 limit 15 to (yr ="1999 -Current" and ("all infant (birth to 23 months)" or "all child (0 to 18 years)" or "all adult (19 plus years)" or "newborn infant (birth to 1 month)" or "infant (1 to 23 months)" or "preschool child (2 to 5 years)" or "child (6 to 12 years)" or "adolescent (13 to 18 years)" or "young adult (19 to 24 years)")) [Limit not valid in Embase; records were retained] (249)	249
Google Scholar	With at least one of the words: Aggressive periodontitis or Juvenile periodontitis or early onset periodontitis or prepubertal periodontitis Where my words occur: anywhere in the article Return articles dated between: 1999-2017 Chosen by relevance (first 10 pages)	100
Total		1876

first molars and incisors), one of which is a first molar with interproximal attachment loss. The generalised form was characterised by interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors.[5]

Even after the change in nomenclature of the 1999 classification, there was considerable contradiction in the diagnosis and reported prevalence regarding this disease. This variation in both diagnosis and reported prevalence could be explained by type and extent of clinical parameters used, inaccurate measurement of clinical attachment levels, limited sample sizes in cross-sectional studies, environmental differences and age ranges tested in studies.[12] To address these issues, a new consensus for classification of periodontal diseases and conditions was published in 2018,[9] and both chronic and aggressive periodontitis are now placed in the same class. The aim of the present study was to assess the reported prevalence of periodontitis in young populations (previously known as aggressive periodontitis) by conducting a thorough review of the literature based on a scope of cross-sectional studies and case reports within the available classification (American Academy of Periodontology 1999 classification) for the included time range (1999 – 2017) and report on the earliest known occurrence of this disease.

MATERIALS AND METHODS

Eligibility Criteria

This review included studies with medically healthy human subjects up to 25 years of age. There was no sample size,

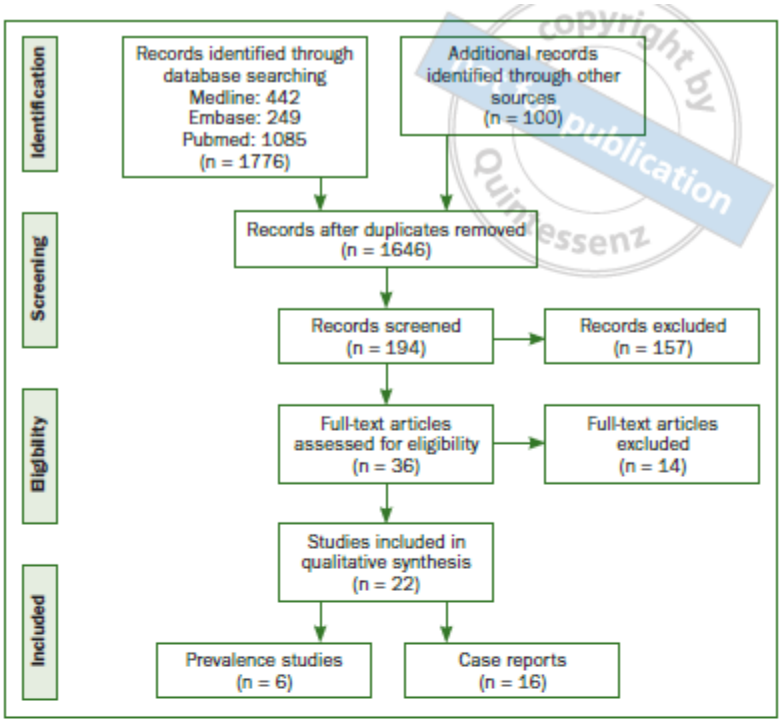

Fig 1 Flow diagram of literature search process according to PRISMA.[35]

country or language restriction. Case reports, case series and prevalence studies were included. Letters, reviews, books, conference/meeting abstracts and duplicates were excluded. We restricted the age up to 25 years as per the recommendation of the AAP Task Force.[20] Even though there is a newer classification available, no studies have been performed following the new classification. Therefore, we restricted our inclusion criteria to studies from 1999 to 2017 only. Hence, we cannot classify the included periodontitis studies according to stages or grades, and have used the American Academy of Periodontology 1999 classification system for the purpose of identifying this disease in young populations.

Information Sources

A database search was performed utilising the following databases: Medline, Embase and PubMed. The search was limited from 1999 (after the 1999 AAP classification was released) to 2017 (before the latest classification of periodontal and peri-implantitis disease and conditions was released).[5]

For the category 'fulfilled AAP criteria', we checked whether the studies included the following: apart from the presence of periodontitis, patients are clinically (medically) healthy, show rapid attachment loss, bone destruction and familial aggregation.

Search Strategy

The databases, medical subject headings (MeSH), search strategy and total findings are shown in Table 1. After downloading all articles from the databases, duplicates were excluded using the 'duplicate checking tool' of RefWorks and the type of match: 'close duplicates'. All the remaining downloaded articles were searched for duplication in Microsoft Excel.

Data Collection and Study Selection

The authors read the titles and abstracts of all selected articles after removing duplicates for inclusion criteria matching. Titles indicating syndrome studies, studies in which patients showed any systemic disorder, or studies that looked at 'attachment loss' or 'periodontal disease' without proper classification were excluded, as were systematic reviews, letters to editors, conference abstracts. Full abstracts of the remaining titles were then read. After reading the abstracts, the authors carefully excluded ones that did not match the inclusion criteria.

Articles selected after this phase were downloaded as full text and then underwent the last stage of selection. The Joanna Briggs Institute (JBI) Critical Appraisal tools for use in JBI Systematic Reviews[51] was used for both case reports and prevalence studies. They consist of an 8- or 9-question checklist with answers 'yes', 'no', 'unclear' and 'not applicable'. Three authors checked to determine if the articles had all the required data elements and fit the eligibility criteria, with disagreements resolved by discussion and consultation with a fourth author.

Table 2 Articles excluded after full-text download and reasons for exclusion

Title of the study	Reason for exclusion
Aggressive periodontitis in children: A 14-19-year follow-up[10]	It did not specify if the subjects were healthy and it was not a prevalence study.
Proximal caries in juvenile periodontitis patients[14]	Aggressive periodontitis classification was based on radiographic findings
Bacterial profile of aggressive periodontitis in morocco: a cross-sectional study[4]	Not prevalence study
Prevalence of aggressive periodontitis in adolescents and young adults[6]	It did not specify if the subjects were healthy
Prevalence of aggressive periodontitis in school attendees in Uganda[1]	It did not specify if the subjects were healthy
Prevalence of Aggregatibacter actinomycetemcomitans in Sudanese patients with aggressive periodontitis: A case-control study[7]	It did not specify if the subjects were healthy
Microbiological characterization in children with aggressive periodontitis[13]	Cohort study
Antibody responses against porphyromonas gingivalis infection in patients with early-onset periodontitis[8]	Not prevalence study
Tnf-alpha and Il-4 levels in generalized aggressive periodontitis subjects[3]	Not prevalence study
Localized aggressive periodontitis treatment response in primary and permanent dentitions[9]	Cohort study
Aggressive periodontitis: laser nd:yag treatment versus conventional surgical therapy[11]	Clinical trial
Combined periodontal and orthodontic treatment in a patient with aggressive periodontitis: a 9-year follow-up report[5]	It did not specify if the subject was healthy
Aggressive periodontitis in a Nigerian teaching hospital[12]	Aggressive periodontitis classification was not according to the AAP criteria
Th1 and Th2 cytokine profile in patients with early onset periodontitis and their healthy siblings[2]	Not a prevalence study

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After all prevalence studies matched our inclusion criteria, an average reported prevalence was calculated according to the Joanna Institutes Reviewers Manual[51] as: average reported prevalence = No. of people with disease at a given point in time / total No. of people in the population.

Risk of Bias in Individual Studies

The risk of bias for prevalence studies was assessed according to a validated tool. It comprised 10 items plus a summary assessment; the first 4 questions aimed to assess the external validity of the study (domains: selection and nonresponse bias) and the remaining 6 aimed to access internal validity (domains: measurement bias and bias related to the analysis). The summary assessment evaluated the overall risk of study bias. This is based on the Grades of Recommendation, Assessment, Development and Evaluation (GRADE)[50] and Cochrane approaches.[26] There was no overall numeric rating of risk of study bias; however, there was an evaluation of the overall risk of study bias based on risk of bias assessment of 10 individual items.[28]

Table 3 Summary of the key prevalence study characteristics

Author	Age	Sex	Sample size total (affected)	Diagnostic criteria	Localised/ generalised	Primary / permanent/ mixed dentition	AAP criteria
Roozegar et al, 2014[39]	14–16	Male: 500 (4)/ female:500 (3)	1000 (7)	The 4 mm or more atrophy in connective tissue attachment at least two permanent teeth (at least one molar) and 2 mm or more bone recession around suffered tooth	Localised	Permanent	Healthy patients
Elamin et al, 2010[17]	13–19	Male: 596 (29)/ female: 604 (12)	1200 (41)	≥ 4 teeth with interproximal sites showing ≥ 4 mm attachment loss, or at least three teeth with interproximal sites showing ≥ 5 mm attachment loss	Localised	Permanent	Healthy patients, rapid periodontal attachment loss
Susin et al, 2005[47]	14–24	Male: 291 (5)/ female: 321 (5)	612 (10)	In the 14- to 19-year group, subjects with four or more teeth with attachment loss ≥ 4 mm were defined as having AgP. In the older group (20 to 29 years), cases were defined as those with four or more teeth with attachment loss ≥ 5 mm.	Not clear	Permanent	Healthy patients
Eres et al, 2009[18]	13–19	Male: 1493 (8)/ female: 1563 (10)	3056 (18)	Involvement of more than one first molar; radiographic evidence of alveolar bone loss ≥ 2 mm on more than one surface of the permanent tooth involved; probing depth at diseased sites > 5 mm; and extensive bone loss with respect to the low levels of plaque and calculus unlike typically observed in chronic periodontitis.	Localised	Permanent	Healthy patients
Levin et al, 2006[32]	18–30	Male: 562 (33)/ female: 80 (5)	642 (38)	The diagnosis of aggressive periodontitis was made on the basis of clinical and radiographic assessment using the classification workshop criteria1 and the specific characteristic radiographic appearance of aggressive periodontitis	Localised (28), generalised (10)	Permanent	Healthy patients Familial aggregation
Funosas et al, 1999[20]	2–12	Male: 80 (0)/ female: 72 (1)	152 (1)	No clear description: 3 mm loss of periodontal insertion in the second primary molar and a lesion. Löe and Silness index: 17.5% for value 1, 46.25% for value 2, 53.75% for value 3. BOP 10% for value 1, 90% for value 2.	Localised	Primary	Healthy

RESULTS

Study Selection

1776 articles were identified from 3 databases and 100 more were identified from grey literature, totaling 1876. The article selection process is shown in the flow diagram according to PRISMA (Fig 1).[35] Thirty-six full text articles were downloaded and reviewed, of which 14 were subsequently excluded due to reasons outlined in Table 2.

Study Characteristics

After the careful search, six prevalence studies and sixteen case reports matched our inclusion criteria. A summary of the key study characteristics and results of the selected articles are presented in Table 3. The included articles were all written in English and Portuguese and were published between 1999 and 2017. The sample size in the prevalence studies ranged from 152[19] to 3056,[17] totaling 6662 patients; patient age ranged from 3 to 25 years old.

All prevalence studies included in this systematic review showed an overall low risk of bias as shown in Table 4. Most studies scored low risk of bias for all criteria, demonstrating that those studies might serve as a representation of the population of the countries studied.

Prevalence Studies

The overall average prevalence of periodontitis in young populations in the 6 evaluated studies was 1.7%. The average prevalence for the localised form of the disease (previously known as LAgP) was 1.42%, which was higher than that of the generalised form (previously known as GAgP), 0.15%. Out of 115 patients total, 95 suffered from the localised form of the disease. Permanent teeth were most commonly affected (114 patients). Only one study mentioned checking for self-reported familial aggregation by patients.[32] There was no difference in disease prevalence between the sexes.

The prevalence studies were performed in 6 countries, and they ranged between 0.6% and 5.9%: Turkey 0.6%; Argentina 0.66%; Iran 0.7%; Brazil 1.6%; Sudan 3.4%; Israel 5.9%.

The studies presented a diverse age group affected by this disease. Therefore, we distinguished age groups ac-

Table 4 Risk of bias assessment

Title	External validity	Internal validity	Overall risk of study bias
The prevalence of localised aggressive periodontitis among students at 14–16 years in Ilam, Iran	High	Low	Low
Ethnic disparities in the prevalence of periodontitis among high school students in Sudan	Low	Low	Low
Aggressive periodontitis in an urban population in Southern Brazil	Low	Low	Low
Periodontal treatment needs and prevalence of localised aggressive periodontitis in a young Turkish population	Low	Low	Low
Aggressive periodontitis among young Israeli army personnel	Low	Low	Low
A case of prepubertal periodontitis and prevalence of gingivitis in a population attending a university clinic in Rosario, Argentina.	High	Low	Low

cording to the World Health Classification and skeletal maturity. This consists of toddlers (10 months – 3 years), prepuberty (4–9 years), early and mid-puberty (10–12 years), adolescents/late or post-puberty (13–19 years), young adults (20–25 years).[21,55] Based on the age ranges present in the data, we had to combine the toddler, pre-puberty and early and mid-puberty into one category (2–12 years). The average prevalences per age group were 0.6% (2–2 years), 1.6% (13–19 years) and 0.8% (20–25 years).

Case Reports of Earliest Incidence of AgP

During the search process, we noted several reports of periodontitis in very young children. Thus, in addition to reporting prevalence, we expanded our review to report the early occurrence of this disease. Sixteen case reports fit our inclusion criteria (Table 5). A total of 18 patients were evaluated. The most frequent age reported was 4 years (3 patients), followed by 5 years (2 patients). The youngest case of periodontitis was diagnosed in a 3-year-old male patient.[38] Most patients were in the pre-puberty age-group (7 cases), followed by adolescents (6 cases) and young adults (3 cases). Toddler and early/mid-puberty age-groups only had 1 case each.

Only one case report presented with all three AAP criteria established in 1999 for the diagnosis of the disease.[46] Only 7[14,29,38,41,44,46,49] out of 13 studies that asked for familial aggregation reported positive familial association. Permanent dentition was most commonly affected.

DISCUSSION

The results of this systematic review show a great diversity in estimated prevalence in the evaluated studies. This can be due to the large variety of screening methods and clinical examination, even though four[17,32,39,47] out of the six studies mentioned the 1999 classification.[5]

Periodontitis in young populations is more often detected among individuals between 10 and 19 years of age.[48] It is still unclear whether this is the most frequently affected group or simply the most frequently studied group, as most of the samples were within this age range. This is interesting, as the earliest occurrence of this disease was noted in primary dentition.

According to our study, the localised form of the disease was more prevalent than the generalised form,[47] which is in accordance with previous prevalence studies that varied from 1.2–4.2% (localised)[2,48] and 0.03–4.3% (generalised).[23,34] The prevalence range in the studies may be influenced by the method of diagnosis, geographic location of the study and study sampling.

Although all studies were performed after the release of the 1999 AAP classification, only four[16,17,32,45] mentioned the use of all diagnostic criteria. These criteria were established to clarify the classification of periodontal diseases and help clinicians differentiate among other periodontal diseases.[5] The low number of studies following all three criteria leaves ambiguity for a possible missed diagnosis. Additionally, the literature[43] reports periodontitis in young populations to be underdiagnosed. This is either due to lack of complementary exams (diagnosing only by radiographs), not performing full-mouth probing (especially in children and adolescents) and difficulties assessing clinical attachment levels.[43] These factors could significantly lower the reported prevalence numbers around the world.

Demographic data from the included study show that the prevalence in Brazil (1.6%) is within the range of previous reports, which varied from 1.3%[4,23] to 5.33%.[12] This wide range in the prevalence of disease reported in Brazil alone could be due to various factors. Brazil is a very large country in terms of both territory and population. The composition of populations examined in these studies could also differ greatly based on the city/state and sampling methods, which in turn can generate discrepant results.[5,20,34,37]

The average prevalence of the disease reported in young Israeli military personnel is 5.9%.[32] This differed from an older report from 1991 that had an average of 0.86%.[6] The included military study followed all 3 diagnostic criteria pro-

Table 5 Summary of case reports

Author	Age (years)	Localised/ generalised	Primary/permanent/ mixed dentition	AAP criteria
Portaro et al, 2008[38]	3 years and 7 months	Generalised	Primary	Healthy, familial aggregation
Spoerri et al, 2014[46]	4	Generalised	Primary	Healthy, familial aggregation, rapid attachment loss and bone destruction
Cunha et al, 2012[14]	4	Generalised	Primary	Healthy
Sharma et al, 2011[44]	4	Generalised		Healthy, familial aggregation, rapid attachment loss and bone destruction
Suzuki et al, 2003[49]	5	Localised	Primary	Healthy, familial aggregation
Vieira et al, 2014[53]	5 and 13	Generalised	Primary and permanent	Healthy
Hilgers et al, 2004[28]	6	Localised	Primary	Healthy, rapid attachment loss and bone destruction
Seremidi et al, 2012[42]	8	Generalised	Mixed	Healthy
Bodur et al, 2001[7]	10	Generalised	Mixed	Healthy, rapid attachment loss and bone destruction
Sant'Ana et al, 2009[41]	14	Localised	Permanent	Healthy, familial aggregation
Bonta et al, 2003[8]	15	Localised	Permanent	Healthy
Haubek et al, 2006[25]	16	Localised	Permanent	Healthy
Ishikawa et al, 2002[29]	17 and 24	Not clear	Permanent	Healthy, Familial aggregation
Vishnoi et al, 2010[54]	19	Generalised	Permanent	Healthy
Livingstone et al, 2015[33]	22	Generalised	Permanent	Healthy
Sivakumar et al, 2014[45]	25	Generalised	Permanent	Healthy

posed by the AAP, whereas the older study with very low prevalence only looked at panoramic radiographs.[6]

In Argentina, various studies have been performed to assess the prevalence of calculus,[15] alveolar bone loss,[11] gingivitis,[11] and clinical attachment loss[11] among adolescents and children. These studies did not specifically examine the prevalence of disease based on the three factors defined by the AAP classification. This may be why the included study reported a prevalence of 0.66% vs the other studies listed above that ranged from 3%[19] to 7.4%.[36] Values from Sudan, Iran and Turkey were in close agreement with other reports.[18,22,23,40]

In this systematic review, we strictly adhered to the classification criteria established by the AAP in 1999 and included studies published only from that date up to 2017, when the latest workshop results were presented. Thus, only 6 studies fit our criteria; so few prevalence studies make it difficult to generalize data to the world population. We have tried to address this by also including region-specific prevalences. However, future studies with the new classification criteria and larger populations would help establish true prevalence patterns.

CONCLUSION

Our results should raise awareness regarding the discrepancies between clinicians and the guidelines in terms of diagnosing periodontal disease in the young population. Although the nomenclature was changed almost 20 years ago, considerable contradiction still exists in diagnoses.[10] Therefore, the current classification of periodontal and peri-implantitis disease and conditions will address the main conflicts present in the literature. New epidemiological studies following the 2018 classification are highly encouraged.

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CHAPTER 6

LITERATURE REVIEW

LORICRIN AND EPITHELIAL RELATED DISORDERS: A SYSTEMATIC REVIEW

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Review

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Loricrin downregulation and epithelial-related disorders: a systematic review

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Summary

Loricrin downregulation has been associated with age-related changes as well as inherited and inflammatory skin diseases. We hypothesize that changes in loricrin could be more related to altered barrier function and consequently disorders that affect epithelial cells, such as psoriasis, atopic dermatitis (AD), erythrokeratoderma, loricrin keratoderma (LK) and periodontitis. The aim of this review is to summarize what is known about the association between loricrin downregulation and epithelial-related disorders (ERDs). A search was performed on the following databases: Medline, Cochrane Library, PubMed, EMBASE, Lilacs, Scopus and Google Scholar, resulting in 16 included articles. Loricrin keratoderma was the ERD most frequently associated with loricrin mutations (730insG, 709insC and 578insG; 5/7 cases – 71.44 %). Atopic dermatitis was the ERD most frequently associated with loricrin downregulation (2/7 cases – 28.6 %). Mutilating palmoplantar keratoderma, progressive symmetrical erythrokeratoderma and a new type of erythrokeratoderma were not associated with any mutations. At the gene level, periodontitis patients showed the highest decrease (–6.89x), followed by AD (–6.5x) and psoriasis patients (–0.5x). In summary, loricrin mutation and downregulation were associated with several ERDs. The diversity in disease presentation is likely related to whether there is a total loss of loricrin, mislocalization and/or if the mutant form of loricrin causes dysfunction of other proteins and/or changes in cornification.

Introduction

The cornified envelope

Mammalian epidermis is a type of epithelial tissue comprised of a stratified squamous epithelium and organized by a finely regulated process of keratinocyte differentiation [1]. It has four layers: stratum basale, stratum spinosum, stratum granulosum, and stratum corneum as shown in Figure 1. The stratum corneum is the outer layer of the epithelium; it is responsible for the permeability of the skin barrier and resilience of the cornified epithelium. It consists of dead, enucleated keratinocytes, devoid of intracellular organelles, called corneocytes [2, 3]. The cornified cell envelope(CE) is the most insoluble structure of the stratified squamous epithelium and is formed below the plasma membrane, replacing it along the terminal differentiation of the keratinocytes [4].

The CE is quite variable in molecular composition, depending on the location of the epithelium, which confers different properties of mechanical resistance, elasticity and impermeability to the tissue. In general, it is composed of several types of cross-linked proteins, such as involucrin, cystatin A, loricrin, small proline-rich proteins, elafin, S100 protein family, profilaggrin, putrescine, spermidine, annexin 1, inhibitor of plasminogen 2 activator, sciellin and some desmosomal components [4].

In the final phases of keratinocyte differentiation, the thickness and stiffness of the CE are increased. The resistance and insolubility of the CE are based on the formation of very stable isopeptide bonds, while the lack of an organized protein structure contributes to its elasticity. In addition to this proteic structure, the CE also has a lipid bilayer that binds to the outer surface of the protein envelope, which is important in its roles in cohesion and protection of the stratum corneum [5].

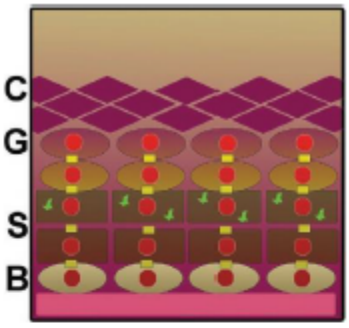

Figure 1 Schematic representation of the cornified epithelium layers. B: stratum basale; S: stratum spinosum; G: stratum granulosum; C: stratum corneum. Green Dots: represents Kerato-hyaline granules.

Loricrin and epithelial disorders

Loricrin is an insoluble protein that constitutes approximately 70–85 % (volume) of the CE in differentiated corneocytes [5–9]. The major function of loricrin is to reinforce the protective barrier. Loricrin is heavily transglutaminated. This means that it forms inter- and intra-protein crosslinks that are highly resistant to proteolysis; they stabilize and strengthen the CE [10].

Loricrin functions as a reinforcement of the CE on its cytoplasmic face. It is a highly insoluble basic protein, rich in glycine (46.4 %), serine (23 %), cysteine (6 %) and glutamine (4 %) [7]. It is expressed exclusively in keratinized epithelia [11] and is closely involved in CE formation [12].

Information for building proteins is encoded by certain genes, and proteins such as loricrin regulate several processes. Protein interactions, even when they are coded by one gene, can interfere with the expression of another gene or with the decrease in expression of a specific protein. Interactions between genes therefore have an important role in the susceptibility to common human epithelial diseases [13].

Recently it was shown that loricrin mutations can induce epithelial conditions by two general processes: anomalies in CE development or flawed apoptosis of differentiating keratinocytes [14]. Correct formation of the CE is essential for the arrangement of a normally keratinized epithelium; it is likely that aberrant expression and/or processing of the protein constituents is involved in epithelial pathologies such as keratodermas and psoriasis [3], or, if bacteria are present, release of the pathogen into the blood stream, causing a destructive reaction as seen in severe forms of periodontitis [15].

Misplacement of mutant *LOR* (loricrin protein) and decreased expression of the most abundant proteins in the CE (involucrin and loricrin) might lead to a breach in the epithelial barrier, resulting in injury and occurrence or exacerbation of certain epithelial disorders.

There are many different types of epithelial disorders that follow certain phenotypic patterns. Some epithelial disorders follow a more genetic pattern, without any phenotypic connection. Several inheritable epithelial diseases have already been associated with *LOR* mutations, the first described being related to a frameshift mutation as an insertion of a G following nucleotide 730 in families with Vohwinkel syndrome [16].

Patients with *LOR* mutations share some characteristics, such as diffuse palmoplantar hyperkeratosis (a characteristic observed in all individuals with skin disorders involving aberrant phenotype mutations in the loricrin gene) [3, 16–22]. Both "honeycombs" and digital constriction bands are usually present [18]. Digital constriction bands known as pseudoainhum are also a common finding and may lead to self-amputation due to restricted circulation at the fingertips [3, 16, 17, 19–22]. Ichthyosis is a cornification process characterized by generalized skin scaling [23] and is also present in all disorders that involve *LOR* mutations [16–22].

The purpose of this review is to systematically profile the current literature to determine what is known about the association between loricrin downregulation and epithelial diseases.

Materials and methods

Eligibility criteria

This systematic review only includes studies of human subjects of any age/sex. There was no restriction of sample size or language. Case reports and case series were included. All epithelium-related disorders that had gene and protein expression analyses were included in our study. Letters, reviews, books, conference/meeting abstracts and duplicates were excluded. Treatments that could affect loricrin expression or studies that did not have a clear ethics statement were also excluded.

Search strategy, data collection and study selection

A database search was performed encompassing the last 20 years and utilizing the following databases: MEDLINE, Cochrane Library, EMBASE, LILACS, PubMed and SCOPUS up to December 2018. The databases, medical subject headings (MeSH), search strategy and total findings are shown in Supplementary Table 1 [online Supporting Information]. Duplicates were excluded after downloading all articles from the databases.

Three authors (RQC, LL, MF) read all titles and abstracts after duplicate removal to ensure that they matched the inclusion criteria. Any disagreements were resolved by consultation with the remaining authors (DC, UR). Articles selected after this phase were downloaded as full texts and were then taken to the last stage of the selection.

Risk of bias in individual studies

The studies were assessed according to the Critical Appraisal tools for use in JBI Systematic Reviews from the Joanna Briggs Institute (JBI) [24]. The checklists were derived from the tools to assess case reports and case series. They consisted of 8–10 questions with answers "yes", "no", "unclear" or "not applicable". The articles were scored according to a percentage scale (0–100 %).

Protocol and registration

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist [25]. Protocol registration is "in process" under the number 49544.

Results

Study selection

One thousand sixty-four articles were identified from five data bases and 100 more were identified from grey literature (Google Scholar). The article selection process is shown in the flow diagram (Figure 2) according to PRISMA [25]. Thirty full-text articles were downloaded and reviewed, of which 14 were subsequently excluded due to reasons outlined in Supplementary Table 2 [online Supporting Information].

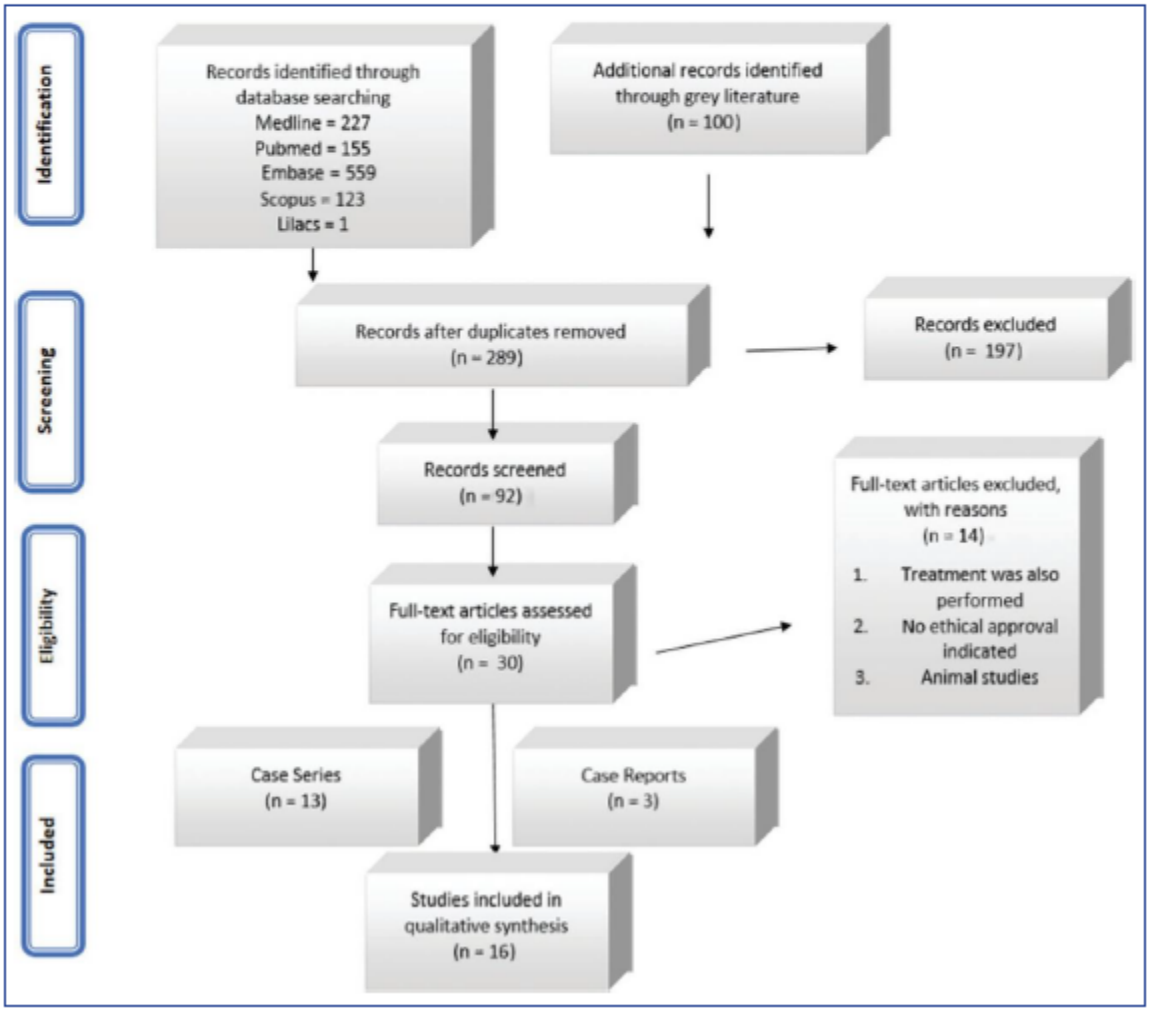

Figure 2 Flow diagram of literature search process according to PRISMA.

Study characteristics

A summary of the key study characteristics and results of the selected articles is presented in Table 1. The included articles were all in English, published between 1996 and 2017 and their sample sizes ranged from one to 48. Three case reports and 13 case series were included in this review. Eight different ERDs were evaluated for loricrin expression or mutation: Vohwinkel syndrome with ichthyotic variant [17, 26], mutilating palmoplantar keratoderma [27], aggressive periodontitis [15], loricrin keratoderma [18, 28, 29], progressive symmetric erythrokeratoderma [19, 20, 30, 31], atopic dermatitis [32–34], psoriasis [19, 34–36] and a novel type of erythrokeratoderma (KLICK; keratosis linearis with ichthyosis congenita and sclerosing keratoderma syndrome) [37].

We used keywords to broadly interrogate the literature and found 16 studies that fulfilled our inclusion criteria: examination of loricrin expression (mRNA or protein) in skin or mucosal disease. Four studies [27, 30, 31, 37] found no changes in loricrin expression while six [15, 19, 32–34, 36] showed changes in mRNA expression and three [18, 20, 26] showed changes in protein expression or localization. Of these, four [18, 20, 26, 36] were associated with DNA mutations, three [18, 20, 26] within the coding sequence and one [36] within the non-coding sequence.

Synthesis of results

The most common methods of mutation detection and expression analysis were polymerase chain reaction (PCR) alone or with quantitative PCR (qPCR). These were complemented by immunofluorescence (IF) histology [18, 27, 31, 34], immunohistochemistry (IHC) and western blot (WB) [30]. A DNA microarray was used alone [33] or in combination with qPCR [15]. The cell/sample investigated influenced the degree of change in loricrin expression, and may have been a limitation in several studies. Downregulation was greatest (– 6.07x) when keratinocytes were included, a cell type in which loricrin is expected to be expressed [15, 34].

Ten articles reported mutation analysis, twelve reported expression analysis and seven included both analyses. Of the ten that presented loricrin mutation analysis, four were negative [27, 30, 31, 37] and the other six found the following mutations: 730insG [15, 16, 20, 21] (rs749909701), 578insG [18] (rs757890729), 709insC [20] (rs997892102), and the variants: -1544 G|A, G>A (a mutation within the 5′untranslated region (UTR)); 64_69dupGGCGGT (a duplication of six nucleotides) (rs150026164); 85A>G, (a single nucleotide substitution); 567_578del, (a 12 nucleotide deletion; *684, A>G (a single nucleotide substitution in the 3′ UTR); and *716A>T (a single nucleotide substitution in the 3′UTR region) (rs886041212) [36].

LOR 709insC, 578insG, and 730insG mutations generated a frameshift, resulting in a protein that had 90, 114 and 82 amino acids, respectively, that differed from the normal protein. The result is an increase in the amino acids arginine and leucine, which have properties that are different from the glycine and lysine residues they replaced, and potentially have severe effects on the structural properties of the CE [23]. These mutations were found in loricrin keratoderma [18, 26, 28, 29], progressive symmetric erythrokeratoderma [20], mutilating palmoplantar keratoderma [17] and psoriasis [36].

The 730insG mutation, which adds an extra G to six normally occurring G residues (codons 230–231), is associated with loricrin keratoderma [29], and produces a frameshift resulting in delayed termination [16, 19, 26]. No alteration in protein expression was observed and this might be due to equal production of mutant and normal loricrin [29]. Since the mutant form is less able to be cross-linked, this could lead to a dominant negative effect [16, 38–40].

In two instances, frameshift mutations resulted in the generation of a nuclear localization signal (NLS) and accumulation of loricrin in the nucleus. 578insG [18] was associated with loricrin keratoderma, and 730insG [26] was associated with Vohwinkel′s keratoderma with ichthyosis (loricrin keratoderma).

Giardina et al. investigated psoriasis, and reported six sequence variations [36], although these were not believed to be causative. Such changes may be tolerated because they do not significantly modify the role of the protein in the CE [41].

Among studies that performed mutation analysis, only Wei et al. included the UTR, all exons and exon-intron boundaries [30]. Although the entire coding sequence of loricrin is contained within the second exon [20], the UTRs are known to play crucial roles in the post-transcriptional regulation of gene expression, including modulation of mRNA transport out of the nucleus and translation efficiency [42]. The UTRs also interact with non-coding RNAs that have key regulatory functions [43]. Thus, studies that limited their mutational analysis to both exons [17, 26, 27, 31, 37], only the second (coding) exon [29] or part of the second exon [18] and did not analyze the UTRs may have missed the critical change in the gene.

Of the twelve studies that analyzed loricrin expression, six found loricrin to be down-regulated [15, 19, 32–34, 36], two did not find any changes [29, 30] and four found anomalous staining [18, 20, 26, 27]. Immunohistochemistry and immunofluorescence studies showed abnormal loricrin staining [20, 34], weak cytoplasmic expression (restricted to lesioned skin) [27] or loricrin only within the nucleus [18, 26]. The finding of parakeratotic cornified cells with loricrin-positive

Table 1 Descriptive characteristics of included articles.

Epithelial-related disorder	Aim	Journal	Loricrin expression	Gene mutation	Method of detection	Number of patients
Mutilating palmoplantar keratoderma (Akiyama et al., 1998)	To study a family of non-Vohwinkel syndrome	Society for Investigative Dermatology	Abnormal distribution of loricrin (weak cytoplasmic expression)	No mutations detected	PCR + IF	2
Loricrin keratoderma (Gedicke et al., 2006)	Report: family with diffuse ichthyosis and honeycomb palmoplantar keratoderma	British Association of Dermatologists	No difference in expression	730insG	PCR + qPCR	13
Aggressive periodontitis (Guzeldemir-Akcakanat et al., 2016)	Investigate molecular biomarkers that play a role in the development of generalized aggressive periodontitis (GAgP)	Journal of Periodontology	Downregulation (6.89x)	Not analyzed	qPCR + DNA microarray	48
Loricrin keratoderma (Song et al., 2008)	Determine the correlation between a loricrin mutation and a heterogeneous phenotype of loricrin keratoderma	British Journal of Dermatology	Loricrin was localized only within the nucleus	578insG	PCR + IF	27
Vohwinkel's syndrome - ichthyotic variant (Korge et al., 1997) (also referred to as loricrin keratoderma)	Characterize ultrastructural and immunocytochemical features of Vohwinkel syndrome and seek evidence of loricrin mutation	Journal of Investigative Dermatology	Loricrin reactive granules were mostly restricted to the nucleus	730insG	PCR + IHC	38
Progressive symmetric erythrokeratoderma (PSEK) (Ishida-Yamamoto et al., 1997)	Report: identification of a frameshift mutation in the loricrin gene on chromosome 1q21 in a family with PSEK	American Journal of Human Genetics	Abnormal loricrin staining/ localization	709insC	PCR + IHC	3
Loricrin keratoderma (Yeh et al., 2013)	Case report	Clinical and Experimental Dermatology	Expression was not analyzed	730insG	PCR	1
Vohwinkel syndrome -ichthyotic variant (O'Driscoll et al., 2002) (also referred to as loricrin keratoderma)	Study: a family originating from the UK with typical features of the ichthyotic variant of VS, identification of a recurrent insertion mutation in the loricrin gene	Clinical and Experimental Dermatology	Expression was not analyzed	730insG	PCR	14

Continued

Table 1 Continued.

Epithelial-related disorder	Aim	Journal	Loricrin expression	Gene mutation	Method of detection	Number of patients
Psoriasis (Takahashi et al., 1996)	Analysis of the expression of B2-adrenergic receptor-mRNA, loricrin-mRNA and involucrin mRNA in the epithelium of patients with psoriasis vulgaris	British Journal of Dermatology	Decreased expression (0.55x)	Not analyzed	qPCR + PCR	5
Atopic dermatitis (AD) (Jarzab et al., 2010)	Compare 1q21 gene expression in lesional versus non-lesional AD skin	International Archives of Allergy and Immunology	Decreased expression (0.5x)	Not analyzed	qPCR	33
Progressive symmetrical erythrokeratoderma (PSEK) (Wei et al., 2011)	Examination of the mutation status of GJB3, GJB4 and LOR in patients with PSEK	Clinical and Experimental Dermatology	No change in expression was found	No mutation detected	PCR + IF+ WB	25
Atopic dermatitis (AD) (Sugiura et al., 2005)	Identify gene expression specific to active AD	British Journal of Dermatology	Down-regulated (6.5x)	Not analyzed	DNA microarray	17
Atopic dermatitis (AD) and chronic psoriasis (CP) (Guttman-Yassky et al., 2009)	Characterize differences between AD and CP in gene expression related to epidermal growth/ differentiation and inflammatory circuits	Journal of Allergy and Clinical Immunology	Down-regulated (AD: 0.015x; CP: 0.687x)	Not analyzed	qPCR + IHC + IF	33
Progressive symmetrical erythrokeratoderma (Akman et al., 2008)	Case report	Clinical and Experimental Dermatology	Not analyzed	No mutation detected	PCR	1
Psoriasis (Giardina et al., 2004)	Verify loricrin's involvement in psoriasis pathogenesis	Annals of Human Genetics	Lower expression in psoriatic lesions	Six sequence variants identified	qPCR + PCR	8
Erythrokeratoderma (novel type) also referred to as KLICK syndrome (van Steensel et al., 2005)	Case report	British Journal of Dermatology	Not analyzed	No mutation detected	PCR	1

Abbr.: PCR, polymerase chain reaction; IF, immunofluorescence; qPCR, quantitative polymerase chain reaction, IHC, immunohistochemistry; WB, western blot.

granules and nuclear retention in the stratum corneum layer was also found in some studies [17, 20, 30].

Of the four studies that did not find any mutation in LOR, only Wei et al., in a case series of patients with progressive symmetrical erythrokeratoderma, did not find any change in protein expression [30]. This was a different conclusion from another case series study by Ishida-Yamamoto et al. that used the same detection methods, but found a frameshift mutation (709insC) [20]. Akiyama et al. found loricrin to be abnormally distributed [27], while Akman et al. and van Steensel et al. did not investigate loricrin expression as a complement to the mutation analysis [31, 37]. Thus, it is possible that alterations in loricrin localization and expression are secondary to other mutations, and still produce the phenotype associated with LOR mutations.

The greatest decrease in *LOR* mRNA was found in patients with aggressive periodontitis (–6.89x) [15]. This is in accordance with previous data from a murine study that investigated *LOR* downregulation in aggressive and chronic periodontitis [44]. In a study of atopic dermatitis, there was a similar decrease in *LOR* expression in involved skin (–6.5x), and even "normal appearing skin" showed reduced LOR expression (–3.5x) [33]. The smallest difference in *LOR* expression was found in psoriasis (decreased by 0.55x). Chronic psoriasis (–0.68x) [34] had twice the decrease in loricrin compared with psoriasis (–0.3x) [30]. Two other studies that investigated psoriasis and atopic dermatitis found similar decreases in loricrin (–0.5x) [19, 32].

The correlation between having a mutation or a downregulation in loricrin and the respective ERD shows that mutations were found in 63 % of the analyzed ERDs (7/11), whereas loricrin downregulation was found in 75 % of the analyzed ERDs (6/8) (Table 2).

Mutilating palmoplantar keratoderma, progressive symmetric erythrokeratoderma and KLICK syndrome were the only ERDs not associated with any mutations. Of the articles that analyzed loricrin expression, only one case series of loricrin keratoderma and one case series of progressive symmetric erythrokeratoderma did not find downregulation. Loricrin keratoderma was the ERD most commonly associated with mutations, with five out of seven cases (71.44 %), while atopic dermatitis was the ERD most commonly associated with loricrin downregulation, with two out of seven cases (28.6 %) (Table 3).

Table 2 Correlation between *LOR* mutation and downregulation and epithelial-related disorders.

Epithelial-related disorder	Mutation	Downregulation
Mutilating palmoplantar keratoderma [17]	No	NA
Loricrin keratoderma [21]	Yes	No
Aggressive periodontitis [18]	NA	Yes
Loricrin keratoderma [19]	Yes	NA
Vohwinkel syndrome – ichthyotic variant [15] (also referred to as loricrin keratoderma)	Yes	NA
Progressive symmetric erythrokeratoderma [22]	Yes	NA
Loricrin keratoderma [20]	Yes	NA
Vohwinkel syndrome - ichthyotic variant [16] (also referred to as loricrin keratoderma)	Yes	NA
Psoriasis [23]	NA	Yes
Atopic dermatitis [26]	NA	Yes
Progressive symmetrical erythrokeratoderma [24]	No	No
Atopic dermatitis [27]	NA	Yes
Atopic dermatitis and chronic psoriasis [28]	NA	Yes
Progressive symmetrical erythrokeratoderma [25]	No	NA
Psoriasis [30]	Yes	Yes
A novel type of erythrokeratoderma (also referred to as KLICK syndrome) [31]	No	NA
Total of positive correlation	63 %	75 %

Abbr.: NA: not applicable.
Not applicable studies were excluded if they did not evaluate mutation or expression.

Risk of bias

The risk of bias in each article was assessed using the Joanna Briggs tool. The answers for all applicable questions from the questionnaires for both the case series (66–100 %) and case reports (100 %) were greater than or equal to 66 %. They were therefore considered of moderate to high quality and were included in this review. Common weaknesses included lack of statistical analysis (case series) or incomplete demographics of the patients.

Table 3 Distribution of ERDs that presented correlation with mutation or downregulation.

Epithelial-related disorder	Frequency	
	Mutation	Downregulation
Atopic dermatitis	0 (0)	2 (28.6)
Aggressive periodontitis	0 (0)	1 (14.28)
Loricrin keratoderma	5 (71.44)	0 (0)
Progressive symmetric erythrokeratoderma	1 (14.28)	1 (14.28)
Atopic dermatitis (AD) and chronic psoriasis	0 (0)	1 (14.28)
Psoriasis	1 (14.28)	2 (28.56)
Total no. (%) with data	7 (100)	7 (100)

Discussion

Based on the importance of loricrin in CE integrity, stability and keratinocyte terminal differentiation, we hypothesized that mutations and changes in expression could underlie epithelial pathologies. Loricrin mutation, downregulation or mislocalization appear to result in fragility of the CE, and consequently lead to a breach in a natural shield and susceptibility of the affected individuals. There are many hypotheses regarding possible mechanisms, and these vary according to the type of alteration in loricrin (mutation and/or mRNA/protein expression reduction or total loss). Many of the gene mutations take the form of frameshifts, which result in a change in the amino acids at the C-terminus, with an increase in arginine. The resulting protein is less likely to be cross-linked. Arginine contributes a positive charge, which can lead to decreased protein stability because of charge repulsion. Charged residues are more hydrophilic, and this could alter another key structural feature of loricrin, its insolubility [45, 46]. This may also lead to a change in protein localization. Changes in DNA/protein sequence can alter interactions with proteins and regulatory RNA, affecting translation efficiency, mRNA stability, protein stability and folding, and protein localization [47].

Other mutations in *LOR* create an NLS that leads to mislocalization, and this seems to interfere with the normal nuclear program of CE formation, especially with regard to apoptosis. This causes late and discontinuous differentiation, resulting in a parakeratotic epithelium in some cases. Guttman-Yassky et al. [34] found a relative absence of loricrin in corneocytes above the granular layer in atopic dermatitis patients, indicating abnormal formation/retention; this may permit infiltration by epicutaneous antigens and an increase in immune responses [48].

Song et al. [18] found a 578insG mutation associated with loricrin keratoderma and restriction of the mutant loricrin to the nucleus (Figure 3). The normal protein was homogeneously distributed in the entire cell, including both cytoplasm and nucleus. This suggests that nuclear concentration results from a change in sequence in the C-terminus of the mutant loricrin protein. Protein translocation to the nucleus is via interaction with proteins from the importin family and is determined by a specific amino acid sequence, known as the nuclear localization signal (NLS). Thus, the frameshift embeds a newly generated NLS as a result of the G insertion. The authors mention that this insertion also leads to a mutant loricrin that is 22 amino acids longer. Our subsequent analysis showed only a difference of one amino acid in length (313 *vs.* 312). The reason for this discrepancy is unclear. Nuclear mutant loricrin could interfere with the regulatory processes of apoptosis and cell division during terminal keratinocyte differentiation [1, 49], and this may explain the phenotype of loricrin keratoderma.

Some studies did not link alterations in *LOR* expression to mutations; other mechanisms, such as mutations in regulatory proteins or pathogen-mediated effects may be at work. Overall, the results are consistent with the notion of a possible truncation in the differentiation program or a change in the structural integrity of the CE as a result of reduced loricrin protein [49]. However, complete loss of expression as a result of genetic mutation may lead to compensation by other proteins, as observed in the knockout mouse, and thus

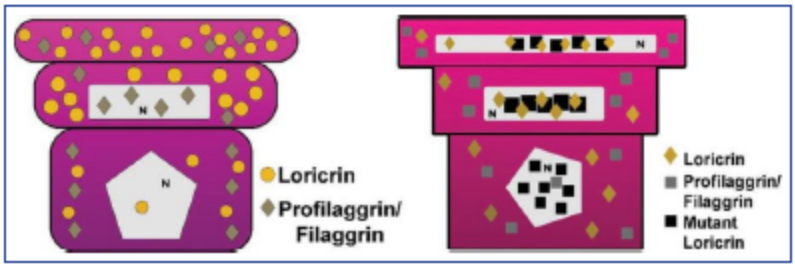

Figure 3 Representation of normal loricrin and profilaggrin distribution and the restriction to the nucleus of the mutant form as seen in loricrin keratoderma.

has little effect on phenotype. The consequences of loricrin downregulation by bacteria and other epithelial pathogens, perhaps secondary to the actions of the host immune system, is an understudied topic and may be an unrecognized mechanism in diseases of the epithelium. Since loricrin is by far the most abundant protein in the CE, pathogen-mediated decrease in expression can affect the formation of the barrier and compromise integrity.

Limitations of the present study include its restriction to case reports and case series, since such studies have less power of extrapolation. The lack of informed consent, treatments performed previous to the case report and *in vitro* studies also restricted the strength of our findings (Supplementary Table 2) [online Supporting Information]. The type of cell investigated influenced the degree of change in loricrin expression, and may have been a limitation in several studies.

Conclusion

There is great heterogeneity among epithelial disorders that have a similar etiological node. Based on our review, we conclude (even with the great diversity of phenotypes among the studied ERDs) that loricrin may play an important role in the cohesion of the keratinized epithelium. Therefore, the characteristics of ERDs are probably related to whether there is a total loss of loricrin, mislocalization of loricrin and/or whether the mutant form of loricrin causes dysfunction of other proteins and/or changes in cornification. The positive relation between loricrin depletion and ERDs may be crucial in the early intervention of certain aggressive diseases and the development of new treatments that focus on specific cellular mechanisms.

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Conflict of interest

None.

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CHAPTER 7
RESULTS

STAT6VT MICE INFECTED WITH PORPHYROMONAS GINGIVALIS SHOW ORAL EPITHELIAL SIMILARITIES TO PATIENTS WITH SEVERE PERIODONTAL DISEASE

Paper submitted to INTERNATIONAL DENTAL JOURNAL in December 2022 Abstract

We investigated the role of the cornified epithelium, the outermost layer of the oral mucosa, engineered to prevent water loss and microorganism invasion, in severe forms of periodontitis (Stage III or IV, Grade C). *Porphyromonas gingivalis*, a major periodontal disease pathogen, can affect cornified epithelial protein expression through chronic activation of Signal Transducer and Activator of Transcription 6 (Stat6). We used a mouse model, Stat6VT, that mimics this to determine the effects of barrier defect on *Porphyromonas gingivalis*-induced inflammation, bone loss and cornified epithelial protein expression, and compared histological and immunohistological findings with tissues obtained from human control, Stage III and IV, Grade C disease patients. Gingival samples were assessed for loricrin, cytokeratin 1, cytokeratin 14, a proliferation marker, leukocyte number, as well as morphological signs of inflammation. In severe periodontal disease patient tissues, there were greater signs of inflammation (rete pegs, clear cells, inflammatory infiltrates), a decrease and broadening of expression of loricrin and cytokeratin 1. Cytokeratin 14 expression was also broader, and decreased in grade IV. *Porphyromonas gingivalis*-infected Stat6VT mice showed greater alveolar bone loss, as assessed by microcomputed tomography, in 9 out of 16 examined sites, and similar patterns of disruption of expression of loricrin, cytokeratins 1 and 14. There were also increased numbers of leukocytes, decreased proliferation and greater signs of inflammation compared with *Porphyromonas gingivalis*-infected controls. Our study provides evidence that changes in epithelial integrity can exacerbate the effects of *Porphyromonas gingivalis* infection, with similarities to the most severe forms of human periodontitis.

Keywords: Loricrin, periodontal disease, epithelial barrier, Stat6VT

introduction

The cornified epithelium (CE) is the outermost layer of skin; in the oral mucosa, this layer is known as the keratinized epithelium[1,2]. It acts as a physical, mechanical, and immunological

barrier against external aggressors and water loss[3,4]. The CE is created through a tightly regulated differentiation process to yield cell bodies without organelles, containing keratin intermediate filaments and lipids[1,2,4]. Proteins from the granular layer of the CE, such as loricrin, involucrin and filaggrin, form a scaffold under the cell membrane in which the keratins are embedded[1,2,4]. Barrier dysfunction has been shown to play a significant role in the pathogenesis of diseases such as atopic dermatitis (AD), psoriasis, loricrin keratoderma, and potentially, periodontitis[5]. Loricrin is vital for the proper functioning of the CE, as it comprises approximately 70-80% of this layer[6].

Research has uncovered a potential mechanism, related to increased levels of T-helper cell 2 (Th2) cytokines, including interleukin-4 (IL-4), to explain the downregulation of loricrin observed in AD[7-11]. IL-4 signaling in keratinocytes is mediated by the Janus kinase (Jak) 2-signal transducer and activator of transcription (Stat) 6 pathway (Figure 1)[11-13]. Stat6 binds to DNA to promote the expression of downstream genes, and in so doing uses a co-factor, p300/Creb-binding protein (CBP); this co-factor is also necessary for loricrin expression[11,12]. When there is chronic IL-4 signaling and Stat6 activation, CBP is sequestered and loricrin gene expression is downregulated (Figure 2)[11,12].

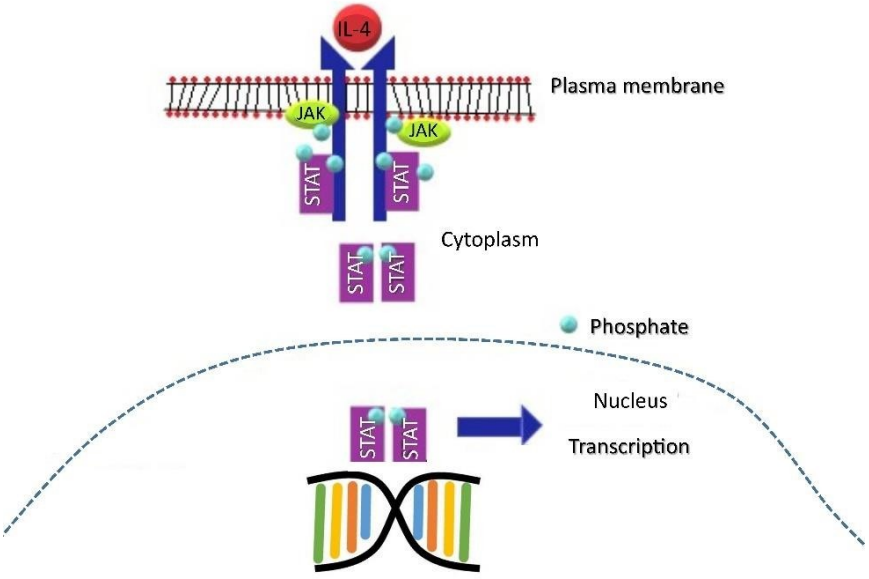

Figure 1 – Diagram of JAK-STAT pathway.

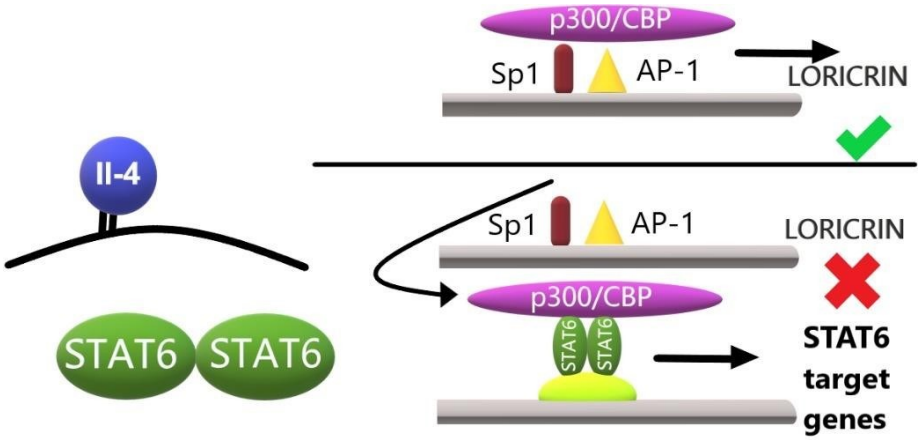

Figure 2 – Chronic IL-4 release, Stat6 uses p300/CREB-binding protein which is also needed for loricrin transcription.

While vastly studied in the skin, there is reason to suspect that dysfunction in the epithelial barrier may also affect the oral cavity[2,11,14,15-23]. Studies have shown that infection with *Porphyromonas gingivalis* (Pg), an important periodontal disease pathogen, can lead to changes in barrier function and CE protein expression by direct and indirect mechanisms[17-23]. Recently, periodontal disease classification has changed to reflect a spectrum disorder, and formerly aggressive periodontitis (or juvenile periodontitis) is now designated as Grade C, Stage III, or IV in this system[24]. For simplicity, we will refer to these forms as severe periodontitis (SvP) IIIC and IVC. Classification as a spectrum disorder implies a similar mechanism, with increased impact according to stage and grade. External (smoking, diabetes) and/or genetic pre-disposing factors may mechanistically combine with the "usual" pathogenesis to create a more severe disease presentation. Previous classifications had considered the pathological mechanism in these severe forms of disease as unique[25]. Like AD and psoriasis, SvP is characterized by a Th2 immune response and studies have shown that in SvP, there is profound downregulation of the CE genes loricrin and filaggrin[26-32]. These studies did not address protein expression or disease manifestation, however.

Mechanistic research to understand AD has yielded multiple mouse models. One example is the Stat6VT transgenic mouse, which has been used extensively in the AD field for over a decade[11,12,32-35]. This mouse was engineered to have a mutant Stat6 transcription factor constitutively expressed in T and B cells via the CD2 locus control region promoter. This mutant form can trigger downstream gene transcription without a stimulus. The decrease in loricrin in

keratinocytes is driven by chronically activated Th2 cytokines[11,12]. These mice manifest lesions over time that resemble human AD. Remarkably, no one has examined the oral cavity of these mice.

Few studies have investigated barrier dysfunction as a mechanism in periodontitis and SvP, and there is no animal model to study the role of the CE in periodontitis[16]. Therefore, our study aims to investigate the localization and expression of CE proteins in human gingival samples of patients that presented with SvP compared to healthy controls, and to determine the impact of CE protein deficiency/mislocalization on periodontal health in the context of infection in mice by Pg.

Methods

Human samples

Gingival epithelium discard samples were collected from 5 patients with gingival health and ten patients with SvP (5 each, Stage III and Stage IV, all Grade C) at the University of Alberta Periodontology clinic following informed consent (Pro00062112). The tissues were collected from gingiva that did not have signs of inflammation. Patients were diagnosed by a second or third year periodontology resident and confirmed by an instructor. There were eight males and seven females ranging in age from 47 to 82 years. All included patients were systemically healthy, not taking any medications and nonsmokers.

Animals

Stat6VT sperm was provided by Mark H. Kaplan (Indiana School of Medicine) for rederivation of Stat6VT mice by the Jackson Laboratory (Bar Harbor, ME). Stat6VT hemizygous mice were mated with wildtype C57Bl/6j mice to yield Stat6VT hemizygotes and littermate controls for our study. Male mice aged 10-12 weeks were used for the periodontal disease study. All procedures were approved by the Animal Care and Use Committee of the University of Alberta (AUP 00002935). Mice were genotyped as previously described [34,48,49].

Sample size

The group number was determined using a power calculation, in which a 25% difference in alveolar bone loss was considered significant. At 80% power, nine mice per group would provide a sufficient sample size. Two extra mice were included per group in case of malocclusion or unexpected morbidities/mortalities (~20%).

Porphyromonas gingivalis infection

Eleven mice per group were orally infected with Pg (ATCC strain #33277) by lavage under anesthesia as previously described[41].

Sample collection and preservation

Five mandibles and masticatory mucosa, from the incisive papilla to the soft palate, were collected from each mouse using a 2.5x microscope for accuracy (DMRE DM2000, Leica). Tissues were initially preserved in 4% formaldehyde at 4ºC for 24 hours and then stored in PBS.

Histology

The mandible was separated into left and right sides by sagittal division. Samples were decalcified in 0.5M Ethylenediaminetetraacetic acid, Disodium Salt Dihydrate, pH 7.4 (Thermo Fisher Scientific) on a rotating platform; the solution was changed every other day for eight weeks [121].

2-5mm thick tissue samples were placed in histologic cassettes and immersed in 50% ethanol for 2 hours before use of the automated processing cycle (Leica Biosystems, TP10). After processing, samples were embedded (EG1160, Leica Biosystems) in Histoplast paraffin (22-900-700, Thermo Fisher Scientific) and positioned using forceps with the aid of a microscope (DMRE DM2000, Leica). Samples were then sectioned (7µm in thickness) using an automated microtome (RM2255, Leica).

For human tissues, the histological process was similar. However, the sample positioning in the cassettes for embedding was different since there was no hard tissue present, no decalcification was needed, and orientation was performed by laying the sample in a way that both epithelium and connective tissue were perpendicular to the metal molding cassette. All further staining described in animal samples applies to humans except from CD45 (53665, Santa Cruz) and Ki67 (SOLA15, Invitrogen), which were not performed in human samples.

Hematoxylin and Eosin staining (H&E)

Tissues were heated at 65°C for 10 minutes to de-paraffinize, and xylene, graded ethanol, and milli-Q water were used to rehydrate the samples (n = 5/group). Slides were immersed in undiluted hematoxylin for 4 minutes, then washed in Milli-Q water and placed in eosin for 30 seconds [122]. Afterward, slides were dehydrated in 95% and 100% ethanol, xylene and then mounted (SP15100, Thermo Fisher Chemical Permount,).

Immunofluorescence

Tissue sections were deparaffinized with xylene and rehydrated with graded ethanol (n = 5/group). Slides were then washed and immersed into antigen retrieval solution (0.6mM citric acid, 10mM trisodium citrate buffer, pH 6.0). After blocking with 10% goat serum (ab7481, Abcam), tissue sections were incubated with primary antibodies against loricrin (85679, Abcam) at 1:100 dilution, filaggrin at 1:100 dilution (NBP1-87528, Novus Biologicals), CD45 (53665, Santa Cruz) at 1:400 dilution, Ki67 (SOLA15, Invitrogen) at 1:300 dilution, CK1 (185628, Abcam) and CK14 (119695, Abcam), both at 1:400 dilution. After overnight incubation with primary antibody, dilutions of 1:500 were used for all secondary antibodies. The secondary antibody for loricrin, CK1, and CK14 was DyLight 488 conjugated goat anti-rabbit (A32731, Thermo Fisher Scientific). The secondary antibody for Ki67 and CD45 was Alexa Fluor 647 conjugated goat anti-rat (A21247, Thermo Fisher Scientific). The stained slides were mounted utilizing SlowFade Gold Antifade Mountant with DAPI to stain nuclei (S36938, Thermo Fisher Scientific) [123].

Alveolar bone measurements

Before decalcification, five fixed heads per group were scanned using three-dimensional micro-computerized tomography (Micro-CT)(Milabs U-SPECT-II/CT) at 25µm voxel size resolution, and scan settings of 70 kVp, 114 µA, 0.5 mm AL filter, and integration time of 500ms. To measure alveolar bone levels, Avizo Software (version 9.1, Thermo Fisher Scientific) and a landmark protocol were employed [36]. Briefly, three landmarks,CEJ, ABC, and root apex (RA) of the first and second mandibular molars on the left side were used to derive our measurements; the distance from the CEJ to ABC was measured in millimeters. Eight measurements were collected per tooth (2 on the sagittal plane – mesial and distal- and six on the coronal plane – distal buccal and lingual, middle buccal and lingual, mesial buccal and lingual). Measurements were obtained by three examiners that were calibrated according to Cohen's kappa inter/Intra-reliability test; the average acceptable error was ≤ 3% or 0.2mm.

Cytokine Array

Relative levels of 96 cytokines were measured using a mouse cytokine antibody array kit (AB193659, Abcam) by comparing five pooled samples from wildtype and Stat6VT mice. After incubation and washes, membranes were developed with a chemiluminescence detection buffer) provided by the manufacturer. Pixel densities were quantified using a ChemiDoc (XRS+, ImageLab software (v 6.1.0), BioRad) with a fixed selection of circular areas placed over the grid-identified spot for each cytokine and chemokine. Each cytokine was measured in duplicate.

Statistical Analyses

Shapiro-Wilk test was used to determine if values were normally distributed. Student's t-test was used for normally distributed data, and Mann Whitney-U test was used if the values were not normally distributed. Significance was set at $p<0.05$.

Results

The expression of CE proteins differs in SvP patients compared to healthy controls.

Tissues from SvP patients, regardless of disease stage, presented with decreased loricrin immunohistochemical signal intensity (Figure 3a-c). The protein was found in unexpected locations, such as spinous or basal layers. Filaggrin was largely contained to the upper layers of the epithelium in healthy and SvP IIIC patients, but became broadly expressed at low levels throughout the epithelium of IVC patients (Figure 3d-f). Cytokeratins in the oral mucosa are stratified according to the differentiation status of keratinocytes. Cytokeratin 1 (CK1) is found in cells entering the differentiation protocol, while cytokeratin 14 (CK14) is found in proliferating basal cells. There was a slight but progressive increase in the distribution of CK1 expression throughout the layers in SvP IIIC tissue samples, accompanied by a decrease in expression (based on comparative fluorescent intensity to healthy control tissue (Figure 3g, h). CK1 distribution was similar to SvP IIIC in SvP IVC patients, but expression fluorescent intensity decreased further (Figure 3i). CK14 was broadly distributed in all layers in SvP IIIC and IVC patients compared to control (Figure 3j-l), and had the greatest expression in SvP IIIC. Hematoxylin and eosin (H&E) staining of human keratinized epithelium showed an increase in clear cells in the SvP IIIC and IVC groups (Figure 3m-o). While the keratinized layer was of similar thickness in disease and healthy tissues, the spinous layer appeared broader in SvP IIIC and SvP IVC.

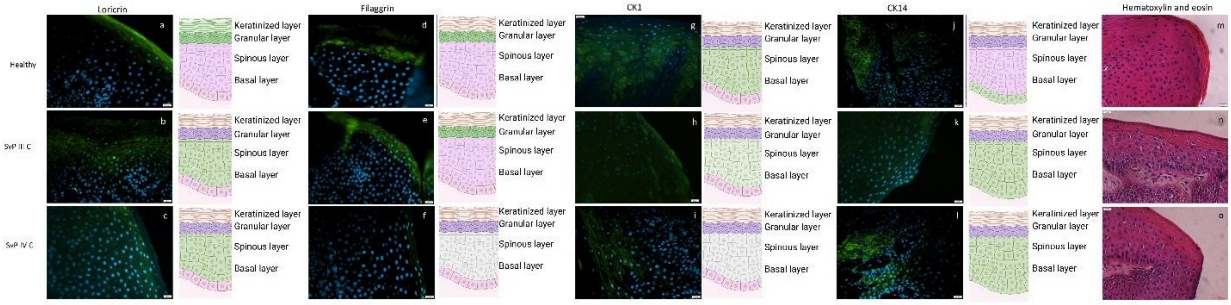

Figure 3 – Gingival samples of healthy and SvP patients stained with loricrin (a-c), filaggrin (d-f), CK1 (g-i), CK14 (j-l) and hematoxylin and eosin (m-o). Scale bar 20μm.

Characterization of Stat6VT mice prior to infection with *Porphyromonas gingivalis*

Stat6VT male mice developed skin lesions at about 10-12 weeks of age; females had earlier and more variable disease onset (data not shown). Faxitron analysis of femurs showed no differences in bone mineral content and bone mineral density for males at this age (Figure 4a-d); females showed significant differences. We assessed alveolar bone loss by a standardized 2-plane landmark microCT protocol[36]. There were 8 measurements each for the 1st and 2nd molars: 2 on the sagittal plane and 6 on the coronal plane. The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was significantly greater at 1 site on the first molar and 4 sites on the second molar in Stat6VT male mice compared with controls (Figure 5a-f, n = 11/group). Neither sex showed any gross signs of oral inflammation. H&E staining of the masticatory mucosa revealed an increase in rete pegs and clear cells that could be indicative of chronic inflammation in male Stat6VT mice. This was confirmed by staining with the pan leukocyte marker, CD45: Stat6VT male mice had a mean of 19.08 ± 0.7432 CD45 positive cells/40x field, compared to control mice, which had a mean of 11.08 ± 1.654 CD45 positive cells/40x field (n = 5/group, p = 0.0012). There was also significantly more proliferation in the epithelium of Stat6VT male mice compared to control, as measured by the number of Ki67 positive cells/40x field (control: 29.57 ± 3.416; Stat6VT: 43.86 ± 3.420, n = 5/group, p = 0.0039). CK1 and CK14 expression were largely similar between the groups (Figure 6a-d). Loricrin expression was equal in intensity, but not confined to the granular layer in Stat6VT mice (Figure 6e, 6f).

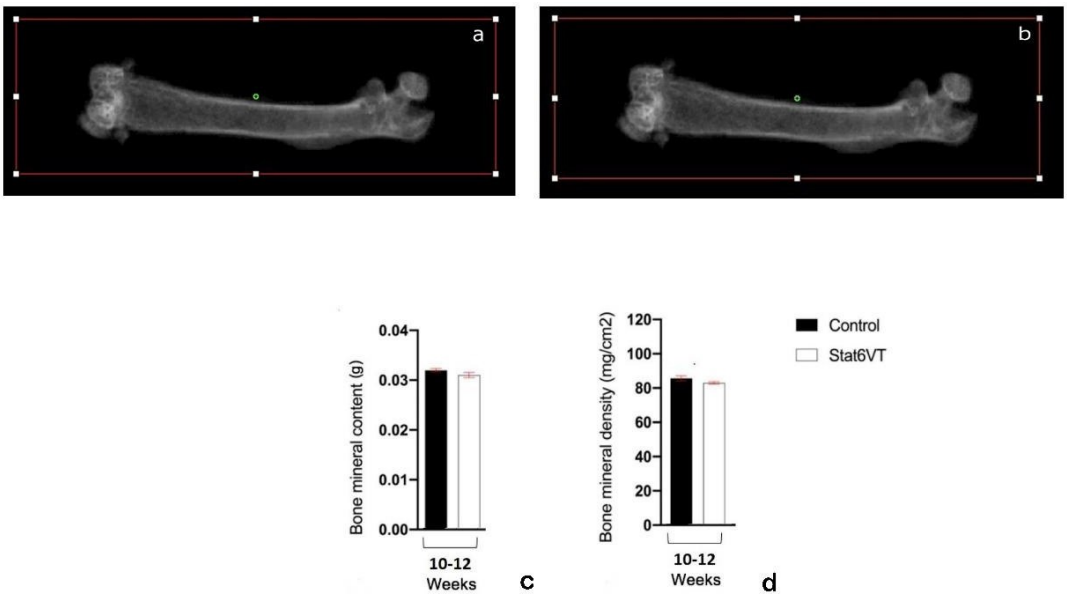

Figure 4 – Bone mineral content and bone mineral density. a: representative control femur (BMC: 0.03230g; BMD: 80.945 mg/cm2); b: representative Stat6VT femur (BMC: 0.03064g; BMD: 80.848 mg/cm2) in the Vision Software (12-week-old male mice; n=11/group); c: graph of femur bone mineral content of male control and Stat6VT (12-week-old male mice; n=11/group).; d: graph of bone mineral density of male control and Stat6VT (12-week-old male mice; n=11/group).

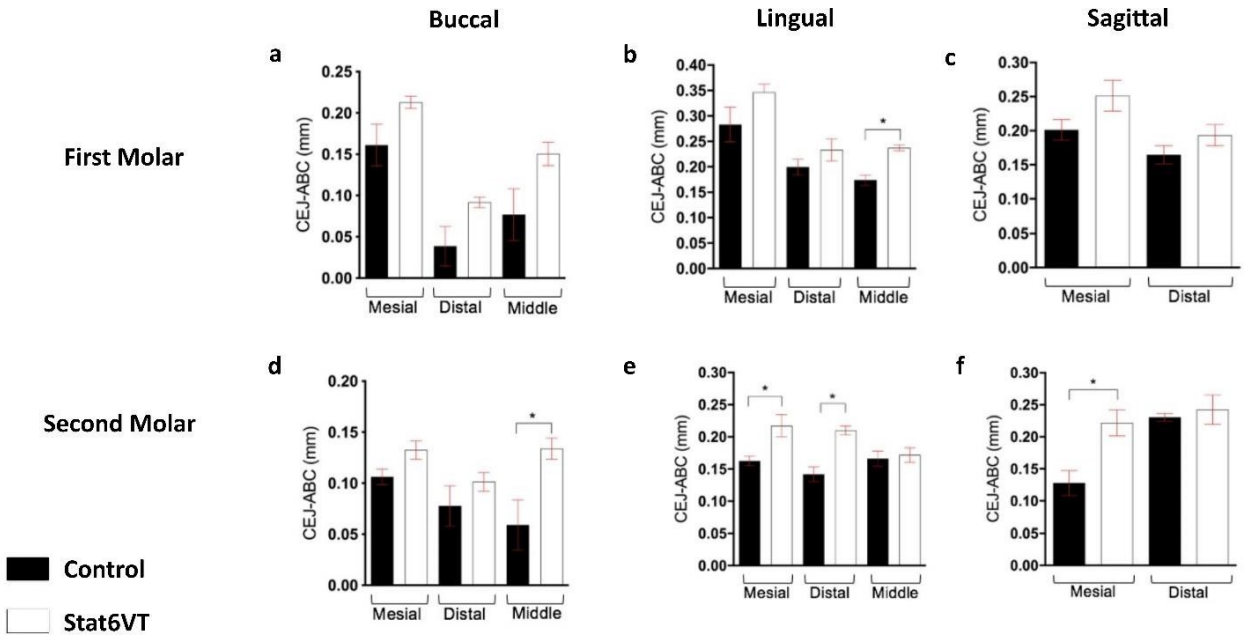

Figure 5 - MicroCT measurements of Stat6VT and controls prior to Pg infection of 12-weeks old male mice (n =5/group) a: first molar buccal aspect, b: first molar lingual aspect, c: first molar sagittal aspect, d: second molar buccal aspect, e: second molar lingual aspect, f: second molar sagittal aspect.

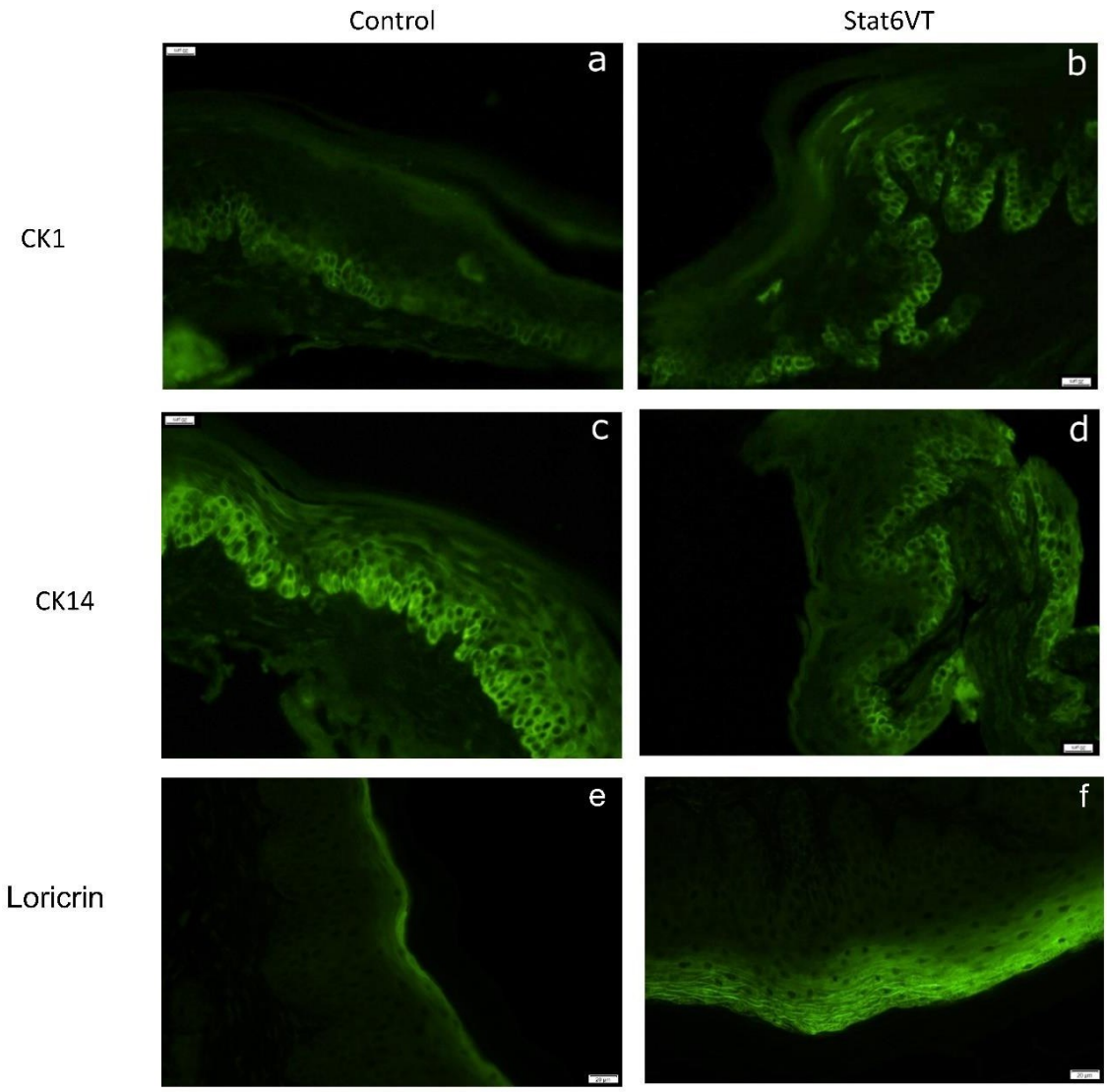

Figure 6 - CK1 positive cells in the masticatory mucosa prior to Pg infection of 12-weeks old male mice (n =5/group) a: control, b: Stat6VT mice. CK14 positive cells in the masticatory mucosa prior to Pg infection of 12-weeks old male mice (n =5/group); c: control, d: Stat6VT mice. Scale bar 20µm. Loricrin staining in the masticatory mucosa prior to Pg infection of 12-weeks old male mice (n =5/group) e: control and f: Stat6VT mice. Scale bar 20µm.

Stat6VT mice have more severe effects of infection with PG

We chose 10-12 week old males for our PD study because disease onset and progression were more uniform, and bone mineral density and content did not differ from controls. We reasoned that skin lesions indicated active disease that could manifest in the oral cavity if

challenged. Pg is a keystone pathogen: it creates a dysbiosis in oral films in low-abundance by orchestrating inflammatory destruction leading to colonization[37-39]. By infecting with this single bacterial strain, studies have shown the development of inflammation and bone loss in animal models consistent with periodontal disease[40,41]. We used a modification of the method of Lalla *et al*., oral lavage with Pg every other day for 2 weeks, to induce infection, and assessed alveolar bone loss by microCT 14 days after the final lavage[42,43]. The distance from the CEJ to the ABC was significantly greater in nine out of 16 assessed sites in Pg-infected Stat6VT mice compared to Pg-infected control mice (n = 5 /group). Three sites were located on the first molar (Figure 7a-c) and six on the second molar (Figure 7d-f).

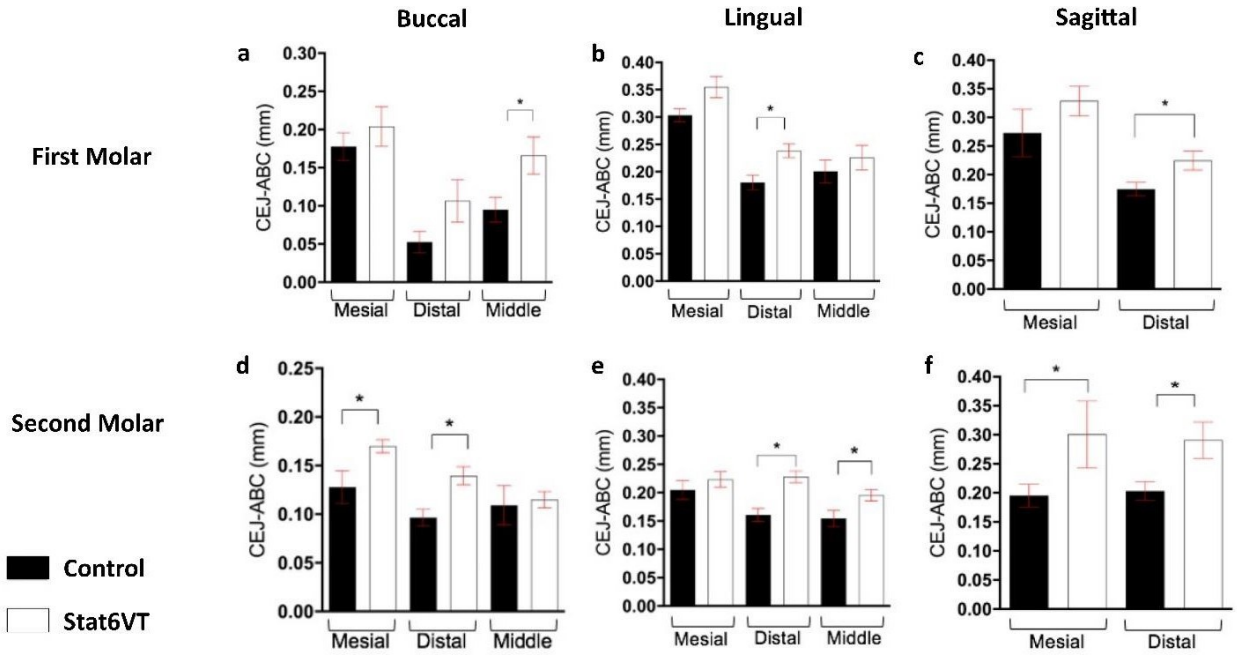

Figure 7 - MicroCT measurements of Pg-infected Stat6VT and controls of 12-weeks old male mice (n =5/group); a: first molar buccal aspect, b: first molar lingual aspect, c: first molar sagittal aspect, d: second molar buccal aspect, e: second molar lingual aspect, f: second molar sagittal aspect.

H&E staining of the masticatory mucosa was again suggestive of increased inflammation in Pg-infected Stat6VT mice compared with controls; there were more clear cells and rete pegs (Figure 8a, 8b). Additionally, Pg-infected Stat6VT mice had patches of cells clustered around the basal layer and across the spinous layer, suggestive of an inflammatory infiltrate. These cells were more sporadic in Pg-infected controls. These findings were confirmed by CD45 staining. Stat6VT

mice had a mean of 35.30 ± 1.539 inflammatory cells/40x field compared with 22.86 ± 1.067 in Pg-infected controls (n = 5/group, p<0.0001) (Figure 8 c-e). These results suggest that the response to infection is greater in Stat6VT mice than in controls.

In normal epithelium, proliferation is greatest in the basal keratinocyte layer, which provides continuous renewal of cells for the upper layers. The nuclear protein, Ki67, usually is expressed exclusively in this basal layer. While this remained the case in Pg-infected controls, in Pg-infected Stat6VT mice, clusters of Ki67 positive cells traveled up the rete pegs (Figure 8f, 8g). This suggests disorganization in the differentiation of the epithelium. The other difference observed was in cell count: there was a greater number of Ki67+ cells in Pg-infected control mice compared with Pg-infected Stat6VT mice (53.97 ± 3.870 *vs.* 38.54 ± 2.637, n = 5/group, p = 0.0013) (Figure 8h).

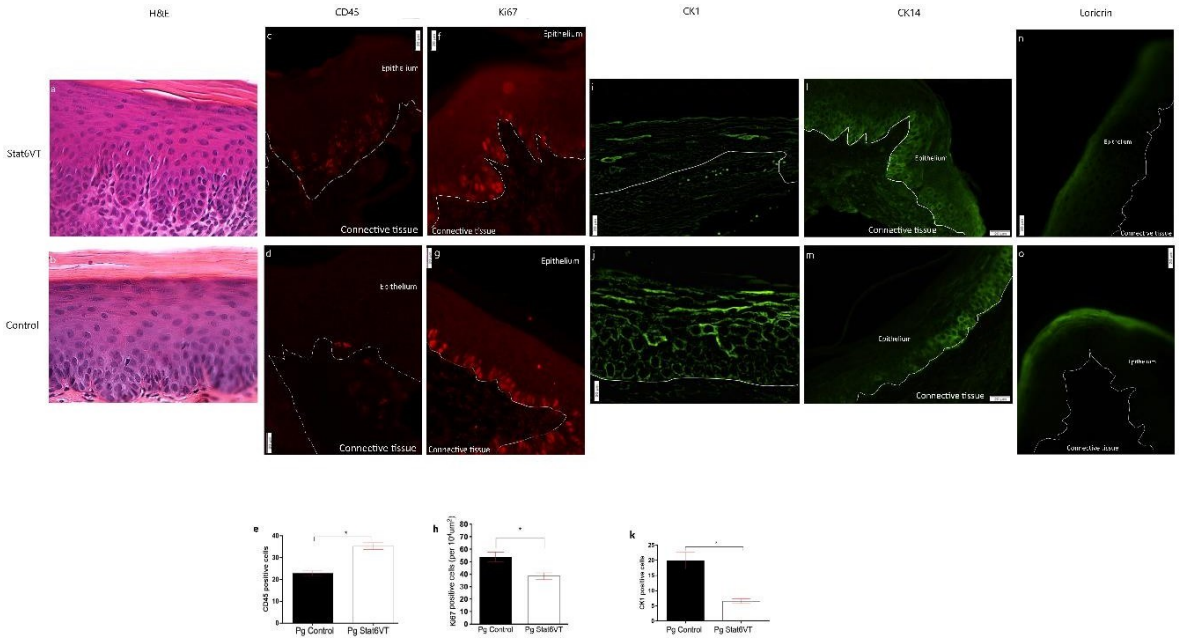

Figure 8 - Hematoxylin and eosin staining of masticatory mucosa of Pg-infected male mice at 12 weeks of age (n =5/group) a: Controls and b: Stat6VT. Scale bar 20µm. CD45 positive cells in the masticatory mucosa of Pg-infected male mice at 12 weeks of age (n =5/group) c: Control, d: Stat6VT mice and e: CD45 positive cell count. The white dashed line demarcates the basal layer of the epithelium for reference. Scale bar 20µm. CK1 positive cells in the masticatory mucosa of Pg-infected male mice at 12 weeks of age (n =5/group) f: Control, g: Stat6VT mice and h: CK1 positive cell count. The white line demarcates the basal layer of the epithelium for reference. Scale bar 20µm. Ki67 positive cells in the masticatory mucosa of Pg-infected male mice at 12 weeks of

age (n =5/group) i: control, j: Stat6VT mice, k: Ki67 cell count. The white line demarcates the basal layer of the epithelium for reference. Scale bar 20μm. CK14 positive cells in the masticatory mucosa of Pg-infected male mice at 12 weeks of age (n =5/group) l: control and m: Stat6VT mice. The white line demarcates the basal layer of the epithelium for reference. Scale bar 20μm. Loricrin staining in the masticatory mucosa of Pg-infected male mice at 12 weeks of age (n =5/group) n: control and o: Stat6VT mice. The white line demarcates the basal layer of the epithelium for reference. Scale bar 20μm.

CK1 expression showed dramatic decrease in Pg-infected Stat6VT mice compared with Pg-infected controls (6.556 $\pm$ 0.76 *vs* 19.94 $\pm$ 2.868 positive cells/40x field, n = 5/group, $p<0.0001$, Mann-Whitney) (Figure 8i-k). CK14, in contrast, was more broadly expressed, extending up into the granular layer and no longer confined to the basal layer as in Pg-infected controls (Figure 8l,8m). Compared with Pg-infected controls, loricrin was not confined to the keratinized and granular layers of the epithelium and was immunofluorescent intensity was decreased (Figure 8n, 8o). These data suggest an overall disruption of the tightly regulated CE differentiation pathway.

Using a mouse cytokine array, 96 cytokines were measured in pooled plasma from 5 mice per group. Stat6VT mice showed 1-fold or greater increase in expression of osteopontin (OPN), intercellular adhesion molecule (ICAM) 1, and insulin-like growth factor binding protein (IGFBP) 2 (there were many cytokines similarly expressed in both groups; these differed between the groups). IGFBP2 was the most increased cytokine (Figure 9).

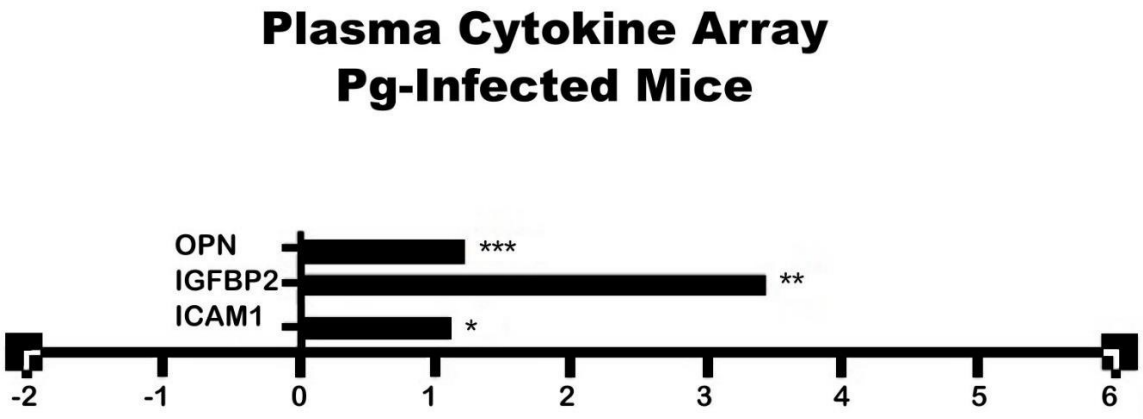

Figure 9 - Plasma cytokine array results expressed as fold change Pg-infected male Stat6VT mice/control at 12 weeks of age (n =5/group).

Discussion

The present study aimed to determine the impact of CE protein deficiency/mislocalization on periodontal health following pathogen challenge in mice, and to compare histological characterization with human SvP. Previous work has shown that deficiencies in CE proteins that affect the epidermis can lead to an intensified host inflammatory response to bacteria involved in disease [44-46]. More specifically, in the oral cavity, loricrin, the most abundant CE protein, is downregulated and is associated with SvP in humans in non-biased gene array studies [26-28,47]. We used a unique mouse model for our studies, the Stat6VT transgenic. This mouse overexpresses a form of the transcription factor Stat6, that is activated in the absence of a ligand, under the direction of the CD2 promoter[48,49]. Elegant mechanistic studies in the AD field showed that constitutive activation of Stat6 mirrors chronic Th2 cytokine stimulation with increased secretion of IL-4[33]. Like AD, SvP also is characterized by a Th2 response. IL-4 receptor-Stat6 signaling requires a transcriptional co-activator, p300/CBP [11]. Sequestration of this co-activator by chronic IL-4 signaling has been shown to negatively affect the expression of keratinocyte CE proteins, loricrin, and filaggrin, which also depend on this co-factor, leading to reduced barrier integrity[33,50,51]. Over time, these mice develop skin lesions similar to human AD. They may serve as a model for SvP, given that recent studies implicate a role for IL-4 in SvP and recognition that a major periodontal pathogen, Pg, can affect CE protein expression via this mechanism[11,52-62]. We were careful to use these mice at a timepoint when there were no differences in bone mineral content, bone mineral density and oral inflammation, but active skin disease.

In addition to the indirect mechanism outlined above, studies have shown that Pg can directly disrupt epithelial barrier function. The most common human isolate of Pg, ATCC 33277, which was used in our study, has been shown to reduce transepithelial electrical resistance in an *in vitro* model of epithelial barrier function[17]. Using the same model, studies implicated a role for gingipains in the degradation of junctional complexes between cells, specifically hydrolysis of E-cadherin[19]. Other studies have shown that exposure of gingival epithelial cells to Pg lipopolysaccharide reduced expression of E-cadherin, occludin and claudin, which are fundamental proteins of the tight junctions[20,21]. Together, this provides evidence that barrier

disruption may be a characteristic of Pg infection. We hypothesized that in the condition of reduced barrier function, the Stat6VT mice, the effects of Pg infection would be exaggerated. Our results revealed enhanced bone loss and inflammation, and dysregulation of CE proteins with disruption of the normal differentiation process. We compared histological findings in the mouse model to human SvP IIIC and IVC. Because periodontal disease is primarily diagnosed at the clinical level, little has been published regarding the histological presentation of SvP. In addition to decreased/disorganized loricrin expression, we found similarities in the change in expression of cytokeratins. Alteration in cytokeratin expression may indicate an oral epithelium under stress[63-66]. A hypothesis for future study is whether SvP patients have already reduced barrier function that is then worsened by Pg infection, leading to greater disease.

Our study made use of refined methodology to measure bone loss. The distance between the CEJ and ABC is one of the most frequent parameters measured to investigate alveolar bone levels in mice[40,67-74]. However, comparisons between studies and human parameters are difficult without a systematic protocol, since measurements are not always performed at specified sites[68,75-81]. In this study, we utilized a recently published standardized protocol to visualize consistent and highly reproducible landmarks for measurement purposes[36]. Across all comparisons, Pg-infected Stat6VT mice demonstrated a greater reduction in alveolar bone levels compared to controls. These findings align with our hypothesis, as we expected an augmented inflammatory response caused by the pathogenic bacteria, with a greater response due to altered barrier integrity in Stat6VT mice. The greatest bone loss observed was in the lingual plane (0.35mm ± 0.1962), followed by the sagittal plane (0.32mm ± 0.02582), which is similar to other studies that used microCT for measuring alveolar bone loss in mice[40,67,68].

H&E staining of oral tissues showed the histopathological changes accompanied by periodontal pathogen infection in Stat6VT and control mice[82,83]. The oral tissues of Stat6VT mice showed an increase in alterations indicative of an inflammatory response, such as clear cells, elongated rete pegs, and areas of basophilic cells grouped from the basal layer to the spinous layer, all above what was noted in controls and uninfected Stat6VT mice[84]. CD45, a pan leukocyte marker, confirmed an increased inflammatory cell presence: there was a 1.59-fold increase in CD45+ cells in Stat6VT tissues compared to controls. These findings mirror what is observed in patients with SvP[25,85].

Cytokeratins constitute the structural foundation of epithelial cells; they participate in mechanical support, maintenance of cell architecture, intercellular transport, junction formation and nuclear integrity, and are ultimately crucial for protein synthesis [63]. Cytokeratins connect with desmosomes and hemidesmosomes to form tight junctions between cells, the extracellular matrix, and surrounding cells. Defects in junctions contribute to various skin and bowel diseases and increase systemic exposure to bacterial lipopolysaccharide[86].

CK1 is typically observed in supra-basal layers of the keratinized oral epithelium and skin, while CK14 is generally observed in the normal stratified epithelium (in both keratinized and non-keratinized tissues)[63-66]. In pathogen-challenged Stat6VT oral epithelium, CK1 immunofluorescence signal was dramatically decreased compared to controls. Similarly, in human oral epithelium from SvP patients, there was a gradual but less dramatic signal decrease that continued to decrease with increased stage of the disease. CK1 expression normally increases during cell turnover and healing processes, since it is involved in epithelial barrier maintenance, repair, and the processing of DNA, cell proliferation, apoptosis, and differentiation[87-89]. Studies show CK1 dysregulation in inflammatory and proliferative diseases[88,89]. Downregulation of CK1 and other important cytokeratins at the gene level have been observed in induced human gingivitis after 21 days, and may be considered a marker of chronic epithelial stress[90]. Given the functions of CK1, reduction could profoundly affect the healing abilities of Stat6VT and human epithelium and contribute to faster periodontal disease progression.

CK14 is fundamental for the normal function of the epithelium and is a marker of proliferating cells; mutations are found to underlie several skin disorders[91,92]. Once cells enter into the differentiation program, CK14 is silenced. Expanded expression of this cytokeratin to supra-basal layers is observed in chronic wound healing or poorly differentiated carcinomas[93]. As in the case of CK1, the mislocalization of CK14 in Stat6VT mice and human SvP patients could be a marker of a stressed and inflamed oral epithelium, and also suggests less terminal differentiation[87,88,93]. The loss of CK1:CK10 in supra-basal layers as a result of replacement by CK14 may impair the differentiation process, reduce epithelial strength and consequently impair barrier integrity[94]. Increased CK14 expression has been associated with greater epithelial fragility, such that even mild trauma can lead to cytolysis[95].

Control and Stat6VT mice both demonstrated loricrin expression in the oral epithelium, and before pathogen challenge, the major difference was broader expression in Stat6VT mice. In

human patients, as well, regardless of disease/healthy state, loricrin could be detected. After Pg infection, Stat6VT mice had both weaker expression and distribution beyond the uppermost layers compared to controls. In control mice, loricrin was confined to the granular layer and above, whereas in Stat6VT mice, loricrin could be detected in most suprabasal layers. This was similar to what was observed in healthy and SvP patients. In healthy oral epithelium, loricrin showed confined expression to the upper layers of the epithelium. In contrast, there was a profound decrease in loricrin immunofluorescent signal intensity in all SvP patients and a broader distribution. Previous studies have shown that loricrin expression is tightly regulated and observed in the granular/cornified layer of keratinized and parakeratinized oral epithelium and, more rarely, in the intermediate layer of non-keratinized epithelium[84,96]. The protein contains several transglutaminase cross-linking sites; cross-linking between loricrin molecules and with other proteins in the CE creates an effective barrier between the outer and inner environment[6]. Further study is necessary to understand whether the wider distribution of loricrin in non-keratinized layers of the oral epithelium has functional consequences. It will also be important to ascertain if loricrin is cross-linked, and interacts with other proteins in these other layers. If loricrin is not expressed appropriately in the CE (amount and localization), the barrier can be compromised, leading to disorders such as Vohwinkel syndrome, psoriasis, AD, and periodontitis[5,45,97,98].

Ki67 is normally present during all active phases of the cell cycle (G1, S, G2, and M) and only absent in resting or terminally differentiated cells (G0)[99,100]. Ki67 positive cells should thus be delimited mainly to the basal layer in the oral epithelium, as observed in our control animals[101]. The appearance of Ki67 in supra-basal layers has been related to oral epithelial dysplasia, which is a change in the maturation of epithelial cells and an increase in the proliferation up to spinous layers[101,102]. This is an interesting finding for Pg-infected Stat6VT mice, since proliferation and differentiation are controlled by autocrine and paracrine elements produced by the keratinocytes, but in alignment with the derangement observed in CE protein distribution and expression[101]. In Pg-infected Stat6VT mice, the number of Ki67+ cells in the basal layer was reduced relative to control (opposite to what was observed in the uninfected mice). This again suggests greater chronic inflammation in these mice[101,102]. These results increase the necessity for more studies involving the role of CE proteins and barrier function in the oral cavity.

Many cytokines were similarly expressed in Stat6VT and control mice infected with Pg, but those that were increased above levels in control mice were quite compelling considering their

function and relationship to periodontal disease. The significantly increased cytokines in the Stat6VT mice were IGFBP-2, OPN and ICAM-1. IGFBP-2 was increased nearly four-fold, whereas OPN and ICAM-1 were increased slightly over one-fold. IGFBP-2 plays a significant role in bone metabolism with differential effects in males and females[103]. It rises with age and strongly predicts decreasing bone mineral density[104-106]. Consistent with our findings, IGFBP-2 expression was shown to be induced by Pg, and studies in humans have shown that greater concentrations of IGFBP-2 correlate with increased probing depth and may be an indicator of periodontal disease progression[107,108]. OPN, also known as bone sialoprotein 1, plays a role in periodontitis since it is essential for bone remodeling and biomineralization during tension and stress[109]. Studies have shown that this cytokine increases in periodontitis and is related to increased proliferation and differentiation of osteoclasts[109-113]. Thus, the increased bone loss observed in Stat6VT mice is consistent with the increased expression of OPN. ICAM-1 has also been implicated in a variety of chronic systemic immune and inflammatory responses and is overexpressed in inflamed gingival tissues[114]. In addition, expression of ICAM-1, an adhesion receptor, on human microvascular endothelial cells has been shown to facilitate invasion by Pg[114-118]. Increased invasion would enhance the speed of progression of the disease, as observed in our mice and SvP[119,120].

In summary, we used a model of epithelial barrier dysfunction in mice to probe its impact if superimposed with Pg infection, and to compare with histological and immunohistological characteristics of SvP IIIC and IVC in human patients. Our data show a change in amount and localization of loricrin, CK1 and CK14, as well as other signs of increased inflammation and epithelial stress in Stat6VT mice that has similarities to changes in patient tissues. Our study provides support for further work on the role of epithelial integrity in all stages of periodontal disease.

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Conflict of Interest

The authors declare no conflict of interest

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CHAPTER 8

RESULTS

ORAL MICROBIOME ANALYSIS AND STEPHAN CURVE KINETICS IN WHITE SPOT LESION DEVELOPMENT IN ORTHODONTIC PATIENTS WEARING FIXED APPLIANCES

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ABSTRACT

Background: White spot lesions (WSLs) are a formidable challenge during orthodontic treatment, affecting patients regardless of oral hygiene. Multifactorial in nature, amongst potential contributors to their development are the microbiome and salivary pH. The aim of our study is to determine if baseline differences in Stephan curve kinetics and salivary microbiome features correlate with WSL development in orthodontic patients with fixed appliances. We hypothesize that non-oral hygiene determined differences in saliva could be predictive of WSL formation in this patient population through analysis of Stephan curve kinetics, and that these differences would further manifest as changes in the oral microbiome. **Methods**: In this prospective cohort study, twenty patients with initial simplified oral hygiene index scores of "good" that were planning to undergo orthodontic treatment with self-ligating fixed appliances for at least 12 months were enrolled. At baseline, saliva was collected for microbiome analysis, and at 15-minute intervals after a sucrose rinse over 45 minutes for Stephan curve kinetics. **Results**: 50% of patients developed a mean 5.7 (SEM) 1.193 WSL. There were no differences in saliva microbiome species richness, Shannon alpha diversity or beta diversity between the groups. *Capnocytophaga sputigena* exclusively and *Prevotella melaninogenica* predominantly were found in WSL patients, while *Streptococcus australis* was negatively correlated with WSL development. *Streptococcus mitis* and *Streptococcus anginosus* were primarily present in healthy patients. Significant moderate correlations with Stephan curve parameters and the following species in WSL developers were found: *Selenomonas spp, Stomatobaculum spp, Butyrivibrio spp, Gemella spp, and Eubacterium nodatum group sulci.* **Conclusions**: While there were no differences in salivary pH or restitution kinetics following a sucrose challenge and no global microbial differences in WSL developers, our data showed salivary pH association with an abundance of acid-producing

bacteria in saliva. The results suggest salivary pH modulation as a management strategy to inhibit the abundance of caries initiators. Our study may have uncovered the earliest predecessors to WSL/caries development.

Keywords: white spot lesion, microbiome, saliva pH

BACKGROUND

The formation of white spot lesions (WSLs) during orthodontic treatment is an aesthetic and morphological adverse effect caused by plaque-mediated demineralization of the tooth surface around brackets. They are associated with changes in enamel morphology and can lead to dissatisfaction with tooth surface appearance. [1, 2] The incidence and prevalence of WSL development during orthodontic treatment vary between 23.4-72.9% and 30-75.6%, respectively, depending on the assessed parameters (age, diagnosis criteria, initial caries assessment). Incidence is generally assessed throughout orthodontic treatment (18-24 months on average), and for most studies that included prevalence, patients were at least 12 years old. [3-14]

Although the expectation is that WSL occurrence in orthodontic patients results from inadequate oral hygiene, Geiger *et al.* showed that even when oral hygiene compliance was moderate to excellent, 42% and 15% of such patients, respectively, still developed WSLs. [4] Another study found that patients with good or moderate oral hygiene still had a prevalence of WSLs ranging from 23-68%, with an average development of 1 new WSL for patients that had good oral hygiene, 1.4 WSLs for patients with moderate oral hygiene and 3.3 WSLs for patients with poor oral hygiene (follow-up 9 to 25 months). [15] These studies suggest that WSL formation is multifactorial and is not solely dependent on competency in performing oral hygiene.

While WSLs share the same demineralization process as caries, their clinical presentation and lack of cavitation are more considered an aesthetic compromise and a precursor to frank dental caries. Fixed orthodontic appliances render oral hygiene more challenging, which, similar to interproximal spaces, favors the creation of new habitats for biofilm accumulation, leading to loss of enamel hydroxyapatite and, finally, the clinical appearance of white spots. A recent study of the microbiota in different types of carious lesions, from WSLs to dentin caries, showed that *Streptococcus mutans,* considered to be highly associated with caries, were low in abundance in WSLs, comprising only 0.73% of the total bacterial community. [16, 17] Interestingly, WSLs had

lower richness and diversity than open dentin cavities. *Streptococcus*, *Rothia*, *Leptotrichia* and *Veillonella* were found at higher levels in carious enamel lesions, whereas *Lactobacillus*, *Shlegelella*, *Pseudoramibacter* and *Atopobium* were associated with dentin lesions. This supports the etiologic contribution of non-mutans bacterial species and their roles in the spectrum of the development of caries. [18-21]

The essential association between pH and caries formation was illustrated by the work of Stephan in 1944. [22] In his classic clinical trial, he showed that a sucrose mouth rinse led to a drop in pH in dental plaque, followed by gradual restoration to baseline over time ("Stephan curve"). The three phases of the Stephan curve are 1. rapid drop in pH, due to fermentation of sucrose by acid-producing bacteria; 2. demineralization of enamel if the pH drop is below 5.5; 3. gradual increase back to baseline within 30 to 60 minutes. In Stephan's study, patients could be classified into caries-free to high caries activity groups based on the association between pH drop and the development of lesions over 12 months. [22] The same pattern of acidification and neutralization occurs in saliva. Saliva has buffering properties that typically act to prevent caries; if the acid neutralization effect of saliva is diminished, this may lead to a higher prevalence of WSLs. [23] In addition, reduced buffering capacity could lead to prolonged low salivary pH, which would effectively select for acidogenic and aciduric bacteria, potentially resulting in saliva acting as a microbial seeding reservoir to accumulating plaque around orthodontic brackets. [24] Saliva buffering capacity is independent of oral hygiene and most likely genetically determined in healthy individuals.

Most orthodontic WSL studies have focused on one aspect of the multifactorial causation, e.g. specific bacterial species, rather than the microbiome as a whole. They have not considered the dynamic protective effects of saliva. [3, 6, 10-13, 25, 26] As such, our understanding of the association between inherent saliva buffering differences in healthy individuals and the formation of WSLs around orthodontic brackets is incomplete. The primary objective of this prospective

cohort study is to determine if baseline Stephan curve kinetics are associated with WSL development, with a secondary objective of investigating the contribution of the salivary microbiome at baseline on Stephan curve kinetics and the development of WSLs.

Methods

Study design, inclusion and exclusion criteria. The sample size for control and WSL patients (cases) was calculated at 80% power with a confidence level of 95% (precision of at least 5% is recommended when expected prevalence ranges between 10-90%). [9] Previous experience in the orthodontic clinic at the University of Alberta showed that 60% of patients developed some signs of a WSL (Flores-Mir, C., unpublished observations). As a result, the number of patients needed was 16 (8 per group). Due to the study length, to allow for possible drop-out of 20%, we set a recruitment target of 10 patients per group, for a total of 20 patients.

Study participants were recruited according to a protocol approved by the University of Alberta Health Research Ethics Board (Pro00099341). Written informed consent or assent was obtained from all participants. Patients were assessed for good oral health before appliance placement based on the simplified oral hygiene index (OHI-S, Green and Vermillion, 1964) prior to start of treatment and every three months. [27] Briefly, six teeth were scored for their debris and calculus indexes (Fédération Dentaire Internationale (FDI) notation: #16: upper right 1st molar, #11: right upper central incisor, #26: upper left 1st molar, #36: lower left 1st molar, #31: left lower central incisor, #46: lower right ^{1st} molar). Debris was scored 0-3 as follows: 0: no debris, 1: soft debris < ⅓ of tooth surface, 2: soft debris >⅓ and <⅔ of tooth surface, and 3: debris covering >⅔ of tooth surfaces. A similar scoring was done for calculus based on crown coverage. The sum for each index was divided by the number of teeth examined (6) and then added together for the total OHI-S index score. The scores related to oral health are as follows: 0.1-1.2: good; 1.3-3.4: fair; 3.1-6.0: poor. [10] Patients with OHI-S scores between 0.1-1.2 (good) that had fully erupted second molars, and a treatment plan for fixed self-ligating orthodontic appliances (at least from

1st molars to 1st molars) for a minimum of 12 months duration, were recruited. There were no restrictions pertaining to age, sex or type of malocclusion. Patients who had any intra-oral appliances other than fixed self-ligating brackets, had any surgery planned during treatment or WSLs of any origin at the beginning of treatment were excluded. Patients that had systemic disease, were smokers or on medication were also excluded. Those enrolled had their hygiene appointments within three months prior to bracket placement. Patients were advised to brush twice a day with a soft-bristled toothbrush and fluoride toothpaste (1450 ppm) and floss daily. All patients also received standard dietary advice, such as to avoid hard/sticky/crunchy foods and sugary drinks. Patients that did not maintain an OHI-S 0.1-1.2 score throughout the treatment were also excluded.

WSL assessment. WSLs were assessed according to the modified WSL index (Gorelick *et al*., 1982) by evaluating the buccal surface of individual teeth for presence or absence and severity. [6] Scores were noted at treatment start (all = 0) and every three months until month 12. The assessment was performed under direct illumination using a dental chair light after drying the teeth with compressed air for 5 seconds.

Saliva collection and Stephan curve kinetics. Stephan curve kinetics were determined before the placement of fixed appliances. Patients were asked to refrain from eating, drinking or chewing gum for 30 minutes. Patients rinsed with water for 30 seconds, and an initial ~1 ml sample of saliva was obtained for pH analysis and ~2 ml for microbiome analysis. Next, patients rinsed with a 10% sucrose solution for 30 seconds, and saliva samples were collected after 5, 15, 30 and 45 minutes. pH was determined using a microelectrode (Cole Parmer pH meter PH6+, Quebec, QC).

DNA isolation and sequencing. Immediately after obtaining the saliva samples, the steps recommended by the manufacturer of the kit used were followed to preserve the DNA (Microbiome DNA Isolation Kit, Norgen Biotek Corp, Thorold, ON). For 16s rDNA amplicon sequencing, primers for two sets of variable regions were used, as one or the other may, in

some cases, more effectively identify genera, and this allowed for the retrieval of a broader microbiome spectrum than what would be achievable with one primer set. The V1-3 region was sequenced using the primers 27F (ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAKRGTTYGATYNTGGCTCAG) and FwR1 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGTNTBACCGCDGCTGCTG). The V4-5 region was sequenced using the following primers: Forward: 515FP4-FwR1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAGTGCCAGCMGCCGCGGTAA) , 515FP3-FwR1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTGCCAGCMGCCGCGGTAA), 515FP2-FwR1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGCCAGCMGCCGCGGTAA), 515FP1-FwR1 (ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA), and reverse: 806RP4-RvR2 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATGGACTACHVGGGTWTCTAAT), 806RP3-RvR2 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGGACTACHVGGGTWTCTAAT), 806RP2-RvR2 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGACTACHVGGGTWTCTAAT), and 806RP1-RvR2 (GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT).*

*Recognition sequences representations in this primer:
W: A or T; H: A or C or G; V: A or C or G; M: A or C; N: A or C or G or T; K: G or T; R: G or A ; B: C or G or T; D: A or G or T

Sequencing was done using the Illumina MiSeq platform. Adaptors were removed using CutAdapt. [28] Parameters for trimming and overlap needed for merging were determined with Figaro. [29] Merged sequences were algorithmically corrected to produce Amplicon Sequencing

Variants (ASVs) using software DADA2. [30] DADA2 was used to bin the nucleotide-corrected ASVs to their identifying taxa using the naive Bayesian classifier against a SILVA rRNA database (v138.1, provided by DADA developer here: (https://zenodo.org/record/4587955#.Ykc0By971jc) using the assignTaxonomy command (Supplementary files 1 and 2). [30] Next, each ASV was condensed based on their taxonomy to yield a table where each taxa is represented only once [phylotoast reference: https://doi.org/10.1038/srep29123]. Analysis was done at this taxonomy level. To avoid double counting the same species, primer averaging was done. [31] This results in forming species-level OTUs (s-OTUs). Next, we condensed the counts of the s-OTUs to species so that each species is unique in our table of features, by summing identical s-OTUs; the function was described here (doi: 10.1038/srep29123).

Statistical analysis. Data were entered into Excel (version 2208, Microsoft), and statistical calculations were done in IBM Statistical Package for Social Sciences (SPSS, version 28). For Stephan curve analysis, the mean and standard error of the mean (mean and SEM) of pH were calculated for each assessment time and were stratified between the case and control groups. Data normality was verified by the Kolmogorov-Smirnov test. The alpha level to determine significance was 5%. Repeated-measures ANOVA was performed to compare pH at the different Stephan curve time points with occurrence of WSLs, using Bonferroni correction for multiple comparisons. For microbiome analysis, alpha and beta-diversity were interrogated using an automated pipeline (Forays into Automating Laborious Analysis of Phylogeny (FALAPhyl): https://github.com /khalidtab/FALAPhyl). Feature-abundance testing was examined via Linear discriminant analysis (LDA) Effect Size (LefSe). [32] Since it was unknown if the effect of pH on bacteria would follow a linear or unimodal function, Spearman correlation and canonical correspondence analysis (using Vegan in RStudio) were performed to interrogate the two relationships, respectively. [33, 34]

Results

Table 1 shows the descriptive statistics of the patients included in this study. The increase in age in the control group was driven by 3 patients that were above 40 years of age. The mean age and SEM of the other 7 controls was 14 + 1.024. Figure 1 shows the aggregate Stephan curves of the WSL group versus control; there were no statistically significant differences at any time points ($p>0.05$, repeated measures ANOVA). In the control group, a comparison of pH at each data point between those greater than 40 years of age with those less than 40 years of age also showed no significant differences.

Table 1. Results from the analysis of variance for the representative terms of the data and collinearity.

	Age (SEM)	Male/female ratio	WSL count (SEM)	Sample size
Control	23.00 (4.638)	1:4	N/A	10
WSL	13.10 (0.3480)	1:1	5.70 (1.193)	10

Alpha diversity rarefaction curves of the observed taxa showed that we had adequate sequencing depth (Supplementary Figure 1). In terms of the saliva microbiome analyses, there were no global differences between the two groups in terms of Chao-1 index, a measure of alpha diversity or species richness ($p>0.05$, Mann–Whitney, Supplementary Figure 2A, B), Shannon diversity index ($p>0.05$, Mann–Whitney), or beta diversity, a measure of the similarity between the groups ($p>0.05$, ADONIS of Phylogenetic Isometric Log-Ratio (PhILR) distances, Supplementary Figure 2C).

Canonical correspondence analysis was performed on the log-transformed rarefied taxa counts to assess the associations between the different terms and species using two models. The first model used raw pH terms and did not result in significant associations ($p > 0.05$). The second model used pH as a function of restitution to baseline. The restitution of pH was coded as delta

pH (ΔpH) between the desired timepoint compared to the initial ($pH_{initial}$ - pH_x) where x = time in minutes (e.g., ΔpH_5, where x= 5 minutes). Therefore, the drop from initial pH was represented as a positive number; higher positive numbers represented a greater difference from baseline pH. The second model resulted in ΔpH5 being the best representation of the explanatory variables for the log transformed taxa counts**,** as shown in Table 2.

Table 2. Results from the analysis of variance for the representative terms of the data and collinearity.

Variables	Degrees of freedom	Chi-Square	F	P-value
ΔpH_5	1	0.03809	1.9524	0.018*
Residual	18	0.35119		

*p < 0.05

Constraining (canonical) the model reduced the inertia from 0.35119 to 0.03809; as such, these variables were responsible for 10.846% of the inertia/variance within the model. The unimodal effect of the terms on species abundances was graphically interrogated (Figure 2). Early pH restitution (ΔpH_5) affected species abundances in agonistic and antagonistic fashions.

The LEfSe method was used to support high-dimensional class comparisons of the taxa (Figure 3). *Capnocytophaga sputigena*, a gram-negative acid-producing bacterium, was exclusively found in WSL patients. Interestingly, *Prevotella melaninogenica,* a pathogen primarily associated with periodontal disease, was also mostly observed in WSL patients. Bacteria such as *Streptococcus mitis* and *Streptococcus australis* were primarily present in healthy patients. *Streptococcus mitis* has been reported as one of the least likely bacteria to contribute to caries, whereas *Streptococcus australis* is considered part of the *Streptococcus mitis* group and is known for arginine hydrolysis and production of alkaline phosphatase. [35]

We then examined the correlations between the pH values and the abundance of the taxa

after centered-log ratio transformation. We similarly investigated the correlation of mean WSL count (WSL group only). Since data normality distribution was violated ($p>0.05$, Shapiro-Wilk test and Kolmogorov-Smirnov test), Spearman correlation was performed. The bacterial species that showed statistically significant correlations ($p<0.05$) are described in Table 3.

Table 3. Significantly correlated variables with rarefied species counts as tested by Spearman correlation ($p<0.05$). Mean and standard error of the mean of the white spot lesion groups and control added to illustrate average counts across the two groups.

Variable tested		**Mean (SEM)**	**Bacteria**	**Correlation**	**p-value**
WSL		**Control**			
Baseline	7.13	7.30	*Eubacterium nodatum group sulci*	-0.4864	0.0296
pH 0	(0.10)	(0.10)			
			Fusobacterium periodonticum	-0.4457	0.0488
			Granulicatella adiacens	0.4892	0.0286
			Lautropia spp	0.4456	0.0489
			Neisseria oralis	0.4896	0.0284
			Stomatobaculum spp	-0.5094	0.0217
pH 5	6.36	6.57	*Eubacterium nodatum group sulci*	-0.5509	0.0118
			Leptotrichia wadei	-0.4999	0.0248
			Selenomonas spp	-0.5656	0.0093
			Stomatobaculum spp	-0.6110	0.0042
pH 15	6.48	6.70	*Eubacterium nodatum group sulci*	-0.4873	0.0293
	(0.16)	(0.09)			
			Butyrivibrio spp	-0.5592	0.0103
			Capnocytophaga leadbetteri	0.4503	0.0463
			Catonella spp	-0.4888	0.0287
			Clostridia UCG-014 spp	-0.4542	0.0442
			Granulicatella adiacens	0.4768	0.0335
			Lactobacillales P5D1-392 spp	0.4863	0.0297
			Mogibacterium diversum	0.4441	0.0497
			Neisseria oralis	0.4677	0.0375

pH 30	6.85 (0.13)	6.93 (0.11)	*Capnocytophaga leadbetteri*	0.4841	0.0305
			Prevotella 7 scopos	-0.4636	0.0395
pH 45	6.90 (0.08)	7.21 (0.11)	*Lautropia spp*	0.4733	0.0350
			Prevotella 7 scopos	-0.4945	0.0266
			Prevotella oris	-0.4670	0.0378
White spot lesion	5.70 (1.193)	N/A	*Aggregatibacter spp*	0.4921	0.0275
			Capnocytophaga sputigena	0.5047	0.0232
			Neisseria mucosa	0.4699	0.0365
			Neisseria sicca	0.5345	0.0151
			Streptococcus australis	-0.6825	0.0009
Age	13.10 (0.34)	23.00 (4.63)	*Fusobacterium periodonticum*	-0.2663	0.0266
			Alloprevotella rava	-0.2170	0.0151
			Lautropia spp	-0.3018	0.0147
			Neisseria mucosa	-0.2540	0.0222
ΔpH5	0.73 (0.11)	0.76 (0.07)	*Gemella spp*	-0.6402	0.0004
			Megasphaera micronuciformis	0.4792	0.0246
			Prevotella oris	-0.4307	0.0423
			Selenomonas spp	0.6548	0.0013
			Streptococcus infantis	-0.3299	0.0306
			Veillonella dispar	0.4988	0.0631
ΔpH15	0.64 (0.10)	0.60 (0.08)	*Fusobacterium periodonticum*	-0.1382	0.0189
			Actinomyces viscosus	0.1210	0.0451
			Neisseria mucosa	-0.1104	0.0076
			Prevotella nanceiensis	0.2462	0.0387
			Prevotella pallens	0.02020	0.0115
			Solobacterium moorei	-0.01193	0.0020
			Stomatobaculum spp	0.1458	0.0076
			Streptococcus cristatus	-0.3668	0.0471
			Streptococcus mitis	-0.2429	0.0113
			Veillonella rogosae	0.3013	0.0329

ΔpH45	0.20 (0.06)	0.26 (0.06)	*Oribacterium sinus*	-0.48500.0301
			Prevotella oris	0.48820.0289
			Tannerella spp	-0.48020.0321

Discussion

Caries result from a dynamic process in which a dysbiotic biofilm facilitates the demineralization of tooth enamel through the production of acid by the microbiota. [36] WSLs are the first clinical indication of this process, hence the importance of understanding initiating and possible inhibiting factors. The increase in WSLs during orthodontic treatment is a significant concern to clinicians, given that even patients with excellent to good oral hygiene still develop some degree of demineralization during treatment. [15] In this study, we compared two groups of patients treated at the same time in a graduate orthodontic clinic: those that did or did not develop WSL, in accordance with the inclusion/exclusion criteria outlined in the methods section of this paper. Our hypothesis was that non-oral hygiene determined differences in saliva, prior to bracket placement, could be predictive of WSL formation in this patient population, through analysis of Stephan curve kinetics, and that these differences would further manifest as changes in the oral microbiome.

In the WSL group, 5.70 (SEM: 1.193) WSLs per patient (range 1-12 WSL) developed. This was despite efforts to minimize WSL formation through patient selection and appliance type. We only enrolled patients with good oral hygiene (OHI-S scores between 0.1-1.2), and this was maintained throughout the study length for patients to be included at the end. Because not all orthodontic devices/approaches (e.g. self-ligating brackets, removable appliances) have the same predisposition to affect oral hygiene, we only included patients with a treatment plan that used archwires with self-ligating brackets and excluded those with elastomeric rings, since they make cleaning more difficult. [36-40] In this way, our study was designed to capture inherent

differences in saliva in an orthodontic setting in a patient pool in which oral hygiene should be well maintained. In line with what has been reported in the literature and previous experience in our clinic, however, ~50% of our patients still developed WSL. [37] This finding alone indicates a need for greater research into the factors that contribute to the development of these lesions.

Saliva Stephan curve kinetics showed no statistically significant differences between the groups across the different time points. Stephan originally performed measurements in plaque, but as in our study, others have focused on salivary pH and pH recovery. [22, 38-41] Salivary pH is a major factor controlling plaque pH. [42] In studies measuring salivary pH in healthy, caries-free/inactive individuals, baseline values ranged from 6.8 to 7.6, similar to our findings. [39, 43] In our study, baseline salivary pH alone was not a good predictor of WSL occurrence in orthodontic patients.

Classical paradigms investigate the effect of pH over time on various tooth structures as related to demineralization properties and microbiome changes compared to a baseline state as a result of some perturbation. In this study, we correlated Stephan curve kinetics with initial patient microbiomes to try to define an at-risk population for WSL formation. Indeed, while the raw pH changes were not significantly associated with any unimodal microbial abundance changes, pH drop at 5 minutes was significantly associated with the abundances of specific species. This suggests that the severity of pH change (at 5 minutes) from baseline was significantly associated with specific bacterial species abundances, confirming that saliva, as an ecological environment, is influenced by such pH perturbation.

CCA and Spearman correlation results suggested that the group of bacteria that moderately negatively correlated with pH 5 in WSL developers, *Eubacterium nodatum* group sulci, *Leptotrichia wadei*, *Selenomonas spp* and *Stomatobaculum spp* are all involved in oral diseases. *Leptotrichia wadei*, *Selenomonas spp* and *Stomatobaculum spp* are acid producers, while *Eubacterium nodatum* group sulci co-isolate with red complex members in periodontitis-causing

plaque. [44] They produce butyrate and acetate, which increases heme production, favoring the growth of some periodontal disease-causing bacteria. [45-47]

Selenomonas spp were related to an increase in ΔpH5 (more severe pH drop). *Selenomonas* are gram-negative, motile bacteria found in the gastrointestinal tract and oral cavity in biofilms. They contribute to plaque structure, produce acetic, lactic and propionic acids as metabolic products, and this may account for their association with increased ΔpH5. [48] They produce acid and thrive in acidic environments. [49-52] Previous reports suggested an association between dentinal caries and subgingival plaque. [48, 53, 54] It is tempting to speculate that these bacteria seed and initiate the pH shift in the biofilm that creates the environment for pathogenic bacteria associated with caries and thus may be essential predecessors.

Gemella spp are gram-positive cocci found on mucous membranes of the oral cavity and upper respiratory tract; they have also been isolated from human dental plaque. In our study, they were negatively associated with ΔpH5. *Gemella spp* have been associated with oral health in children and young adults. [53] Another study; however, found that *Gemella sanguinis* and *Gemella haemolysans* were associated with gingivitis in an adolescent orthodontic patient population, and a recent metagenomic analysis included *Selenomonas spp* and *Gemella spp* as co-prevalent with *Streptococcus*, *Veillonella* and *Actinomyces* in the saliva of patients with caries. [55, 56] *Gemella spp* ferment glucose, sucrose and sugar alcohols to yield acid in anaerobic and aerobic conditions. [57] Similar to *Selenomonas spp,* they are adapted to an acidic environment.

At time 15, *Butyrivibrio spp* (anaerobic butyric acid-producing) showed a moderate negative correlation. This group is metabolically adaptable; they ferment a variety of sugars and cellodextrins. This allows some important *Streptococcus* species (including *Streptococcus mutans* and *Streptococcus sobrinus*) to produce extra-cellular polymers and adhere to dental surfaces; in addition, they can grow favorably in the presence of cellodextrins. [58, 59]

For WSL count, there was a moderate positive correlation with *Capnocytophaga sputigena* and *Neisseria sicca*, and a negative correlation with *Streptococcus australis* (see later). *Neisseria spp* have been shown to account for >15% of the microbiome of caries-active saliva, but have also been associated with caries-free status. [60, 61] LEfSe analysis showed that *Capnocytophaga sputigena* was exclusive to WSL patients. This differed from the findings of Tanner *et al.* Generally considered oral commensals, *Capnocytophaga spp* require CO_2 and ferment carbohydrates to succinate and acetate. They have been associated with gingivitis, periodontal disease, halitosis, diabetes and pre-diabetes. [66-69] Their relationship with caries remains equivocal; one study found *Capnocytophaga spp* to be associated with caries-active individuals, while several suggest they are more often a sign of a caries-free state. [62-64] In a study of microbial succession in biofilms following professional cleaning, while no differences in bacterial species colonization were uncovered between healthy and periodontitis patients, *Capnocytophaga sputigena* was found in subgingival biofilms seven days after initial colonization by *Streptococcus mitis, Veillonella parvula* and *Capnocytophaga gingivalis*. [65] *Prevotella melaninogenica,* another pathogen that is commonly associated with periodontal disease, advanced carious lesions and active-caries saliva, was found mostly in WSL patients in our study. [66-69] Together with the presence of *Capnocytophaga sputigena*, these results suggest a more mature plaque environment reflected in the saliva of WSL developers. This is interesting because these parameters were assessed more than two months before the first WSL developed in one of our patients.

Streptococcus mitis and *Streptococcus australis* were primarily present in healthy patients. *Streptococcus mitis* was reported to be one of the least likely bacteria to contribute to caries. After the introduction of 16S sequencing, this species could be differentiated from *Streptococcus mutans* and shown to be one of the least resistant to low pH when compared to other *Streptococcus* species. [70] *Streptococcus australis* can hydrolyse arginine to ammonia, a base

that can neutralize acid and plays a key role in plaque homeostasis by inhibiting the growth of aciduric bacteria. [71] In this way, *Streptococcus australis* antagonizes dental caries pathogenesis. It can produce alkaline phosphatase, which increases the calcium and phosphate content of saliva and plaque, promoting remineralization. [35, 72]

Stomatobaculum spp showed a moderate negative correlative relationship with baseline pH in WSL developers. These anaerobic gram-positive bacteria have been found to be associated with dentin caries and root canals. [73-75] They ferment glucose to butyrate, lactate, isovalerate and acetate. Similar to *Capnocytophaga spp, Stomatobaculum spp* have been shown to be more abundant in pre-diabetics.

Overall, our data indicate an increased abundance of acid-producing bacteria in the saliva of WSL developers, but interestingly, not the usual suspects, *Streptococcus mutans Streptococcus sobrinus,* and *Lactobacillus acidophilus,* which are considered to be the major caries initiators. Our data also differ from others that researched the microbiota of WSLs. This is likely because our microbiome analysis was done prior to WSL detection. Thus, our study may have uncovered the earliest predecessors to WSL/caries development.

Limitations

Although we tried to standardize the patients' bracket system, oral hygiene and enamel conditions pre-treatment, dietary habits, socio-economical background and lifestyle could not be fully controlled in this study. The control group was older compared to the case group, but our analyses did not detect any differences between the 3 control patients that were greater than 40 years of age (43, 44, 45) compared to the 7 that were less than 40 years of age. The mean age of these 7 control patients (14, SEM:1.024) was very close to the 10 patients in the WSL group (13.10, SEM:0.3480).

It should be emphasized that at time of saliva collection and Stephan curve kinetics, all patients had just had a professional dental cleaning and all had equivalent good OHI-S scores. Collection of saliva and dental plaque are simple, non-invasive procedures that can provide a breadth of information to mechanistically address enamel demineralization and identify susceptibility in subjects. In our study, we only evaluated saliva prior to orthodontic treatment, and this parameter alone could not predict individuals more likely to develop WSLs. We did not measure dental plaque pH or plaque microbiome. These two factors can be a point for future investigation as dental plaque and saliva possess different microbial compositions and dental biofilm is known to play an important role in the progression of dental caries [18, 76, 77]. Previous studies point at smaller differences in microbiome plaque from orthodontic patients that developed WSLs *vs* controls, however this information, combined with the saliva microbiome data could potentially result in stronger associations, especially when associated with pH curves/drops [56].

It is possible that Stephan curve kinetics in plaque would be more definitive and that Stephan curve kinetics change during orthodontic treatment, which may then define a WSL group. Other studies have used a higher concentration of sucrose in their rinse, which may more effectively uncover differences in saliva buffering capacity. Nonetheless, the fact that many bacteria which are known to be associated with caries were found to be related to pH restitution provides a compelling rationale for further studies.

Conclusions

In our population of healthy subjects with initial good oral hygiene, there were no differences in initial salivary pH or restitution potential following a sucrose challenge and no global microbial differences between WSL developers and healthy patients. However, to our knowledge, this is the first time that changes in salivary pH (either absolute pH or recovery) have been found to be associated with the abundance of acid-producing bacteria in saliva. The results suggest

saliva pH modulation as a therapeutic strategy to inhibit the abundance of caries initiators. The earliest predecessors to WSL/caries development may have been identified.

List of abbreviations

ASV: Amplicon Sequencing Variants

FALAPhyl: Forays into Automating Laborious Analysis of Phylogeny FDI: Fédération Dentaire Internationale

LDA : Linear discriminant analysis LefSe: Effect Size

OHI-S: Simplified oral hygiene index

SPSS: Statistical Package for Social Sciences WSL: White spot lesions

ΔpH: delta pH

FIGURE LEGENDS

Figure 1. Stephan curves at baseline for those with and without WSL at 12 months.

Figure 2. Bimodal relationship between species abundances: the linear gradient of the statistically significant continuous representative term of the data, ΔpH_5 (blue line). The solid line represents a positive increase in the term, while the dashed line represents a decrease in the term. The placement of species/genera (points) is indicative of their association to the continuous term, when an imaginary-line originating from the continuous variable at a right-angle (the solid, or dashed line) intersects with that point. Intersections at the peripheries, as opposed to those closer to the middle, indicate increased strength of association.

Figure 3. Visualization of differential features ranked by effect size

Supplementary Material

Supplementary Figure 1. Alpha rarefaction curves (observed features): depth of coverage for each patient sample (each colour represents one patient).

Supplementary Figure 2. Violin plots of alpha diversity representing richness and diversity: A: Chao 1 index; B: Shannon index; C. Principal coordinates analysis (PcoA) of PhILR distances between WSL patients (red) and controls (green). The ellipses represent the standard deviation of the dispersion in the two groups, with each sample connected to the centroid (larger dot) by a grey line.

Supplementary File 1: cutadap V13 and V45 **Supplementary File 2**: libraries

DECLARATIONS

Ethics and consent to participate

All methods were performed in accordance with the relevant guidelines and regulations. The study was approved by the University of Alberta Health Research Ethics Board, Edmonton, Alberta, Canada (Approval number: Pro00099341).

Consent for publication

Not applicable

Availability of data and materials

The dataset used and/or analyzed during the current study is available on https://www.ncbi.nlm.nih.gov/bioproject/901626

Competing interests

The authors declare that they have no competing interests in this section.

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Biomedical Research Award – American Association of Orthodontists.

Author contributions

RQC was involved in data collection, data analysis, original draft writing of the manuscript. CFM and MF were involved in the study design, data analysis and manuscript revision. KA was involved in data curation, data analysis, original manuscript drafting and revision. All authors read and approved the final manuscript.

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FIGURES

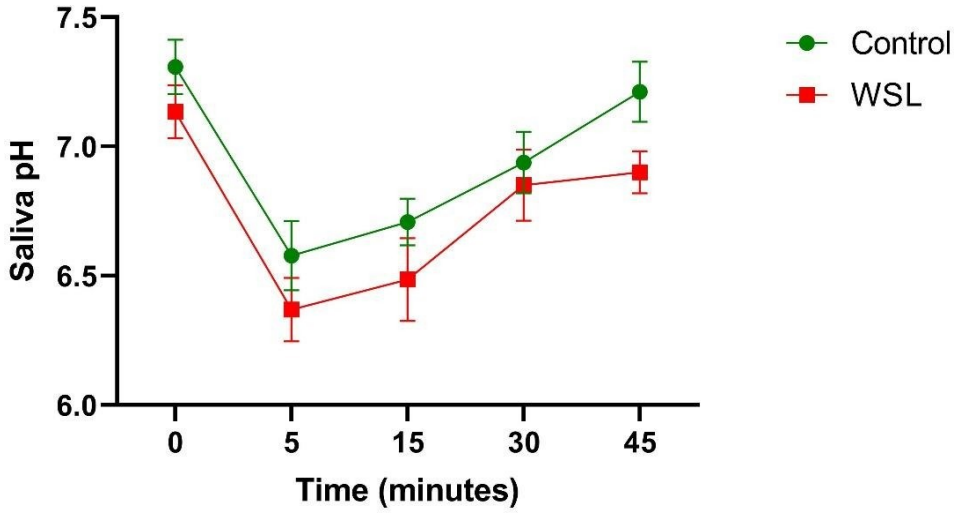
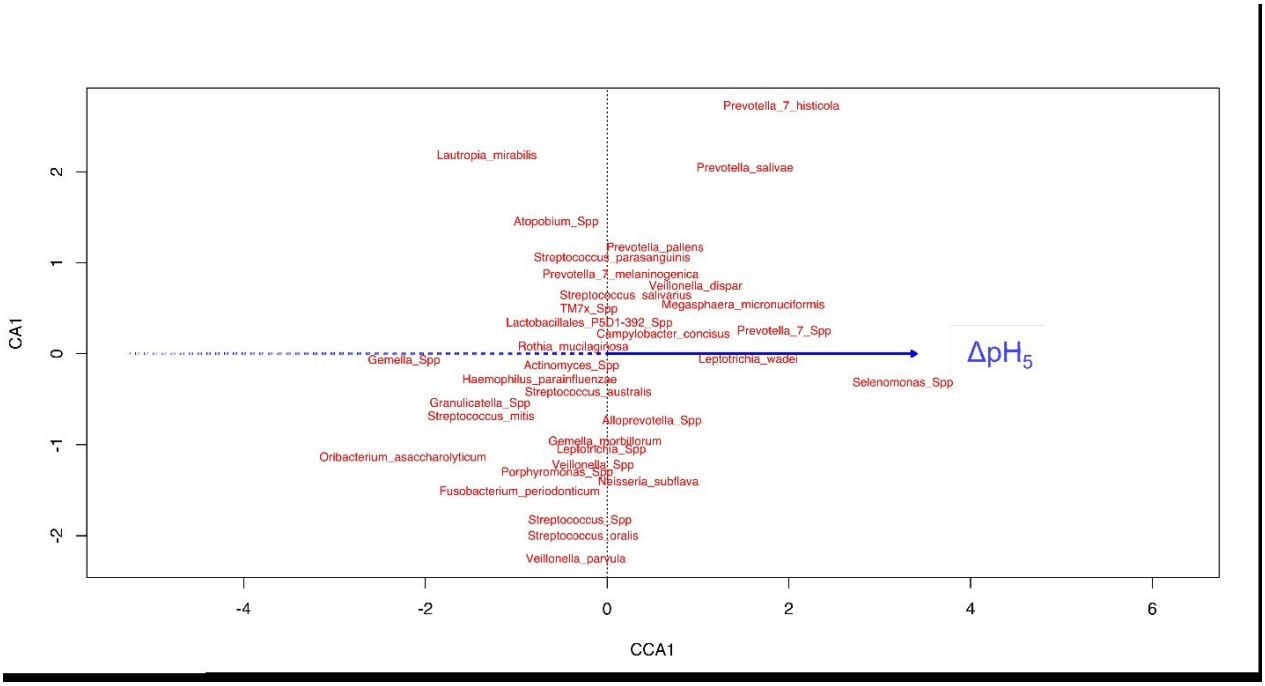


Figure 1. Stephan curves at baseline for those with and without WSL at 12 months.



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when an imaginary-line originating from the continuous variable at a right-angle (the solid, or dashed line) intersects with that point. Intersections at the peripheries, as opposed to those closer to the middle, indicate increased strength of association.

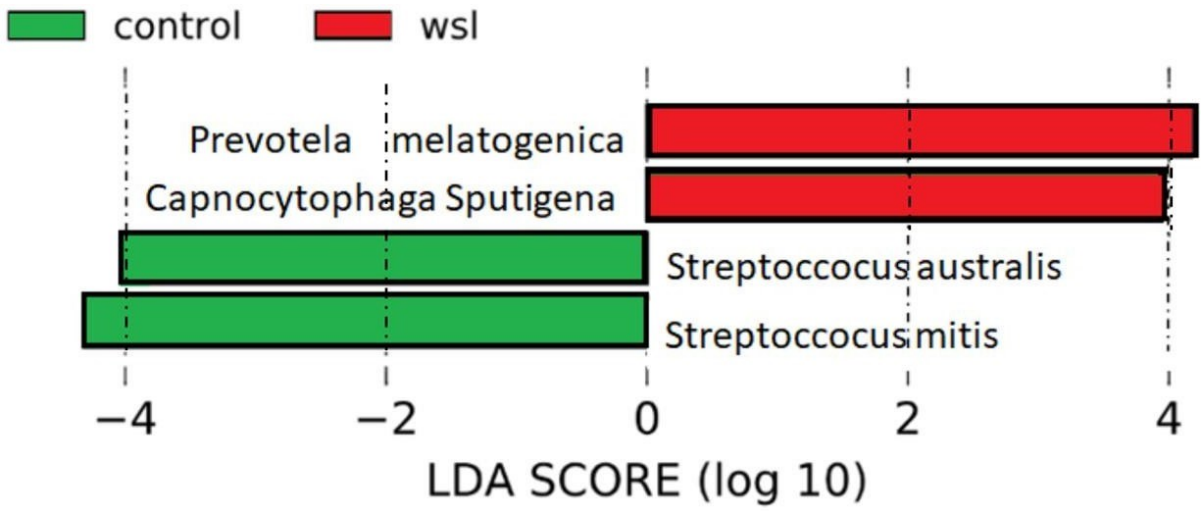
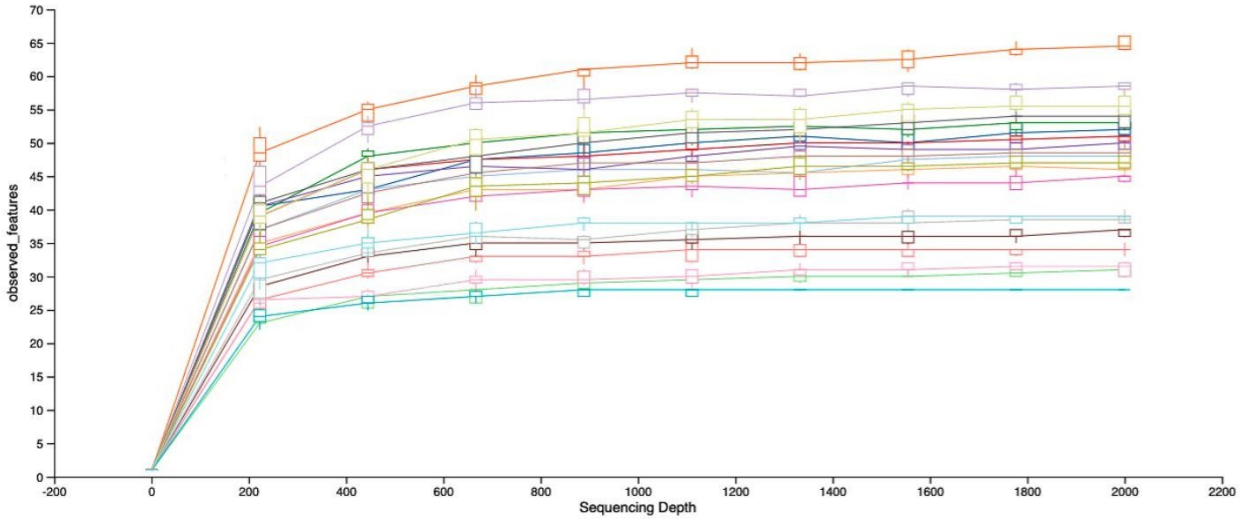
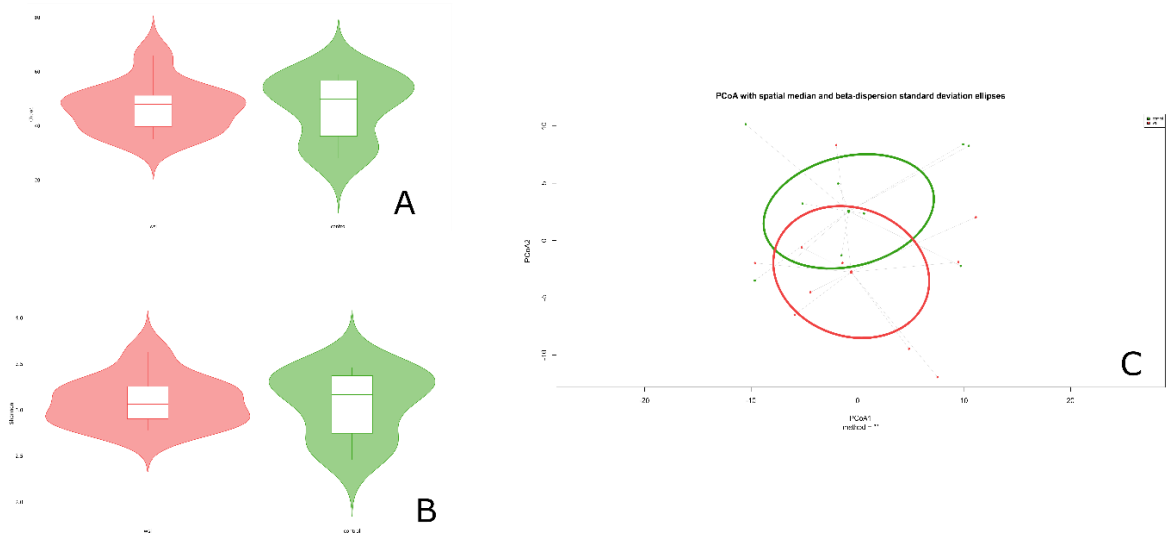


Figure 3. Visualization of differential features ranked by effect size

SUPPLEMENTARY FILES



Supplementary Figure 1. Alpha rarefaction curves (observed features): depth of coverage for each patient sample (each colour represents one patient).



Supplementary Figure 2. Violin plots of alpha diversity representing richness and diversity: A: Chao 1 index; B: Shannon index; C. Principal coordinates analysis (PcoA) of PhILR distances between WSL patients (red) and controls (green). The ellipses represent the standard deviation of the dispersion in the two groups, with each sample connected to the centroid (larger dot) by a grey line.

Supplementary File 1: cutadap V13 and V45

Cutadapt for removing the adaptors

V13

Adaptors used in this case are 27F and FwR1
(ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAKRGTTYGATYNTGGCTCAG) and
(GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGTNTBACCGCDGCTGCTG)

I ran the cutadapt.sh file as follows: bash cutadapt.sh

V45

Multiple adaptors were used in this case with variable lengths. This makes things a little more complicated to process

Adaptors used are:

- Forward: 515FP4-FwR1, 515FP3-FwR1, 515FP2-FwR1, 515FP1-FwR1
(ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAAGTGCCAGCMGCCGCGGTAA ,
ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGTGCCAGCMGCCGCGGTAA ,
ACACTCTTTCCCTACACGACGCTCTTCCGATCTTGTGCCAGCMGCCGCGGTAA ,
ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTGCCAGCMGCCGCGGTAA)

- Reverse: 806RP4-RvR2 , 806RP3-RvR2 , 806RP2-RvR2 , 806RP1-RvR2
(GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCATGGACTACHVGGGTWTCTAAT ,
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACGGACTACHVGGGTWTCTAAT ,
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGACTACHVGGGTWTCTAAT ,
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT)

Based on the regions that are shared across the samples, and the fact that cutadapt will trim all the information past the region in the '3 primers, I have truncated the primers to the following:

-V45 adaptor 5' GTGCCAGCMGCCGCGGTAA

-V45 adaptor 3' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

I ran the cutadapt.sh file as follows: bash cutadapt.sh

Next steps, each was run the same way through DADA2

Forward for V13 will be trimmed at 250 which is the estimation where the quality begins to crash

Reverse for V13 is much worse quality, which is expected, but this is the most errors I've seen. Will trim at 200 and will see if the two will align or not

Turns out the fastq files that we got from them already had the adaptors pre-trimmed, or at least there is an error with the files after we remove the adaptors with cutadapt that they error out the file, so I ran the fastq files we got from them directly after renaming them in the normal Illumina convention ("_S#_L001_R1_001.fastq"). This is most likely the real scenario because V4-V5 has multiple kinds of primers so it would be very weird if they give you the unfiltered result

After running Figaro for best parameters for trimming, this is the command we should run in DADA2

Command used in DADA2 docker environment

V13 (assumption in Figaro that V13 has a maximum of 490 bp in length) library(dada2)

path <- "/data"

fnFs <- sort(list.files(path, pattern="_R1_001.fastq", full.names = TRUE)) fnRs <- sort(list.files(path, pattern="_R2_001.fastq", full.names = TRUE)) sample.names <- sapply(strsplit(basename(fnFs), "_"), `[`, 1)

```
filtFs <- file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz")) filtRs <- file.path(path,
"filtered", paste0(sample.names, "_R_filt.fastq.gz")) names(filtFs) <- sample.names
names(filtRs) <- sample.names
# out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(299,273),maxN=0, maxEE=c(5,8), truncQ=2,
rm.phix=TRUE,compress=TRUE, multithread=TRUE)
out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(270,250),maxN=0, maxEE=c(8,8), truncQ=2,
rm.phix=TRUE,compress=TRUE, multithread=TRUE)

errF <- learnErrors(filtFs, multithread=TRUE) errR <- learnErrors(filtRs, multithread=TRUE)
dadaFs <- dada(filtFs, err=errF, multithread=TRUE) dadaRs <- dada(filtRs, err=errR, multithread=TRUE)
mergers <- mergePairs(dadaFs, filtFs, dadaRs, filtRs, verbose=TRUE) seqtab <-
makeSequenceTable(mergers) write.table(seqtab,"/data/seqtableV13.tsv")
seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE,
verbose=TRUE)
sum(seqtab.nochim)/sum(seqtab)

# Then I took the output file, took all the sequences. Made them into a separate text file. Split them to 1
sequence at a time (issues with the computer not having enough resources to assign taxonomy) through
the following shell command: split -l 1 v13_sequences.txt. Next I assigned taxonomy to each one
separately

taxa <- assignTaxonomy(, "data/silva_nr99_v138.1_train_set.fa.gz",multithread=TRUE)

## V45 (assumption in Figaro that V13 has a maximum of 390 bp in length) library(dada2)
path <- "/data"
fnFs <- sort(list.files(path, pattern="_R1_001.fastq", full.names = TRUE))
```

fnRs <- sort(list.files(path, pattern="_R2_001.fastq", full.names = TRUE)) sample.names <-

sapply(strsplit(basename(fnFs), "_"), `[`, 1)

filtFs <- file.path(path, "filtered", paste0(sample.names, "_F_filt.fastq.gz")) filtRs <- file.path(path,

"filtered", paste0(sample.names, "_R_filt.fastq.gz")) names(filtFs) <- sample.names

names(filtRs) <- sample.names

Figaro determined this to be the best: c(178, 144) c(1, 1) 95.87 95.86624 300

out <- filterAndTrim(fnFs, filtFs, fnRs, filtRs, truncLen=c(178,144),maxN=0, maxEE=c(1,1), truncQ=2, rm.phix=TRUE,compress=TRUE, multithread=TRUE)

errF <- learnErrors(filtFs, multithread=TRUE) errR <- learnErrors(filtRs, multithread=TRUE)

dadaFs <- dada(filtFs, err=errF, multithread=TRUE) dadaRs <- dada(filtRs, err=errR, multithread=TRUE)

mergers <- mergePairs(dadaFs, filtFs, dadaRs, filtRs, verbose=TRUE) seqtab <-

makeSequenceTable(mergers) write.table(seqtab,"/data/seqtable.tsv")

seqtab.nochim <- removeBimeraDenovo(seqtab, method="consensus", multithread=TRUE, verbose=TRUE)

sum(seqtab.nochim)/sum(seqtab)

Supplementary File 2: libraries

Hide

```
library(vegan)
library(tidyverse)
library(ggvegan)
library(gginnards)
library(ggrepel)
```

Load the OTU table in the format that Vegan approves of

Note that this rarefied document have had 1) OTUs condensed manually 2) mitochondria and chlorophylls removed

Hide

```
OTUtable = read_tsv("/Volumes/GoogleDrive/My Drive/Lab/saliva_stephans_curve/trial2/whitespots_rarefied_4931.tsv", col_names = TRUE, skip=1, show_col_types = FALSE) %>% as.data.frame(.)

titles = OTUtable[,1]

rownames(OTUtable) = titles

OTUtable[,1] = NULL

OTUtable = t(OTUtable)

head(OTUtable)
```

Hide

```
map = read_tsv("/Volumes/GoogleDrive/My Drive/Lab/saliva_stephans_curve/trial1/map/whitespots_rarefied.txt") %>% as.data.frame(.)

titles = map[,1]

rownames(map) = titles

map[,1] = NULL

map[,9] = NULL # Removes the color columns

map[,9] = NULL # Removes the color columns

map[,2] = NULL # Removes the sex column

nams=names(map)

head(map)
```

The following steps have been modified from the following tutorial (https://rfunctions.blogspot.com/2016/11/canonical-correspondence-analysis-cca.html)

Hide

```
OTUlog = decostand(OTUtable, "log")


ccamodel1 = cca(OTUlog ~ Disease + Age + Time0 + Time5 + Time15 + Time30 + Ti
me45 , map)


ccamodel0 = cca(OTUlog ~ 1, map)


mod = step(ccamodel1, scope=list(
 lower=formula(ccamodel0),
 upper=formula(ccamodel1)),
 direction="both") #AIC=125.59
```

```
mod_standard = step(ccamodel0,scope=formula(ccamodel1),test="perm") #AIC=125.
59


# So basically nothing is really fitting a CCA model
```

I think its time to remove the variables that are no longer needed Let's redo the analysis but this time with Time as a delta from Time0 Hide

```
map = read_tsv("/Volumes/GoogleDrive/My Drive/Lab/saliva_stephans_curve/trial
1/map/whitespots_rarefied_delta.txt") %>% as.data.frame(.)

titles = map[,1]

rownames(map) = titles

map[,1] = NULL

nams=names(map)

head(map)
```

Let's do the same thing again now but with the new variables Hide

```
OTUlog = decostand(OTUtable, "log")


ccamodel1 = cca(OTUlog ~ Disease + Age + Time0  + delta_time5 + delta_time15
+

delta_time30 + delta_time45, map)


ccamodel0 = cca(OTUlog ~ 1, map)


mod = ordistep(ccamodel1, scope=list(

 lower=formula(ccamodel0),

 upper=formula(ccamodel1)),

 direction="both",

 permutations=1000,

 steps=1000) #In this case, we have delta_time15 being significantly differen
t. What about interaction terms?


ccamodel3 = cca(OTUlog ~ Disease + Age + Time0  + delta_time5 + delta_time15
+ delta_time30 + delta_time45 +
```

```
 Disease*Age + Disease*Time0 + Disease*delta_time5 + Disease+delta_time15 + D
isease*delta_time30 + Disease*delta_time45 + Age*Time0 + Age*delta_time5 + Ag
e*delta_time15 + Age*delta_time30 + Age*delta_time45 + Time0*delta_time5 + Ti
me0*delta_time15 + Time0*delta_time30 + Time0*delta_time45 + delta_time5*delt
a_time15 + delta_time5*delta_time30 + delta_time5*delta_time45 + delta_time15
*delta_time30 + delta_time15*delta_time45 + delta_time30+delta_time45 , map)


mod2 = ordistep(ccamodel3, scope=list(
 lower=formula(ccamodel0),
 upper=formula(ccamodel3)),
 direction="both",
 permutations=1000,
 steps=1000,
 Pin=0.05,
 Pout=0.06)


anova.cca(mod2,by = "margin")
print(mod2)
vif.cca(mod2)
```

From the looks of it, interaction terms are the most important components here. That is, Time0 with delta_time15, Time0 with delta_time30, and interaction between delta_time5 and delta_time45. But, the collinearity between Time0:delta_time15, Time0:delta_time30, delta_time5:delta_time45, delta_time30, and delta_time15 are very high. Will need to figure out what to do with that. Based on https://sites.google.com/site/mb3gustame/warnings/warning-confounding-variables-and-multicollinearity, removal of collinear terms is acceptable in exploratory analysis.

Hide

```
ccamodel4 = cca(OTUlog ~ Disease + Age + Time0  + delta_time5 + delta_time15
+ delta_time30 + delta_time45 +
 Disease*Age + Disease*Time0 + Disease*delta_time5 + Disease+delta_time15 + D
isease*delta_time30 + Disease*delta_time45 + Age*Time0 + Age*delta_time5 + Ag
e*delta_time15 + Age*delta_time30 + Age*delta_time45 + Time0*delta_time5 + T
ime0*delta_time30 + Time0*delta_time45 + delta_time5*delta_time15 + delta_tim
e5*delta_time30 + delta_time5*delta_time45 + delta_time15*delta_time45 + del
ta_time30+delta_time45 , map) # Removed the most collinear variable, which i
s Time0:delta_time15, next step was remove delta_time15*delta_time30




mod3 = ordistep(ccamodel4, scope=list(
```

```
lower=formula(ccamodel0), upper=formula(ccamodel3)), direction="backward",
permutations=1000, steps=1000,
Pin=0.05, Pout=0.06)

anova.cca(mod3,by = "margin") print(mod3)
vif.cca(mod3)

This model looks good, only 4 terms, and all are not collinear. Moving on

Time to graph them
```

<!-- rnb-text-end -->

<!-- rnb-chunk-begin -->

<!-- rnb-source-begin eyJkYXRhIjoiYGBgclxubXljY2EgPSBjY2EoZm9ybXVsYSA9IE9UVWx
vZyB+IGRlbHRhX3RpbWU1ICsgZGVsdGFfdGltZTE1ICsgZGVsdGFfdGltZTQ1ICsgZGVsdGFfdGlt
ZTUqZGVsdGFfdGltZTQ1LCBkYXRhID0gbWFwKVxuXG5hbm92YS5jY2EobXljY2EsYnk9XCJtYXJna
W5cIilcbnZpZi5jY2EobXljY2EpXG5cblxucGxvdChteWNjYSlcblxubXlnZyA9IGF1dG9wbG90KG
15Y2NhKVxuXG5sYWJlbHMgPSBteWdnJGxheWVyc1tbMV1dJGRhdGEkTGFiZWxcbmNjYTEgPSBteWd
nJGxheWVyc1tbMV1dJGRhdGEkQ0NBMVxuY2NhMiA9IG15Z2ckbGF5ZXJzW1sxXV0kZGF0YSRDQ0Ey
XG5sYWJlbHNfY29vcmRzID0gY2JpbmQobGFiZWxzLGNjYTEsY2NhMilcbnNhbXBsZU5hbWVzID0gc
m93bmFtZXMoT1RVdGFibGUpXG5sYWJlbHNfY29vcmRzMiA9IHN1YnNldChsYWJlbHNfY29vcmRzLC
AhbGFiZWxzICVpbiUgc2FtcGxlTmFtZXMgKSAlPiUgYXMuZGF0YS5mcmFtZSguKSAjIFJlbW92ZSB
0aGUgc2FtcGxlIG5hbWVzIGFuZCBjb29yZGluYXRlc1xubGFiZWxzX2Nvb3JkczIkY2NhMSA9IGFz
Lm51bWVyaWMobGFiZWxzX2Nvb3JkczIkY2NhMSlcbmxhYmVsc19jb29yZHMyJGNjYTIgPSBhcy5ud
W1lcmljKGxhYmVsc19jb29yZHMyJGNjYTIpXG5cbm15Z2cyID0gbXlnZyArICBnZW9tX3RleHRfcm
VwZWwoc2l6ZSA9IDUsIG1heC5vdmVybGFwcyA9IDEwMCxcbiAgYWVzKHggPSBsYWJlbHNfY29vcmR
zMiRjY2ExLCB5ID0gbGFiZWxzX2Nvb3JkczIkY2NhMiwgbGFiZWwgPSBsYWJlbHNfY29vcmRzMiRs
YWJlbHMgKSkgKyB0aGVtZV9saWdodCgpICsgZ2VvbV9qaXR0ZXIoKVxuXG5zdmcoXCJ+L0Rlc2t0b
3AvdGVzdC5zdmdcIiwgaGVpZ2h0ID0gNDAsIHdpZHRoID0gNDApIFxubXlnZzJcbmRldi5vZmYoKV
xuXG5teWdnMyA9IGRlbGV0ZV9sYXllcnMobXlnZzIsIFwiR2VvbVBvaW50XCIpXG5cbmdnc2F2ZSg
--
>
```
```

```r
mycca = cca(formula = OTUlog ~ delta_time5 + delta_time15 + delta_time45 + de
lta_time5*delta_time45, data = map)


anova.cca(mycca,by="margin") vif.cca(mycca)



plot(mycca)


mygg = autoplot(mycca)


labels = mygg$layers[[1]]$data$Label cca1 = mygg$layers[[1]]$data$CCA1 cca2 =

mygg$layers[[1]]$data$CCA2

labels_coords = cbind(labels,cca1,cca2) sampleNames = rownames(OTUtable)

labels_coords2 = subset(labels_coords, !labels %in% sampleNames ) %>% as.data
.frame(.) # Remove the sample names and coordinates labels_coords2$cca1 =

as.numeric(labels_coords2$cca1) labels_coords2$cca2 =

as.numeric(labels_coords2$cca2)


mygg2 = mygg + geom_text_repel(size = 5, max.overlaps = 100,

  aes(x = labels_coords2$cca1, y = labels_coords2$cca2, label = labels_coords
2$labels )) + theme_light() + geom_jitter()


svg("~/Desktop/test.svg", height = 40, width = 40) mygg2

dev.off()


mygg3 = delete_layers(mygg2, "GeomPoint")
```

Let’s see if bioenv has any solutions for what environmental variables are correlated with the distance matrix

Hide

```
# This time, let's try the full sequences, in PhILR format

OTUtable = read_tsv("/Volumes/GoogleDrive/My Drive/Lab/saliva_stephans_curve/
trial2/white_spots_primer_averaged_with_code.tsv", col_names = TRUE, skip=1,
show_col_types = FALSE) %>% as.data.frame(.)

titles = OTUtable[,1] rownames(OTUtable) = titles OTUtable[,1] = NULL

OTUtable = t(OTUtable)


suppressWarnings(suppressMessages(library(philr)))

suppressWarnings(suppressMessages(library(phyloseq)))

suppressWarnings(suppressMessages(library(ape)))


mytsv = otu_table(OTUtable, taxa_are_rows = FALSE)

mytree = suppressWarnings(dist(t(mytsv),method="euclidean")) %>% hclust(.,met
hod="ward.D") %>% as.phylo(.)


phylo = suppressMessages(merge_phyloseq(mytsv,mytree))



isRooted = suppressWarnings(ape::is.rooted(phy_tree(phylo))) isBinary =

suppressWarnings(ape::is.binary(phy_tree(phylo)))

if(!isRooted){stop("Tree needs to be rooted.",call.=FALSE)} #if not rooted

if(!isBinary){phy_tree(phylo) = multi2di(phylo@phy_tree)} #if not binary tree


phy_tree(phylo) = ape::makeNodeLabel(phy_tree(phylo), method="number", prefix
= 'n')


# Add 1 to avoid fractions on a zero denominator


data.no0 = transform_sample_counts(phylo, function(x) x+1)

phylot = merge_phyloseq(data.no0,phylo@phy_tree,phylo@sam_data,phylo@tax_tabl
e) # make your new GP phyloseq object based on the newly created matrix


myMatrix = phylot@otu_table@.Data tree = phy_tree(phylot)
```

```
gp.philr = philr::philr(myMatrix, tree, part.weights='uniform', ilr.weights='
uniform') %>% suppressMessages(.)
```

```
map_continuous = map
map_continuous[,1] = NULL
```

```
bioenv_results = bioenv(gp.philr ~ Age + Time0 + Time5 + Time15 + Time30 + Ti
me45,map_continuous, metric = "euclidean",method="spearman",index="euclidean"
)
bioenv_results
```

So only Time5 is correlated with the matrix. Even still, very weak correlation.

CHAPTER 9

DISCUSSION

For a proper diagnosis and treatment, as well as research into the etiology, pathogenesis, and natural history of periodontal disease, a classification of periodontal and peri-implant diseases and conditions is necessary [1]. In 2017, a new classification system that emerged from population studies, basic research and prospective studies that evaluated environmental factors and systemic risk factors, was proposed [1]. This one system presents substantial differences from the previous classifications and is the current guideline for periodontal diagnosis [1].

Periodontitis is currently defined as a "*disease-causing the progressive destruction of the tooth-supporting apparatus, characterized by a CAL, a radiographically assessed alveolar bone loss, the presence of periodontal pockets and gingival bleeding*" [1]. However, in the past, a great diversity of descriptions was utilized to define this unique disorder and up to this date, some of them are still in use and may cause confusion. The introduction for the most severe type of periodontitis, classified then as "juvenile periodontitis", was done by Butler in 1969 (Chapter 4) [2]. There was also segmentation of juvenile periodontitis into two groups: the localized form, which would affect the first molars and incisors, and the generalized form, which would affect most of the dentition [3]. Other nomenclatures also used were "pre-pubertal periodontitis", early-onset periodontitis and finally "aggressive periodontitis" [4-6]. Despite refinements to these previous classifications, there remained widespread disagreement as to how to narrow down specific characteristics that were unique to these patients.

In our literature review, we found that there was a great variety of criteria being used by different authors to define determinants of categories of periodontal disease (Chapter 4). They differed in standards for pocket depth and CAL, and some used periapical and interproximal radiographs for diagnosis [7-10]. Also based on our review, we found that another cause for miscategorization was the clinical examination protocol itself, for example, the number of teeth included (full mouth vs half-mouth), number of periodontal sites assessed for each tooth (e.g. 4 *vs* 6) and even the specific periodontal probing device [11]. A limitation of some of these studies was also the sample size of the studied population [12, 13].

We also assessed the prevalence of the then-defined "aggressive periodontitis" in young populations (Chapter 5). Only six studies fit our inclusion criteria for review. We found a great diversity in the estimation of the prevalence due to the large variety of screening methods and clinical assessment criteria. Overall, we found that the localized form of the disease was more

prevalent than the generalized form, which was in agreement with other prevalence studies that showed the localized form varied from 1.2–4.2% and the generalized form from 0.03–4.3%

The differences observed in the prevalence range for aggressive periodontitis could be affected by the method of diagnosis, study sampling, socioeconomic status and ethnicity of the population. From the countries included in the review, Argentina and Turkey reported the lowest prevalence (0.66% and 0.6%, respectively), followed by Iran (0.7%), Brazil (1.6%), Sudan (3.4%) and Israel (5.9%) [18]. The average was 1.7%, and there are several explanations for the variation found. One explanation could be the population studied; some countries are territorially large, such as Brazil, therefore the composition of populations examined in these studies could also differ greatly based on the city/state and sampling methods. Population composition in several countries can vary in ethnicity, socioeconomic status, age and sex. For example, some states in Brazil, such as Rio de Janeiro, have a higher female population (3.4% higher, equivalent to almost to 600.000 people). As women are more likely to seek oral health treatment, this could be a potential explanation for the finding of increased prevalence when compared to countries that have more males [19, 20]. Another factor that could play an importance role is access to health care. Approximately 16% of Brazilians live in rural areas with poor or no access to oral/general health support or are uninsured. In comparison, the populations of Argentina and Israel are mostly urban (about 92%) [20, 21]. Generally, populations that have greater access to health care were less prone to aggressive periodontitis [22]. Israel seems to be an exception that will be discussed later in this chapter.

As the diagnosis of aggressive periodontitis also has age as a criterion (younger than 35), countries with younger populations may show increased incidence. Approximately 43.9% of the Brazilian population is younger than 35 years of age, and this is similar to Israel (42%). In comparison, the under 35 years of age group in Argentina accounts for 37.2% of the population, and in Iran, it accounts for 35.2% of the population [21]. Other factors made comparisons between studies difficult: in the study from Israel, which showed the highest prevalence of aggressive periodontitis, the sample consisted of a young male military population (a very specific group). In Argentina, there was not a clear standardization in the method of periodontal assessment to compare with previous studies [8, 14, 17, 23]. Sudan, Iran, and Turkey showed a prevalence similar to what had been reported in the literature [8, 14, 24, 25]. Our review made clear that even after

all the guidelines that accompanied the 1999 classification, many studies still lacked standardization. The discussion as to how to properly classify this severe disease remained. There were suggestions for some periodontal parameters to be included as standards: probing pocket depth, bleeding on probing, furcation involvement, papilla bleeding index and plaque index. Others suggested the addition of the gingival index, calculus index and CAL [23, 26].

The development of a worldwide standardized classification system is important for categorizing diseases to facilitate the establishment of diagnosis, treatment, and prognosis. It is also important to inform clinical trials, as well as research into etiology, pathogenesis, natural history and treatment. There was broad agreement that the previous periodontal disease classification system no longer aligned with new clinical and scientific evidence, and a new classification system for periodontitis was developed at the World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions, which took place in Chicago from November 9th to 11th of 2017 [1]. The classification of the different presentations of periodontal disease was changed to reflect a spectrum disorder. A spectrum disorder can be defined as a condition that has a variety of other conditions associated, presenting unique symptoms, features and a range of severity [27]. This term is often used to clarify that there is not a single cause, but rather several subgroups [27]. The formerly recognized “chronic” and “aggressive” forms of periodontitis are currently classified based on a multidimensional staging and grading system. The severity, complexity and distribution of the disease are included in the staging (I to IV). Grading includes the time frame of progression of the disease (A to C). For this thesis, we have used the term “severe forms of periodontitis” (SvP) for patients diagnosed as Grade C, Stage III or IV [1].

One of the biggest challenges with SvP is understanding its etiology, as it is multifactorial and represents both host and biofilm mediated destruction of gingival tissues [28]. Some reports in the literature point to suppression of chemotaxis of neutrophils and monocytes as contributors to periodontal destruction, as it could impair the normal host response to neutralize infection [29]. Another theory is that the neutrophils may become hyperactive, impairing their phagocytosis capacity and resulting in an excessive production of superoxide (an oxygen radical that can cause connective tissue breakdown) [30]. Consequently, this may lead to periodontal attachment loss. In addition, bacterial endotoxin can increase oxidative stress in gingival fibroblasts, which can then

cause tissue damage, vascular barrier dysfunction and continuation of the inflammatory cascade [31-34].

Another more recent hypothesis about the etiology of SvP relates to the function of the epithelium in the homeostasis of the oral environment [35, 36]. Our epithelium is the first line of defense against mechanical and chemical harms. Our understanding of the association between impaired epithelial function and epithelial-related disorders is starting to grow. In this thesis, we have explored the connection between epithelial-related disorders (full body and oral) and loricrin downregulation (Chapter 6). We chose to focus on loricrin because it makes up ~70% of the protein in the CE, and plays an important role in its structural organization. Loricrin mutation, downregulation or mislocalization resulted in weakness of the CE and subsequently, disruption in a biological protection resulting in vulnerability to affected individuals [37]. We found that the underlying mechanisms of disease varied and were dependent on the type of loricrin alteration. For example, gene mutations could lead to changes in amino acids affecting protein structure, cross-linking or localization, mRNA or protein expression, and reduction or complete loss of loricrin [38-42].

In diseases involving loricrin, some gene mutations took the form of frameshifts which are defined as "*an insertion or deletion of nucleotide bases in numbers that are not multiples of three*" [43, 44]. The result was a protein that was less cross-linked (change in the amino acids at the C-terminus, with an increase in arginine) [43]. Additionally, since arginine is positively charged, this could lead to a more unstable protein due to charge repulsion [37]. Charged residues are more hydrophilic, and this alters a fundamental trait of loricrin, its insolubility [45, 46]. This, then, may play a role in changing loricrin localization within the cell [43, 47].

The systematic review uncovered that patients with loricrin mutations may have different diagnoses but share certain features such as diffuse palmoplantar hyperkeratosis, "honeycombs", digital constriction bands (pseudoainhum), and ichthyosis (cornification process characterized by generalized skin scaling) which are all characterized by excessive epidermal thickening [48-52]. Similarly, disorders with a seemingly common loricrin-related feature displayed different disease characteristics. Eight different epithelial-related disorders were connected to loricrin downregulation either by assessment of protein or gene expression, with different clinical presentations: Vohwinkel syndrome with ichthyotic variant, mutilating palmoplantar keratoderma,

aggressive periodontitis, loricrin keratoderma, progressive symmetric erythrokeratoderma, atopic dermatitis, psoriasis, and a novel type of erythrokeratoderma (KLICK; keratosis linearis with ichthyosis congenita and sclerosing keratoderma syndrome) [37]. This suggests that there is great complexity in the interplay of loricrin with other proteins in the CE, that may also be dependent on the timing of loss of expression, the exact alteration of the protein (folding defect, change in charge, etc.), and other unknown factors.

The correlation between epithelial integrity and periodontitis may have been overlooked in the past by dermatologists, as dermatological signs/symptoms are not usually associated with oral conditions, but more recently, investigation into this association has increased [53-57]. For example, the association between psoriasis and severity of periodontitis has been investigated in 4 recent studies [53, 55-57]. A meta-analysis suggested that patients with psoriasis presented with worse periodontal health (more gingival inflammation, alveolar bone loss and more missing teeth) than those without psoriasis [56]. In 2019, loricrin was associated with chronic periodontitis (term used by the authors) and at least a 2-fold downregulation at the transcript level in gingival samples from these patients [58]. When investigating aggressive periodontitis, Guzeldeimir-Akcakanat *et al*. back in 2016 found a 7-fold decrease in loricrin gene expression, while Nowak, in 2013, found a significant 25% decrease in loricrin gene expression for aggressive periodontitis patients, but no significant change in loricrin expression in chronic periodontitis patients [59, 60]. These studies led to our hypothesis that downregulation of loricrin could result in a change in barrier integrity in the oral cavity, and, like the manifestations in the skin, lead to greater inflammation and bone loss.

Aiming to gather further evidence on our hypothesis, we investigated an animal model to determine how downregulation of the main epithelial proteins plays a role in alveolar bone loss and inflammation in mice (Chapter 7). As previously mentioned, the Stat6VT transgenic mouse has been used widely in AD research for over a decade [61-66]. A mutant Stat6 transcription factor is constitutively expressed in T and B cells via the CD2 locus control region promoter. Downstream gene transcription can be activated by this mutant form, even with no exogenous stimulus. There is then a decrease in loricrin and filaggrin in keratinocytes, which is driven by Th2 cytokines [61, 62]. The lesions observed in these animals resemble human AD. To the best of our knowledge, we are the first to investigate their oral cavity. Our goal was to determine the impact of CE protein deficiency/mislocalization on periodontal health following periodontal pathogen

challenge. In addition to exploring the animal model, we also collected human gingival samples from SvP patients and healthy controls to compare the localization and expression of four epithelial proteins (CK1, CK14, filaggrin and loricrin).

In order to investigate mice alveolar bone levels, we used a systematic protocol by measuring distance from the CEJ to the ABC (Chapter 3). This is an important refinement in our study compared to others. The distance between the CEJ and ABC is one of the most frequent parameters measured to investigate alveolar bone levels in mice [67-75]. However, comparisons between studies and human parameters are difficult without a systematic protocol, since measurements are not always performed at specified sites [68, 76-82]. Our protocol was unique as it incorporated two different planes in the measurements: coronal and sagittal [83]. This addressed previous limitations, as most studies only looked at mesial/distal of either the sagittal plane or coronal, but not both [69, 70, 84-87]. The biggest drawback in looking at only one plane, for example the sagittal, is that losses that occur on the lingual/buccal sites could be underestimated or completely missed.

We first applied this protocol in the longitudinal study on unchallenged Stat6VT mice and controls. In our assessment of unchallenged 10-12 week old male mice, the distance from CEJ to the ABC was significantly greater at 1 site on the first molar (mid-lingual site) and 4 sites on the second molar (mid-buccal site and mesial and distal sites) in Stat6VT compared with controls. At this time point, our results showed that there were minimal changes between the groups.

Bone mineral content and bone mineral density were also analysed via Faxitron. There were no differences in bone mineral content or density in Stat6VT male mice compared to controls at 10-12 weeks of age, whereas at 8 weeks and 18 weeks, some difference were observed. In females, differences between Stat6VT and control mice were observed at all time points. There are several potential reasons for the difference in results observed between sexes. Females presented with early onset of skin lesions, which can cause significant stress to the mice at an early age, which in turn can predispose to problems related to maturation, nutrition and bone development. Another reason, that could affect both sexes, is that constitutive activation of Stat6 may augment secretion of cytokines that activate a Th2 immune response in a continuous manner. The Th2 immune response may impact bone composition via upregulation of Th2 cytokines, which, in addition to downregulating CE proteins, can induce RANKL-mediated osteoclastogenesis through effects on B lymphocytes [88]. Continuous B-cell activation can

increase IL-1 secretion, which may cause tissue destruction [88]. Th2 cytokines are able to upregulate pro-inflammatory cytokines such as IL-19, IL-20, IL-25, IL-27, IL-12Rβ2, IL31RA and nitric oxide synthase 2 [62, 89]. Thus, the determination of BMC and BMD measurements allowed us to have a better understanding of bone health in this mouse model, and plan experiments accordingly. As we were investigating the effects of a potential downregulation of a protein localized in the keratinized epithelium, assessing systemic bone conditions was of fundamental importance, as this will also be reflected in the way alveolar bone levels may respond to a pathogen. If, for example, these animals were to present with an intrinsic bone condition such as osteoporosis, this could potentially skew our results.

Neither sex showed any visible signs of oral inflammation at any time point in our longitudinal study, even when there was significant bone loss. However, on histological investigation, using a pan-leukocyte marker (CD45), a classic method of inflammatory cell assessment, at 10-12 weeks of age, Stat6VT male mice showed an increased number of CD45 positive cells when compared to controls (almost 2-fold difference).

We next endeavored to directly compare loricrin protein levels in the oral cavity of Stat6VT mice with controls. Initially, two methods of protein detection were used to quantify differences in loricrin in our test groups. The first was sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis with Western blotting. A verified polyclonal antibody against loricrin was used. Due to loricrin's highly cross-linked nature, we found that our sample preparation buffer, which included reagents to break crosslinks, could not fully resolve loricrin to one species. This resulted in a series of bands of different molecular weights, that further differed for each preparation, with no predictable pattern. This was discussed by a previous student in the lab, Danielle Clark in her thesis [90]. As a result of the lack of reliability of Western blots to quantify loricrin, we explored using an enzyme linked immunosorbent assay (ELISA) to quantify loricrin expression in keratinized tissue. There were a few complications that occurred when using this protein detection modality. First, due to the very small area of keratinized tissue available in mice, it was difficult to isolate enough tissue to obtain enough loricrin protein to produce a discernable signal. In addition, often times the sample was contaminated by non-attached mucosa and connective tissue. With the complications encountered with Western blotting and ELISA, the next option was to use antibodies to detect loricrin in tissue sections by immunofluorescence. We chose the indirect method (with a primary and a secondary antibody)

because of its higher sensitivity and signal amplification [91]. This method of protein detection offered several advantages: it allowed us to visually distinguish non-keratinized from keratinized tissue, the polyclonal antibody used could bind the crosslinked forms of loricrin, and precise localization of loricrin could be completed within the keratinized epithelial architecture.

There are some drawbacks to this method as well. Unlike Western blotting and ELISA, immunofluorescence is not quantifiable. It only allows making qualitative comparisons visually between sections. In an attempt to control sectioning parameters to make this comparison more reliable, we took a number of steps in sample processing and sample analysis (as outlined in the Methods section of this thesis). Briefly, one quadrant of the mandible was embedded sagitally, using the lower incisor to position the sample within the paraffin block. Sections were only considered for analysis if they contained, the first or second molar including crown, roots and root apex as well as alveolar bone crest mesial and distal to the chosen tooth. In embedding soft tissue, sagittal slices of approximately equal thickness were aligned according to color differences between keratinized and non-keratinized mucosa. Antibody concentrations, as well as microscope settings were also kept the same to avoid any discrepancies in signal intensity.

We initially needed to determine at what time point there would be protein down-regulation of oral loricrin, if that would correspond with skin disease development and if there were differences between males and females. To our knowledge, loricrin protein expression in the oral epithelium of these mice was never investigated before. Therefore, our first finding was that control and Stat6VT mice both demonstrated loricrin expression in the oral epithelium before pathogen challenge via immunofluorescence staining, with the major difference being a broader expression in Stat6VT mice, with similar signal intensity in both groups. In regards to the skin lesions, Stat6VT male mice developed skin lesions at about 10-12 weeks of age; females had earlier and more variable disease onset, approximately at 6-8 weeks [92]. We bred each Stat6VT mouse (both sexes) with a C57Bl/6j wild-type control mouse to ensure that all mice were hemizygous for Stat6VT. However, the sex-based differences in the phenotypical skin presentation of females may suggest a hormonal or physiological difference in female Stat6VT breeders compared to male Stat6VT breeders, as the female animals that had early skin lesions were mostly from female breeders [92].

In the unchallenged mice, we were able to detect a significant increase in oral keratinocyte proliferation in Stat6VT male mice compared to controls by measuring the number of Ki67

positive cells. Keratinocytes play an important role in the control of autocrine and paracrine elements in the proliferation and differentiation processes of the epithelium. Ki67 is one of the most used markers as it is able to show proliferation alterations even at early stages of tumors [96, 97]. It is usually present during all active phases of the cell cycle (G1, S, G2, and M) [93, 94]. In the oral epithelium, these cells are delimited mainly to the basal layer, as observed in our control mice [95]. In Stat6VT mice, there were a greater number of positive cells and they were not confined to the basal layer. The appearance of Ki67 in supra-basal layers has been associated with epithelial dysplasia (alteration in the maturation of epithelial cells and an increase in the proliferation up to spinous layers) [95, 98]. On the other hand, CK1 and CK14 expression were largely similar between the groups. These results suggest that overexpression of Stat6 led to changes in the oral epithelium consistent with an inflammatory response. Further study needs to be done to determine if this inflammatory response is due to a lower threshold for the detection of pathogenic bacteria in the oral cavity due to a change in barrier integrity.

We chose 10–12-week-old males for our *Porphyromonas gingivalis* (Pg) infection model because skin disease onset and progression were more uniform, and bone mineral density and content did not differ from controls. Control and Stat6VT mice were orally infected with Pg (Chapter 7). When assessing their oral epithelium post-infection, Stat6VT mice had an apparent weaker expression and a more widespread distribution of loricrin. In Stat6VT mice, loricrin was not confined to the granular layer and above, as observed in control animals. CK1 protein was also decreased in Pg-infected Stat6VT mice compared to infected controls. CK1 is known to increase during natural cell turn over and healing processes, as it has a known role in epithelial barrier maintenance, repair, DNA processing, cell proliferation, apoptosis and differentiation [99-101]. Given its important role, a decrease in CK1 expression could interfere with epithelial recovery from aggressions and consequently interfere with the epithelial barrier integrity. CK14 is a known proliferation marker [102]. When cells enter the differentiation program, CK14 is silenced whereas Ki67 starts to be expressed in the S phase, and increases in a progressive manner through S and G2 phases, then reaches a plateau at mitosis. CK14 was expanded to surprabasal layers and Ki67 also presented an increase in cell count. Both proliferation markers showing such an increase could potentially indicate a dysplastic event [95, 98].

Across all comparisons, Pg-infected Stat6VT mice demonstrated a greater reduction in alveolar bone levels compared to controls. These findings align with our hypothesis, as we

expected an augmented inflammatory response caused by the pathogenic bacteria, with a greater response due to altered barrier integrity in Stat6VT mice. The greatest bone loss observed was in the lingual plane (0.35mm ± 0.1962), followed by the sagittal plane (0.32mm ± 0.02582), which is similar to other studies that used microCT for measuring alveolar bone loss in mice [60-62].

We also investigated the cytokines present in the blood collected from both animal groups (Chapter 7). The cytokines that were significantly increased in the Stat6VT mice were OPN, IGFBP-2 and ICAM-1. The most significantly increased was IGFBP-2, which showed nearly a four-fold increase. This cytokine plays a significant role in bone metabolism, it increases with age and has been shown to be a strong predictor of decreasing bone mineral density [103-106]. In agreement with our findings, IGFBP-2 expression is induced by Pg and studies in humans have demonstrated that increased concentrations of IGFBP-2 associate with increased probing depth and could be a marker of periodontal disease progression [107, 108].

OPN (also known as bone sialoprotein 1) plays a role in periodontitis, as it is crucial for bone remodeling and biomineralization. Studies show that this cytokine is increased in periodontitis and is related to an increase in osteoclasts proliferation and differentiation [109-113]. Therefore, the increased bone loss observed in Stat6VT mice in our microCT results is consistent with the increased expression of OPN.

ICAM-1 was increased by a little over one-fold. This cytokine has been implicated in several chronic systemic immune and inflammatory responses, and it is known to be overexpressed in inflamed tissues of gingival origin [114]. In addition, the expression of ICAM-1, an adhesion receptor, on human microvascular endothelial cells, can facilitate the invasion by Pg, subsequently enhancing the speed of progression of disease, as observed in our mice and SvP patients [114-120].

Pertaining to humans, deficiencies in CE proteins that affect the epidermis can lead to an intensified host inflammatory response to bacteria involved in disease (Chapter 6) [121-123]. We demonstrated that loricrin appeared to be decreased and not confined to the granular layer and above as observed in controls when assessed via immunofluorescence in the oral epithelium of SvP patients, using constant methodological and image capture parameters (Chapter 7). Loricrin, as well as filaggrin, were also found in unexpected locations, such as the spinous or even basal layers. Our results show a downregulation of loricrin at the protein level, which is in agreement

with the current literature. It has been shown to be downregulated at the gene level in SvP patients, in at least 4 independent studies [57, 58, 60, 124]. The most recent of the gene expression studies not only found a profound decrease in loricrin (20-fold), but also in CK1 (20-fold) and filaggrin (6.58-fold) [124]. These findings support our human study results, as we also found a qualitative decrease in CK1 at the protein level, based on immunofluorescent intensity. Downregulation of CK1 at the gene expression level, along with other important genes, such as CK10, occludin and filaggrin have also been observed in an induced human gingivitis trial (approximately 21 days post-induction) [125]. Although gingivitis does not directly affect the underlying alveolar bone, it has the potential to develop into periodontitis. Thus, a decline in CK1 could greatly impair the healing abilities of the periodontium and contribute to progression of disease.

A major finding of our studies, in both human and mouse tissues (Chapter 7), was the change in localization of CK14. Mutations in this protein are related to the etiology of several skin disorders, such as bullous congenital ichthyosiform erythroderma, epidermolysis bullosa simplex, epidermolytic palmoplantar keratoderma, pachyonychia congenital and white sponge naevus [126, 127]. Expanded expression of CK14 to supra-basal layers is observed in chronic wound healing or in poorly differentiated carcinomas [100]. Therefore, the mislocalization of CK14 observed in human SvP patients could be an indication of an inflamed and stressed oral epithelium [99, 100, 128]. The lack of CK1:CK10 in supra-basal layers, because of CK14 replacement, may also diminish the protective abilities of the epithelium and consequently, weaken barrier integrity [129]. This supports our hypothesis that a barrier defect could explain the enhanced inflammatory reaction and increase in bone loss in response to pathogenic bacteria in SvP.

Limitations and future directions

The classification system for periodontitis is not a static model, it has been undergoing modifications over the years as the understanding of the etiology has been expanded in light of new research [1, 11, 26, 130-132]. The 1996 World Workshop on Periodontology indicated the need to review older classifications. Thus, a committee met to revise the classification system for periodontal diseases, which was presented in 1999 [4]. This classification proposed numerous changes. Periodontitis was classified as chronic and aggressive (localized or generalized), necrotizing ulcerative periodontitis and periodontal disease as a manifestation of systemic diseases

[132]. The subsequent classification released in 2017, eliminated the distinction between aggressive and chronic periodontitis. The reason was that the evidence had yet to identify a specific pathophysiology that would distinguish the two forms of periodontitis. The proposed system notes that "*there is evidence of multiple factors, and interactions among them, that influence clinically observable disease outcomes (phenotypes) at the individual level*" [28].

This research began prior to the 2017 Classification of Periodontal Diseases and Conditions. At that time, aggressive periodontitis was still a distinct form of periodontitis. The subjects for this study diagnosed with aggressive periodontitis presented with a severe form of disease, were young (< 35 years of age), had a family history of periodontitis, and no underlying systemic diseases. As the study advanced, the 2017 classification was released. This included a new model where periodontitis was defined using a staging and grading qualifier. The staging describes the severity of the disease where Stage I is mild, progressing to Stage II and then Stage III and IV are more severe forms [28]. In addition, grading describes the progression of the disease from the slow rate of Grade A to the rapid rate of Grade C. To align with the 2017 update, the study included subjects with either Stage III Grade C or Stage IV Grade C periodontitis as this is the more severe and rapid progressing form of the disease. Of note, the grading system could have influenced sample collection as it includes modifiers such as diabetes and smoking. These grade modifiers could have assigned a patient to a higher grade based on these systemic considerations (even if temporarily). Therefore, only patients that were systemically healthy and non-smokers were included in this study. Further, the age limitation was no longer considered in the patient pool. The reason is that the grading system can use "*indirect evidence of progression*" through a radiographic bone loss ratio defined by the bone loss percentage over patient age (ranging from <0.25 to >1) [28]. In a way, this would be similar to what was observed in aggressive periodontitis: if for example, these patients were younger than 35 and had more than 35% of bone loss, they would be Graded as C (ratio in this case being at least 1). However, even in a systemically healthy individual, physiological mechanisms may differ from a Grade C who is 35 to a Grade C who is 60 (same Stage). In older patients, alveolar bone may lose mineral density, have reduced trabeculae and reduced bone formation, there is thinning of cortical plates, a decrease in vascularity and water content and thickened collagen fibers [133-136].

The workshop, however, did expect novel research investigating disease-specific mechanisms that looked at the diverse biological connections in unique phenotypes. Therefore, future research could focus on the impact of ageing and periodontitis where patients having similar diagnoses but different ages are compared. This may bring some answers as to how aging could affect susceptibility and prognosis. In addition, an evaluation of the different CE proteins could be studied across the different Stages and Grades of Periodontitis. This may clarify mechanisms that give rise to various phenotypes observed across the populations with different severity of periodontitis.

Another important area for future research is barrier function. We know that both oral and skin epithelium have common functions and histological characteristics, such as tight junctions, CE, stratification of layers by gene/protein expression and major cell type. Deficiency/mislocalization of CE proteins underlie several skin pathologies related to barrier function. Therefore, in the beginning stages of our project we tried to build a 3D human cornified epithelium model (also known as 3D skin equivalents) generated from epidermal keratinocytes to test barrier function after inducing loricrin deficiency by genetic knockdown. We employed two different assays: transepithelial electrical resistance (also known as TEER) and dextran migration. We used commercially available keratinocytes (primary cells) to build the 3D skin equivalent. Primary cells more accurately represent the biology of normal cells, however they are usually more technique sensitive and harder to manipulate than cell lines [137, 138]. Unfortunately, we were not successful in building the 3D skin model to perform the planned experiments, as we were not able to grow more than two layers. Some companies nowadays sell 3D reconstructed human epidermis, human buccal and gingival phenotypes, allowing researchers to request specific gene knockdowns. Therefore, utilizing proteins that showed some sort of downregulation or misplacement in our study in a knockdown *in vitro* model could assist in the determination of barrier function.

An additional field for future exploration is *in vivo* barrier function assays. Our findings suggested downregulation or dislocation of several CE proteins in the epithelium of our animal model. This opens doors to experiments that look directly at barrier function *in vivo,* such as dye diffusion assays or measurement of transepidermal water loss (also known as TEWL) [137].

From a clinical perspective, the possible disruption of differentiation of the oral epithelium due to changes in CE protein expression could give insight that would lead to new avenues for treatment. Research into barrier dysfunction had led to the development of new pharmaceuticals. Nowadays, there are commercially available body washes and moisturizers that contain filaggrin which are advertised to help improve skin barrier function for patients with AD or psoriasis [139, 140]. As research enlightens more pathways involved in the epithelium organization, differentiation and function, more can be translated to into medicinals.

A limitation of the Stat6VT model, as previously explained, is that they overexpress Stat6. This may be potentially confounding, as it is an important factor for lymphocyte proliferation, gene expression (e.g. IL-4 induced genes) and T-cell differentiation [63, 64, 141, 142]. Previous studies show that about 5% of Stat6VT mice develop lymphoproliferative disorder, causing splenomegaly and promoting alterations in splenic cell populations [63, 141]. Some studies suggest that this is dependent upon differences in animal facilities, and that the activation of the immune system occurs in response to environmental antigens that are able to disturb peripheral lymphocyte homeostasis [63, 141]. No animals in our study had signs of lymphoma.

There is an alternative/complementary animal model that can be explored for future research: a transgenic mouse overexpressing IL-4 [143-145]. These mice have some similarities with our Stat6VT transgenic animal: both have been used as an established atopic dermatitis model, they have increased expression of IL-4-stimulated surface markers and percentages of peripheral B cells, and decreased loricrin expression [63-65, 144-147]. As in the Stat6VT model, IL-4 is expressed in epithelial cells. It is known to have less effects on the lymphoid organs and it downregulates the skin immune system against common pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, therefore this could also occur in the oral cavity [144]. In terms of skin lesion development, these animals show skin lesions a bit earlier than the Stat6VT model [143, 144]. Systemic and alveolar bone condition of this transgenic have not been published, so this would also be a point of investigation prior to considering it for the Pg-infection model [143, 144].

WHITE SPOT LESIONS AND ORTHODONTICS

Oral health starts with preventing possible diseases that may affect the oral cavity. Caries results from a dynamic process in which the presence of microorganisms in dental plaque can lead to a balance disorder between the mineral phase of the tooth and the oral environment surroundings through the production of acids by the microbiota [148]. WSL are one of the first indicators of enamel demineralization, hence the importance of understanding the initiators and possible preventors of this process. The increase in WSL during orthodontic treatment is a continuous concern to clinicians, given that even patients with excellent to good oral hygiene still develop some sort of demineralization during treatment [149].

In this study, the oral hygiene of patients had to be maintained as at least "good" according to the OHI-S throughout the treatment. The diagnosis of WSL as well as oral hygiene checks were performed by at least two orthodontic residents and recorded via pictures and chart notes. We found an average of 5.70 WSL per patient (range 1-12 WSL, standard error of mean ± 1.193), which is very similar to what has been reported in the literature [149-152]. In our study, we included only orthodontic treatment with self-ligating brackets, since elastomeric rings may increase the amount of microorganisms around each bracket [153, 154]. The fact that patients maintained good oral hygiene was a fundamental inclusion criteria, as previous studies showed that patients could still develop WSL despite compliance [155]. As patients had good oral hygiene from beginning to end of treatment and still presented with WSL, there is room for more exploration on the diversity of factors that could be contributing to the development of these lesions, such as microbial and salivary environments.

In a pioneering work, in 1944, Robert Stephan evaluated patients' dental plaque pH after carrying out a mouth rinse with a glucose solution, in individuals free of caries and with different caries extent. Based on this study, a "Stephan curve" was created. The curve has basically 3 phases. in the first phase, there is a rapid drop in pH due to the acid production from fermentation of dental plaque bacteria. In the second phase, normally the pH remains below the critical pH, 5.5, when the dissolution of calcium and phosphorus ions try to prevent the action of acids, and the onset of remineralization. In the last phase, gradual increase in pH occurs, until reaching the initial level. The last phase occurs between thirty to sixty minutes after rinse, and the pH tends to remain at this level between meals [156, 157]. In Stephan's study, *plaque* pH in caries-free patients reached a

minimum of 6, whereas in the extreme caries activity group, the minimum pH reached was 4.5 [157].

Studies that looked at *saliva* pH showed that baseline/resting pH values ranged from 6.8 to 7.6 [158, 159]. The main reason for such variation was the standardization in the collection method (if patients had brushed before, utilization of mouth rinse or eating food at least 30 minutes prior to their appointment), since these values are measured prior to any dietary challenge. In our study, we found quite similar results [159-162].

Only one study looked at saliva pH drop after a 10% sucrose challenge as ours; most studies utilized acidic drinks or milk to test for saliva pH, which makes a clear comparison to our study more challenging [162]. Nevertheless, when comparing to the study that used sucrose to evaluate the Stephan curve, saliva had an average pH of 6.6 at the 5 minute time point, 6.8 at the 15 minute time point, and then pH 7 for both 30 and 45 minute time points, which compared well with our study [162].

Changes in salivary pH on the enamel can be related to demineralization properties and microbiome variations compared to a baseline or resting state because of a variety of perturbations. In our study, we investigated the correlation between Stephan curve kinetics with patient microbiomes at the beginning of treatment to try to define an at-risk population for WSL formation. In fact, the absolute pH changes were not significantly associated with any unimodal microbial abundance changes, however, pH drop at 5 minutes was significantly associated with the abundances of specific species. This suggests that the severity of pH change (first drop or 5 minutes timepoint) from baseline was significantly associated with specific bacterial species abundances, confirming that saliva, as an ecological environment, is influenced by such pH perturbation. This also leads us to believe that oral hygiene within the 5 minute window post-sugar challenge might be key to reduce some acid producing species.

It is known that the relative abundance of species in groupings certainly varies in space and time corresponding to how species react to environment conditions [163]. Therefore, in terms of statistical techniques that allow us to perform a multivariate analysis of datasets, we opted to do a canonical correlation analysis (CCA); this type of analysis finds linear transformations for two multivariate datasets so that the correlation between converted datasets is augmented [164]. As our

results showed, when we considered the pH drop at 5 minutes, constraining the model showed 10.846% of inertia within the model [165].

We found correlations with absolute pH at different time points, and with ΔpH at 5 minutes. At baseline, the bacteria that showed a moderate relationship ($0.5 < r < 0.75$) with pH was *Stomatobaculum Spp* (anaerobic gram positive), presenting a negative correlation. This bacterium has been related to *H. pylori* biopsies, with very little documentation as to its functions or metabolic characteristics [166]. Then at 5 minutes after sucrose rinse, *Eubacterium nodatum group_sulci* (anaerobic Asaccharolytic gram positive), *Selenomonas Spp* (anaerobic acidogenic gram negative bacteria) and *Stomatobaculum Spp* presented a negative correlation. *Selenomonas* are known to have as metabolic products acetic, lactic and propionic acid, to have a negative correlation given the drop in the pH [167-170]. On the other hand, known metabolic products of *Eubacterium nodatum* are butyrate and acetate, which increase heme production (allowing the growth of some bacteria involved in periodontal disease) and may act synergistically to promote the growth of certain bacteria over others (such as *Pg* promoting the growth of *Treponema denticola*) [171-173].

15 minutes after sucrose rinse, only *Butyrivibrio Spp* (anaerobic butyric acid-producing) showed a moderate negative correlation. This group is metabolically adaptable, as they are able to ferment a great variety of sugars and cellodextrins [174, 175]. Sugar fermentation by *Butyrivibrio Spp* allows some important *Streptococcus* species to produce extra-cellular polymers and adhere to dental surfaces utilizing this polymer; in addition they can grow favorably in the presence of cellodextrins [175, 176].

For the comparison of bacteria and ΔpH at 5 minutes, a moderate negative correlation was observed with *Gemella Spp* and a positive correlation with *Selenomonas Spp*. For the *Selenomonas* species, as previously explained for absolute pH at 5 minutes, the correlation makes sense, given that this species thrives in an acidic environment. *Gemella* are gram-positive cocci which have been previously isolated from human dental plaque; they can produce acetic and lactic acids in the absence of oxygen and acetic acid and CO_2 in the presence of oxygen [177]. The current literature on *Gemella* shows that it has been associated with oral health in children and young adults [178]. Another study, however, found that *Gemella sanguinis* and *Gemella haemolysans* were associated with gingivitis in an adolescent orthodontic patient population, and a recent metagenomic analysis included *Selenomonas spp* and *Gemella spp* as co-prevalent with *Streptococcus, Veillonella* and

Actinomyces in the saliva of patients with caries [179, 180]. *Gemella spp* ferment glucose, sucrose and sugar alcohols to yield acid in anaerobic and aerobic conditions [177]. Similar to *Selenomonas spp*, they are adapted to an acidic environment. Interestingly, the fact that they have been associated with health in one study could be due to the bacterial configuration, as it varies from subject-to-subject (some individuals even with severe caries do not present with detectable levels of *S. mutans*, for example) and this supports the ecological microbiome hypothesis (caries results from an alteration in the equilibrium of resident microflora determined by shifts in local environment) [181].

For WSL count, there was a moderate positive correlation with *Capnocytophaga sputigena* and *Neisseria sicca*, and a negative correlation with *Streptococcus australis*. Studies suggest that *Neisseria* spp account for >15% of the saliva microbiome in active caries patients, however they have also been associated with caries-free status [182, 183]. The difference in patients' age in the study that found an increased prevalence of *Neisseria* spp in caries-free group (3-5 years of age) compared to ours could explain a shift later in life (as our patients were predominantly adolescents), as diet, dentition, oral hygiene habits of a young child differ from adolescents/adults. *Neisseria* spp also include many different pathogens, with the list continuously revised. There are currently 10 associated with humans, which means that *Neisseria* spp include several groups that may have more impact in the oral environment than necessary *Neisseria Sicca* singularly [184].

LEfSe analysis showed that *Capnocytophaga sputigena* was exclusively found in WSL patients. They are normally considered oral commensals [180]. *Capnocytophaga spp* require CO_2 and ferment carbohydrates to succinate and acetate. They have also been associated with gingivitis, periodontal disease, halitosis, diabetes and pre-diabetes [66-69]. Their relationship with caries remains ambiguous; one study found *Capnocytophaga spp* to be associated with caries-active individuals, while several suggest they are more often a sign of a caries-free state [185-187]. The difference could be due to multiple species of the same genus (spp) being investigated (with no clear distinction of the species level) and age-group. The studies that found association of *Capnocytophaga* with health investigated *Capnocytophaga gingivalis*, especially in aggregates with *Actinomyces Israeli.* These studies, however, did not specify the age-group, whereas the study that found an association with caries investigated an adolescent population (average age 17) [188, 189]. One study that observed microbial succession in biofilms following professional cleaning

found that species such as *Capnocytophaga sputigena* specifically were more prevalent in subgingival biofilms of periodontally compromised patients seven days after initial supragingival colonization by *Streptococcus mitis*, *Veillonella parvula* and *Capnocytophaga gingivalis,* which were present in both healthy and diseased individuals. This suggests a more mature biofilm in patients with WSL [190].

Prevotella melaninogenica, another pathogen that is commonly associated with periodontal disease, advanced carious lesions, and active-caries saliva, was found mostly in WSL patients in our study [191-194]. Along with the presence of *Capnocytophaga sputigena*, these results suggest a more mature plaque ecosystem displayed in the saliva of patients that developed WSL. This is remarkable because these parameters were assessed more than two months before the first WSL developed in one of our patients.

Streptococcus mitis and *Streptococcus australis* were primarily present in healthy patients. *Streptococcus mitis* was reported to be one of the least likely bacteria to contribute to caries. After the introduction of 16S sequencing, this species could be differentiated from *Streptococcus mutans* and shown to be one of the least resistant to low pH when compared to other *Streptococcus species* [195]. *Streptococcus australis* can hydrolyse arginine to a base, ammonia [196]. As such, it can neutralize acids and play a significant part in plaque homeostasis by preventing the growth of aciduric bacteria [196]. In this manner, *Streptococcus australis* is able to antagonize dental caries pathogenesis as it can produce alkaline phosphatase, which boosts the calcium and phosphate availability in saliva and plaque, supporting remineralization [197, 198].

A moderate negative correlative relationship with baseline pH was observed for *Stomatobaculum spp* in WSL patients. These bacteria are anaerobic gram-positive and have been previously associated with dentin caries and teeth that need endodontic treatment [199-201]. They are able to ferment glucose to butyrate, lactate, isovalerate and acetate [199-201]. Similar to *Capnocytophaga spp*, *Stomatobaculum spp* have been shown to be more abundant in pre-diabetics individuals [201-203].

In conclusion, our results suggest an increased abundance of acid-producing bacteria in the saliva of WSL patients, but without usual “expected” bacterial profile (*Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus acidophilus*, known as the most important caries initiators). One reason for the change in the typical bacterial profile is the fact that our microbiome

analysis was done prior to WSL detection. Thus, our study may have uncovered the earliest predecessors to WSL/caries development. This data could be important as initial bacteria profile may be a key factor for the future development of WSL. The current results also shed light on potential saliva pH modulation as a therapeutic method to manipulate the oral cavity's abundance of caries initiators.

Limitations and future directions

We were able to control for several factors that are listed in the inclusion criteria, some of those were: patients' bracket system, length of treatment, oral hygiene before and throughout treatment, enamel conditions pre-treatment and patients' body temperature. However, dietary habits, ethnicity and socio-economical background could not be fully controlled in this study.

At the University of Alberta, all patients receive basic dietary advice, written and verbally, on braces bonding day. When talking about diet to patients, it is suggested that they avoid chewy, crunchy, sticky and hard foods and the ingestion of anticariogenic or cariostatic foods, such as milk, soft cheeses, cooked meats, eggs and most vegetables is promoted. However, we do not make direct reference to avoid sugar rich foods/drinks. This could have led to a skew in our results as it is known that there is a bidirectional association between oral wellbeing and diet/nutrition as the intake of sugars has been linked to an augmented risk of developing caries [204]. A new diet advice pamphlet could be created with the purpose of directly addressing the need to avoid specific cariogenic foods and motivation for cariostatic/anticariogenic foods. There are food journals used in pedodontics that aim to limit caries risks by controlling the patients' diet, as they have to report every meal as they would with a dietician. Patients can still refuse or forget to enter certain foods, but having a system like this in place could help address this limitation. Previous studies, however, have shown that the validity of diet questionnaires and journals is very short term. We thoughtfully considered a questionnaire, but given out study length and we decided it would most likely not be useful.

It is also known that there is an inequality in dental caries experience in different ethnic groups [205]. Even though our group consisted of Canadian citizens that lived in Edmonton, Alberta, their ethnic background could not be tracked. Hispanic and black individuals experience

a higher prevalence of dental caries than the white population in different countries [205, 206]. As we had a very specific population niche, this could have also limited our results and potentially explain the findings observed between the WSL and the healthy group. According to the 2013 National Household Survey, 22% of Canadian residents are first generation immigrants, 17.4% are second generation immigrants (born in Canada but at least one parent was born outside of Canada), and 60.7% are third generation or more (some generations of ancestors born in Canada) [207]. Even though self-declared information can have its own bias, adding ethnicity questions may be useful in a future study [207-209].

Common socioeconomic status indicators used in epidemiological research are parental education, rural residency, access to dental care and frequency of brushing and flossing [210]. Amongst those, we were only able to control for oral hygiene. Higher level of mother/father education is negatively associated with dental caries experience, whereas rural area residency is positively associated with increased occurrence of dental caries [210]. This limitation could be addressed by adding a survey at initial patient screening that included some of these factors and they could be added to the study inclusion/exclusion criteria.

The control group was older compared to the WSL group, but our analyses did not detect any differences between the 3 control patients that were greater than 40 years of age (43, 44, 45) compared to the 7 that were less than 40 years of age in terms of Stephan curve kinetics. Without these 3 patients, the mean age of the remaining 7 control patients was 14 ± 1.024, therefore very close to the 10 patients in the WSL group (13.10 ± 0.3480).

It should be emphasized that at time of saliva collection and Stephan curve kinetics, all patients had just had a professional dental cleaning, and all had equivalent good OHI-S scores. In addition, strict oral hygiene protocol was taught and monitored. It included: brushing and flossing at least three times a day (at least 3 minutes in total), flossing could be done with the assistance of floss threaders/superfloss or proxabrushes or waterflosser. All patients were advised to use toothpastes that contained at least 1450ppm fluoride.

Some factors that we did not measure were salivary buffering capacity and salivary flow. These are important measurements, as they may provide us with the quantity and quality of the saliva, and when increased contribute to decreased susceptibility for caries [211, 212]. Saliva buffering capacity is the property that allows saliva to maintain a constant pH (blocking the excess

of acids and bases), which also assists in the maintenance of teeth and oral mucosa integrity [161, 211, 213]. Many bacteria require a specific pH for their maximum growth, therefore the buffering capacity of saliva prevents potentially pathogenic microorganisms from colonizing the oral environment by inhibiting their optimum conditions [213]. Salivary flow is usually measured in mL/min and can be affected by age, sex, time of day and possibly genetic disorders [214]. Therefore, these factors could be confounding variables that need to be further explored. Orthodontic appliances do not influence these factors, so this measurement could be performed at the beginning or during treatment and be correlated to microbiome findings and salivary pH/curve [213].

We also did not measure dental plaque pH or plaque microbiome. These two factors can be a point for future investigation, as dental plaque and saliva possess different microbial compositions and the dental biofilm is known to play an important role in the progression of dental caries [215-217]. Previous studies suggest smaller differences between microbiome plaque in orthodontic patients that develop WSL *vs* controls, however this information combined with the saliva microbiome data could potentially result in stronger associations, especially when associated with pH curves/drops [180].

In regards to pH of dental plaque, it is highly variable according to the tooth surface from where it was collected, even in the same patient. On the other hand, collection of saliva is a very simple and non-invasive procedure that can provide a range of information to address enamel demineralization and identify susceptible subjects. Nonetheless, we only evaluated saliva prior to orthodontic treatment, and this parameter alone could not predict individuals more likely to develop WSL. It is possible that Stephan curve kinetics in dental plaque would be more informative, as well as assessing both at different time points during treatment and after treatment. This may demonstrate more distinct differences between the groups. Another modification that can be considered for future studies is the concentration of the sucrose rinse. We used 10% in our study, but other studies have used higher concentrations, which may more effectively uncover differences in saliva buffering capacity. Nevertheless, the fact that several bacteria which are normally related to caries were found to be associated with ΔpH provides a convincing justification for additional investigation.

We assessed WSL clinically on the enamel by drying the surface with a regular triple air syringe, also known as direct visual assessment (DVA). The assessment was done by an orthodontic resident and confirmed by a second resident via pictures. We understand that this is a limitation of our study, as this is not an easy assessment given the bracket surface coverage and studies that use DVA or pictures (or even the combination of the two) tend to show a smaller prevalence than ones that look at light-induced fluorescence [218, 219]. Light-induced fluorescence is a method that can be more reproducible, more objective and quantifiable when assessing WSL [218]. The fluorescence image can detect incipient lesions, which can be digitalized and the loss of fluorescence can be quantified when compared to sound enamel (fluorescence radiance level) [220, 221]. Therefore, this method of assessment would allow researchers to detect even initial enamel lesions, making the WSL diagnosis more accurate not only throughout treatment, but even at the inclusion criteria stage.

Discussion References

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