Loricrin and Aggressive Periodontal Disease

by

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Abstract

Background: Aggressive periodontitis (AgP) is often associated with specific features, such as a unique microbial profile or pattern of host-response (localized to first molars and incisors in some of the cases). Nonetheless, a unifying mechanism has not been established for this condition. AgP patients are typically diagnosed under the age of 35 years and present with plaque accumulation that does not correlate with the degree of bone destruction present. The rapid bone loss ultimately leads to early tooth loss and complex restorative treatment for patients. AgP patients have also been shown to have an increased expression of Th2 cytokines. Although controversial, Th2 cytokines have been observed in the active or "progressive" lesion of chronic periodontal disease. Th2 cytokine expression has been shown to play a role in loricrin downregulation and other studies have shown downregulation of the mRNA for loricrin in AgP patients. Loricrin comprises 70-80% of the total protein mass of the cornified epithelium and its complex cross-linked structure helps maintain the barrier between the external and internal environment. The importance of loricrin as a barrier protein is illustrated in a different inflammatory disease of the skin: atopic dermatitis. Atopic dermatitis patients experience a downregulation of loricrin protein in their skin and as a result, are more susceptible to pathogenic bacteria penetration and a consequential inflammatory response. Research in the area of atopic dermatitis has shown that an increased Th2 response can result in loricrin downregulation via the transcription factor Stat6. As AgP patients exhibit a Th2 response, it is possible that Th2 cytokines activate the Stat6 pathway in AgP patients which leads to downregulation of loricrin in the oral cavity. This downregulation in loricrin could compromise barrier function in the

epithelium of patients with AgP and thus, may explain the dramatic inflammatory response they experience.

Aims: **Aim 1**: Determine if downregulation of loricrin at the protein level is associated with AgP in human patients. **Aim 2**: Determine if Stat6VT mice are a potential model of AgP.

Methods: Gingival tissue samples were collected from periodontally healthy patients and AgP patients undergoing routine periodontal surgeries in which tissue is normally discarded, in clinics in Alberta, Canada, and Sao-Paulo, Brazil. Western blot and ELISA techniques were used as loricrin protein detection methods. These methods determined if AgP patients experience a downregulation in loricrin. Loricrin protein concentration was measured in Stat6VT and wild type mice; Stat6VT mice are engineered to overexpress Stat6, and as a result, have higher levels of Th2 cytokines and consequently, loricrin downregulation. ELISA was used to determine any difference in loricrin protein expression in mice oral tissue samples. MicroCT analysis measurements were performed to compare bone loss between the two mouse groups. Sections were obtained and stained with hematoxylin and eosin for histological evaluation.

Results: A total of 12 samples from AgP patients and 11 samples from healthy control patients were collected. An ELISA was performed to compare the amount of loricrin protein in the samples. The average concentration of loricrin was significantly higher in the healthy group (9.240±1.572ng/ml) than in the AgP group (2.813±0.8583ng/ml) ($p=0.0008$, MannWhitney test). A total of 6 Stat6VT mice were compared to 6 wild type mice by ELISA. The difference in loricrin protein expression in the tissue of Stat6VT and wild type mice was not statistically significant ($p=0.537$) (The means ± S.E. were 0.14 ± 0.03 for wild type and 0.10 ± 0.03 for Stat6). In this first trial, mice were not gender matched, nor scored for dermal lesion severity, which may have affected our outcome. Interestingly however, the Stat6VT mice had

significantly more bone loss than wild type mice, even in the absence of specific pathogen challenge. Furthermore, Stat6VT mice showed signs of inflammation and bone resorption in the histological sections.

Conclusions: These results suggest that patients with AgP have less loricrin protein expression, consistent with gene expression studies. Based on these results we now hypothesize that decreased loricrin protein may result in a compromised oral barrier by disrupting normal epithelial differentiation, and this may explain why biofilm bacteria cause such a dramatic inflammatory response in AgP patients. Additionally, Stat6VT mice showed increased inflammation and bone resorption compared to wild type. Our results suggest that this mouse strain deserves further study to determine its utility as a model for AgP studies.

Preface

This thesis is an original work by Danielle Clark. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, "The role of loricrin in aggressive periodontal disease", No. 00062112.

Chapter 1 of this thesis has been published as Clark D, Febbraio M, Levin L, "Aggressive Periodontitis: the Unsolved Mystery" Quintessence International, vol. 48, issue 2, 103-111. I was responsible for the literature review and manuscript composition. Both Febbraio and Levin were the supervisory authors and were involved with concept formation and manuscript composition.

Dedication

I dedicate my dissertation work to my parents who taught me the value of hard work and dedication while providing me with unconditional support throughout my educational career as well as to my dear fiancée who truly believed in me and who continues to make the bad days good, and the good days even better.

Acknowledgments

This work was done in collaboration with my two supervisors Maria Febbraio and Liran Levin, along with Raisa Catunda, Marcelo Faveri, and Magda Feres. Other acknowledgements extend to lab members Yuli Broner, Konrad Lehmann, Julia Piche, Umar Rekhi, and Maria Alexiou. Funding acknowledgment extended to the University of Alberta Hospital Foundation Fund, the University of Alberta Dental Hygiene Research Fund and the School of Dentistry Fund.

I would also like to give special consideration to both of my supervisors Dr. Febbraio and Dr. Levin. As a dental hygienist, I had very limited experience in research, especially in a laboratory setting. Nonetheless, both Dr. Febbraio and Dr. Levin took the risk and accepted myself as a master student in oral biology. My supervisors were able to increase my skill set exponentially. From learning how to do simple techniques such as using a pipette, they guided me to eventually be able to understand and perform laboratory experiments like western blotting. Before I knew it, both of them were coaching me through presentations for the International Association for Dental Research Conference in San Francisco, CA and the European Federation of Periodontology Congress in Amsterdam, The Netherlands. With their motivation and support, I was able to achieve several prestigious awards and funding. My experience has inspired me to continue my research career and pursue a PhD.

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Chapter 1: Introduction

Aggressive Periodontal Disease-Background

Aggressive periodontal disease (AgP) was classified in 1999 as a specific form of periodontal disease characterized by a non-contributing medical history, rapid attachment loss and bone destruction and familial aggregation (Albander 2014). The progression of disease in AgP is thought to occur at a more rapid rate than in the chronic form of the disease. This rapid destruction of bone and attachment structures might lead to the subsequent loss of teeth. The aggressive nature of AgP can lead to the devastating early loss of teeth in a very young patient population (Levin, 2011). The exact etiology of AgP remains mysterious. Although it is doubtless that bacteria are a major player in the disease, it is unclear why there is an exaggerated response to the apparently minimal plaque accumulation. Genetic predisposition is another influence in the manifestation of AgP and familial aggregation is one of its defining characteristics (Susin 2014). Genes have been identified that are associated with AgP, with many also associated with the host immune response, including those that affect the expression of IL-1, IL-6, IL-10, tumour necrosis factor-α, among others (Rescala 2010, Zhang 2016, Duarte 2015). It should be noted, however, that many studies have specifically looked at this category of genes only, thus there is an intrinsic bias. According to Stabholz et al., there are no specific genes that differentiate AgP and chronic periodontal disease, however some research has alluded to the possibility of different polymorphisms of one gene being responsible for the differential presentation of the diseases (Stabholz 2010).

An important clinical finding in AgP in contrast to chronic periodontitis is the presence of only a thin plaque biofilm and little if any calculus, which does not correlate with the degree of damage and intensity of the inflammation (Albander 2014, Herbert 2015). Thus, it appears as if

the host is responding to a much greater peril than present and patients require early diagnosis and frequent professional appointments for scaling and oral hygiene instruction in order to maintain their teeth. An alternative hypothesis is that the host response is appropriate for the threat, but there is some other factor that increases the threat. This response may be attributed to a compromised protective barrier. For example, if the epithelial barrier of the sulcus is healthy and functioning normally, pathogenic bacteria will not be able to penetrate with ease (Figure 1). However, if for some reason the epithelial barrier is compromised, it may result in easier penetration of bacteria, leading to an inflammatory response from the host (Figure 2). With so many unknowns, AgP remains a mystery and warrants essential further research to both allow for definitive diagnosis and to increase our basic understanding so that effective new early detection and treatment strategies can be developed for better outcomes for patients.

Figure 1: A healthy sulcus with an intact barrier preventing bacterial penetration. When the epithelial barrier of the sulcus is healthy and functioning normally, pathogenic bacteria will not be able to penetrate with ease

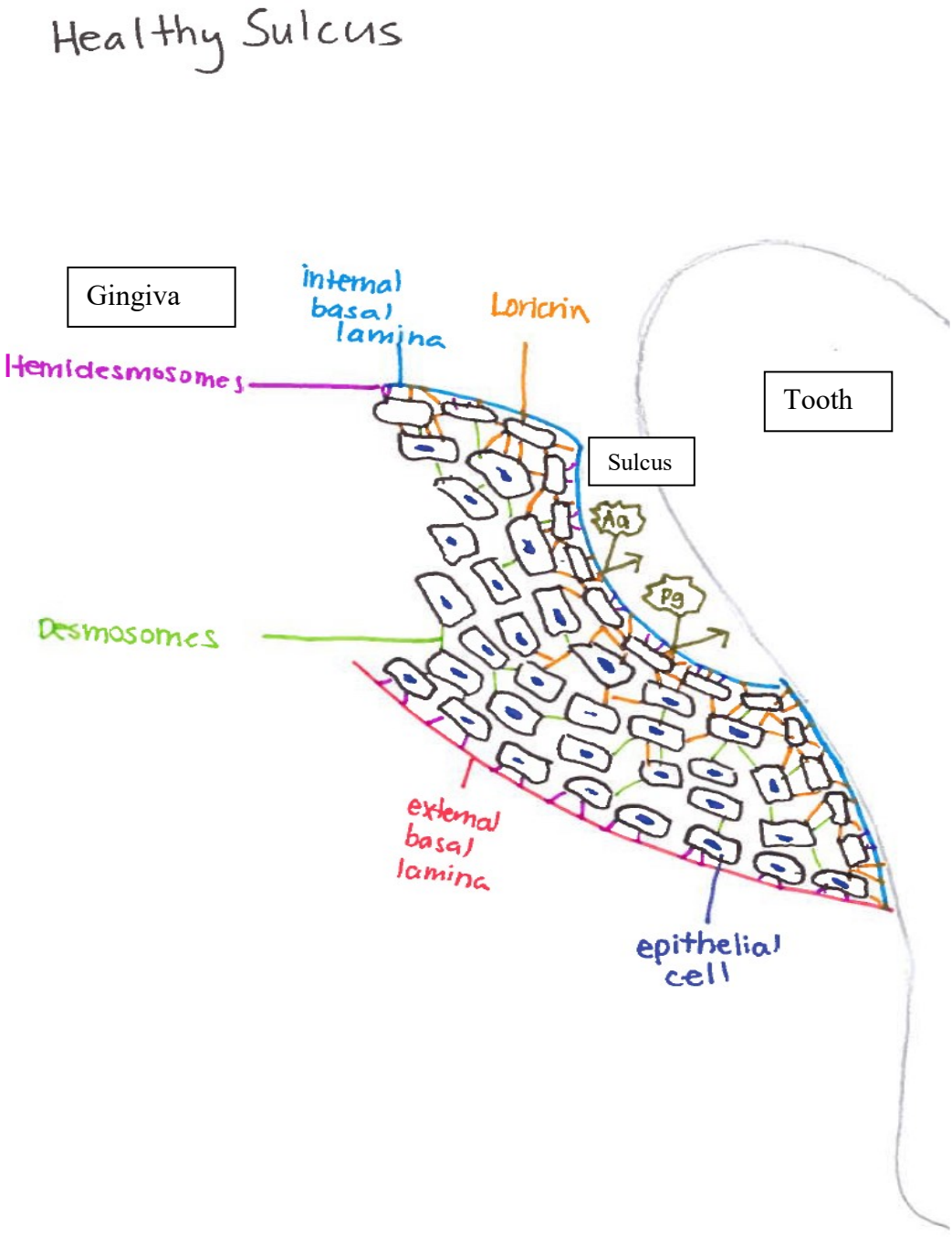
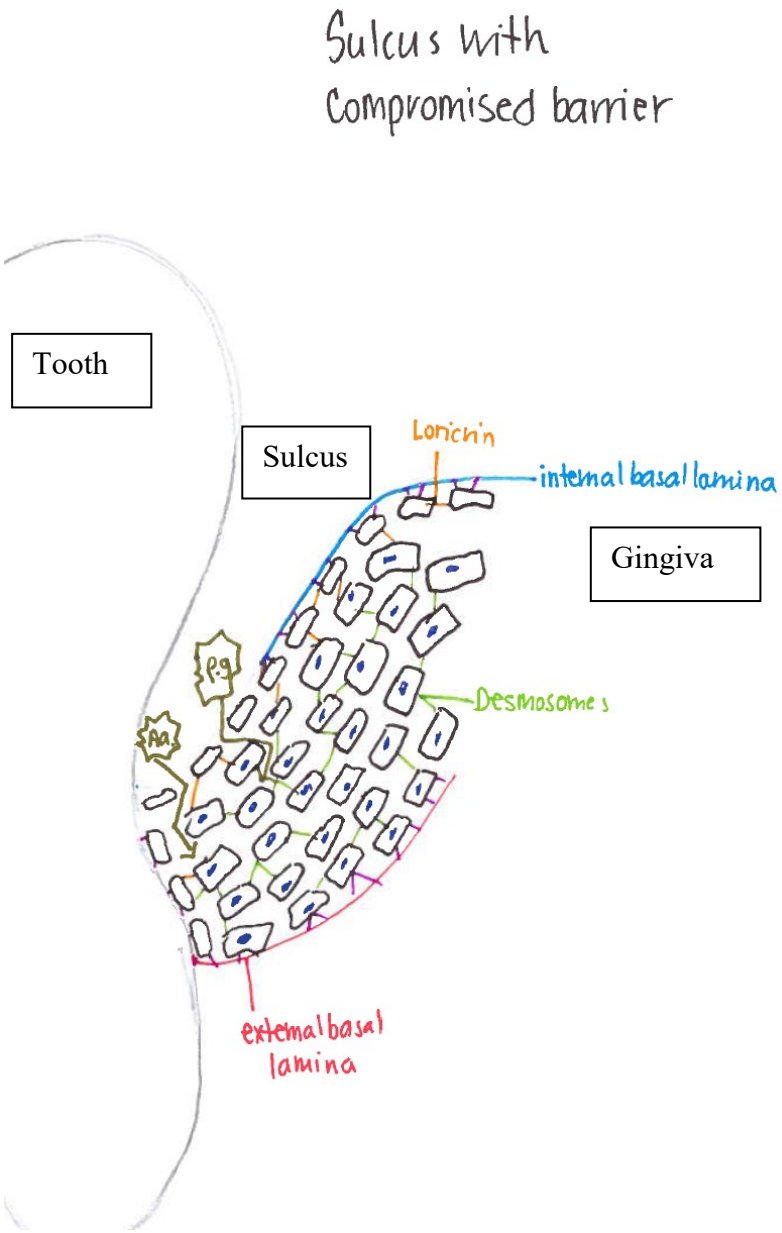


Figure 2: A sulcus with a compromised epithelial barrier due to less loricrin allowing easy penetration of a small amount of bacteria.



The Role of the Cornified Epithelium in Barrier Function

The cornified epithelium (CE) is the outermost layer of the skin and oral mucosa, optimized for barrier function (Darlenski 2011). Figure 3 provides an illustration of the different layers of the epithelium, while Figure 4 illustrates a more detailed drawing of the CE. In the CE, keratins give mechanical strength, while proteins tightly join adjacent cells to create a barrier. The CE proteins responsible for this barrier function include: involucrin, cystatin A, loricrin, small proline-rich proteins, elafin, proteins of the S100 family, profilaggrin and some desmosomal components (Proksch 2008). Loricrin is a 26 kilodalton (kDa) insoluble protein that constitutes approximately 70-85% (volume) of the CE in differentiated corneocytes (Hohl 1991, Steven 1994, Steinert 1995, Kalinin 1991, Steinert 1998). Loricrin is heavily transglutaminated which means it has isopeptide bonds in-between free amine groups and acyl groups found at the end of side chain proteins. The formation of these bonds creates stable structures that are relatively insoluble. The major function of these bonds is to help reinforce the protective barrier made by loricrin. The inter- and intra-protein crosslinks are highly resistant to proteolysis and stabilize and strengthen the CE (Candi 1995). Therefore, if loricrin expression is downregulated, it is reasonable to suspect a potential impact on the barrier function of the CE.

Figure 3: Stratified layers of an intact epithelium (derived from Pocket Dentistry, Chapter 12)

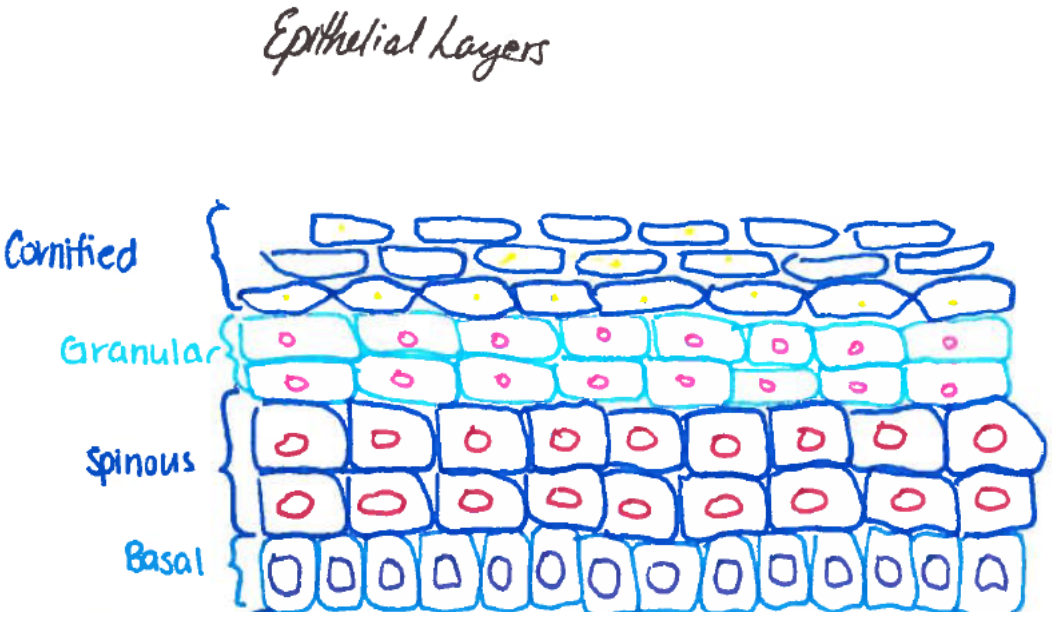
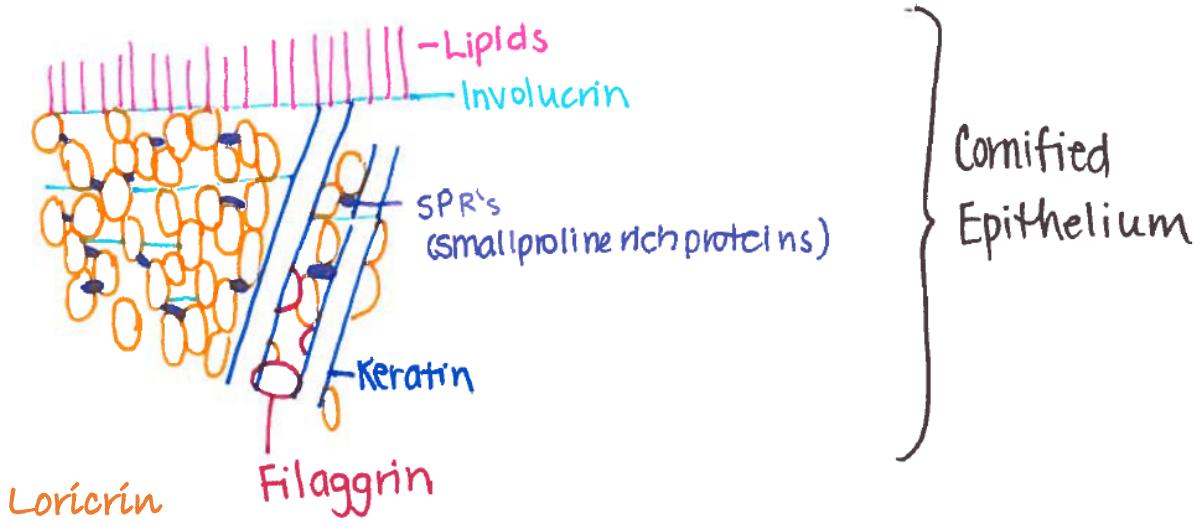


Figure 4: Visual representation of loricrin in the cornified epithelium (derived from Pocket Dentistry, Chapter 12).



Barrier Function in Skin Diseases

Barrier function plays an important role in inflammatory skin diseases. Psoriasis and atopic dermatitis are two such diseases in which patients present with loricrin downregulation (O'Driscoll 2002, Koch 2000). Psoriasis is a chronic disease involving an immune mediated response in the skin (Bilal 2018). Although its exact mechanism remains a mystery (similar to AgP), studies suggest TNF-alpha is dysregulated and as a result, anti-TNF-alpha therapies are one strategy used to treat the disease. Another target currently being investigated is psoarisin, a protein which may regulate epidermal cell differentiation (Son 2016). Psoriasin is upregulated in both psoriasis and atopic dermatitis and has been shown to inhibit epidermal differentiation by reducing the expression of key proteins like loricrin (Son 2016). The lack of loricrin and other epidermal proteins may compromise the epithelial barrier and consequently, lead to the chronic inflammatory disease, psoriasis. Interestingly, psoriasis has been recently associated with chronic periodontal disease (Sarac 2017, Su 2017, Painsi 2017, Nakib 2013, Holmstrup 2017). Despite the evidence for association, it is unclear if there is a causal mechanism between the two diseases. It is also important to recognize that although to date there may be no evidence of an association between AgP and psoriasis, it could be due to the low prevalence of AgP in the populations studied.

With regard to atopic dermatitis, its association with periodontal disease has not been established, however it is possible that these inflammatory diseases may have barrier dysfunction in common. Atopic dermatitis is a prevalent skin disorder in children characterized by patches of itchy, red and scaly skin (Nutten 2015). Atopic dermatitis may share similar characteristics to AgP, in that the skin of individuals with atopic dermatitis seems to dramatically react to a greater threat than what is actually present. Atopic dermatitis also parallels AgP and chronic periodontal disease in that the predominant/acute phase of atopic dermatitis is a Th2 response (similar to AgP)

while the chronic lesions are characterized by a Th1 response (similar to chronic periodontal disease) (Fiset 2006) (see later for a discussion of Th1/Th2 responses). Impaired skin barrier function is a suspect in the etiology of atopic dermatitis, as a compromised skin barrier would allow more pathogens to penetrate the epithelium eliciting an immune response (Nutten 2015). Loricrin specifically has not been thoroughly investigated with regards to periodontal diseases, however the parallel observed between barrier dysfunction and bacteria in other diseases supports the potential for an association (Schleimer 2017). Barrier dysfunction has been associated with several other inflammatory conditions, such as rhinosinusitis, eosinophilic esophagitis and allergic rhinitis (Schleimer 2017). Whether therapies, specifically immune modulators, used in atopic dermatitis or other diseases could be used for AgP remained an open question.

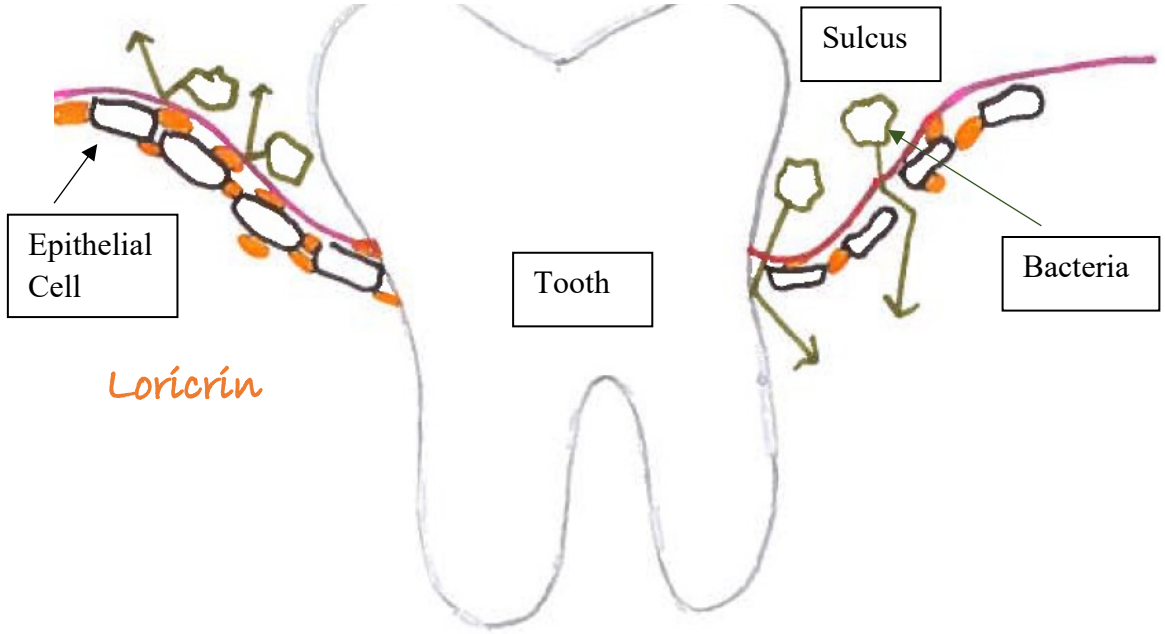
Chronic rhinosinusitis, characterized by nasal inflammation leading to loss of smell, facial pain and nasal discharge, is another inflammatory disease where epithelial barrier dysfunction is suspected (Soyka 2012, Rom 2014). Pro-inflammatory cytokines, IL-4 and IFN-gamma, decrease expression of occluden and zonula occludens 1, which are critical in mediating the tight junctions of the epithelium. With decreased expression of these tight junction proteins, a complete barrier cannot be formed, resulting in an inflammatory response.

Barrier Dysfunction and AgP

This association of barrier function and inflammation may be applicable to AgP. Since loricrin is the most abundant protein in the cornified epithelial layer, it follows that its expression in gingival epithelium may be important to the functionality of the cornified epithelium. In two unbiased studies of genes regulated in AgP, loricrin has been found to be significantly downregulated. One study compared showed loricrin was decreased 7-fold in AgP patients compared to healthy controls and a second study showed loricrin was decreased by 25% in AgP

patients compared to chronic periodontal patients (Nowak 2013, Guzeldemir E 2015). A consequence of downregulation of loricrin may be compromise of the cornified epithelial integrity, resulting in a smaller number of bacteria necessary to elicit a dramatic inflammatory response. Figure 5 provides a graphic illustration of how barrier dysfunction due to decreased loricrin may allow pathogenic bacteria to penetrate more easily.

Figure 5: A visual comparison of a systemically healthy patient with bacteria challenge. The left represents a patient with normal expression of loricrin. The right represents an otherwise systemically healthy patient with lowered loricrin expression.



The Role of Cytokines in AgP

The exact role of different cytokines in AgP remains unclear. Cytokines are messengers that help to regulate cell-mediated immunity and can be produced by many cells including T lymphocytes (Berger 2000). T cells contain receptors on their cell surface that allow them to recognize foreign antigens (Berger 2000). A subset of T cells associated with periodontal disease are T helper cells. Two types of T helper cells, Th1 and Th2, are evident in periodontal disease. Th1 cells produce Th1 cytokines that include IFN-gamma and IL-2, which induces production of IL-1, TNF-alpha and prostaglandin E-2 (Bascones 2005). Th2 cells produce Th2 cytokines, which include IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 (Bascones 2005). The role of T helper cells in AgP and chronic periodontal diseases is controversial. One hypothesis suggests that active lesions of periodontal diseases are characterized primarily as a Th2 response (Seymour 1993, Bartova 2000, Zein 2017), while the counter hypothesis suggests that the active lesion is characterised by a Th1 response (Ebersole 1994, Dennison 1997). The Th1 hypothesis is based on the evidence that the pro-inflammatory cytokine IFN-gamma is present, which then triggers macrophage activation and an IL-1 response, leading to periodontal destruction (Ebersole 1994). Interestingly, Dennison et al. suggest that there is a downregulation of IL-4 and IL-1 which leads to periodontal destruction (Dennison 1997). However, there is significantly less support for this specific theory. On the contrary, Seymour et al. initially proposed the Th2 hypothesis based on the increased B cell population in periodontal disease versus gingivitis (Seymour 1993). The data behind this hypothesis was the shift from predominantly T cell populations in gingivitis to mainly a B cell population in periodontal disease. The Th2 response is suspected to produce cytokines for B cell activation and subsequently, the production of B cell IL-1 (Seymour 1993). IL-1 has been extensively researched and there is strong evidence that it is necessary to stimulate bone

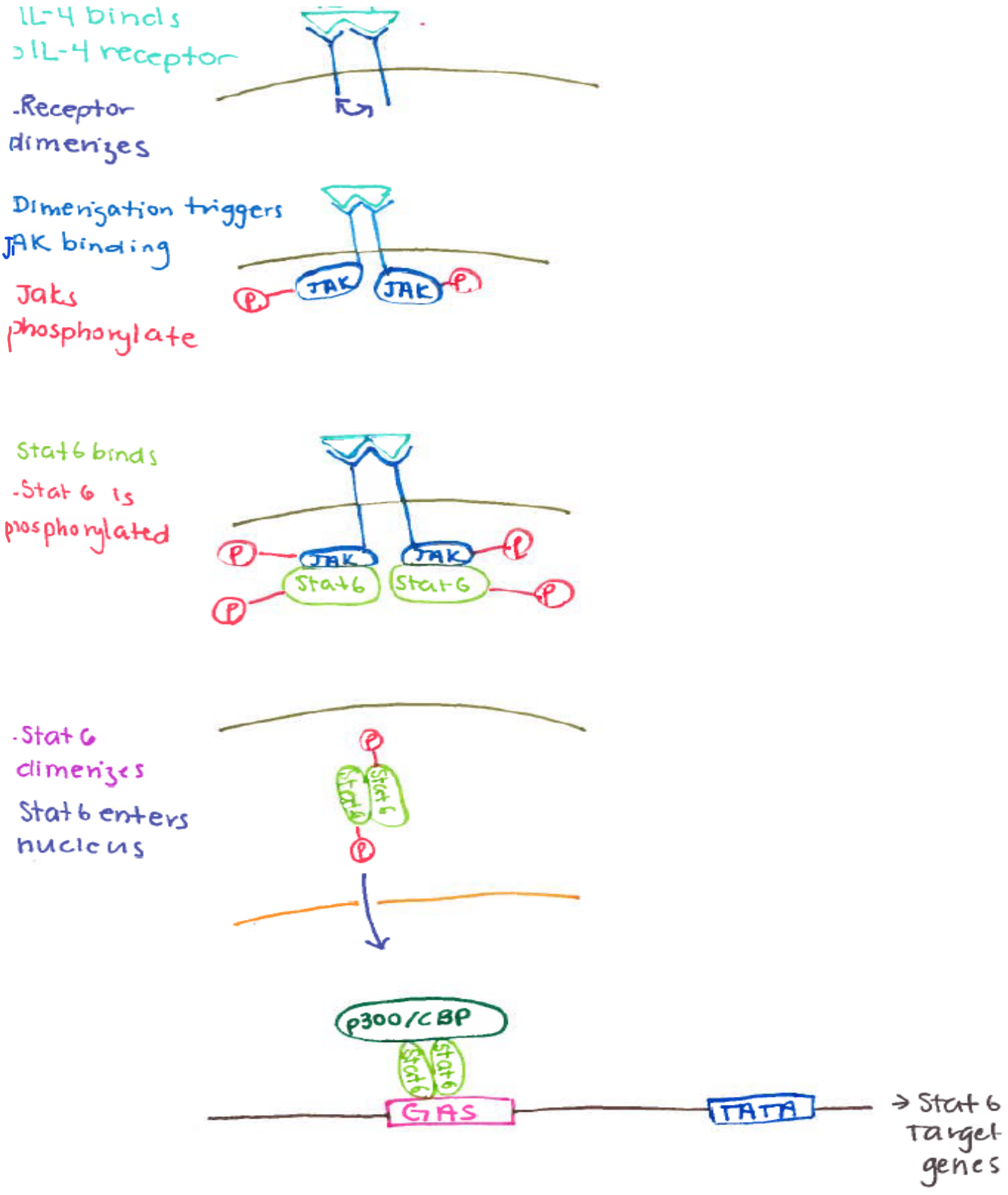
destruction in periodontal disease (Wang 1993,Kamei 2014, Gonzales 2014). Therefore, despite the controversy, there is more evidence to suggest that the inflammation of AgP is characterized by a Th2 response as Th2 cytokines are expressed during the progressive lesion phase of periodontitis (Nath 2011, Bartova 2000, Zein 2017).

Link Between Th2 Cytokines, Stat6 and Loricrin

Interestingly, it has been shown that Th2 cytokines, predominately IL-4 and IL-13, can activate Signal Transducer or Activator of Transcription (Stat) 6 (Kim 2008, Bruns 2003, Kotanides 1993, Hou 1994, Schindler 1994). Stat6 is a protein, a transcription factor, that binds to promoter sequences of specific genes to stimulate expression; many of these promoters belong to Th2 cytokines (Mitchell 2005). IL-4 specifically has been shown to activate the Stat6 transcription factor via the Janus kinase (Jak)-Stat signalling pathway. Upon IL-4-IL-4 receptor binding, the tyrosine residues on the receptor are phosphorylated by the Jaks leading to the recruitment of Stat6. Stat6 is then phosphorylated, stimulating dimerization, which allows the transcription factor to enter the nucleus and activate specific gene transcription. Fascinatingly, loricrin has been shown to be downregulated by Th2 cytokines via a Stat6 pathway in patients with atopic dermatitis (Bao 2017, Kim BE 2008). This downregulation of loricrin has been observed at the protein and gene level in both affected and non-affected sites of atopic dermatitis (Kim 2008). Until recently, the mechanism of how Stat6 downregulates loricrin protein was unclear. In 2017, Bao *et al*. demonstrated loricrin downregulation occurred through IL-4 (Bao 2017). They found that loricrin transcription requires a co-activator known as p300/CBP. Similarly, p300/CBP is recruited as a coactivator for IL-4 activated Stat 6. As a result, both IL-4 activated Stat6 and loricrin transcription compete for p300/CBP (Bao 2017). This competition leads to less transcription of loricrin which could result in the downregulation of loricrin observed at the protein

level. A visual representation of the IL 4-Stat6 pathway which mediates downregulation of loricrin via p300/CBP sequestration is illustrated in Figure 6. Presently, a Stat6VT transgenic mouse model is used to research the role of loricrin downregulation in atopic dermatitis (Da Silva 2018). The constitutively activated Stat6 in the Stat6VT mouse leads to an activated phenotype in T lymphocytes and increased Th2 differentiation (Bruns 2003). Furthermore, these mice have increased IgE serum levels similar to the phenotype of human atopic dermatitis (Bruns 2003). The Stat6VT mice were developed using the CD2 locus control region for specific targeting of expression to B and T lymphocytes (Bruns 2003). Two amino acids, valine and threonine are converted to alanine residues in the Stat6 gene, resulting in Stat6 being constitutively phosphorylated (Bruns 2003). This then mimics the activated form of Stat6. As a result of this mutation there is constant sequestration of the cofactor P300/CBP that is also important to loricrin protein expression, and consequently, this mutation leads to loricrin downregulation (Bruns 2003). Therefore, the Stat6VT mouse model is interesting to explore in the context of our hypothesis.

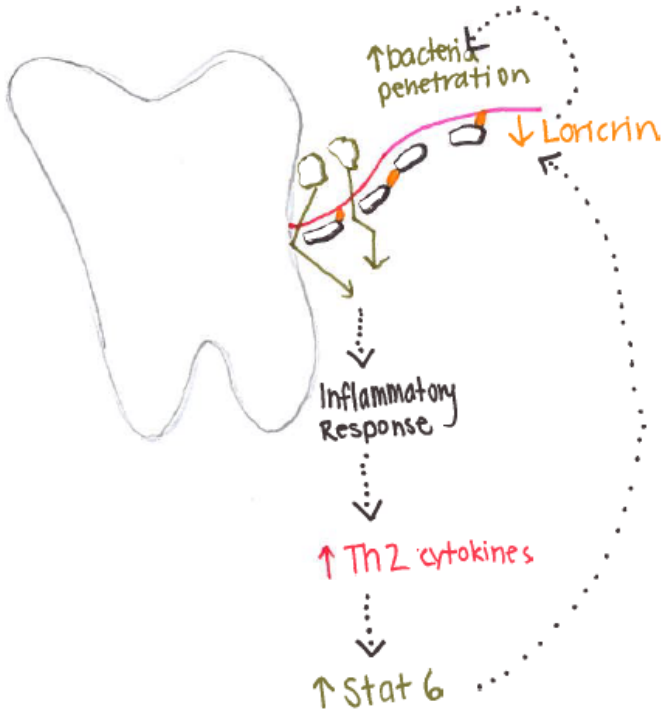
Figure 6: Downregulation of loricrin protein mediated by IL4 activated Stat6 mechanism.



No IL-4 →

Previous studies showed that AgP patients experienced a 25% and 7 fold decrease in loricrin gene expression (Nowak 2013, Guzeldemir 2015). A potential hypothesis to explain AgP is that patients may be somehow predisposed to lower than normal loricrin levels (perhaps via different genetic polymorphisms). These "AgP susceptible" patients could then experience a further regulation of loricrin via a bacterial-mediated Th2/IL4 response, pushing the level to a critical low threshold. Thus, a combination of genetic and environment factors leads to the "perfect storm". This idea is illustrated in Figure 7. When these patients are exposed to pathological bacteria, such as those found in AgP, even at low levels, they may be highly susceptible to the consequences.

Figure 7: A visual representation of a further downregulation of loricrin via a Th2 mediated Stat6 response.



HYPOTHESIS

Based on the current literature, our overall hypothesis is that pathogen-mediated loricrin downregulation could result in an impaired oral epithelial barrier which could then lead to the increased inflammatory response and profound damage observed in AgP-susceptible patients. The downregulation of loricrin in AgP is hypothesized to be greater than that which occurs in chronic periodontitis, and to occur as a result of two conditions: The first is a genetic predisposition to loricrin downregulation and the second is pathogen mediated. This hypothesis would then explain the familial component of the disease, and the need for the pathogen to also be present. The primary objective of the present study is to compare loricrin protein expression in healthy control and AgP patients. The second objective of this study is to explore the use of a transgenic constitutively active Stat6 mouse model for AgP studies, to give insight regarding the role of loricrin in AgP.

Chapter 3: Materials and Methods

Chemicals and Supplies

Unless specified, general chemicals, plasticware and supplies were from Fisher Scientific.

Human Subjects

Healthy and AgP patients were identified and diagnosed by a periodontist. Patients who met provided informed consent prior to entering the study (Appendix). Healthy patients were diagnosed as periodontally healthy patients and showed periodontal pockets no greater than 4mm and no evidence of radiographic bone loss. Healthy patient tissues were collected during crown lengthening procedures. AgP tissues were collected during periodontal surgery. Tissues from patients were collected, placed in RNAlater (Quiagen, Cat No.16706) or formalin and stored at -80ºC. All 11 of the healthy samples and one AgP sample were from Canada while the remaining AgP samples were from Brazil. Ethical approval was obtained from the University of Alberta Ethics Board (Pro00062112) and from the Universidade Federal de Pernambuco (66860717.8.0000.5208).

Mice

Oral tissue and skulls of Stat6VT transgenic mice were generously provided by Dr. Mark Kaplan (Indiana University School of Medicine), who created the model for atopic dermatitis studies (Bruns 2003, DaSilva 2018). Stat6VTmice are congenic with C57Bl/6 mice and have been maintained by the lab, with periodic backcrossing to control genetic drift, for more than a decade. The male mice carry the transgene to increase litter production, and offspring are therefore hemizygous for the transgene. Both male and females are susceptible to atopic dermatitis and were used in the present study. Mice show atopic dermatitis lesions at approximately 3 months of age.

Western Blot Analysis

Western blot protocols were assessed to determine if this would be an effective method of detection and quantification of loricrin protein. The objective was to establish a consistent protocol to be able to quantify loricrin in both mice and humans. As an initial pilot, we used skin samples, which we knew should be positive for loricrin, from both mice and humans, and mouse oral tissues.

Protein Extraction

Dithiothreitol (DTT) and radioimmunoprecipitation assay buffer (RIPA) were independently used to homogenize mouse skin and oral tissue, as well as human cuticle and human skin tissue. The purpose of the extraction buffers was to lyse the cell membrane to subsequently expose loricrin protein for detection. The DTT extraction buffer uses Tris-Hydrochloric acid (Tris-HCl) as the buffering ingredient while the RIPA buffer used Tris and sodium chloride (NaCl). The detergent component is used to separate membrane proteins from the membrane itself (Linke 2009). Some detergents work by completely denaturing the protein. This was the purpose of the sodium dodecyl sulfate used in both the DTT and RIPA buffers. The purpose of the DTT in the extraction buffer is to function as a reducing agent and break disulfide bonds while the ethylenediaminetetraacetic acid (EDTA) acts as a chelating agent (Posch 2014). This is important as the EDTA chelates Mg ions which are critical cofactors in degradation enzymes (Posch 2014). In the RIPA buffer specifically, two additional detergents are present. The first is sodium deoxycholate which is a bile acid detergent used for disrupting and dissociating protein interactions (Ji 2010). The second, NP-40 (nonyl phenoxypolyethoxylethanol) is a non-anionic detergent used for solubilizing membrane proteins (Ji 2010).

Table 1: DTT Extraction Buffer Recipe

Stock	**Extraction Buffer**	**50 ml**	**100ml**
1 M Tris HCl	0.1 M Tris HCl pH=8.5	5 ml	10 ml
20% SDS	1% SDS	2.5 ml	5 ml
200 mM DTT	20 mM DTT	5 ml	10 ml
500 mM EDTA	5 mM EDTA	0.5 ml	1 ml
n/a	Mili Q Water	37 ml	74 ml

Table 2: RIPA Extraction Buffer (Purchased from ThermoFisher Scientific (Cat. No. 89900))

5 M NaCl
1.0% NP-40
10% sodium deoxycholate
10% SDS
1 M Tris
0.5 M EDTA

Sample Preparation

Oral tissue from mice was weighed prior to homogenization. The oral tissues were then homogenized using a 7ml dounce homogenizer over ice. The lysis buffer used was a DTT extraction buffer. The ratio of sample to lysis buffer was 2mg:1ml. The sample was homogenized for 10 minutes. Next, the sample was boiled for 30 minutes at 100℃ to break disulfide bonds and change the protein from its tertiary to its primary structure. The sample was centrifuged at for 15 minutes at 12000xg to remove debris and stored at -20℃ .

Gel Electrophoresis

10% and 12% Tris-Glycine SDS polyacrylamide gels, followed by western blotting were the first techniques evaluated for loricrin protein detection (Table 3). Tris-Glycine SDS polyacrylamide gel electrophoresis (PAGE) is used for fine resolution of a broad range of molecular weight proteins. The 10% Tris-Glycine gel did not result in good resolution of loricrin protein (data not shown). Therefore, the higher percentage Tris-Glycine gel (12%) was trialed. A higher percentage of acrylamide helps resolve smaller proteins. This is because a higher percentage of acrylamide results in an increased cross-linking which ultimately leads to smaller pore size in the gel. However, the 12% Tris-glycine gel did not result in better resolution (Figure 14). Next, both 10% and 16% Tris-Tricine SDS gels were evaluated. The 16% Tris-Tricine gel was tested first. However, the samples did not run past the stacking gel. This may have been due to the small pore size of the 16% gel. Finally, the 10% Tris-Tricine SDS page was tested and showed the best resolution of loricrin protein (Figure 15).

Table 3: Tris-Glycine Gel Recipe

	Separating Gel 10%	**Separating 12%**	**Stacking Gel 5%**
Mili Q water	9.4 ml	8.2 ml	6.85 ml
Acrylamide/Bis 40%	5 ml	6 ml	1.25 ml
Tris	5.2ml (1.5M Tris)	5.2ml (1.5 M Tris)	2.5ml (0.5M Tris)
10% SDS	200 ul	200 ul	100 ul
10% Ammonium Persulfate (APS)	200 ul	200 ul	100 ul

TEMED	20 ul	20 ul	10 ul

Table 4: Tris-Tricine Gel Recipe

	Separating Gel 10%	**Separating Gel 16%**	**Stacking Gel (4%)**
Acrylamide/Bis 29:1	6 ml	10 ml	1 ml
Gel Buffer 3x (3M Tris, 1M HCl, 0.3% SDS)	10 ml	10 ml	3 ml
Glycerol	3 ml	3 ml	-
Add water to final volume	30 ml	30 ml	12 ml
10% APS	150 ul	100 ul	90 ul
TEMED	15 ul	10 ul	9 ul

Samples were thawed and centrifuged for 5 minutes at 10000 x g to ensure only supernatant was collected from the samples. Samples were then mixed with 5x loading buffer. The purpose of the loading buffer is to reduce disulfide bonds immediately prior to electrophoresis; the glycerol increases the density of the samples so that they do not float away in the running buffer. The SDS component of the buffer also denatures proteins and gives them a negative charge so the proteins will stack based on size during the electrophoresis. Samples were boiled for 10 minutes at 100℃. The BioRad Mini-Protein Tetra System was used for electrophoresis. Samples were

loaded along with a molecular weight ladder (Spectra Multicolor Broad Range Protein Ladder, Product No. 26634). We also loaded a known amount of bovine serum albumin (BSA) in a control lane so that we could determine whether the transfer of proteins was successful (by staining the membrane with using Ponceau S) following western blotting. The electrophoresis chamber was filled with 1x running buffer.

Running Buffer

Table 5: Tris Glycine Gel Recipe

	10x Glycine Running Buffer (1L) pH=8.3
25 mM Tris Base	30 g
200 mM Glycine	144 g
0.1% SDS	5 ml of 20% stock
Mili Q Water	Up to 1000 ml

Table 6: Tris Tricine Gel Recipe

	Anode Buffer (10x)	**Cathode Buffer (10x)**	**Gel Buffer (3x)**
Tris (M)	1.0	1.0	3.0
Tricine (M)	-	1.0	-
HCl (M)	0.225	-	1.0
SDS (%)	-	1.0	0.3
pH	8.9	8.25	8.45

The gel was run at 80V until samples traversed the stacking gel. The voltage was then increased to 120V until samples neared the end of the gel. Upon completion of the gel electrophoresis, the

separated proteins were transferred onto a Polyvinylidene difluoride (PVDF) membrane (Immobilon-P, 0.45 um, Millipore, Cat No. IPVH00010).

Protein Transfer

Four pieces of filter paper (ThermoScientific, Product No. 88600) and Immobilon-P were cut to the size of the gel prior to each transfer. Six sponges were soaked in water for five minutes and then in transfer buffer for 5 minutes. The membrane was soaked in methanol for ten seconds and rinsed in water for 60 seconds. The membrane was then soaked in transfer buffer for two minutes. Next, two pieces of filter paper were soaked in transfer buffer and then placed on the gel. The gel and filter paper were together soaked in transfer buffer for an additional five minutes. Immobilon-P membrane was aligned to the other side of the gel. The last two pieces of filter paper were soaked in transfer buffer and placed on top of the membrane. A pipet was rolled over the layers of filter paper, membrane and gel to remove any bubbles. These layers were then compressed in the transfer system (BioRad Mini-Protean System) with the six sponges. The apparatus was filled with transfer buffer with an ice-pak inside. Additionally, ice was packed around the apparatus.

Table 7: Transfer Buffer Recipe

	Transfer Buffer
25 mM Tris Base	
193 mM Glycine	
10% MeOH	

The transfer voltage was set at 100V for one hour. After the transfer was complete, the success of the transfer was determined by incubating the membrane in Ponceau S solution for 5 minutes, and then destaining with methanol. If bands of protein were visualized, the transfer was deemed successful and antibody detection was attempted. Our BSA control, which resolved as a single known molecular weight band, was helpful in determining our success as well. The membrane was then blocked with 5% milk in 1x TBST (Tris buffered saline with 0.05% tween) overnight. The purpose of this step was to prevent non-specific binding of antibodies and reduce background. Milk proteins bind to the membrane and block these sites, allowing the antibody to bind to the specific target antigen (loricrin).

Table 8: Ponceau S Solution Recipe

	Ponceau Solution
Ponceau	0.5 g
Glacial acetic acid	1ml
Mili Q Water	Up to 100 ml

Table 9: 10x TBS Recipe

	10x TBS 1L
250 mM Tris Base	30.28 g
14 M NaCl	81.86 g
30 mM KCl	2.24 g
Mili Q Water	Up to 1000 ml

Table 10: Blocking Solution Recipe

	Blocking 5% milk in 1x TBST (0.05% Tween)
1x TBST	500 ml
Non-fat dry milk powder (Carnation – Nestle)	2.5 g

Protein Detection

In order to detect loricrin, we used a MilliPore Snap ID 2.0 apparatus for incubation of the Immobilon-P membrane with antibodies. This apparatus uses suction to create a tight interface between membrane and antibodies, and in this way increases the effective concentration of antibodies. The procedure is also shorter than used for a typical western blot. The membrane was removed from the 0.05% milk in 1x TBST and placed in the MilliPore Snap ID 2.0 cassette. This system uses vacuum suction to pull the antibody through the membrane. This method was used over traditional western blot method because the traditional method involves incubating the membrane in antibody for several hours. On the contrary, the vacuum suction is more efficient and protein detection only takes 1-2 hours. The membrane was equilibrated with 1x TBST for ten minutes. The 1x TBST was removed via vacuum suction and 10ml of a 1:2000 dilution of primary antibody was added for fifteen minutes. The primary antibody was removed and re-added for 15 minutes. The primary antibody was then removed, and the membrane was washed with 10ml of 1x TBST 6 times. The same procedures was used with 1:10000 dilution of secondary antibody. After, the membrane was washed again 6 times with 10 ml of 1x TBST.

Table 11: Preparation of primary and secondary antibody for loricrin protein detection.

Primary Antibody 1:2000	1 x TBST w/ Tween
5 ul anti-loricrin (Cat # PA5-30583 Invitrogen)	10 ml

Secondary Antibody 1:10000	1x TBST w/ Tween
1 ul Goat anti-rabbit IgG HRP (Conjugated to horse radish peroxidase) (**Cat #** 31460 Invitrogen)	10 ml

Imaging

Protein imaging was done using a BioRad Chemi Doc MP Imaging System. The membrane was placed in between a clear plastic sheet and coated with chemiluminescent reagent (ThermoScientific, Super Signal Chemiluminescent Substrate Product No. 34095). The Chemi Hi-Sensitivity setting was used, and the exposure was first set as signal accumulation and then manual exposures were taken. Time was adjusted according to the quality of signal.

Enzyme Linked Immunosorbent Assay (ELISA)

Human Sample Preparation

Human tissue samples were prepared according to the Cloud Clone Corp protocol for human loricrin (Cloud Clone Corp, SEC568Hu). Samples were weighed in 1.5 ml microfuge tubes. Lysis buffer, which was included in the ELISA kit, was added. The amount of lysis buffer added was in a ratio of 1:50 as recommended in the Cloud Clone Corp protocol. The amount of each sample was 50mg/ml. The tissue was then minced and sonicated on ice in a 4℃ cold room until the tissue was completely homogenized. Samples were centrifuged at 10,000 g for 5 minutes for removal of debris and stored at -20℃ .

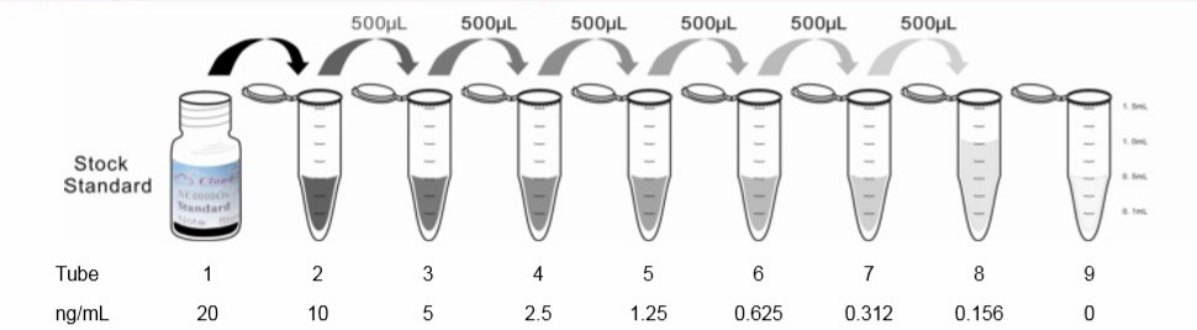
Mouse Sample Preparation

Tissues were homogenized according to the Cloud Clone Corp protocol for mouse loricrin (Cloud Clone Corp, SEC568Mu). Mouse oral samples were collected by the lab of Dr. Mark Kaplan, a collaborator from the Indiana University of Medicine, and provided frozen. Samples were thawed and centrifuged at 10,000 x g for 5 minutes. Tissue samples were weighed in microfuge tubes and then lysis buffer, which was included in the ELISA kit, was added in a ratio of 1:20, as recommended in the protocol. The concentration of each sample was 20mg/ml. The tissue was then minced and sonicated until the tissue was completely homogenized. Samples were centrifuged at 10,000 x g for 5 minutes and stored at -20℃.

ELISA Procedure

The contents of the kit and samples were brought to room temperature. A standard curve was created by serial dilution of loricrin, provided in the kit.

Figure 8: Procedure for making serially diluted loricrin from standard provided by Cloud-Clone Corp. Image courtesy of Cloud Clone Corp.



The Cloud Clone Corp kit included a 96 well plate pre-coated with loricrin antibody. We designed our assay to include blank wells, control wells with solely lysis buffer, and the diluted standards, in addition to our samples (all in duplicate). 100 ul of control/standard/sample were

added to each well. The 96 well plate was then covered and incubated for two hours at 37℃. After the incubation was completed, the liquid was pipetted from each well followed by the addition of 100ul of Detection Reagent A. The reagent likely contained the detection antibody for loricrin, which was now bound to the capture antibody coated on the bottom of the 96 well plate. Detection Reagent A was incubated with the samples for one hour at 37℃. Next, the wells were washed three times with wash solution provided in the ELISA kit. Then, 100ul of Detection Reagent B was added to each well. Although the kit does not provide specific details regarding the ingredients of either detection reagents, Detection Reagent B likely contains horse radish peroxidase (HRP)-conjugated secondary antibody. This enzyme-linked antibody binds to the Detection antibody in Reagent A and will facilitate color change which ultimately allows detection of the loricrin protein. The samples were incubated for thirty minutes with Detection Reagent B and following the incubation, the wells were washed five times. Then 90ul of tetramethylbenzidine (TMB) substrate solution was added to each well. TMB is a substrate is for HRP; HRP converts TMB into an insoluble blue product. The degree of conversion and subsequent blue color is correspondent to the amount of loricrin that originally bound the pre-coated plate. The plate was the covered and incubated for 15 minutes at 37℃ with the substrate solution. After 15 minutes, a stop solution was added to stop the color change. Stop solution contains sulfuric acid which alters the pH change and inhibits HRP. The addition of the stop solution changes the color from blue to yellow.

Data Collection

After the stop solution was added to the plate, the plate was read using a microplate reader (Synergy H1 Hybrid Multi-Mode Reader). The absorbance was set at 450nm in order to detect the yellow wavelength. The duplicate results were averaged for each well. The average optical density of the blank was subtracted from the standard, control and samples wells. A standard curve was constructed using the mean optical density and concentration from each standard. A line of best fit was plotted to make a standard curve. Statistical analysis was performed using Prism Software. The data were tested for normal distribution using the Shapiro-Wilk test.

Because the data were found to not be normally distributed, a Mann Whitney test, was performed to determine whether they differed.

Measurement of Alveolar Bone Loss

Animals were imaged using MiLabs U-SPECT-II/CT (Utrecht, The Netherlands), scanned with a voxel size of 25μm, and scan settings of 70 kVp, 114 μA, 0.5 mm AL filter, and integration time 500 ms. 3D reconstructions were performed using Avizo software. The measurement protocol used to measure alveolar bone loss was developed by Dr. Raisa Catunda. Periodontal bone height analysis was performed by measuring the distances from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) as a ratio of the total root length (TRL) in the first and second mandibular molars. Eight measurements were established: two on the sagittal plane and six on the coronal plane. Image orientation was initially done on the coronal plane of the first molar to make the sagittal measurement (mesial and distal) as follows: the sagittal plane is defined as the one passing through the long axis of the first molar in the root apex (RA) and middle of the pulp chamber (MPC) (Figure 9); the landmarks were CEJ, ABC and RA (Figure 10). For the coronal measurements (mesial-buccal, middle buccal, distal-buccal, mesial-lingual, middle-lingual, distal-lingual, Figure 11), the plane used for orientation was the sagittal and for the mesial and distal measurement (either buccal or lingual) the plane was defined as the middle of the floor of the pulp chamber in the designated root and the middle of the last third of the root (Figure 12). For the middle buccal and lingual measurements, the plane was defined as the middle of the pulp chamber and the middle of the distance between the last third of the two roots (Figure 13). This step allowed comparison of similar slices and removed bias of any rotation the teeth could have had. The results are presented in millimeters and as a percentage of TRL (CEJ – ABC / TRL x 100) relative to wild type.

Figure 9: Illustration of the sagittal plane defined as one passing through the long axis of the first molar in the root apex and middle of the pulp chamber.

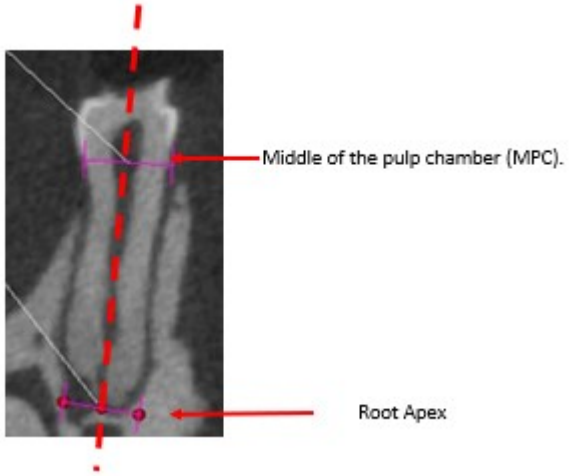


Figure 10: Land marks from the sagittal cut were the root apex, alveolar bone crest (ABC) and the cemento-enamel junction (CEJ).

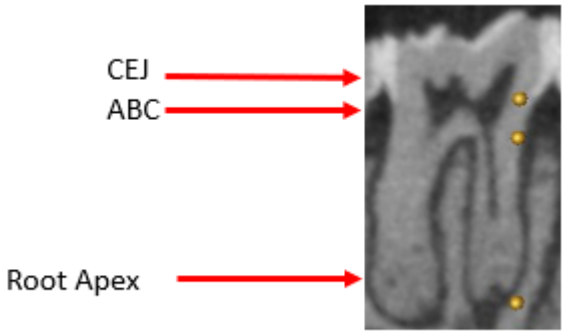


Figure 11: Measurements taken from the image of the coronal plane.

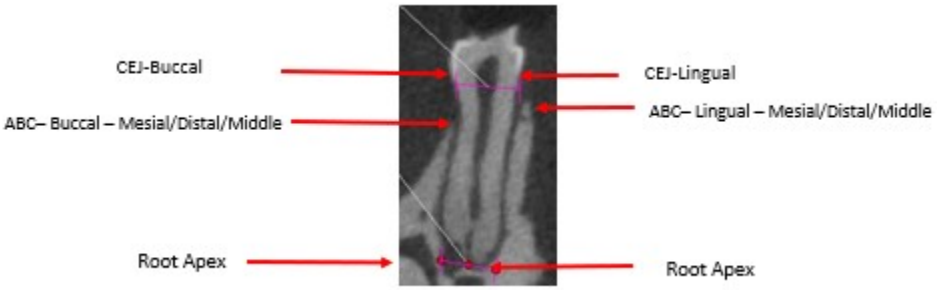


Figure 12: Definition of the coronal plane in the sagittal plane for mesial and distal measurements (A: Orientation plane for Lingual and Buccal Coronal Distal Measurements B: Orientation plane for Lingual and Buccal Coronal Mesial Measurements).

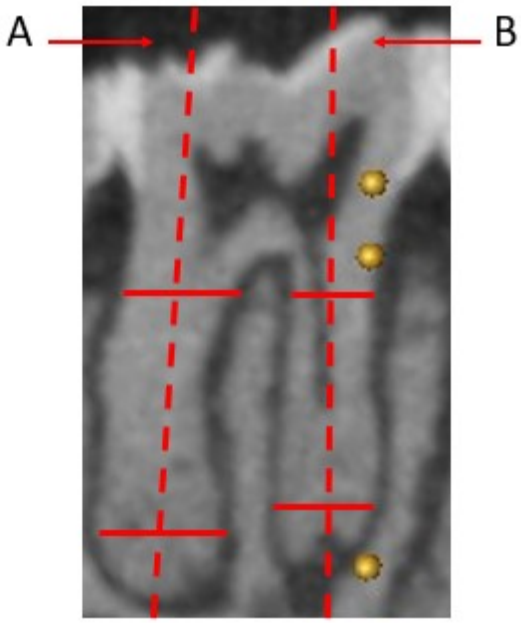
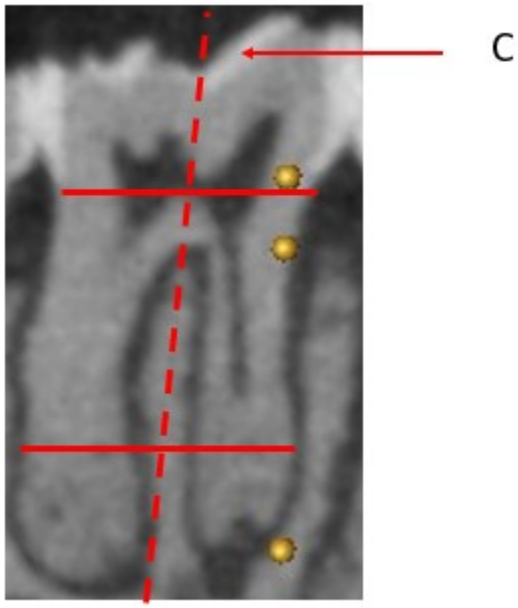


Figure 13: Definition of the coronal plane in the Sagittal plane for middle measurement (C: Orientation plane for Lingual and Buccal Coronal Middle Measurements).



Histology of Stat6VT and Wild Type Mice

Decalcification and Embedding

Mouse heads were obtained from Dr. Mark Kaplan from the Indiana University of Medicine. The heads were fixed in formalin prior to their arrival to the University of Alberta. The mouse heads were stored in PBS at 4℃. They were then decalcified for histology preparation. The skin was removed and each head was cut into two halves (right and left) in order to increase the surface area during the decalcification process. After cutting, the two halves were transferred into 0.5M EDTA (Disodium Salt Dihydrate, Fisher Chemical), pH=7.4 solution for decalcification. Each head half was kept in a single 50mL conical tube, with 10mL EDTA, shaking. The solution was changed every three days. After a month, when the hard tissue became soft, each half of the head (R and L sides) was further cut into two halves - upper and lower quadrant. This resulted in a total of four samples (four quadrants) from each mouse: Q1 (right upper/maxilla), Q2 (left upper/maxilla), Q3 (left lower/mandible), Q4 (right/ lower/mandible). The maxilla was excluded from our analysis, and the mandibular first molars were the teeth chosen to assess alveolar bone loss. This is because the first molars of mice exhibit the least variation in tooth morphology. The incisor was kept only in the mandible, for fast orientation purpose. Each mandible and its surrounding tissue was embedded in a paraffin block.

Sectioning and hematoxylin and eosin (H&E) staining

We used the services of the HistoCore, Alberta Diabetes Institute at the University of Alberta for sectioning (5 microns) and hematoxylin & eosin (H&E) staining processes.

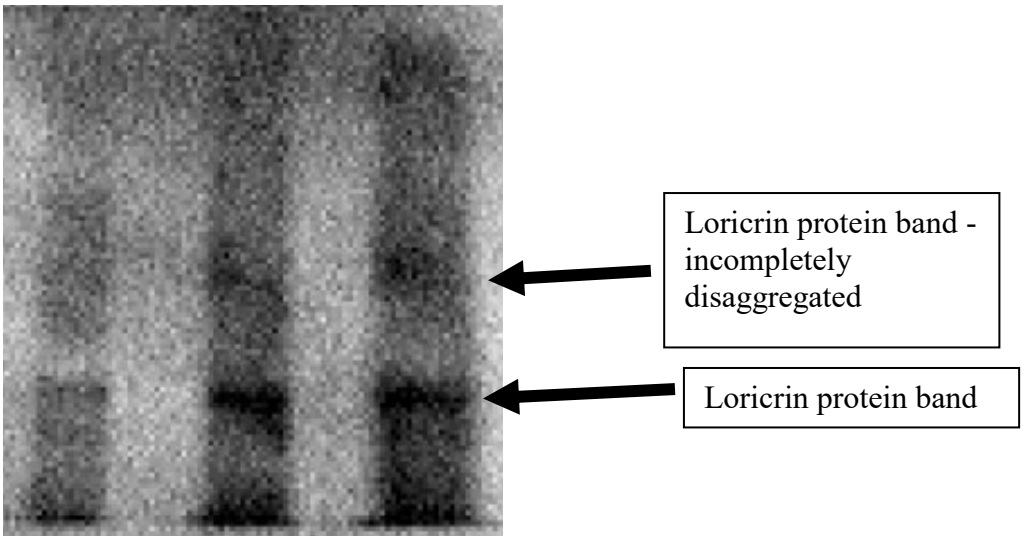
Chapter 4: Results

Western blot is an inappropriate method to quantify loricrin.

The expected molecular weight for human loricrin is 26 kDa and for mouse loricrin 38 kDa. Loricrin is a highly crosslinked protein and as a result, we tried multiple methods to break these crosslinks and resolve the protein into a single band for quantification. A single, discrete band at the expected molecular weight would be the most convincing for quantification. If several bands of differing molecular weight are present, it would lead to doubt as to the authenticity of the protein in that band (it could be argued it was a cross-reacting protein) and would make it much more difficult to accurately quantify. In order to disaggregate loricrin two different lysis buffers, RIPA and DTT were used. Ultimately, the DTT extraction buffer was chosen as it resulted in more discrete bands of loricrin than the RIPA buffer. This is likely due to the DTT's ability to break disulfide bonds.

Ultimately, we were able to detect loricrin using western blot analysis of healthy oral tissues from C57Bl/6 mice homogenized in DTT extraction buffer. We diluted our sample several times, reasoning that accurate detection would show a corresponding reduction in signal. Figure 14, shows this loricrin protein concentration-dependant series of mouse oral samples using a 12% Tris Glycine gel. As the sample amount loaded increased, so did the intensity. Unfortunately, accurate molecular weight analysis could not be performed for this experiment because the molecular weight ladder did not transfer onto the membrane. A disadvantage of this western blot analysis was the degree of background signal. This may be due to poor resolution of the low molecular weight proteins (and subsequent poor detection). In addition, as shown in this example, a single discrete band was not obtained.

Figure 14: Western blot analysis of mouse oral epithelium. Identically prepared samples were loaded at different concentrations (10 ul, 20 ul, 30 ul in order).

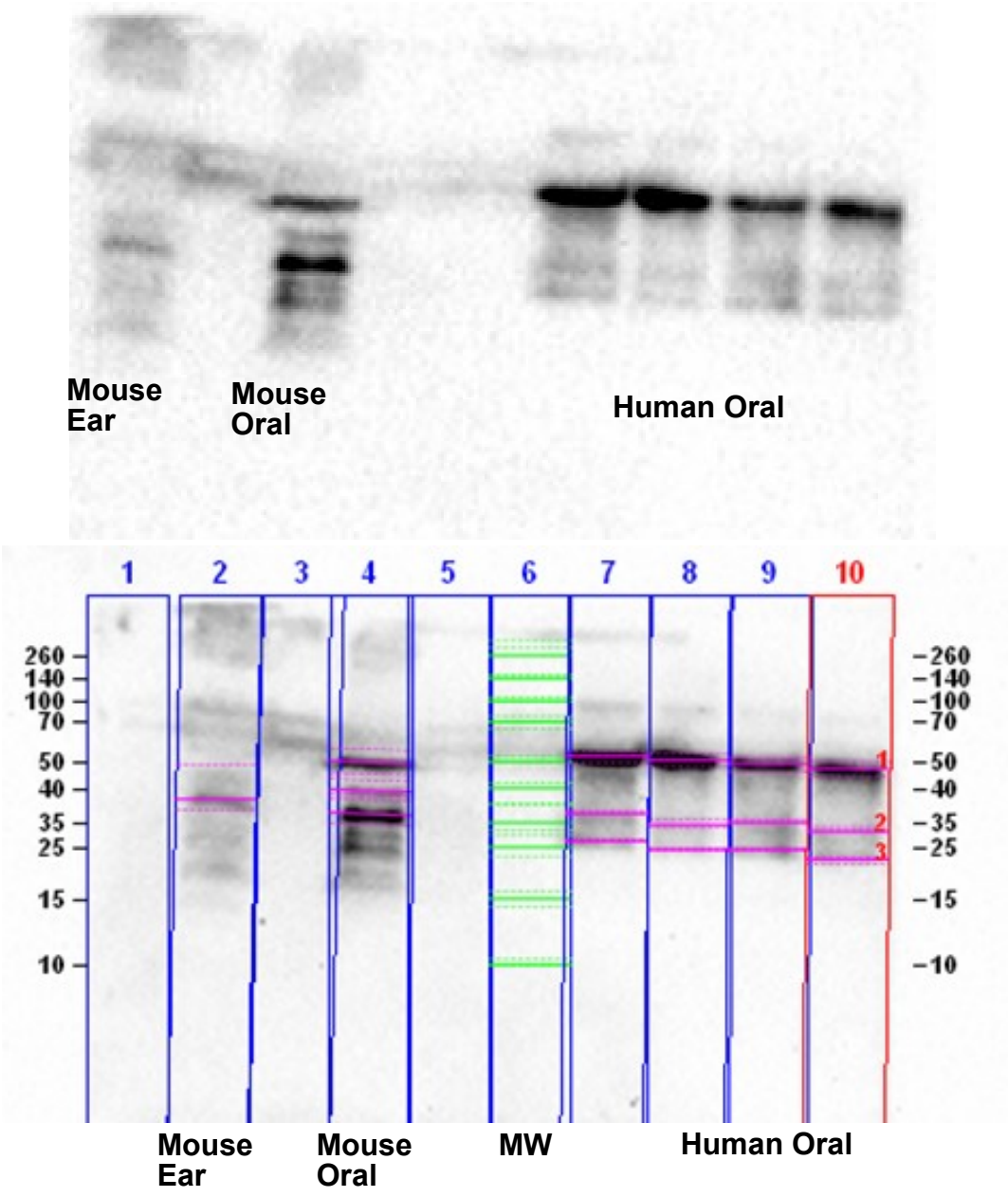


Due to poor detection of loricrin using the Tris-Glycine gel, resulting in low signal:noise ratio (Figure 14), a Tris-Tricine SDS PAGE protocol was evaluated (Schagger, 2006). The purpose of using this was to improve the separation and thus the resolution of the proteins of the sample, and perhaps allow better transfer and detection. Tris-Tricine SDS PAGE can resolve smaller proteins (3-200 kDa) (Haider 2011).

The samples (mouse ear and oral tissues, human oral tissue) were homogenized using DTT extraction buffer. The membrane showed several faint bands in the range of 20 to over 260 kDa. Mouse oral tissue presented with two heavy bands at approximately 35 kDa and 50 kDa. Human oral tissue had a major band of about 50 kDa. These results are illustrated in Figure 15. Multiple bands were consistently detected using western blot analysis. As loricrin is a heavily cross-linked protein, it is tempting to hypothesize that these are multimers, but without more extensive analysis, it is not proven. Thus, while western blot analysis was able to detect loricrin protein in the oral epithelium and skin of healthy C57Bl/6 mice, and human oral tissue, due to

the presence of multiple bands as a result of the extensive crosslinking of the protein, we concluded it was an inappropriate method for quantification.

Figure 15: Western blot analysis of mouse ear, human oral mucosa, and mouse oral mucosa using a 10% Tris-Tricine SDS page. The lower part of the figure shows overlay of MW markers and quantification.

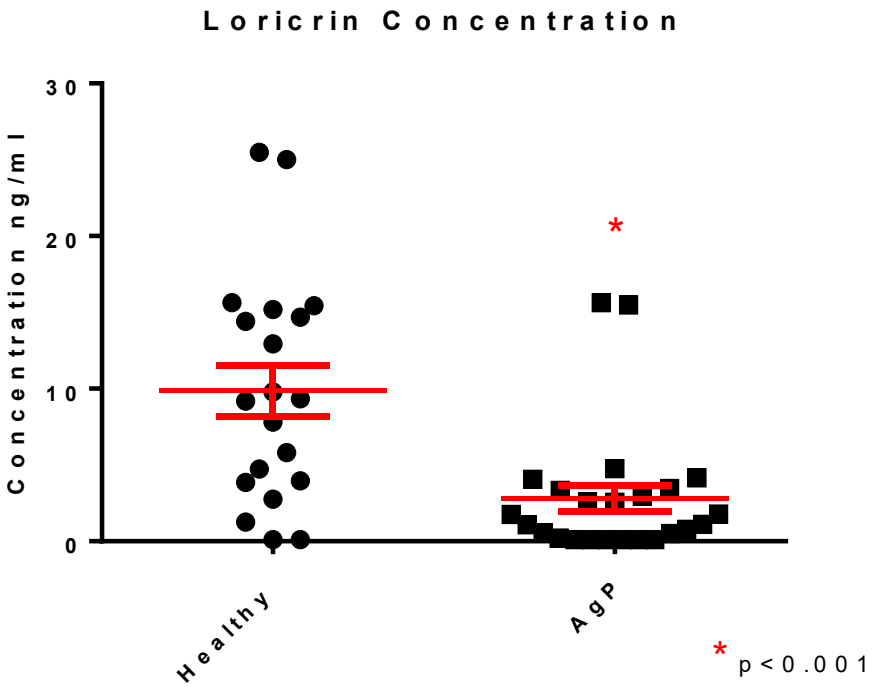


ELISA demonstrates that loricrin protein is decreased in AgP versus Healthy Controls

Because of the issues with the western blot technique and the detection of apparent multimers, we decided to try a validated ELISA for human loricrin. The sample preparation for the ELISA involved less manipulation and thus, we reasoned, could be a better approach for loricrin quantification.

An initial pilot ELISA was performed to determine an approximate dilution of the human samples that would be within the standard curve range, according to the Cloud Clone Corp protocol. One healthy human sample was thawed, centrifuged at 10,000 x g for 5 minutes and diluted 1:5 in PBS. Loricrin was successfully detected at this 1:5 dilution level. Subsequent samples were then prepared. All samples were analyzed using the same plate and ELISA protocol at the same time. To quantify and compare loricrin protein expression in AgP and healthy patients, a standard curve was created using serially diluted loricrin that was provided by the manufacturer. AgP samples were from patients under 35 years of age. Healthy patient samples were from individuals whose age averaged 51.5 $\pm$ 7.60 years. Each sample was assayed in duplicate. As shown in Figure 16, the mean $\pm$ S.E. was 9.874 $\pm$ 1.665 for healthy patients and 2.813 $\pm$ 0.8583 for AgP patients. This is approximately a 61% reduction. The results were significant ($p<0.001$, Mann Whitney test). The Mann Whitney test, a non-parametric test, was indicated as the data were not normally distributed, as assessed by the Shapiro-Wilk normality test.

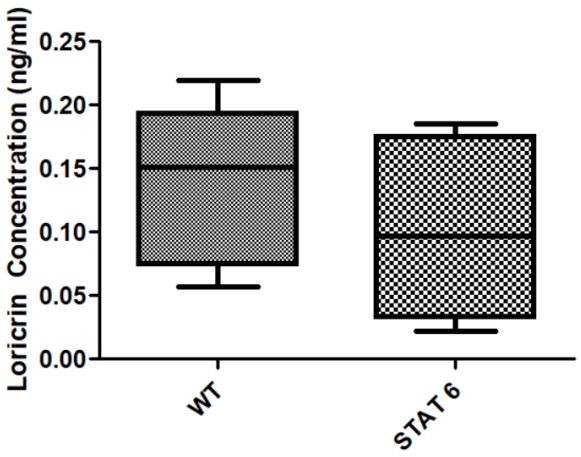
Figure 16: ELISA of 11 healthy and 12 AgP gingival tissue samples.



Loricrin Expression in Stat6VT Mice

A mouse loricrin-specific ELISA was used to detect loricrin protein in Stat6VT and wild type mouse samples. The ELISA did not show a significant difference between loricrin protein expression in Stat6VT mice versus wild type (Mann Whitney test). The means $\pm$ S.E. were 0.14 $\pm$ 0.03 for wild type and 0.10 $\pm$ 0.03 for Stat6VT; p=0.537).

Figure 17: ELISA of 6 Stat6VT mice and 6 Wild Type mice. (The means ± S.E. were 0.14 ± 0.03 for wild type and 0.10 ± 0.03 for Stat6VT; p=0.537).



Assessment of Bone Loss in Stat6VT Mice

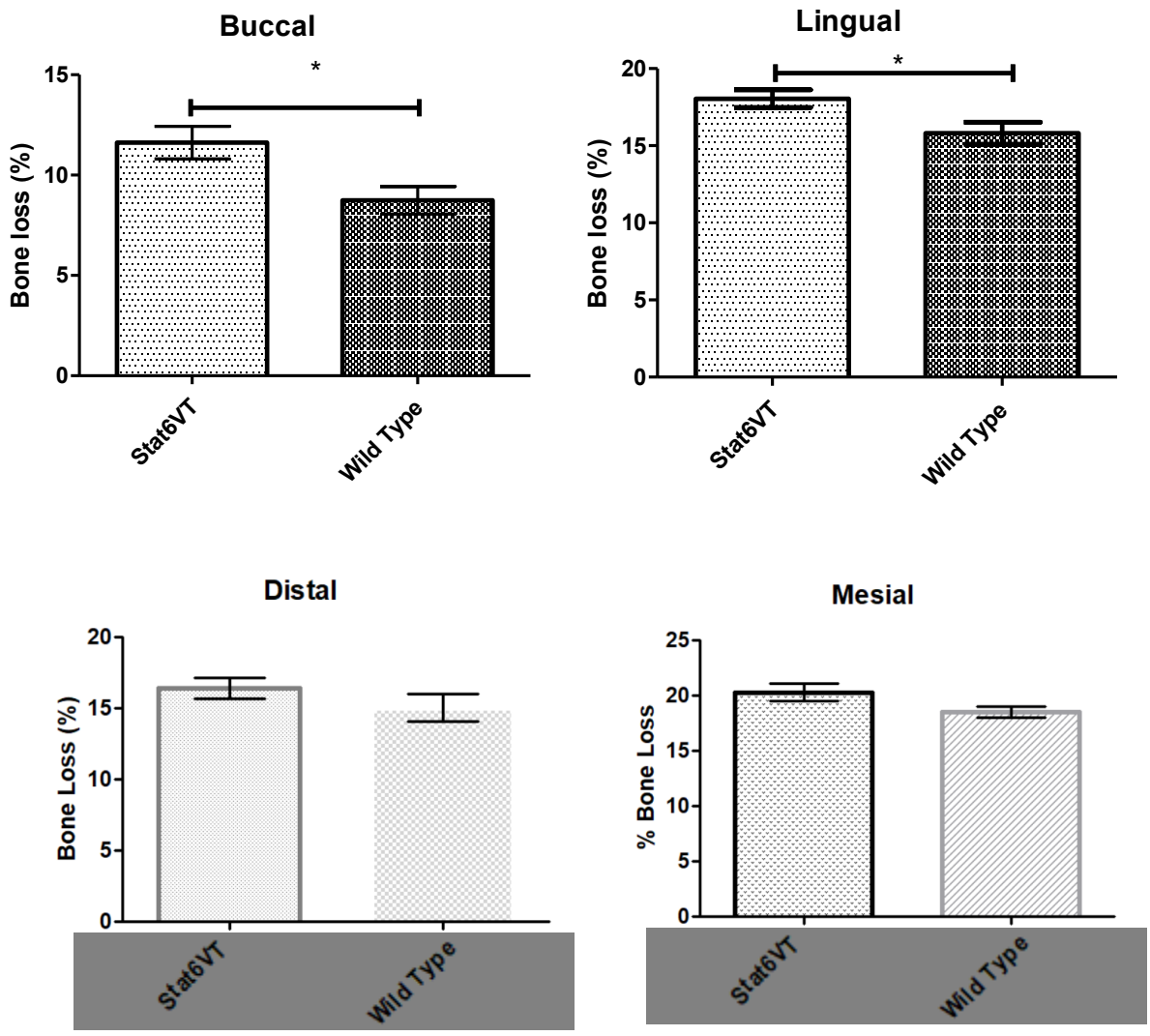
A total of 6 Stat6VT and 6 Wild Type mice were assessed for bone loss around their first molars. First molars in mice are often used to assess bone loss as they exhibit less variation in tooth morphology allowing for consistent measurements. The mice were 5-6 months old but unfortunately were not gender matched. Characteristics of the mice are detailed in Table 10. An assessment of bone loss from the microCT analysis of the Stat6VT and wild type mouse jaws was performed to determine if the loricrin downregulation influenced bone loss in the mice. We reasoned that since Stat6VT mice experience loricrin downregulation in their skin, leading to atopic dermatitis, they may experience loricrin downregulation in their oral tissues, which may make them more susceptible to inflammation-induced alveolar bone loss, even from endogenous bacteria. The measurement protocol was developed by a fellow graduate student in the lab, Raisa Catunda. The protocol compared alveolar bone loss around all aspects of the tooth

(buccal, lingual, mesial and distal). Bone loss was only significantly different between wild type and Stat6VT mice in the buccal and lingual aspects; there were no differences in the mesial and distal aspects. The results are presented in millimeters and as a percentage of TRL (CEJ – ABC / TRL) relative to wild type.

Table 12: Descriptive characteristics of the Stat6VT and Wild Type Mice.

Date of Birth	Date of Sacrifice	Age (months)	Sample ID	Sex
11/23/16	5/1/17	6	Stat6VT1	Male
11/23/16	5/1/17	6	Stat6VT2	Male
11/23/16	5/1/17	6	Stat6VT3	Female
11/30/16	5/1/17	6	Stat6VT4	Male
12/8/16	5/1/17	6	Stat6VT5	Male
12/22/16	5/1/17	5	Stat6VT6	Female
11/23/16	5/1/17	6	Wild Type1	Male
11/23/16	5/1/17	6	Wild Type2	Female
12/8/16	5/1/17	6	Wild Type3	Male
12/22/16	5/1/17	5	Wild Type4	Male
12/22/16	5/1/17	5	Wild Type5	Male
12/22/16	5/1/17	5	Wild Type6	Male

Figure 18: Bone loss analysis of Stat6VT and wild type mice.

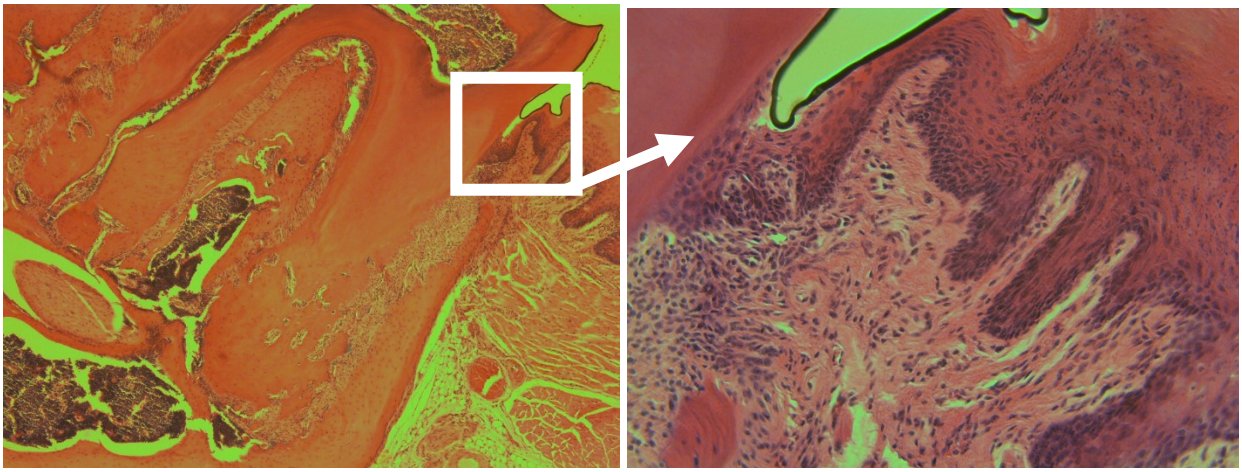


Histology of Stat6VT and Wild Type Mice

In a unblinded preliminary study, one Stat6VT and one wild type mouse were randomly chosen for histological analysis. The Stat6VT mouse was a male and the wild type mouse was female; both were 6 months of age at sacrifice. The purpose of this was to evaluate if the epithelium around the first molars demonstrated any signs of inflammation or changes in epithelial morphology. As loricrin is present in the cornified epithelium, and is downregulated in the skin

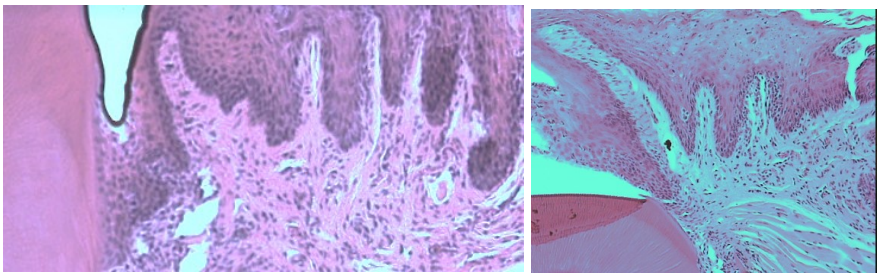
of Stat6VT mice, it is possible that the Stat6VT mice may experience spontaneous increased inflammation in the oral cavity, as they do in the skin. Histological analysis revealed that Stat6VT mice may indeed experience increased inflammation when compared to wild type. Figure 19 shows a histological section of a Stat6VT mouse. These sections were examined in consultation with Raisa Catunda, a periodontist, DDS, MSc. In the sulcular epithelium, ovoid cells are present which may be suggestive of lymphoplasmacytic infiltrate.

Figure 19: Ovoid cells in the sulcus of the Stat6 mouse suggestive of inflammation.



Other findings unique to the epithelium of Stat6VT mice were the presence of disorganized layers of the epithelium and fewer rete pegs. This is suggestive of dysfunction in the differentiation process of the epithelial layers. In comparison, the wild type mouse exhibited refined layers of the epithelium and several rete pegs (Figure 20).

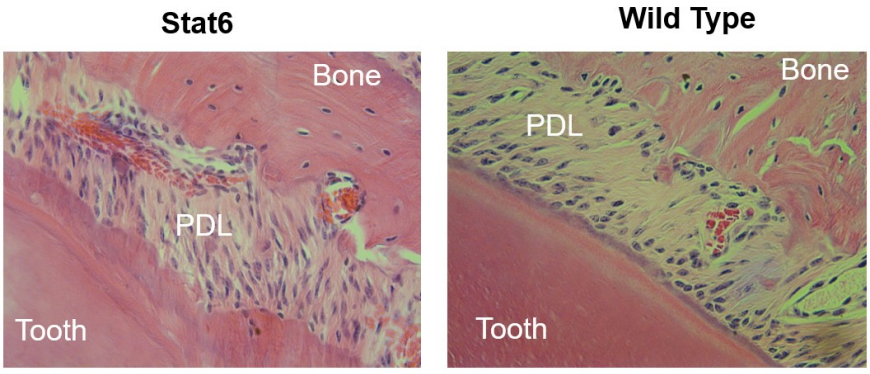
Figure 20: Well differentiated layers of epithelium and rete pegs in the wild type mouse compared to poorly differentiated layers in the Stat6VT mouse and few rete pegs.



Wild Type Stat6VT

Stat6VT mice also showed evidence of bone resorption in the area of the periodontal ligament (PDL). This finding was unique to the Stat6VT mouse and was not found in the wild type. As shown in Figure 21, there is an increased number of osteoid cells indicative of bone resorption. As loricrin has been shown to be downregulated in skin epithelium of Stat6VT mice, these data suggest a similar downregulation in the oral cavity. Although this was not proven in the ELISA, we believe this was due to fundamental flaws in the experimental design of the ELISA and will be repeated with a better study design in the future. With the caveat that we have just examined a single mouse, this preliminary data gives rationale to continue our study. We suspect that similar to what is observed in the skin of these mice, the oral epithelium is more susceptible to bacterial challenge and thus, experience increased inflammation around their teeth.

Figure 21: Active resorption in the alveolar bone in Stat6VT mice.

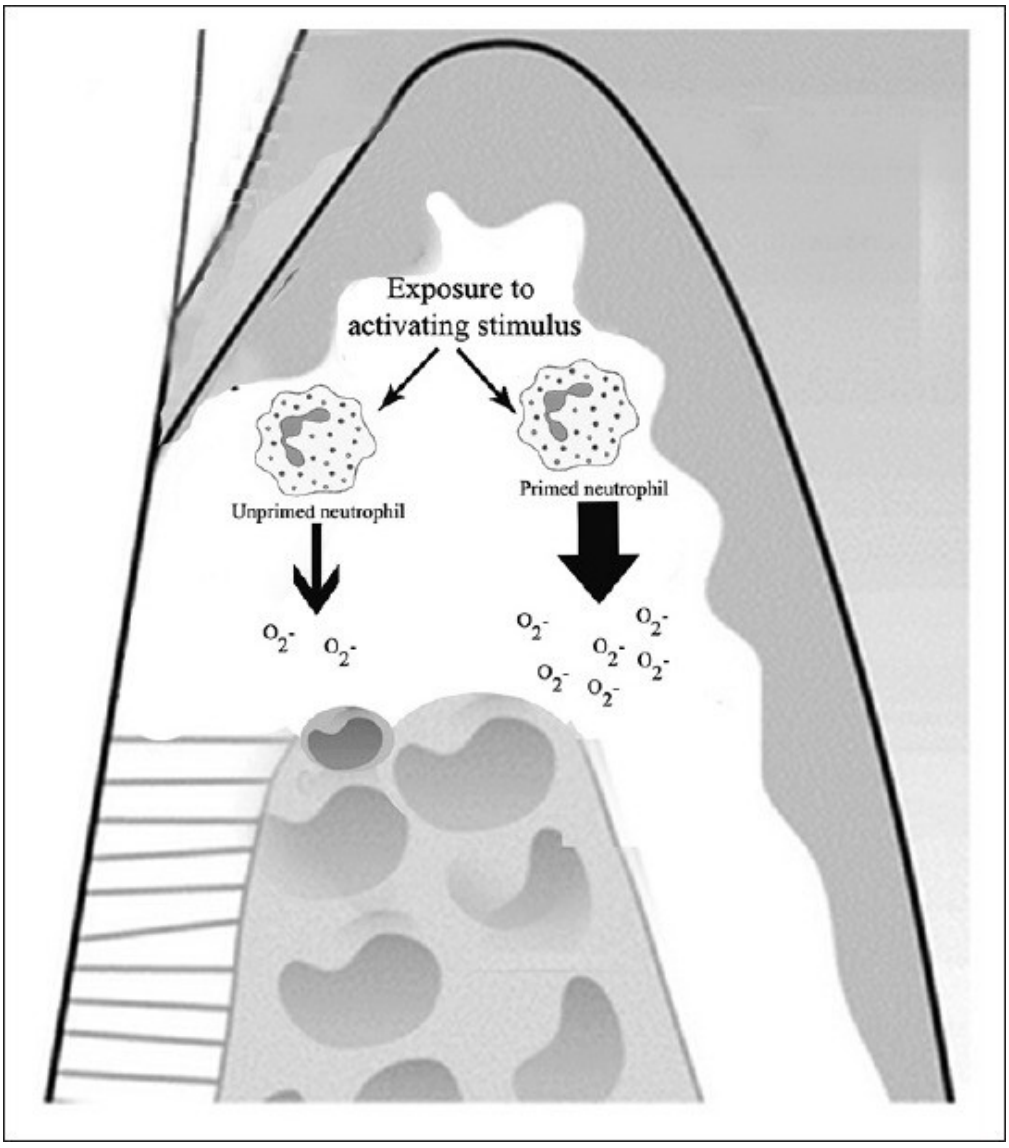


Chapter 5: Discussion

AgP is characterized as having familial aggregation. A systematic review of AgP epidemiological studies showed high prevalence (1-5%) of localized AgP in the African population and African-Americans (2.6%) (Susin 2014, Catunda 2018). The risk of developing AgP is as high as 10% if relatives have been previously diagnosed with the disease (Nibali 2008). The evidence for genetic susceptibility has lead to the investigation of possible genes that may be responsible for the disease. The expression of IL-1 has been previously associated with AgP, along with neutrophil dysfunction (Stabholz 2010, Kaneko 2004, Lindhe 2008). IL-1 is a pro-inflammatory cytokine that can cause bone resorption which makes it a cytokine of interest for periodontal disease progression (Graves 2003, Taubman 2001). Studies have shown that inflamed tissue of chronic periodontitis patients have increased levels of IL-1 and it is suspected that some patients have inherently increased levels of IL-1 which make them more susceptible to chronic periodontitis (Stashenko 1991, Ishihara 1997, Gore 1998). Nonetheless, the role of IL-1 was challenged by Mark et al. who showed no difference in IL-1 response to bacterial challenge by culturing monocytes taken from age matched chronic periodontitis patients and healthy controls (Mark 2000). Although IL-1 plays a role in the immune response and is associated with bone resorption in periodontal disease, it is accepted that its role in periodontal disease remains unclear (Loos 2005). Furthermore, due to the relative rarity of AgP, it is difficult to compare the nature of AgP and chronic periodontal disease and even explore AgP itself. Regardless, periodontal disease is also hypothesized to be a spectrum disease in which some individuals experience advanced rapid progression of bone loss, while most individuals experience a comparably slow progression.

Another theory is that patients with AgP have hyperactive neutrophils which are responsible for the observed mass tissue destruction (Kantarci 2003, Kurihara 1993). AgP patients are hypothesized to have "primed" neutrophils. This means that they have an enhanced ability to react (McPhail 1984). As a result of priming, these neutrophils can mediate the increased tissue damage, have prolonged retention, increased recruitment and prolonged lifespan. Priming also results in an increase in free radical generation (O_2-) (Figure 22). The destructive nature of the primed neutrophil is suspected to play a role in the aggressive tissue inflammation and bone resorption observed in AgP (Albander 1998, Kaner 2006, Figuerdo 2000, Gomez 1994).

Figure 22: Unprimed versus primed neutrophil function in the gingival sulcus (Figure taken from Shah 2017).



Despite the available literature about the pathogenesis of both chronic and aggressive periodontal disease, the conflicting evidence does not provide support for a concrete etiology for the disease. An intriguing clinical characteristic of AgP patients in contrast to chronic periodontitis patients is the presence of only a thin plaque biofilm (Albander 2014, Herbert 2015). AgP patients present with little to no calculus and the bacterial load does not appear to correlate with the intensity of the inflammation. This exaggerated response makes it seem as if the host is responding to a much greater challenge than what is actually present. The hypothesis of this study was that the host response may instead be appropriate for the threat. This dramatic

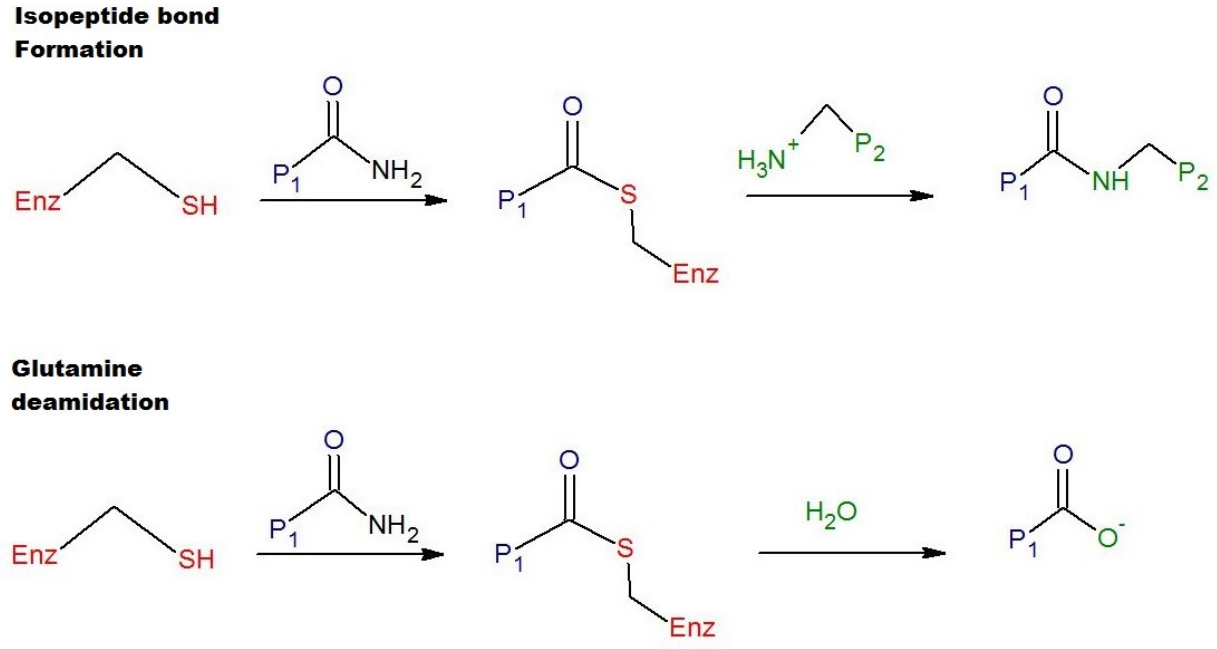
response may be attributed to a compromised protective barrier. If the epithelial barrier is compromised, it may result in easierinclusi penetration of bacteria leading to an inflammatory response from the host (Figure 2).

Loricrin, the most abundant protein in the cornified epithelium responsible for barrier function was a protein of interest for a few reasons. Firstly, loricrin has been evidenced to play a critical role in atopic dermatitis (Kim 2007). Patients with this inflammatory skin disease have decreased loricrin protein in their skin and as a result, are more susceptible to pathogen induced inflammation (Kim 2007). Secondly, loricrin has been previously seen to be downregulated in patients with AgP. More recently, a link between Th2 cytokines and loricrin mRNA transcription was uncovered. Because AgP is characterized by a Th2 response, this gave further support for initiation of our studies. The overall hypothesis of this study is that pathogen-mediated loricrin downregulation could result in an impaired oral epithelial barrier which could then lead to the increased inflammatory response and profound damage observed in AgP-susceptible patients. The primary objective of the present study was to compare loricrin protein expression in healthy control and AgP patients and the second objective was to explore the use of a transgenic constitutively active Stat6 mouse model (Stat6VT) for AgP studies.

Loricrin protein is decreased in AgP versus Healthy Controls

The theoretical kDa of human loricrin is 26 kDa and 38 kDa for mouse (Mehrel 1990). However, loricrin is a highly transglutaminated and cross-linked protein rich in glycine, serine and cysteine (Hohl, 1993). Transglutamination is the process where the enzyme, transglutaminase, catalyzes the formation of an isopeptide bond with a free amine group as well as an acyl group at the end of a protein side chain (Klock 2012) (Figure 23).

Figure 23: Process of transglutamination where by tranglutaminase form isopeptide bonds between the free amine group and acyl group (Klock 2012).



The cross linking and transglutamination of loricrin makes it difficult to completely disaggregate the protein into its primary structure. Therefore, it is not surprising that loricrin has been reported to be approximately 60 kDa in rodents and 35 kDa in humans by SDS polyacrylamide gel electrophoresis (Hohl 1993). The molecular weight of loricrin is likely greater than predicted due to crosslinking/transglutamination and how the sample is prepared and evaluated. For example, whether the sample is prepared with reducing agents may result in different-sized loricrin multimers, which could explain the different molecular weights or even multiple bands, as we observed in our results. Our results showed the presence of loricrin in skin and human samples with a molecular weight of 25, 35 and 50 kDa, while the mouse tissue samples presented bands at 35 and 50 kDa (Figure 15). In order to attempt to better dissociate loricrin into its primary structure, two different extraction buffers were used: RIPA and DTT. Despite using different extraction buffers, extensive boiling and reducing conditions, detection of

loricrin protein was poor using the Tris Glycine gel (Figure 14). We then switched to a Tris Tricine gel to try to improve loricrin protein resolution and detection, since Tris Tricine gels provide better resolution of smaller proteins. Although this protocol led to better resolution (Figure 15), multiple bands remained. Because of the inability to detect loricrin as a discrete band consistently, it was concluded that western blot was an inappropriate method to measure loricrin protein.

Therefore, an ELISA was used to compare AgP and healthy samples for loricrin protein expression. The ELISA showed a significant reduction in loricrin protein expression in AgP patients versus healthy controls. This is noteworthy because, to our knowledge, this is the first study to compare loricrin protein expression in AgP and healthy patients. A previous study evaluated gene expression in both healthy and AgP patients (Guzeldemir 2016). The study compared gene expression by microarray following RNA isolation from gingival biopsies from 23 AgP patients and 25 healthy individuals (Guzeldemir 2016). The results of this study showed that loricrin gene expression was downregulated almost 7-fold in patients with AgP (Guzeldemir 2016). Thus, these findings at the mRNA level are validated at the protein level by our present study. These data provide support for the hypothesis that AgP patients have significantly less loricrin protein expression than healthy patients, and this could impair epithelial barrier function.

Cytokine Expression in AgP

Th2 cytokines stimulate the production of B cell proliferation and differentiation (Seymour 1996). The balance between Th1 and Th2 cytokines are suspects in the mechanism of periodontal disease. Th1 cytokines enhance cell mediated responses while Th2 cytokines suppress cell mediated responses and instead, enhance antibody production (Modline 1993). Although controversial, Th2 cytokines are suspected to contribute to the destructive nature of

AgP (Bartova 2000, Sigush 1998). This role of Th2 cytokines was first described by Seymour et al., who proposed that the progressive lesion was a Th2 cytokine response based on the increased B cell population (Seymour 1996). The role of Th2 cytokines was further studied and Yamakazi et al. showed that the progressive lesion had increased IL-4 expression, which is also indicative of a Th2 response (Yamakazi 1994). Manhart et al. later confirmed these findings using a cell blot analysis to compare early onset periodontitis (now known as AgP) with gingivitis patients (Manhart 1994). Cells from the early onset periodontitis lesions showed significantly more IL-4 production than the patients with gingivitis (Manhart 1994).

Th2 Response, Stat6 and Loricrin

The Th2 response is not only indicative of a progressive lesion in periodontitis but also plays a role in another inflammatory disease linked to the epithelial barrier. Atopic dermatitis is an inflammation of the skin and is associated with loricrin downregulation (Kim 2007, Bao 2017). The mechanism of loricrin downregulation is mediated by Th2 cytokines via the transcription factor Stat6 (Kim 2007). Kim et al. compared loricrin expression in healthy patients and those with atopic dermatitis. They used qPCR and immunohistochemistry and found a statistically significant difference in loricrin protein and gene expression (Kim 2007). The mechanism behind the loricrin downregulation was further explored by Bao et al. Their study showed that loricrin was suppressed in IL-4 transgenic mice, which is a classic mouse model used in the study of atopic dermatitis. Using cultured keratinocytes, they found that IL-4 downregulation of loricrin was decreased when a pan-Jak inhibitor was added. This suggested that the Jak-Stat pathway was involved in the IL-4 downregulation of loricrin. Bao and colleagues then knocked-out Stat6 to find that loricrin was no longer suppressed. We hypothesize that in the event a susceptible patient with low levels of loricrin encounters a

periodontal pathogen, activation of the Stat6 pathway could result in further downregulation of loricrin, to a critical state that triggers the massive host response to even a small bacterial threat. The downregulation of loricrin may explain the exaggerated response AgP patients have to minimal bacteria.

Stat6VT Mouse Model

Currently, there is no animal model for AgP, which greatly impedes study. Our group decided to investigate the feasibility of using mice with decreased loricrin for oral biology research. The loricrin knockout mouse, while viable, is not a good candidate, as there is a compensatory upregulation of filaggrin, which can substitute for loricrin. As a result, these mice only have a slight transient skin phenotype (Koch 2000)

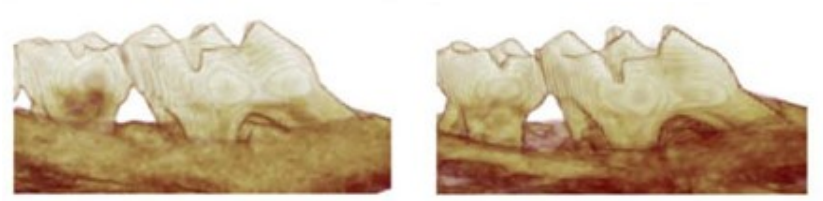
Both Stat6 constitutively activated transgenic (Stat6VT) and IL4 transgenic mice have been created and have been utilized for atopic dermatitis research (Kim 2007, Bao 2017, Koch 2000). We were fortunate that Dr. Mark Kaplan agreed to provide us with samples from Stat6VT mice for preliminary studies. In the Stat6VT mouse, Stat6 is constitutively active causing a chronic Th2 response. This Th2 response leads to impaired loricrin protein expression resulting in atopic dermatitis, which occurs spontaneously at about 12 weeks of age. These dermal lesions are evidence of disease. If these lesions are evident in the skin, we hypothesized it may also be present in the oral cavity. It is unknown whether male and female mice have different levels of loricrin expression and thus, both sexes were tested.

We hypothesized that these mice would also experience a downregulation of loricrin in the gingival epithelium. Our hypothesis suggests that as loricrin decreases, a critical threshold exists where even a normal level of bacteria could provoke inflammation. Although the results of the ELISA showed no statistical difference in loricrin protein expression, this may have been due

to the small sample size (12 mice), and the lack of gender and age matching. In addition, the mice were also not graded for atopic dermatitis lesions. It could be that if similarly affected mice were used, the variability in the results would have been less. Thus, although these results were not different, we believe there are multiple caveats that warrant a repeat of this experiment with a better study design. (We had asked our collaborator to provide us with mice that he had recently euthanized for some preliminary studies).

MicroCT analysis of the first molars revealed significant bone loss in the buccal and lingual sites, but not the mesial and distal sites, of Stat6VT mice compared with wild type. This pattern was not necessarily expected, as previous studies using mice in periodontal disease studies showed bone loss around all aspects of the tooth (Hiyari 2015). However, our study differs from most in the literature as we have not induced the disease in any way. It may be that as a result, the overall bone loss is less, and therefore any differences at the mesial and distal sites may have been too small for us to detect. As exemplified by the results of Hiyari and colleagues shown in Figure 24, LPS-induced bone loss occurred all around the tooth, however, the differences in the mesial and distal aspects appear less dramatic than the buccal aspect, which is similar to our results.

Figure 24: LPS induced bone loss around all aspects of the teeth. Figure taken from Hiyari et al. 2015.



Control LPS Injected

Our microCT evidence of bone loss in Stat6VT mice was supported by preliminary histological sections of the first molar and surrounding tissues. The poorly differentiated epithelium observed in the Stat6VT mouse may be indicative of a weakened barrier function. If bacteria are penetrating the sulcular epithelium, it is reasonable to expect the increased inflammatory infiltrate observed in the sulcus. Correspondingly, the evidence of bone resorption by the presence of osteoid in the periodontal ligament would explain the alveolar bone loss detected by microCT in the Stat6VT mice.

As previously noted for the ELISA section, a limitation to this study is that Stat6VT mice do not all have the same level of phenotype penetrance and the timing at which the phenotype (atopic dermatitis) appears varies. In this pilot, the collaborator provided samples that were not gender matched nor controlled for phenotype. Therefore, there is probably great variation between the mice. In future studies, mice of similar phenotype will be used to better control for variation.

It could be argued that the bone loss observed in the Stat6VT mice is not actually a result of bone resorption. Instead, the formation of the bone itself could be influenced by the effect of Stat6 on bone formation. However, previous studies have demonstrated that Stat6 knockout and wild type mice have a bone phenotype that is completely indistinguishable (Kaplan 1996, Takeda

1996, Shimoda 1996, Li 2013). This suggests that the bone loss we detected in the Stat6VT mice is more likely attributed to loricrin downregulation, however, this remains to be established.

Overall, the preliminary results from this mouse model showed that Stat6VT mice experience alveolar bone loss and have evidence of bone resorption by MicroCT and histological analysis. These results are likely attributed to loricrin downregulation. These data are exciting because they may potentially lead to the use of these mice as a model for AgP. As described in Chapter 2, loricrin is essential for the barrier function of the cornified epithelium. If this layer is impaired due to loricrin downregulation, even a small amount of bacteria could theoretically trigger a dramatic inflammatory response. This does not preclude other contributing factors that have been established as important in AgP. For example, the bacteria species involved in AgP may also have qualities that further contribute to the extreme host response in the circumstance of reduced barrier integrity. As demonstrated by the ELISA of AgP and healthy patients, AgP patients have a significant downregulation in loricrin protein expression. Therefore, decreased loricrin expression aligns with the known theory of AgP and may be considered a risk factor for the development of AgP.

New classification of periodontal diseases

In June 2018, at the EuroPerio Congress hosted by the European Federation of Periodontology, a new classification of periodontal disease was released. Current evidence does not justify chronic and aggressive periodontal disease as two different entities (Papapanou 2018). Instead, periodontal disease is now categorized based on stage and grade. The stage of periodontal disease evaluates the amount of bone loss that has occurred. Stage one involves clinical attachment loss of 1-2 mm while stages three and four involves attachment loss of 5 or more milometers (Papapanou 2018). The nature of the bone loss is described in the grade of

periodontal disease. Grade A and B are slow and moderate rates of disease progression, respectively. Grade C is on the end of the spectrum and describes rapid bone loss that does not seem to correlate with the amount of microbial deposit present (Papapanou 2018). Thus, AgP is now classified as Grade C periodontal disease. This strengthens are current study as we hypothesize the amount of loricrin is related to the degree of barrier dysfunction and thus disease susceptibility. This would explain the variation seen in the human loricrin ELISA.

Limitations

Due to limited resources, sample size of both the AgP samples and Stat6VT mouse samples are a limitation to the study. However, another cohort of AgP and healthy patient samples to repeat the ELISA. Dr. Mark Kaplan has also agreed to send more Stat6VT and wild type mice samples. None the samples used were gender matched and the mice did not necessarily demonstrate an atopic dermatitis phenotype. This may explain the lack of a difference in loricrin protein expression. The next cohort of mice will be gender matched and controlled for phenotype. Another limitation to our study was that only one mouse from each group was evaluated for histology and immunohistochemistry was not performed limiting the information available from the histological cuts. Going forward, all the mice will be sectioned and stained and a protocol will be used to quantify differences between the mice. Additionally, the limited tissue samples of AgP patients did not allow us to perform a protein assay, and the normalization was done by tissue weight. Furthermore, tissue samples were also retrieved from one spot in the patient's mouth which may lead to sample bias and the ages of the control and AgP patients were not matched. Loricrin expression can change with age and this is a confounding factor. Another cohort of samples will allow us to see if loricrin downregulation is a consistent finding.

Conclusion

Loricrin, the most abundant protein in the cornified epithelium, plays an important role in maintaining barrier function. The results of this study demonstrate that western blot analysis is an inappropriate method for loricrin protein detection due to its complex cross-linked structure. Nonetheless, a validated human loricrin-specific ELISA showed that loricrin protein expression was significantly reduced in patients with AgP. This reduction in loricrin may compromise the barrier function of the cornified epithelium, which may correspond to what seems to be a overexaggerated response to plaque. Although there are other factors contributing to the progression of this disease, decreased loricrin protein expression could be considered a risk factor for AgP. Furthermore, preliminary results demonstrate that a Stat6VT mouse model is a potential model for further studies on aggressive periodontal disease. Although there were limitations to the sample sizes for all experiments, the Stat6VT mice demonstrated bone loss and inflammation despite no intentional bacterial challenge. Overall, the results of this study support the hypothesis that loricrin downregulation in the Stat6VT mouse may make it an appropriate model for future AgP studies and that patients diagnosed with AgP experience a downregulation in loricrin protein.

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Appendix

Aggressive periodontitis: The unsolved mystery

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Aggressive periodontal disease is an oral health mystery. Our current understanding of this disease is that specific bacteria invade the oral cavity and the host reacts with an inflammatory response leading to mass destruction of the alveolar bone. Aggressive periodontal disease is typically observed in a population under the age of 30 and occurs so rapidly that it is difficult to treat. Unfortunately, the consequence of this disease frequently involves tooth extractions. As a result, the aftermath is chewing disability and damage to self-esteem due to an altered self-image. Furthermore, patients are encumbered by frequent dental appointments which have an economic impact in regards to both personal financial strain and absent days in the workplace. Aggressive periodontal disease has a tremendous effect on patients' overall quality of life and needs to be investigated more extensively in order to develop methods for earlier definitive diagnosis and effective treatments. One of the mysteries of aggressive periodontal disease is the relatively nominal amount of plaque present on the tooth surface in relation to the large amount of bone loss. There seems to be a hidden factor that lies between the response by the patient's immune system and the bacterial threat that is present. A better mechanistic understanding of this disease is essential to provide meaningful care and better outcomes for patients.
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Key words: bone, bone loss, gingival health, juvenile periodontitis, plaque

Aggressive periodontitis (AgP) is a form of periodontal disease, classified in 1999 at the International Classification Workshop; the other common types are chronic periodontitis and necrotizing periodontitis.[1] There are three main characteristics that set AgP apart from other forms of periodontitis:

- a noncontributory medical history
- rapid attachment loss and bone destruction
- familial aggregation of the cases.[2]

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At the same workshop, AgP was further categorized into localized and generalized forms.[1] Localized AgP is described as having circumpubertal onset, a strong serum antibody response, and localized first molar/incisor presentation, with interproximal attachment loss involving at least two permanent teeth (one being a first molar), but comprising no more than two other non-first molar/incisor teeth.[1] Generalized AgP is defined as affecting individuals under 30 years of age with generalized interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors. Generalized AgP has pronounced episodic destruction of attachment structures and alveolar bone, and poor serum antibody response to infecting agents.[1] AgP is unique in that it typically has an early age onset; however, it can develop later in life as well.[1] In North America, it is estimated that 0.1% to 0.2% of Caucasians, 0.5% to 1.0% of Hispanics, and 2.6%

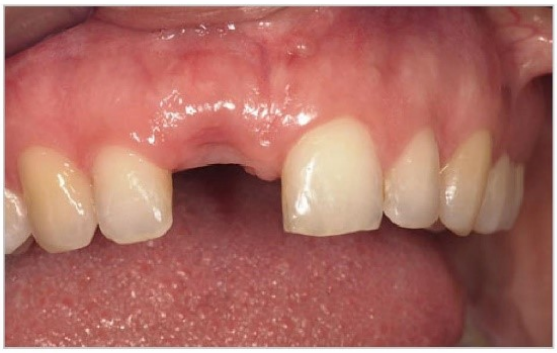


Fig 1 A 12-year-old male patient who lost the maxillary right incisor due to aggressive periodontal disease.

of African-Americans are affected by AgP,[2] but studies from other locales and in other ethnic groups have demonstrated much higher rates of occurrence.[3,4] Although relatively rare in North America, AgP has significantly devastating results because oral health is associated with an individual's quality of life (Fig 1).[5] The tooth loss experienced by children and adolescents due to this disease has both social and emotional implications, and could influence ability to learn in school and function in society. As a result, it is important that we address AgP as early as possible through the development of better diagnostics, and that we direct more research into understanding its underlying etiology.

HISTORY OF THE DISEASE AND PREVIOUS DEFINITIONS

Periodontal disease was initially scientifically investigated in the early 1920s.[6] Gottlieb was one of the first to formulate definitions of different clinical forms of periodontal disease.[6] He defined what is probably AgP as "diffuse atrophy of the alveolar bone" and related the nature of the disease to defective cementum development.[6] Gottlieb's theory was that tooth loss experienced from this form of disease was a result of the body attempting to remove malfunctioning teeth.[6] Later, in 1942, Orban and Weinmann furthered Gottlieb's work on this specific form of the disease and renamed it "periodontosis".[7] They suggested that periodontosis was a noninflammatory degenerative disease resulting from poorly functioning fibers leading to the remodeling of the alveolar bone.[7] In spite of this definition, varying amounts of gingival inflammation were observed in periodontosis.[8]

The role of plaque bacteria in inflammatory processes in the gingiva and the potential association with periodontal disease began to be investigated by Newman et al[9] in 1976. Newman and colleagues discovered that *Actinomyces naeslundii*, *Actinomyces viscosus*, and *Streptococcus mutans* were associated with increased plaque and bone loss.[9] In 1971, Baer[10] introduced a new definition schema and separated "periodontosis" into generalized and localized forms. The disease was described as localized when the incisors and first molars were the only teeth affected.[10] Baer[10] also highlighted that the degree of bone loss was not proportionate to "the local irritants present." In 1989, periodontal disease was differentiated into two main categories: early onset periodontitis, and adult periodontitis.[11] Early onset periodontitis aimed to describe a younger population that experienced an especially aggressive form of the disease.[12] Rapidly progressive periodontitis was also a category that was included in this workshop.[12] There was a lack of clarity in this classification system, however, as the definitions were not specific, and applying them to clinical practice was difficult.[12] This led to another workshop in 1999 where the term early onset periodontitis was changed to AgP and the category "rapidly progressive periodontitis" was removed.[12] Today, AgP remains categorized into generalized and localized.[1]

DISEASE NATURE

Bacteria are the culprit for many oral health complications and AgP is not excluded. According to Lang et al,[1] general features of AgP include a noncontributing medical history, rapid attachment loss, and bone destruction as well as familial aggregation. A secondary feature of the disease includes increased numbers of *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*.[13] The extent to which bacteria influence the disease remains a mystery. Clinically, patients

who are diagnosed with AgP present with minimal plaque accumulation. This discrepancy raises a question about the extent to which bacteria play a role in disease.

The host response is critical in AgP. The interplay between bacteria and the inflammatory response initiated by the host leads to destruction of the supporting alveolar bone. Interleukin 10 (IL-10), an anti-inflammatory cytokine that modulates the activity of type 1 T-helper cells, natural killer cells, and macrophages, has been shown to have a significant role in regulating the host response.[14-16] One theory suggests that an antigen on *A actinomycetemcomitans* has the ability to bind the IL-10 receptor and act as an antagonist or agonist.[17] This affects production of IL-1β, which is an important pro-inflammatory mediator.[16] Teles et al[16] hypothesized that an imbalance between pro- and anti-inflammatory cytokines may be responsible for the onset of AgP. Effective periodontal therapy, which includes plaque bacteria removal, has been demonstrated to lower IL-1β and increase IL-10 levels. These data suggest that these cytokines have a role in the pathogenesis of AgP.[18] According to Rescala et al,[19] the immunologic and microbiologic characteristics of generalized chronic periodontitis and generalized AgP are similar in nature. In this study, levels of IL-1β, IL-2, IL-4, IL-8, interferon-γ, and elastase activity were measured and there were no statistically significant differences between the levels in the two diseases.[19] A recent study (2016) also showed no significant difference in the biomarkers IL-6, IL-10, tumor necrosis factor-α, C-reactive protein, and alkaline phosphatase obtained from gingival crevicular fluid from patients with chronic periodontitis and AgP.[20] A systematic review also came to the conclusion that there is a lack of evidence demonstrating a distinct cytokine profile that distinguishes between patients with AgP and chronic periodontal disease.[21]

Disease progression rates

The progression of disease in AgP is thought to occur at a more rapid rate than in the chronic form of the disease. This rapid destruction of bone and attachment structures is what causes the loss of teeth (Fig 1). A systematic review, published by Nibali et al,[22] revealed that the rates of tooth loss were similar for both AgP and chronic periodontal disease. It is important to note that this study only considered tooth loss and did not study rates of attachment loss. Due to patient variables, such as age, professional maintenance, homecare routines, secondary diseases, and smoking, studying the rate of disease is not straightforward. Baer[10] estimated that AgP progressed three to four times more rapidly than chronic periodontal disease, based on radiographic evidence of alveolar bone loss. Chronic periodontal disease has been shown to progress at a rate of ~0.2 mm per year in terms of clinical attachment loss.[23] A longitudinal study of AgP demonstrated progression rates of up to 0.46 mm per year.[24] Despite the lack of systematic evidence to confirm disease progression rates of AgP, case studies suggest that clinical attachment loss occurs more rapidly compared to chronic periodontal disease.

TREATMENT

Early diagnosis

In order to provide the most successful treatment, early diagnosis is a priority. Once AgP is confirmed, treatment interventions can be implemented. This will also minimize the degree of destruction, allowing conservative interventions, if the disease is diagnosed at the earliest point. These interventions include oral hygiene instruction together with reinforcement by regular scaling and root planing appointments at the dental office. These appointments are important as studies have demonstrated that patients diagnosed with AgP who undergo regular scaling and root planing experience less tooth loss.[25] A recent retrospective follow-up study showed that patients undergoing regular scaling and root planing had annual tooth loss rates of 0.10 ± 0.1825. These findings were comparable to those found by a systematic review performed by Nibali et al,[22] which observed annual tooth loss rates of 0.14 in AgP patients receiving active or maintenance/supportive periodontal therapy. In contrast, Dopico et al[26] revealed higher tooth loss rates (0.27 teeth per year) in

their follow-up study of patients who did not undergo strict scaling/root planing treatments. Therefore, the earlier AgP is definitively diagnosed, the earlier regular scaling and root planing interventions can be implemented in order to prevent further destruction of the periodontium.

Plaque control

A common feature of patients diagnosed with AgP is that plaque accumulation does not seem to correlate with the degree of inflammation and bone loss. Therefore, the role of good oral hygiene in the treatment of AgP may be questioned. Bacterial plaque has a causative role in inflammation of the gingival and periodontal tissues and, therefore, it is important that effective homecare techniques are emphasized to patients diagnosed with AgP. Although the exact etiology of AgP remains unknown, it is clear that plaque has a role and needs to be removed effectively from the oral cavity for better outcomes.[27]

Systemic antibiotics

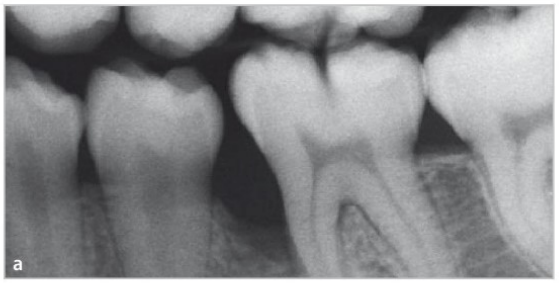
Antibiotics are a controversial treatment for AgP. Systemic antibiotics can be prescribed to patients in an effort to control the bacteria present and reduce the destruction that is occurring. Antibiotic therapy is typically provided in combination with scaling and root planing procedures. Commonly prescribed antibiotics include amoxicillin and metronidazole. A systematic review and meta-analysis conducted by Keestra et al[28] suggested that the combination of antibiotics with scaling and root planing had a statistically significant benefit compared with scaling and root planing alone, with regards to pocket depth reduction, clinical attachment levels, and bleeding upon probing. The study also concluded that a combination of amoxicillin and metronidazole was the most effective combination of antibiotics. Another meta-analysis found a similar result: the use of amoxicillin and metronidazole in combination with scaling and root planing had a significant positive impact on clinical attachment levels and pocket depth reduction.[29] Due to the increased concern about antibiotic resistance, dental professionals should remain cautious when prescribing antibiotics; however, the use of systemic antibiotics has been demonstrated to be effective in the treatment of AgP.

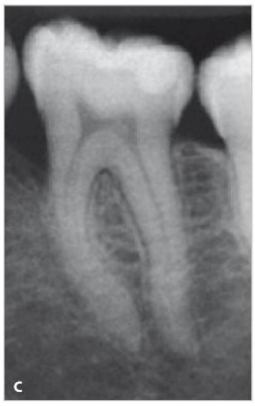
Because of the concern over effects of systemic antibiotics, local strategies are also being used. Minocycline hydrochloride (Arestin, AAIPharma Services) is an antibiotic in a powder form that is administered locally into the periodontal pocket itself, with the expectation of slow release (21 day time period) during which it reduces bacterial presence. There is currently no systematic review analyzing the effectiveness of this treatment, but case reports suggest that local antibiotics may be effective in treating AgP.[30] Like minocycline hydrochloride, doxycycline hyclate (Atridox, Tolmar) is another suitable antibiotic, available in the form of a slow release (21 days) gel.

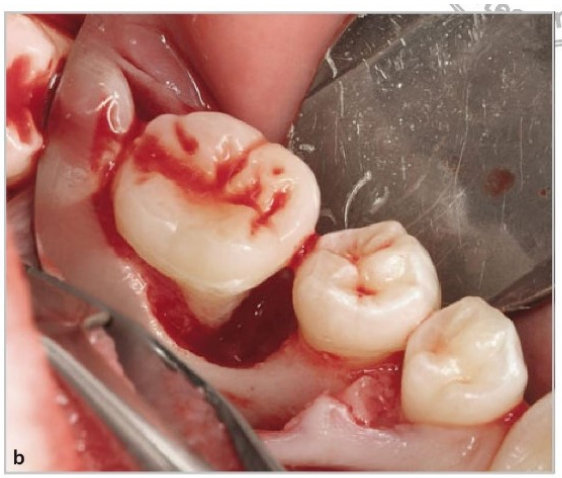
Periodontal surgeries

In some cases, periodontal pockets can be difficult to access if they are sufficiently deep and characterized by excessive inflammation. When instruments used to debride the root surface are unable to reach to the depth of the pocket, periodontal surgery may be required. Such surgery allows the site to be easily accessed and ensures the complete debridement of the entire tooth (Figs 2 and 3). Since *A actinomycetemcomitans* is able to manifest within the epithelium of the periodontal pocket, the removal of diseased tissue might also contribute to better outcomes in the treatment of AgP.[31] The combination of antibiotics with periodontal surgery for the successful treatment of AgP has been documented.[32-35] For example, one study demonstrated that a combination of Widman flap surgery, tetracycline, and professional maintenance led to reduced pockets depths and radiographic bone fill after a 5-year follow-up.[35]

Periodontal surgery can also be utilized for bone grafting techniques. Although there is not extensive evidence surrounding the success of bone grafting procedures specifically for patients with AgP, case reports have been published that demonstrate bone grafting as a viable treatment option. Mabry et al[31] performed bone grafting in a split-mouth approach in 16 patients





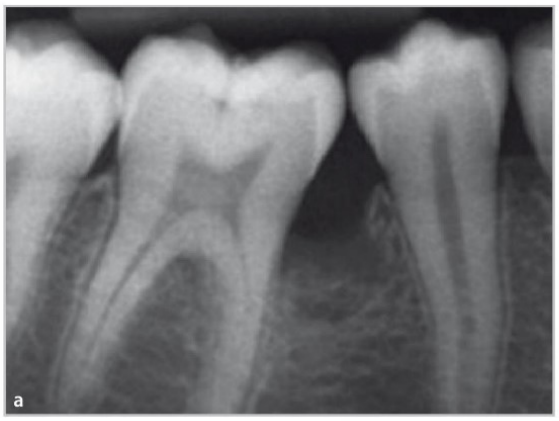


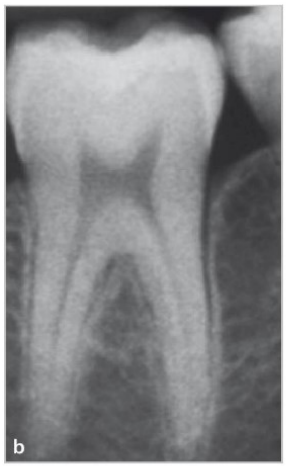
Figs 2a to 2c Radiographic *(a)* and intraoperative *(b)* views of localized AgP in a 13-year-old female patient; note the dramatic bone loss mesial to the first molar. *(c)* 1-year postoperative radiograph showing bone regeneration next to the first molar.

diagnosed with AgP. The results revealed that bone density and bone fill were significantly greater in pockets treated with bone grafting techniques than in pockets treated with scaling and root planing treatments only.[31] Periodontal surgery can also be utilized with respect to guided bone regeneration. Sirirat et al[36] also performed a split-mouth approach but compared bone grafting with guided bone regeneration using a polytetrafluoroethylene membrane. Although both treatments were successful, the areas that were treated with membrane had a greater reduction in pocket depth and greater clinical attachment.[36] Today, there is a wide range of available material for both bone grafting and bone regeneration techniques.

Immune modulation

A major conclusion from research in the field is that the tissue damage caused by AgP is not solely a result of bacterial products. The host response to the bacteria is a driving force in the intense inflammation that leads to rapid tissue destruction.[37] It is well accepted that





Figs 3a and 3b Pre- *(a)* and 18 months postoperative *(b)* radiographs of a 14-year-old with localized aggressive periodontal disease demonstrating the bone healing following periodontal treatment.

inflammatory mediators that are involved in periodontal disease, such as cytokines and metalloproteinases, are dysregulated in a way that causes increased production in the extracellular matrix of the oral epithelium.[37] Due to the importance of the host response in AgP, treatment options focused on modulating the host immune response are a potential treatment option for patients. Although this option has not been extensively studied, anti-cytokine agents may be used to regulate the formation of osteoclasts, the cell type that breaks down bone. One study analyzed the effects of an IL-1 inhibitor in a non-human primate model and showed reduced inflammation and bone resorption.[38] Kinases, which are essential enzymes involved in host signaling and production of the inflammatory response, have also been tested for their ability to modulate osteoclast formation. Drugs designed to target specific kinases have been shown to reduce the formation of osteoclasts and consequently reduce bone loss.[39,40] Host immune-modulation may be a promising future route for the treatment of AgP, pending more research in the area.

LONG-TERM PROGNOSIS

Many factors are involved in AgP. Whether the disease is localized or generalized, or is in the primary or permanent dentition can also affect the prognosis of the disease. One study analyzed the response to scaling and root planing in combination with systemic antibiotics in the permanent and primary dentitions. Although the therapy was effective in both dentitions, the periodontal improvement in the primary dentition was more significant than that demonstrated in the permanent dentition.[41] This emphasizes the importance of early definitive diagnosis, and the need for more research in this area.

Active and supportive periodontal therapies are imperative when patients present with AgP. Active periodontal therapy involves scaling and root planing, as well as open flap surgeries when necessary.[42] Supportive periodontal therapy includes oral hygiene education, frequent dental cleanings, and frequent reevaluation of the patient's periodontal status.[42] Patients with AgP are more likely to experience a relapse during supportive periodontal therapy.[43] Importantly, it has been shown that patients with AgP do not lose more teeth than patients with chronic periodontal disease if they are treated appropriately over the long term.[42] A study by Graetz et al[42] analyzed affected teeth in patients with AgP and found that 88.2% of teeth labelled questionable and 59.5% labelled hopeless survived for 15 years when undergoing supportive periodontal therapy. Therefore, both active and supportive periodontal therapy have been shown to lead to better clinical outcomes and are essential to effective treatment and maintenance of AgP to prevent loss of teeth.

DENTAL IMPLANTS IN AGP PATIENTS

Implant success may be a concern for patients who have been diagnosed and treated for AgP. A recent study showed dental implant survival rates for patients previously treated with AgP as high as 97.3%.[44] Four years after placement, there was no significant difference in the amount of keratinized tissue around the dental implant in both healthy and AgP patients.[44]

Another study showed implant survival rates in patients with AgP to be 96%.[45] However, implant success rates for AgP patients were considerably lower, at 33%.[45] AgP patients are also more susceptible to peri-implant diseases, as 56% presented with peri-implant mucositis and 26% presented with peri-implantitis.[45] Comparatively, 40% of periodontally healthy patients presented with mucositis, and 10% presented with peri-implantitis.[45] This likely explains the lower observed success rates for dental implants in patients with AgP.

Nonetheless, a 10-year prospective cohort study comparing implant status of patients with AgP and periodontal healthy patients concluded that AgP patients undergoing treatment can have missing teeth successfully restored with dental implants.[46] These patients did experience significantly more bone loss than periodontally healthy patients at the 10-year mark, however, despite 3-month recall intervals.[46]

Mengel and Flores-de-Jacoby[47] performed a prospective study analyzing the success of dental implants in areas with regenerated bone in patients with AgP. They found that the 3-year survival rate was 100%, but cautioned that the potential for bone loss should not be ignored and the long-term prognosis should still be questioned.[47]

In summary, there is potential for dental implant success in AgP patients; however, further research and more specific protocols should be outlined to ensure the best outcomes for these patients, since bone loss is more significant than in healthy patients.

MYSTERIES TO BE SOLVED

The exact etiology of AgP remains mysterious. Although it is doubtless that bacteria are a major player in the disease, it is unclear why there is an exaggerated response to minimal plaque accumulation. Genetic predisposition is a major influence in the manifestation of AgP and familial aggregation is one of its defining characteristics.[1] Genes have been identified that are associated with AgP, with many also associated with the host immune response, including those that affect the expression of IL-1, IL-6, IL-10, and tumor necrosis factor-α, among others.[48] It should be noted, however, that many studies have specifically looked at this category of genes only, thus there is an intrinsic bias. According to Stabholz et al,[48] there are no specific genes that differentiate AgP and chronic periodontal disease; however, some research has alluded to the possibility of different polymorphisms of one gene being responsible for the differential presentation of the diseases.[48]

In contrast to genes affecting cytokines, another possible genetic contribution includes dysregulation of phagocytosis of invading bacteria.[49] Analysis of neutrophils from patients with AgP and healthy controls showed a significantly higher number of Fc-gamma receptor polymorphisms.[49,50] Neutrophils are the body's "first responders" to bacterial invasion. Patients with Fc-gamma receptor polymorphisms had neutrophils that were less efficient at phagocytosis of periodontal bacteria.[49] On the other hand, some studies have shown hyperactive neutrophil function that may be responsible for rapid attachment loss.[51] The exact contribution of neutrophils in AgP thus remains unclear. These studies suggest that there may be multiple different etiologies of AgP that present with similar clinical symptoms. The mystery of AgP can be partly attributed to the complexity of the host immune response; however, further research in this area is necessary to increase our understanding of the pathophysiology.

Another interesting question that arises is the clinical presentation of both localized and generalized forms of the disease. The pathophysiologic processes behind only molar and incisor teeth being affected in the localized form of the disease remain incompletely understood.

Other theories regarding the etiology of AgP are being developed based on unbiased studies of gene expression, and include a focus on the barrier function of the periodontal tissue.[52-55] Perhaps the oral epithelial barrier is impaired, leading to greater exposure of the host to bacterial plaque, minimal as it may seem, and thus the observed "excessive" immune response and tissue destruction are actually in accordance to the exposure. Changes in oral epithelial barrier function represent a new direction of study.

With so many unknowns, AgP remains a mystery and warrants essential further research both to allow for definitive diagnosis and to increase our basic understanding so that effective new treatment strategies can be developed for better outcomes for patients.

CONCLUSION

AgP is an impressively destructive disease that is accompanied by devastating loss of self-esteem and costly dental procedures. Its complex etiology and pathophysiology contribute to many unsolved mysteries. In order to further prevention and treatment, the scientific and clinical communities need to address these mysteries. Detailed mechanistic study of AgP might lead to advancements in its treatment and, consequently, minimize its devastating effects.

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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: Danielle Clark

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 I be paid to be in the research?

Participants will receive a reimbursement for parking, at the rate of $20.00.

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 Danielle Clark 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: Danielle Clark **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