The Role of Chemokines in Cyclosporine Induced
Gingival Overgrowth

BY

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THESIS

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LIST OF ABBREVIATIONS

CsA.............................................Cyclosporine (Cyclosporin A)
ECM..............................................Extracellular matrix
ELISA........................................Enzyme-linked immunosorbent assay
EMT...........................................Epithelial mesenchymal transition
EndMT.........................................Endothelial mesenchymal transition
FOXO1.......................................Forkhead box protein O1
GO..............................................Gingival overgrowth
HGF...........................................Human gingival fibroblast
HLA..........................................Human Leukocyte Antigen
IL...............................................Interleukin
LPS..........................................Lipopolysaccharide
MMP..........................................Matrix metalloproteinase
NFAT.........................................Nuclear factor of activated T-cells
PAMPS......................................Pathogen-associated molecular patterns
PDGF..........................................Platelet derived growth factor
PCR..........................................Polymerase chain reaction
SOS-1..........................................Son of sevenless-1
TGFβ..........................................Transforming growth factor beta
TIMP.........................................Tissue inhibitor of matrix metalloproteinase
TNFα...........................................Tumor necrosis factor alpha
SUMMARY

This study investigated the role of chemokines in Cyclosporine (CsA) induced gingival overgrowth (GO). 14 patients with renal transplants undergoing CsA treatment and 3 healthy subjects were selected for this study from a larger subpopulation. 11 individuals on CsA treatment experienced GO, while 3 subjects did not have clinical evidence of GO. Gingival biopsy samples were taken for all individuals in the three groups (control, healthy n=3; CsA NO GO n=3; CsA GO n=11). RNA was extracted from the biopsy samples and gene array analyses for differential expression were performed.

CsA treatment was associated with increased chemokine expression globally; this was more evident in the CsA GO group. Chemokines reported in the literature to be profibrotic (CCL2, CCL3, CCL5) were significantly increased in the CsA GO group. Additionally, the chemokine ligand-receptor axis of CXCL8/CXCR1 and CXCL16/CXCR6 were notably increased in the CsA GO group alone. These ligand-receptor interactions are increased in fibrosis of the liver and kidney. CsA NO GO subjects had increased chemokine ligand production without the paired receptor. This may represent a protected immune response, resulting in no GO in certain individuals.

Investigation of upstream toll-like receptor (TLR) signaling revealed an increase in TLR1, TLR2, and TLR4, which may drive chemokine potentiation in addition to CsA tissue specific effects. The process of CsA GO involves active fibroblast transformation from pericytes, fibrocytes, or the epithelial mesenchymal transition. Chemokines are intimately involved in the aforementioned cells and events. Therefore, CsA treatment may alter chemokine levels and function through some or all of these pathways to produce a GO response in certain individuals.
1. INTRODUCTION

1.1 Anatomy & Function of the Gingiva

The purpose of the gingiva is to surround the teeth and provide protection for the underlying tissues and alveolar bone. The gingiva is divided into three distinct types based on anatomical characteristics. These are the marginal gingiva, the attached gingiva, and the interdental gingiva. Each type of gingiva demonstrates distinct physiological and cellular characteristics dependent upon the specific function in preventing mechanical and microbial damage. The gingiva is composed of overlying layer of stratified squamous epithelium and an underlying connective tissue.

The keratinocyte is the principle cell type of the gingival epithelium; other non-keratinocytes or clear cells include Merkel cells, Langerhans cells, and melanocytes. Cells within the gingival epithelium are essential for the host response to microbial and viral agents through activation and potentiation of the innate and acquired immune pathways. The gingival epithelium protects the underlying structures and permits cellular interchange for the purposes of host defense. Proliferation and differentiation of the keratinocyte is responsible for its host response function. The gingival epithelium can be subdivided into three characteristic areas: outer epithelium, sulcular epithelium, and junctional epithelium. These three epithelial zones represent different adaptations of the gingival epithelium to the tooth and the alveolar bone. The sulcular and junctional epithelium are non-keratinized, while the oral (outer) epithelium is keratinized. The oral and sulcular epithelium have a protective function, while the function of the junctional epithelium is to regulate tissue health. Histologically, all types of gingival epithelium
have a stratum basale, stratum spinosum, and stratum granulosum; however, the oral epithelium has an additional stratum corneum layer.\textsuperscript{2}

The fibroblast is the principal cell type of the gingival connective tissue; other cells of the gingival connective tissue include macrophages, mast cells, and undifferentiated mesenchymal cells.\textsuperscript{2} Fibroblasts have an important role in the development, repair, and maintenance of connective tissue. A cardinal role of the fibroblast is to create and maintain the extracellular matrix of the gingival connective tissue through synthesis and degradation of collagen, elastic fibers, glycoproteins, and glycosaminoglycans.\textsuperscript{3} By volume, the gingival connective tissue consists of 5% fibroblasts, 60% collagen fibers, and 35% nerves, vessels and matrix. Collagenous proteins are predominant in the gingival connective tissue. Type I collagen, the main collagen in all layers, is seen in dense bundled, thick fibers mostly in the lamina propria. Type III collagen appears in thinner fibers, in reticular patterns, mostly near the basement membrane.\textsuperscript{3} Type IV collagen is nonfibrillar, and together with adhesive glycoproteins composes the basement membrane. The basement membrane separates the epithelium and underlying lamina propria.\textsuperscript{4} Historically fibroblasts were thought to be uniform in response to stimuli and function. However, recent studies have shown variable phenotypes of fibroblasts having altered responses to cytokines. Gingival fibroblasts have also been found to express different cytokines and cytokine receptors (i.e. interleukins 1, 6, and 8).\textsuperscript{5} The components of the connective tissue are actively involved in cellular signaling to allow for growth, wound healing, and fibrosis.\textsuperscript{4}
1.2 **Gingival Overgrowth**

Gingival overgrowth (GO) refers to the enlargement of the gingival structures within the oral apparatus. GO has been mostly reported to be a condition affecting the keratinized components of the periodontium. Causes of GO reported in literature include systemic genetic conditions with gingival manifestations (i.e. Hurler syndrome, Niemann-Pick disease, Sturge Weber syndrome, etc.), genetic mutations in isolated GO (SOS1), and drug (Cyclosporine, Phenytoin, etc.) induced forms. Previous terms used synonymously with gingival overgrowth represented histological observations, such as gingival hypertrophy or gingival hyperplasia. These terms represent a microscopic characterization of the gingival apparatus. Hypertrophy is defined as an increase in the size of cells, while hyperplasia refers to an increase in the cell number. Both cellular descriptions can occur together and are a result of normal or pathologic processes. Gingival overgrowth (GO) will be the terminology of choice for this paper.

1.2.1 **Tissue Fibrosis**

Fibrosis is a tissue specific response resulting in the deposition of connective tissue related proteins. It may involve an increase in collagen accumulation and extracellular matrix proteins. In liver fibrosis, there is an increase in the extracellular matrix due to an increase in synthesis and decrease in degradation. Similar responses are noted with idiopathic pulmonary fibrosis and fibrosis of the kidney. Most types of GO have been designated as tissue fibrosis based on clinical characteristics. The histological appearance of gingival overgrowth exhibits an increase in cellular fibroblasts, type I and type III collagen, extracellular matrix proteins, and epithelial components. The
exact mechanism of the fibrotic changes is unknown. Proposed etiologic explanations for the changes in fibrotic components have been an increase in normal growth, an overproduction of gingival fibroblasts, a decrease in collagenase activity, or select fibroblast transformation.\textsuperscript{13,14}

1.2.2 Drug-Induced

Gingival overgrowth from the consumption of medications was first observed in 1939 with the usage of Phenytoin.\textsuperscript{15} With the introduction of additional pharmaceutical agents since that time, the incidence of drug induced GO has increased. While a variety of drugs have been reported to be associated with GO, most reports are related to three main drug categories: anticonvulsants, immunosuppressants, and calcium channel blockers.\textsuperscript{8,13} It is estimated that 5% of the geriatric population takes these medications. Phenytoin is the most common anticonvulsant known to cause GO. Its continued use for the treatment of partial and secondarily generalized seizures makes Phenytoin a common drug for the treatment of epilepsy. Similar to other drugs that can cause GO, the exact mechanism by which Phenytoin causes GO is complex and poorly understood as a whole.\textsuperscript{16,17} Calcium channel blockers may also cause GO. These drugs provide an antihypertensive effect and are commonly used to treat patients with mild, moderate or severe hypertension.\textsuperscript{18} Calcium channel blocker induced GO has been reported to occur, with a wide range of 6.3%-83\%.\textsuperscript{19,20} Studies have also shown that calcium channel blockers, when used in combination with immunosuppressants, may increase the appearance of GO.\textsuperscript{21}

Proposed mechanisms of drug induced GO are thought to involve calcium signaling. Although all three categories of GO inducing drugs are functionally different,
the common signaling pathway involves the antagonism of calcium. Intracellular calcium regulates collagen phagocytosis through α2β1 integrin and is important in the maintenance of normal tissue integrity. Drug induced alterations of this pathway, as well as many other pathways, may explain the tissue changes with GO.

1.2.3 Indices

The degree of GO is classified into three categories based on the hyperplastic index reported by Angelopoulos and Goaz. This classification system was modified by Pernu et al., and then modified again by Ingles. All three indices score GO from zero to three based on the extent of vertical proliferation and soft tissue characteristics of the gingiva as follows:

"Score 0 = no gingival overgrowth
Score 1 = mild gingival overgrowth; thickening of the marginal gingiva, lobular granulation of the gingival pocket, overgrowth covering the gingival third of the crown or less
Score 2 = moderate gingival overgrowth; overgrowth extending to the middle of the crown
Score 3 = severe gingival overgrowth; overgrowth covering two thirds of the crown or the whole attached gingiva is affected."

1.3 Cyclosporine: History

Cyclosporine (Cyclosporin A, CsA) is a classical immunosuppressant that is derived from the fungus *Tolypocladium inflatum*. It is regarded as one of the first
immunosuppressive drugs to select for T cells without cytotoxicity.\textsuperscript{26} The history of CsA began with its serendipitous isolation from soil samples by Dr. Jean Borel in 1970.\textsuperscript{26} At first, the costs of production were too high for clinical use at a time when organ transplantation was in infancy. However, as organ transplantation became more advanced and common in modern medicine, CsA was re-introduced to increase survival rates. Pre CsA 5-year success rates were 20\% for heart transplants and 50\% for renal transplants. Moreover, available immunosuppressants at that time were cytotoxic for T cells leading to increased post-operative infection rates. CsA human trials increased in the late 1970’s with advancing transplantation technology; it was officially approved for all transplant patients by the FDA in 1983. The 5-year success rate of organ transplantation post CsA therapy was above 90\%.\textsuperscript{8}

1.4 \textbf{Cyclosporine: Pharmacokinetics}

Cyclosporine is available in both oral and intravenous forms. The pharmacokinetics of CsA is dependent upon the patient’s disease severity, type of organ transplant, patient age, and concomitant use of other medications. The therapeutic dose of CsA is 10-20 mg/kg for oral consumption.\textsuperscript{27} After administration, CsA peak concentrations are seen in blood/plasma after 1 to 8 hours,\textsuperscript{27} with serum concentrations ranging from 100-400 ng/ml.\textsuperscript{28} Most well controlled studies note peak concentrations 3-4 hours after absorption in the small intestines when taken orally.\textsuperscript{28} CsA bioavailability ranges from 5\%-89\%, with lower absorption rates noted in kidney, liver, and bone marrow transplant patients. CsA binds strongly to plasma proteins, particularly to lipoproteins and is eliminated via bile salts because it is insoluble in water. The
metabolism of CsA is thought to be primarily due to cytochrome P450 isozyme CYP3A4. These specific cytochromes are expressed in both the liver and small intestine, and may have tissue specific and individual variations. Cytochrome P450 isozyme CYP3A4 is also noted to be expressed in human gingiva. Therefore, P450 isozyme CYP3A4 variations may be responsible for the different metabolism rates of CsA.

1.5 **Cyclosporine: Mechanism of Action**

Early studies on the mechanism of CsA reported the ability for CsA to inhibit T cell activation and proliferation. The predominant theory is that CsA inhibits transcription of cytokine genes (i.e. IL-2, IL-4), which in turn suppresses the function of T cells. Calcineurin is a cytosolic protein that is tightly regulated by calcium/calmodulin signaling. During normal T cell function, a calcineurin/calmodulin complex is established through calcium signaling, which causes dephosphorylation of nuclear factor of activated T-cells (NFAT). Dephosphorylation of NFAT creates an active molecule, which translocates to the nucleus and binds to the interleukin-2 promoter site.

With CsA therapy, CsA molecules enter T cells and bind to cytosolic cyclophilins forming a cyclophilin-CsA complex. Consequently, the cyclophilin-CsA complex inhibits the formation of the calcineurin/calmodulin complex and subsequent dephosphorylation of NFAT. The phosphorylated NFAT cannot enter the nucleus and promote the proliferation of T cells through transcriptional activation of interleukins. Other biologic mechanisms inhibited by CsA treatment include the JNK and p38 pathways. Transcription factors such as, AP-1 and NF-κB, are also altered by CsA. Additionally,
recent studies note CsA involvement in the inhibition of macrophages and IL-1 receptors on the surface of T-helper cells.

1.6 Cyclosporine: Side Effects

The side effects of CsA treatment are numerous and include immunological, renal, hepatic, and neurological issues. Reported side effects of CsA use are nephrotoxicity, neurotoxicity, hypertension, hepatotoxicity, anemia, secondary infections, and gingival overgrowth. More rare side effects are malignancies of the kidney and liver, as well as Kaposi’s sarcoma. CsA mediated fibrosis of tissues has been reported in the literature since its introduction as a “wonder drug” for post transplantation use. Fibrosis of the renal, pericardial, pulmonary, and hepatic tissues has been reported with CsA therapy and may lead to increased morbidity and mortality for immunosuppressed patients. Chronic nephrotoxicity and renal fibrosis is a realistic consequence of long term CsA treatment, and is a major challenge for transplant physicians. Although the mechanism of CsA mediated renal fibrosis is unknown, it is believed to be dose related and/or genetic in nature.

One of the most common side effects of CsA treatment is GO. CsA induced GO was first reported in literature in 1983. It was described as generalized gingival enlargement, especially pronounced in areas of poor oral hygiene or defective marginal restorations. The clinical symptoms resembled phenytoin induced GO, with an increase in keratinized tissue volume, mostly on the buccal surfaces. CsA induced GO is usually observed after 6 months of treatment in certain individuals. GO usually self resolves after removal of an affected tooth or discontinuation of CsA. CsA induced GO is
reported to occur, in susceptible individuals, with a range of 13-86%.\textsuperscript{53} Its presence is thought to be influenced by factors such as, plaque control, age, drug serum concentration, length of therapy, and overgrowth classification.\textsuperscript{53} The wide range of CsA GO occurrences reported in literature may be a result of a lack of standardization during assessment and reporting. Recent, well-controlled studies report a range of approximately 30% for CsA GO.\textsuperscript{21} It is important to note that CsA GO susceptibility remains unidentified, but maybe genetic in nature.

1.7 **CsA GO: Clinical Appearance**

The clinical presentation of CsA induced gingival overgrowth is characteristic for papillary enlargement, more prominent on the buccal or labial aspects of the gingiva.\textsuperscript{54} The palatal or lingual surfaces may be affected, but usually to a lesser extent. The main features of GO are limited to the gingiva and do not extend to the alveolar mucosa. CsA GO is seldom reported in complete edentulous patients, or in partially edentulous areas. The degree or severity of GO can range from minimal increases in tissue contours to full coverage of the occlusal or masticatory surfaces. Gingival tissues in CsA GO are noted to have a thickened marginal gingiva, a lobular appearance of the gingival pocket, and overgrowth of gingiva past the cemento-enamel junction. Additionally, the gingiva is reported to have an erythematous, soft, fragile appearance.\textsuperscript{28} Although similar to Phenytoin induced GO, CsA induced GO is more hyperemic and easily bleeds upon manipulation.\textsuperscript{28}
1.8 CsA GO: Histology

Histologic characteristics of CsA GO are similar to those of other drug induced GO. Initial histological studies note a marked difference in the epithelial and connective tissue layers. The epithelial layer consists of smooth, blunted papillary projections that are interdigitated with narrow crypts. The majority of the surface has a parakeratinized, squamous epithelium with elongated rete pegs.\(^\text{55}\) The stratum spinosum layer appears to be thicker than normal gingiva. Additionally, the overall mean volume of density of the oral epithelial cells is significantly higher in CsA GO (74.1\%) compared to no GO (54.8\%), \((p<0.001)\).\(^\text{56}\)

The connective tissue layer shows an increase in vascularity, and localized areas of inflammatory cell infiltrate (plasma cells).\(^\text{48,55,56}\) The overall connective tissue has a decrease in collagen volume and an increase in the volume of the non-collagenous matrix. The mean volume of collagen density within the connective tissue layer is significantly lower in CsA GO (66.2\%) compared to no GO (76.2\%), \((p<0.001)\); the mean volume of density of the extracellular matrix is significantly higher in CsA GO (14.4\%) compared to no GO (11.3\%), \((p<0.001)\).\(^\text{56}\) Additionally, the amount of cells and vessels is increased in the connective tissue layer.\(^\text{56}\) Most studies explain the clinical depiction of CsA GO as predominately connective tissue in nature, with an increase in the extracellular matrix, ground substance, plasma cells, and vasculature. However, there is contention regarding the volume of fibroblasts in CsA GO. Some studies note an increased number of fibroblasts with ovoid vesicular nuclei, whiles others report no such increase in fibroblast density.\(^\text{57}\)
1.9 **CsA GO: Risk Factors**

Several risk factors have been theorized to increase the presence and severity of GO with CsA treatment. These include drug dosage, concomitant medications, age, oral hygiene (presence of inflammation), and genetic polymorphisms. The therapeutic dosage of CsA has been studied in detail in relation to the presence of GO. It appears that a threshold concentration is needed for GO to occur, with higher dosages causing a greater severity of GO.\(^5^8\) A correlation may exist between serum CsA concentration and GO.\(^5^9,^6^0\) However, in certain individuals, GO is still seen at lower concentrations.\(^6^1\) The concomitant use of other medications has shown to both increase and decrease the incidence of GO. Calcium channel blockers are regularly used with CsA to offset immunosuppressant-induced hypertension. However, the combination of both medications can increase the risk of developing GO.\(^6^2,^6^3\) Alternatively, the concomitant use of azathioprine or prednisolone can decrease the presence or severity of GO, possibly due to the decrease in concentration of immunosuppressant needed.

Age has also been indicated as a risk factor for CsA GO. Studies have shown children and adolescents display a higher incidence of GO after CsA treatment.\(^6^4,^6^5\) Although the exact mechanism is unknown, it may relate to the overall healing ability and immune status of younger individuals.\(^6^6\) The role of plaque and inflammation in CsA GO has been of much debate. It has been noted by some authors that the incidence of higher plaque scores are associated with an increased exacerbation of CsA GO.\(^5^3,^6^0,^6^7\) These findings suggest that oral hygiene and maintenance are important in the formation of GO.\(^2^0,^2^4,^6^0\) Interestingly, higher concentrations of CsA are noted in gingival crevicular fluid and plaque than in blood samples from CsA treated patients.\(^6^8\) Accordingly, the
presence of plaque induced gingival inflammation may increase the bioavailability of CsA. Therefore, studies have attempted to show that plaque control can lower the severity of GO, but not fully eliminate the occurrence. Examination of the prevalence of putative periodontal pathogenic bacteria has shown *T. forsythia*, *T. denticola*, and *P. gingivalis* to be significantly increased in CsA GO. However, it is important to note that plaque accumulation may not necessarily cause GO, but may be a consequence of challenging hygiene from the overgrowth.

A genetic susceptibility to CsA GO has been theorized since the first reports of the condition were recorded. The heterogeneity of the gingival fibroblast has been noted in literature and has been hypothesized as the cause of GO. Alterations in fibroblast activity are thought to be directly related to fibroblast: sensitivity, proliferation, synthesis, and/or degradation. Previous studies have reported the role of human leukocyte antigens (HLA) phenotypes as a risk factor for CsA GO. HLA-DR2 and HLA-A19 are thought to be inductive of CsA-induced GO, while HLA-DR1 and HLA-B37 are thought to be protective. Most of these studies are underpowered, and after correcting for multiple significance, HLA-B37 appears to have a significant inverse correlation with GO.

Nevertheless, CsA GO is a variable condition with individuals being “responders” or “non-responders”. Moreover, the degree of response is variable with different degrees of severity. The genetic susceptibility to CsA GO appears to involve complex gene alterations and may be related to a multitude of factors; it does not appear to be a single gene interaction.
2. LITERATURE REVIEW

2.1 Proposed Mechanisms of CsA GO

The etiology and mechanism for CsA GO remains unknown, but prior studies have investigated different biologic pathways and cell signaling molecules thought to be involved. Studies have evaluated alterations in collagen and extracellular matrix breakdown, fibroblast proliferation and apoptosis, cytokine and chemokine levels, and growth factor imbalances. Deliliers et al. proposed the term “individual hypersensitivity” as a plausible explanation for patients exhibiting signs of CsA GO. Nevertheless, the exact mechanism has not been identified, as CsA GO appears to be a complex disorder.

2.1.1 Fibroblast Proliferation & Apoptosis

Collagen consists of multiple groups of fibrous structure proteins found in connective tissue and the extracellular matrix. Collagen is the most abundant protein in the human body. The collagen family of molecules are linked together to form a triple helix structure. There are at least 19 types of collagen that share this motif, but differ in high order structure and function. Collagen formation is influenced by many different molecules, including cytokines and growth factors. For proper function, a homeostasis exists between collagen synthesis and degradation; a disturbance in this equilibrium can lead to conditions with increased collagen production.

Studies have looked at the influence of CsA on collagen metabolism through examination of human gingival fibroblasts. The exposure of gingival fibroblasts to CsA showed a potential decrease in collagenase activity; however, this was true for only certain strains of fibroblasts. It was concluded a collagenolytic response to CsA
involved variability and susceptibility of gingival fibroblasts. Alternatively, another study reported a pro-stimulatory effect on collagen synthesis with fibroblast exposure to CsA.\textsuperscript{81} Cell culture studies on the effects of CsA on human gingival fibroblasts have been replaced with biopsy studies in recent years.

Nishikawa et al. and Kantari et al. studied the influence of cellular apoptosis on GO. Kantarci et al. noted a decrease in fibroblast apoptosis in gingival biopsies of GO.\textsuperscript{82} Apoptosis cellular markers caspase-3 and Forkhead box protein O1 (FOXO1) transcription factor were notably altered with all forms of GO. A decreased in caspase-3 (mediator of apoptosis in mammalian cells) and an increase in FOXO1 (transcription factor involved in cell cycle arrest) were related to decreased apoptosis of gingival fibroblasts and subsequent GO. Also, noted by Kantarci et al. was an increase in fibroblast proliferation.\textsuperscript{82} Therefore, GO was described as a proliferative phenomenon, with a greater decrease in apoptosis than proliferation of fibroblasts. Budubeli et al. also examined the role of cellular apoptosis and cell division with CsA GO in gingival tissues.\textsuperscript{83} Ki-67 antigen was used as a maker for cell proliferation and biotin-labeled 16-dUTP was used as a marker for apoptosis. It was noted that the level of apoptosis was greater than the level of cellular proliferation in GO. Similar studies have also used p53 (pro-apoptotic protein) and bcl-2 (anti-apoptotic protein) for assessment of CsA GO without statistical significance.\textsuperscript{84}

Histology and biopsy studies on CsA GO patients have reported varying results. A study by Dannewitz et al. reported an increase in type I collagen with CsA GO in renal patients.\textsuperscript{85} These results were confirmed with indirect immunofluorescence and quantitative polymerase chain reaction. The same author also compared the role of
fibroblasts and keratinocytes in CsA GO and noted an increase in mRNA expression of collagen type I in gingival fibroblasts only.\textsuperscript{85} Other studies have reported contrary findings; a study by McGaw et al. noted no differences in the density of fibroblasts or extracellular collagen when comparing CsA GO renal patients and health or inflamed gingival tissues.\textsuperscript{57}

Authors have also reported an increase in extracellular matrix proteins. Newell et al. reported that CsA stimulated glycosaminoglycan (GAG) production in the majority of gingival fibroblast cell lines and certain normal cell lines, indicating CsA as cell-strain dependent.\textsuperscript{86} Gnoatto et al. also found an increase in non-collagenous extracellular matrix proteins, heparin sulfate proteoglycans. mRNA transcription was increased in the CsA GO group for betaglycan, syndecan-2, syndecan-4.\textsuperscript{87}

2.1.2 \textbf{Matrix Metalloproteinases & Tissue Inhibitors of Matrix Metalloproteinases}

Matrix metalloproteinases (MMPs) are a category of enzymes responsible for the degradation of collagen and proteins of the extracellular matrix.\textsuperscript{88} Tissue inhibitors of matrix metalloproteinases (TIMPs) are a family of four protease inhibiting enzymes responsible for the regulation of MMP activity in different tissues.\textsuperscript{89} Disruption of the MMP-TIMP axis has been reported in conditions such as rheumatoid arthritis, atherosclerosis, tumor growth, metastasis, and fibrosis.\textsuperscript{90} The MMP-TIMP interaction has also been studied in CsA GO.

In a study by Dannewitz 2006 et al., MMP-1, MMP-10, and TIMP-1 had an increased gene expression of 1.9-fold, 2.3-fold, and 4.8-fold, respectively, in CsA GO patients compared to healthy gingiva sections.\textsuperscript{72} The increase in expression of these
MMPs indicate an increased breakdown in collagen and extracellular matrix proteins, but a higher expression increase in TIMP-1 represents a greater inhibition of MMPs leading to an overall accumulation of the extracellular matrix. *In-vitro* studies have also been conducted to understand the effects of CsA GO on MMPs and TIMPs. Yamada 2000 et al. cultured gingival fibroblasts with and without phenytoin or CsA treatment to study expression changes. The authors reported that both drugs inhibited expression of MMP-1, TIMP-1, and cathepsin L; however, cathepsin B was not significantly inhibited. Hyland 2003 et al. noted similar findings in an *in-vitro* study. Cultured gingival fibroblasts in CsA resulted in inhibition of MMP-1 in a time dependent manner, but no clear TIMP-1 inhibition. Both authors concluded that CsA GO may lead to an accumulation of tissue due to a decrease in collagen and extracellular turnover by MMPs. Sonmez et al. compared MMP-1 expression in CsA GO and CsA no GO patients. As the concentration and duration of CsA was increased in cell media, gingival fibroblasts demonstrated significantly decreased MMP-1 levels in both CsA GO and CsA NO GO groups. The conclusion was that CsA treatment did not significantly influence MMP-1 protein expression. Sukkar 2007 et al. studied the relationship between MMP-1 and TIMP-1, and pro-inflammatory cytokines (IL-1, IL-17, TGF-β, and oncostatin M) with CsA and nifedipine treatment. Fibroblasts from patients with CsA GO had significantly lower levels of MMP-1 (*p < 0.001*) and lower levels of TIMP-1 (*p < 0.05*) than control fibroblasts. Overall, the ratio of MMP-1 to TIMP-1 was reduced leading to greater accumulation of collagen and extracellular matrix proteins. No individual pro-inflammatory cytokine had a significant effect on MMP-1 or TIMP-1. However, the
combination of IL-1 and oncostatin M had a synergistic effect on increasing MMP-1, which was reduced by the addition of CsA.

2.1.3 Gene Polymorphisms

Genetic polymorphisms in the promoter regions of alleles result in altered transcriptional activity. These genetic variations may be mainly or partially responsible for the CsA GO phenotype. Polymorphisms have been noted for MMP-1, MMP-3, IL-6, SPARC (secreted protein, acidic, and rich in cysteine), and TGF-β. The majority of these studies are published by a single group of authors comparing patients on CsA with GO and without GO from a Polish population. The results show that the MMP-1 polymorphism (-1607 1G/2G) does not reach statistical significance ($p < 0.073$). The MMP-3 polymorphisms (-1171*A and -1171*dupA) also did not reach statistical significance ($p > 0.05$). A study on IL-6 gene polymorphisms (-174G/G, -174G/C and -174C/C) showed that the risk of GO was not statistically different between the genotypes. Similarly, no statistical difference was noted in the SPARC gene polymorphism (c.998C>G SNP) or TGF-β polymorphisms (+869T>C in codon 10, +915G>C in codon 25, -800G>A, and -509C>T). Overall, studies on individual genetic polymorphisms have proved inconclusive as the etiology for CsA GO.

2.2 Cytokines

Cytokines are wide-ranging group of protein molecules involved in cell signaling. This family of proteins is made up of chemokines, interleukins, interferons, and tumor necrosis factors. Cytokines are predominately studied in the field of immunology and
inflammation; although, multiple other functions have been noted in literature. Cytokine involvement has been noted in growth and differentiation, chemotaxis, and cell signaling. Many cells possess the ability to secrete or stimulate cytokine production, including macrophages, activated lymphocytes, epithelial, and endothelial cells. The function of cytokines appear to be pleotropic, affecting different cells with different responses. An important function of cytokines in relation to CsA GO is the ability to enhance chemotaxis for fibroblasts and the ability to encourage a fibrogenic response. The fibrogenic process is thought to be more dependent on the chemokine sub-family of cytokines.

2.2.1 Interleukins, TNFα, PDGF, TGF-β

Interleukins are immuno-regulatory proteins that exhibit a tissue specific effect with autocrine and paracrine signaling. They are responsible for cell activation, proliferation, and differentiation. Several studies have reported the upregulation of certain interleukins with CsA treatment. Williamson et al. reported that CsA treatment increased expression of IL-6 protein and IL-6 mRNA in CsA GO human tissues. A study by Chae et al. revealed CsA therapy increased IL-6 and TGF-β1, which was related to an increase in fibroblast proliferation. Further investigation also revealed that CsA induced ERK, p38 MAPK, and PI3K signaling pathway activation, potentially leading to gingival fibroblast proliferation. In another similar in-vitro study, the ability of CsA to upregulate IL-6 in response to the presence of bacteria, IL-1β, or tumor necrosis factor α was examined. The authors noted that gingival fibroblasts, stimulated with CsA, demonstrated increased expression of IL-6. Moreover, IL-6 expression had further
upregulation when CsA and TGF-β were combined. In contrast, a study by Atilla et al. found that CsA increases IL-6 and IL-1β, in gingival crevicular fluid, with the presence of inflammation. This effect was similar in patients with CsA GO and CsA no GO.\textsuperscript{105} Therefore, it is plausible that CsA may not have a direct effect on IL-6 and IL-1β, but the subsequent inflammation and/or overgrowth associated with CsA may be the predominant factor.\textsuperscript{105}

Suzuki et al. assessed the effects of CsA on CD54 and toll-like receptors (TLR) in response to stimulatory TLR ligands (lipid A and Pam\textsubscript{3}CSK\textsubscript{4}).\textsuperscript{106} CsA increased CD54 expression in gingival fibroblasts stimulated with lipid A or Pam\textsubscript{3}CSK\textsubscript{4}. Additionally, CsA increased the production of IL-6 and IL-8 induced by Pam\textsubscript{3}CSK\textsubscript{4}, and IL-8 induced by lipid A. In general, CsA stimulated gingival fibroblasts in combination with TLR ligands, but not alone.\textsuperscript{106}

Platelet-derived growth factor (PDGF) is a protein that influences angiogenesis to control cell growth and division. PDGF-B is important in fibroblast migration and proliferation.\textsuperscript{107} The role of PGDF-B in CsA GO has been observed by some authors. Studies show that CsA GO upregulates the expression of PDGF-B, which is predominately secreted by macrophages; this phenomenon is not seen in normal control gingiva.\textsuperscript{108,109}

Transforming growth factor beta (TGF-β) is a cytokine group of proteins with many tissue specific functions: proliferation, regulation, differentiation, and cell cycle events. Specifically, TGF-β1 and Smad7 have been associated with CsA GO. TGF-β1 is one of the most studied proteins based on its role in wound healing and inflammation. Studies have shown increased TGF-β1 levels in fibroblasts treated with CsA.\textsuperscript{5,110} Increased
levels of TGF-β1 have been noted in fibrotic diseases of certain tissues. Smad7 is a protein that antagonizes TGF-β type 1 receptor signaling, inhibiting TGF-β1 function. Sobral et al. details how Smad7 overexpressing gingival fibroblasts effectively block synthesis and production of type I collagen and MMP-2. Hence, variations in individual TGF-β proteins may be responsible for CsA GO presence and severity.

### 2.3 Chemokines (CC, CXC, XC, CX3C)

Chemokines were first discovered over four decades ago in the field of inflammation. The initial discovery was related to scientists’ curiosity to understand leukocyte migration into other tissues. It was shown that certain molecular attractants, such as interleukin 8 (IL-8), were fundamental to the process of leukocyte migration and diapedesis. It was noted that molecules similar to IL-8, the chemokines, were important in inflammation and immunology. The process by which leucocytes are attracted to a site for a host-generated response is controlled in part by chemokines. Chemokines are a sub-category of cytokines, which are also involved in host-response and immunology. The multifunctional role of chemokines has led to an increased interest and focus on the topic. More than 50 human chemokines have been identified by genomic methods.

Chemokines are formed from four cysteines molecules creating two di-sulphide bonds. Chemokines exist in two predominate forms, CXC and CC, and can be identified based on the position of the first two cysteines amino acids. If the two cysteines are adjacent to each other, then the chemokines are categorized as CC; if there is another amino in between the cysteines, then the chemokines are categorized as CXC. This group has 17 different mammalian chemokines reported. CXC chemokines are further
divided into two categories dependent upon the existence of the amino acid sequence ELR (glutamic acid, leucine, arginine). Those with an ELR motif have functions such as signaling neutrophils to enter the surrounding tissues from the blood stream.\textsuperscript{113} Those without the ELR motif function as a chemoattractant for lymphocytes. The CC chemokine subfamily has 27 different mammalian chemokines reported. Their role is to signal for migration of monocytes, natural killer cells, and dendritic cells.\textsuperscript{114} The other 2 lesser-known subfamilies are the C chemokines and the CX\textsubscript{3}C chemokines. The C chemokines are made up of only two cysteines. They function as chemoattractants to lymphocytes and natural killer cells. The CX\textsubscript{3}C chemokine proves to be an integral membrane protein that is often referred to as fractalkine. It commonly functions as a chemoattractant and an adhesion protein.\textsuperscript{113}

2.3.1 Chemokine Receptors

Similar in nomenclature, the receptors for the chemokine ligands were identified as “chemokine receptors”.\textsuperscript{115} In humans, it was determined that chemokine receptors bind to multiple chemokines, and that individual chemokines may bind to multiple receptors. The redundancy of chemokine ligand-receptor interactions may represent an evolutionary mechanism to offset loss of function of the chemokine ligand and/or receptor. However, all chemokine receptors are members of the G-protein coupled receptor family.\textsuperscript{116} The mechanism by which these receptors function is still not fully understood, but cAMP inhibition and signal transduction are involved. Similar to the chemokine ligands’ four subcategories, chemokine receptors have two subcategories based on amino acid location and function. The chemokine receptors exist as CC and
CXC receptors; accordingly, the ligands have to have identical amino acid structures to properly bind.

Throughout the human evolutionary process, the number of functional human chemokines ligands has increased. Interestingly, co-evolution of chemokine receptors occurred to allow these ligands to have appropriate bindings sites.\textsuperscript{117} Goh et al. investigated the concept of gene linkage within the chemokine complex and found that chemokines with shared sequences or in close proximity to other chemokines on the same chromosome were more likely to bind to related receptors.\textsuperscript{117} Therefore, CC chemokine ligands will only bind to CC chemokine receptors, with CXCR following the same pattern.

2.3.2 Chemokine Ligand-Receptor Axis (e.g. CXCL8/CXCR1)

One of the first discovered and most studied of the chemokines is interleukin 8 (IL-8, CXCL8). Its founding was related to its involvement in leukocyte migration and inflammatory response in individuals with certain diseases.\textsuperscript{118} As described previously, the redundancy of chemokine ligand-receptor interactions may represent an evolutionary mechanism to offset loss of function of the chemokine ligand and/or receptor. IL-8 and its receptor IL-8RA (CXCR1) are prime examples of ligand and receptor promiscuity.\textsuperscript{119} Promiscuity represents the ability for ligands to have more than one binding receptor, and the ability for receptors to have more than one ligand interactions. For the most part, in humans, the majority of receptor promiscuity is seen in neurotransmitters.\textsuperscript{120} This biologic advantage is due to the importance of neurotransmitters in normal function of healthy individuals. There are few other examples of receptor promiscuity; however, IL-
8RA (CXCR1) has shown a similar phenomenon. The IL-8 receptor exists (or used to exist) in three predominate forms, IL-8RA, IL-8RB, and IL-8RAP. However, recent studies have shown that the predominate chemokine receptors only exist in the IL-8RA (CXCR1) and IL-8RB (CXCR2) forms. The human genome has increased the amount of chemokine ligands, but reduced the amount of variable receptors available to bind, which leads to receptor promiscuity.

<table>
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<tr>
<th>Ligand</th>
<th>CXCL family</th>
<th>Corresponding CXCR</th>
<th>Ligand</th>
<th>CCL family</th>
<th>Corresponding CCR</th>
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<tr>
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Figure 1. Chemokine ligands and corresponding receptors. Note the greater amount of ligands than receptors found in humans is a function of receptor promiscuity. (Adapted from Harrison et al.)

2.3.3 **Functions of Chemokines**

In humans, chemokines have been noted to function in many biological processes, as well as diseases. Figure 2 represents a few of the processes that past studies of chemokines have revealed. The inflammatory and immunologic pathways have been some of the most extensively studied fields related to chemokines. Chemokines have also been shown to have varying degrees of tissue specificity, with
some chemokines present in many cells and other chemokines limited to certain tissues. Chemokines are responsible for leukocyte modulation, neutrophil migration to areas of inflammation, and granule exocytosis during certain stages of inflammation. During the inflammatory process, many cellular components up-regulate the production of the certain chemokines. These components include IL-1, TNF-α, and LPS. Another biologic function of chemokines is the regulation of hematopoiesis. Chemokines regulate the proliferation and differentiation of stem and progenitor cells through alterations of migration patterns into bone.

Figure 2. Biologic processes influenced by chemokines. The main focus of the diagram is wound healing; however, more research on chemokines has broadened the scope of interactions into many other biological processes such as fibrosis. (Figure modified from Rossi et al.)

2.3.4 Chemokines in Disease

The role of chemokines in disease is diverse, reflecting the multiple biologic pathways effected by chemokines in normal human physiology. Debate exists onto whether chemokines are causative in disease, or whether they are downstream mediators. However, since most chemokine pathways are autocrine, even down-stream
effects can signal an increase in chemokines up-stream in the pathway.\textsuperscript{125} It is proposed that the redundancy of chemokine ligands offers more protection from mutations and variations in certain chemokines. Nevertheless, not all chemokines have redundancy. The majority of diseases associated with chemokines are due to dis-regulation of inflammation and the related host response. Since most cellular processes can induce chemokines, a major shift in control can cause disruptions of the immune response.\textsuperscript{125}

Autoimmune diseases such as rheumatoid arthritis and multiple sclerosis have established chemokine involvement. Many CC chemokines are notably increased in both of these conditions, and significant amounts of CCL-2, CCL-7, and CCL-8 have been found in brain biopsies of multiple sclerosis patients.\textsuperscript{126} New pharmacologic management approaches have attempted to alter CC chemokine binding in order to reverse or postpone the symptoms of multiple sclerosis.\textsuperscript{126}

In mice studies, knockout mutations of CCR-2, CCR-5, and CXCR-3 have increased the survival of organ grafts.\textsuperscript{127} Mutations in CCR-5 tripled the survival time and mutations in CXCR-3 added an additional 50 days. Moreover, mutations in these chemokine receptors decreased the amount of immunosuppressant (e.g. CsA) medication needed post-operatively.

Chemokines also promote vascular proliferation that may assist neoplastic growth. Chemokines may directly alter certain tumor growth factors, as reported in non-small cell lung cancers.\textsuperscript{128} Moreover, tumor cells are also noted to increase autocrine production of chemokine and chemokine receptors. The proposed theory is that an increase in chemokine ligand and receptor directly produced by tumor cells leads to malignant transformation and metastasis.\textsuperscript{129}
2.4 **Fibrosis**

Fibrosis is characterized by the overgrowth, hardening, and/or scarring of various tissues. It results in the excess deposition of extracellular matrix components (e.g. collagen).\(^{101}\) Fibrosis may be the end product of chronic inflammatory reactions, such as infections, autoimmune disorders, allergic reactions, chemical/radiation injury, and tissue wounding. Research indicates that mechanisms driving fibrogenic responses may be distinct from those of inflammation.\(^{101}\) The myofibroblast, when activated, is the primary mediator of fibrosis through production of collagen. Myofibroblasts are derived from different sources: resident mesenchymal cells, epithelial and endothelial cells within the epithelial/endothelial-mesenchymal (EMT) transition, circulating fibrocytes and pericytes.\(^{130,131}\) Drug-induced and certain genetic forms of gingival overgrowth have fibrotic characteristics according to clinical and histological appearances.\(^7,8,28,132\) In response to injury, wound healing results in fibrosis through distinct processes: angiogenesis, migration/proliferation of fibroblasts, deposition of extracellular matrix, and remodeling.\(^4\) Cytokines, chemokines, growth factors, (VEGF, PDGF), caspases, and many other cell signaling regulators have been considered modulators of fibrosis. Chemokines act as chemoattractants for immune signaling, but are also noted in fibroblast activation.\(^{101}\)
2.4.1 Chemokines & Fibrosis

Chemokines are also intimately involved in fibrosis. Fibrosis is related to the end stages of inflammation and occurs in both normal physiology and pathology. In the early stages of wound healing prior to fibrosis, chemokines are responsible for recruiting macrophages, plasma cells and T lymphocytes. These cells promote a pro-fibrotic response through direct and indirect interaction with chemokines. Sahin et al. investigated chemokines and tissue fibrosis and provided a basic understanding of the mechanism of fibrosis mediated by chemokines. Chemokines are considered to be responsible for fibroblast activation and angiogenesis, which are key factors in fibrosis of many different organs. A commonality seen throughout chemokine-mediated fibrosis is the up-regulation of CCL2, CCL3, and CCL5 in most types of fibrosis. Other chemokines involved in fibrosis appear to be differentially expressed in a tissue specific manner. The up-regulation of CCL2, CCL3, CCL5 may indicate a common biologic pathway responsible for fibrosis.

In liver tissues, chemokines of the CC and CXC families have been implicated in fibrotic diseases. Resident hepatic cells express chemokines, which are responsible for leukocyte chemotaxis, subsequent production of inflammatory mediators, and ultimately fibrosis. In murine liver fibrosis, CCL3 and CCL5 along with their receptors CCR1 and CCR5, respectively, attenuate liver fibrosis. Absence of the CCL3 and CCL5 ligand leads to a reduction in hepatic fibrosis due to stellate cell activation and immune cell infiltration. CCL2 and CCR2 are also involved in hepatic fibrosis. CCR2 deficiency in mice has been shown to reduce infiltration of inflammatory macrophages and pro-fibrotic effects of hepatic stellate cells. Appropriately stimulated hepatic stellate cells (HSCs)
can produce extracellular matrix proteins and are responsible for liver fibrosis. HSCs are considered pericytes, pre-cursors to contractile cells throughout endothelial cells, which respond to stimulation by differentiation into collagen-producing cells. HSCs express many chemokines, such as CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, and CXCL12.

Pulmonary fibrosis is related to pro-inflammatory cells (monocytes, neutrophils, mast cells and leukocytes) and also by alveolar epithelial cells. Similar to liver chemokines, CCL2 and CCR2 are involved in pulmonary fibrosis. Human studies have shown increased expression of protein and mRNA of CCL2 in lung epithelial cells from subjects with idiopathic pulmonary fibrosis, and higher levels of CCL2, CCL3, and CCL4 in bronchoalveolar lavage fluid. Alveolar epithelial cells express CCL2, CCL3, CCL5, CCL11, CXCL2, CXCL5, and CXCL12.

Renal fibrosis is a pathologic process involving tubular epithelial cells, vascular endothelial cells, and fibroblasts. With the initiation of the fibrotic pathway, renal fibroblasts deposit extracellular matrix proteins responsible for end-stage renal disease. The chemokines known to be involved in renal fibrosis are CCL2, CCL5, and CXCL10.

Fibrosis of the skin (e.g. scleroderma) is a process that involves inflammation, angiogenesis, and fibrosis. Leukocytes, endothelial cells, and components of the epithelial mesenchymal transition are thought to be involved in the process of dermal fibrosis. A number of chemokines are reported to be etiologic in the process: CCL2, CCL3, CCL5, CCL7, CCL17, CCL22, CCL27, CXCL1, CXCL8, and CXCL16.
Chemokines are involved in multiple, diverse pathways and physiological processes, including inflammatory cell migration; fibroblast and endothelial cell activation, as well as production and activation of TGF-β. Chemokines enable angiogenesis to occur via endothelial cells; subsequent events allow for the influx of fibroblasts and inflammatory cells needed for fibrosis to occur.¹³⁸

2.5 Epithelial-Mesenchymal Transition

The epithelial-mesenchymal transition (EMT) is a tissue specific, highly regulated, complex mechanism that involves cell-cell and cell-extracellular matrix interactions.¹⁴⁸ EMT allows for the release of epithelial cells and reorganization of the cytoskeleton; additionally, transcriptional regulation may be altered. The EMT pathway is important in the progression of many epithelial tumors.¹⁴⁹ Chronic inflammation or conditions that encourage tissue disruption may stimulate fibrosis, including EMT, which disrupts tissue integrity and function.¹⁴⁸ Sume et al. reported that epithelial cells undergoing EMT can alter the basement membrane and allow for pro-fibrotic signaling markers to promote gingival overgrowth.¹² In addition to the disruption of the basement membrane, the EMT appears to promote discontinuous collagen type IV expression and decreased laminin-5.¹⁵⁰ Overall, the EMT is reported to be a mechanism through which interactions between the epithelium and connective tissue can promote a fibrotic pathology.¹⁵¹
2.6 **MAPK Signaling**

Mitogen-activated protein (MAP) kinases are responsible for signal transduction pathways controlling many intracellular events. MAP kinases are a component of other kinase cascades and biologic process. Through signaling cascades, MAP kinases regulate many different biological events such as proliferation, differentiation, and apoptosis. The Son of Sevenless-1 (SOS-1) gene is located within the MAP kinase signaling cascade. SOS-1 gene mutations are known to cause hereditary gingival fibromatosis through constant activation of MAP kinase signaling, which drives nuclear events that result in cell proliferation and differentiation.\(^7\) CsA GO has also been indicated as a consequence of gingival fibroblast proliferation, possibly through MAP kinase activity (p38 MAPK and PI3K).\(^5\)

2.7 **Toll-Like Receptors**

Toll-like receptors (TLR) are proteins that are crucial for the innate immune system.\(^{152}\) TLRs are expressed on the membranes of leukocytes (macrophages, dendritic cells, natural killer cells), lymphocytes (T and B), and non-immune cells (epithelial cells, endothelial cells, fibroblasts).\(^{152}\) Specific ligands bind to TLRs to initiate an immune response, such as lipopolysaccharide (LPS), peptidoglycan, lipoproteins, and bacterial RNA and DNA. In periodontal disease, oral pathogens stimulate the TLRs through binding of bacterial byproducts. This initiates the destructive process caused by signal transduction and downstream events. The binding of LPS from gram-negative bacteria to TLR stimulates protein kinases that amplify signals for inflammatory mediators (e.g. cytokines, chemokines) or transcription factors.\(^{152}\) Similarly to chronic
periodontitis, in CsA GO TLR4 is noted to be upregulated and expressed at higher levels than healthy gingiva. Furthermore, TLR4 expression is localized mainly to the epithelium—connective tissue border area, near the basal cell layer.\textsuperscript{153} Recent studies have shown that CsA inhibition of calcineurin may cause a decrease in TLR signaling in mouse macrophages.\textsuperscript{154}

TLR modulation by CsA, through its interaction with calcineurin, may initiate a signaling cascade leading to increased cytokine and chemokine production.\textsuperscript{154} Such autocrine mediators are known to contribute to fibrosis either directly or indirectly through inflammatory processes. This complex mechanism involves multiple biologic pathways and may be etiologic for CsA GO in susceptible individuals.
3. HYPOTHESES & OBJECTIVE

3.1 Hypotheses

CsA acts as an immunosuppressant through reduction of T cell activity, and studies have reported differential expression of numerous genes along its therapeutic pathway.\textsuperscript{155,156} Transcriptional regulation results in the intended and unintended effects of cyclosporine therapy.\textsuperscript{46} Certain individuals on CsA therapy experience side effects, such as, gingival overgrowth, nephrotoxicity, and epithelial cancers. These side effects are seen in a minority of patients and are believed to occur as the result of altered gene expression due to CsA acting on a genetically susceptible individual. CsA is reported to be a pro-fibrotic agent in the fibrosis of the lung, liver, kidney, and skin.\textsuperscript{44,45} Moreover, studies have established a role for the chemokine ligand-receptor axis in the etiology of fibrosis.\textsuperscript{100,125,134,137,157} The complexity and redundancy of the chemokine ligand-receptor interaction leads to a tissue specific response. CsA may have a similar pro-fibrotic role in CsA associated GO.

We hypothesize that CsA therapy induces differential gene expression of the chemokine ligand-receptor axis, which is etiologic in formation of CsA GO in susceptible individuals. The objective of this study is to analyze differentially expressed chemokine ligands and receptors in CsA GO and non-GO tissues.
4. MATERIALS & METHODS

4.1 Subjects

The protocol of the study was reviewed and approved by the institutional ethics committee. Informed consent was acquired from the subjects according to the Helsinki Declaration for sample tissue collection. From a previous study group, n = 125, 14 CsA therapy patients were selected. Of the 14 CsA therapy patients, all were renal transplant subjects, ages 23-43 years old, who were seen at Ege University School of Dentistry, Department of Periodontology. All 14 patients were treated with CsA for at least 6 months and had no relevant pre-existing medical or drug histories. The CsA serum concentration was individualized per patient for a therapeutic effect and was maintained between 80-300ng/ml. Some CsA therapy patients also were medicated with azathioprine and prednisolone in accordance with transplantation protocols. Patients concurrently taking other medications reported in literature to cause GO were excluded and not selected for this study. Each patient received a dental cleaning every 2 weeks to ensure the gingival tissues were free from plaque-induced inflammation.

Clinical parameters of this study population have been described in detail previously:

“Assessment of clinical periodontal parameters was made of the full dentition, excluding third molars. All subjects had a clinical periodontal examination including the measurement of periodontal probing depth using a Williams probe. Dichotomous measurement of supragingival plaque
accumulation and bleeding on probing and hyperplastic index scores were also recorded. The degree of gingival overgrowth was classified into four categories based on the criteria of Angelopoulos and Goaz, modified by Pernu et al. The patients were dichotomized into a gingival overgrowth-negative (GO-) group, those with no signs of gingival overgrowth (score 0), and a gingival overgrowth positive (GO+) group, those with signs of overgrowth (scores 1-3) for analysis.

Radiographic examination was completed to identify the presence of proximal alveolar bone loss (measured from the cemento-enamel junction to the crest of bone at >95% of sites with ≤ 3mm) using panoramic films. Patients with no radiographic bone loss were included in this study.

4.2 Gene Expression Studies

4.2.1 Tissue Collection

Gingival tissue samples were obtained from maxillary anterior interdental areas exhibiting a characteristic appearance of GO through a gingivectomy procedure (CsA GO, n = 11). The remaining three subjects on CsA therapy without GO had biopsy samples collected in a similar manner from the anterior interdental areas (CsA no GO, n = 3). Additionally, normal gingival tissues were obtained from healthy individuals (Normal
Gingiva, n = 3) during routine crown lengthening surgery. The collected tissues were excised as an objective of customary periodontal surgery, and retained in which the tissues would have been normally discarded. The samples were stored and stabilized in RNAlater® to analyze the RNA for gene expression and profiling.

4.2.2 **Isolation of RNA**

TRIzol solution was used for total RNA extraction from gingival tissue samples. Electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was completed to assess and verify RNA quality by the presence of robust 18S and 28S peaks.

4.2.3 **Microarray Preparation**

Gene expression from gingival tissue samples was investigated using CodeLink™ Whole Human Genome Gene arrays (Applied Microarrays, Inc. Temple, AZ). This particular microarray provides expression profiling of approximately 57,000 human transcripts. The microarrays are constructed on UniGene, RefSeq and Ensembl public databases. The reported sensitivity of detection is as low as 1: 2,000,000 mass ratio (< 0.3 copies/cell), with predictable reproducibility.¹⁶¹
4.2.4 **Data Analysis**

GenePix Series B Scanner (Axon Instruments, Union City, CA) was utilized for recognition of probes within the microarray. Probe quantitation was accomplished through CodeLink™ Expression Analysis v5.0. The microarrays were individually scaled to express intensity, and normalized to the mean for statistical examination.

4.2.5 **Gene Ontology and KEGG Pathway Analysis**

GeneSifter software (Geospiza, Seattle, WA) was employed to quantify and compare expression of normalized values; certain genes exhibiting significant upregulation and down regulation were selected for further review. Kyoto Encyclopedia of Genes and Genomics (KEGG) pathways\(^{162}\) and ontological pathways\(^{163}\) were examined. Specific genes were selected based on previously reported CsA GO literature and statistical analysis showing a significant critical value \(p < 0.05\).

4.3 **Histological Analysis**

Tissue samples from two patients, under CsA treatment with GO, were selected for histological evaluation. Tissue samples were fixed for 3 days in neutral buffered formalin. The specimens were then prepared by routine methods and embedded in paraffin. Serial sections (5μm) were taken and mounted on slides for haematoxylin and eosin staining. The samples were viewed by light microscopy with magnification up to 50X.
5. RESULTS

5.1 **Subjects**

Gingival biopsy samples were acquired from 14 patients on CsA treatment and 3 control subjects not on CsA or other GO causing medications. The recorded hyperplastic index indicated, 3 CsA treatment patients did not show clinical signs of GO, while 11 patients did have a GO presentation.

5.2 **Microarray Analysis**

Codelink™ Human Whole Genome microarrays evaluated the expression of >54,000 probes. Mean-normalized expression data was evaluated using GeneSifter software. Differentially expressed genes were selected for further evaluation through the use of KEGG and STRING to provide potential biologic pathways and mechanisms.

5.2.1 **Selection of Differentially Expressed Genes in CsA GO**

Genes previously reported in the literature to be associated with CsA GO were selected for evaluation. Expression data for particular genes within biologic pathways and mechanisms reported to be involved in GO were also investigated. Additionally,
chemokines reported to be involved in fibrosis of other tissues were analyzed, along with all other chemokines available in the probe set.

5.2.2 Statistical Testing

Analysis of these microarrays may not fit the assumption of a standard distribution required by parametric testing (e.g. Student’s t-test). Additionally, outliers, data noise, or a low sample population may influence the use of parametric testing. Non-parametric tests do not have such assumptions about distribution of the data and will be more useful when a non-normal distribution of data exists. A study by Troyanskaya et al. examined non-parametric tests for identifying differentially expressed genes in microarrays. Non-parametric tests were considered convenient and robust methods for examining differentially expressed genes for biological or clinical analysis. For the purposes of this paper, non-parametric testing was completed for the aforementioned reasons.

5.3 Chemokines

The three test groups, normal gingiva control (no CsA treatment), Cyclosporine treatment without overgrowth (CsA NO GO), and Cyclosporine treatment with gingival overgrowth (CsA GO) were analyzed in order to identify differences in genetic expression. A search of chemokine ligand and chemokine receptor genes using the Genesifter software identified 65 genes for further review. After removal of duplicates due to probe ascertainment, a total of 57 genes remained for investigation. A cursory review of expression profiles based on quantitative expression (heat mapping) revealed
relatively more upregulation of chemokines than downregulation in the CsA GO group (appendix A).

Statistical testing, Kruskal-Wallis, was completed to indicate statistically significant differences (p <0.5) in gene expression for chemokine ligands and chemokines receptors. Expression of 25 chemokine ligands and receptors were noted to be significantly altered, with the majority demonstrating upregulation in expression (figure 3).

Figure 3. Heat map of chemokine expression. Illustration of chemokines significantly upregulated between the three groups. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
5.4 **Chemokines Involved in Fibrosis**

Certain chemokines (CCL2, CCL5, and CXCL8) have been noted in literature to be consistently upregulated in tissue fibrosis of the kidney, liver, lung, and skin. These chemokines as well as others reported to be pro-fibrotic are displayed in table 1. As noted previously, CsA appears to increase expression of particular chemokines in all subjects receiving CsA therapy. However, other specific chemokines are only significantly upregulated in individuals demonstrating CsA GO. CsA treatment is associated with significant upregulation of CXCL2 and CXCL8 in all patients. In contrast, individuals with CsA GO show significantly more expression of CCL2, CCL3, CCL5, CXCL1, CXCL2, and CXCL8 than in controls. CXCL16, a mediator of liver fibrosis is significantly upregulated in the CsA GO group.
Table I
Relative Expression of Potential Fibrogenic Chemokine Ligands

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>CsA NO GO</th>
<th>CsA GO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expression</td>
<td>Fold Δ</td>
<td>Expression</td>
</tr>
<tr>
<td>CCL2</td>
<td>0.510±0.150</td>
<td>1</td>
<td>0.936±0.150</td>
</tr>
<tr>
<td>CCL3</td>
<td>0.152±0.025</td>
<td>1</td>
<td>0.213±0.004</td>
</tr>
<tr>
<td>CCL5</td>
<td>0.347±0.045</td>
<td>1</td>
<td>0.437±0.077</td>
</tr>
<tr>
<td>CCL7</td>
<td>0.152±0.006</td>
<td>1</td>
<td>0.162±0.028</td>
</tr>
<tr>
<td>CCL11</td>
<td>0.061±0.005</td>
<td>1</td>
<td>0.054±0.014</td>
</tr>
<tr>
<td>CXCL1</td>
<td>1.369±0.194</td>
<td>1</td>
<td>1.501±0.043</td>
</tr>
<tr>
<td>CXCL2</td>
<td>0.098±0.016</td>
<td>1</td>
<td><strong>0.909±0.183</strong> *</td>
</tr>
<tr>
<td>CXCL4</td>
<td>0.031±0.010</td>
<td>1</td>
<td>0.149±0.005</td>
</tr>
<tr>
<td>CXCL5</td>
<td>0.026±0.009</td>
<td>1</td>
<td>0.015±0.005</td>
</tr>
<tr>
<td>CXCL8</td>
<td>0.165±0.028</td>
<td>1</td>
<td><strong>6.259±1.709</strong> *</td>
</tr>
<tr>
<td>CXCL10</td>
<td>1.108±0.075</td>
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<td>0.583±0.095</td>
</tr>
<tr>
<td>CXCL12</td>
<td>1.409±0.505</td>
<td>1</td>
<td>1.196±0.293</td>
</tr>
<tr>
<td>CXCL16</td>
<td>0.986±0.029</td>
<td>1</td>
<td>1.206±0.064</td>
</tr>
</tbody>
</table>

Table I. Relative expression of chemokine ligands reported in literature to be involved in fibrosis. Results from Cyclosporine treatment patients without gingival overgrowth (CsA no GO) and Cyclosporine treatment patients with gingival overgrowth (CsA GO). *P< 0.05.

Figure 4. Differential gene expression of CCL2.
CCL2 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CCL2. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Figure 5. Differential gene expression of CCL3.
CCL3 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CCL3.
CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Figure 6. Differential expression of CCL5.
CCL5 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CCL5.
CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Figure 7. Differential expression of CXCL1.
CXCL1 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CXCL1. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Figure 8. Differential expression of CXCL2.
CXCL2 is significantly upregulated in all subjects receiving CsA treatment (CsA NO GO and CsA GO).
Intensity= mean-normalized quantitative expression of CXCL2. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Figure 9. Differential expression of CXCL8 (Interleukin 8, IL8).
CXCL8 is significantly upregulated in all subjects receiving CsA treatment (CsA NO GO and CsA GO).
Intensity= mean-normalized quantitative expression of CXCL8. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Figure 10. Differential expression of CXCL16.
CXCL16 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CXCL16. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
5.5 Chemokine Receptors Involved in Fibrosis

Previous studies have reported both the CC family and CXC family of chemokine receptors to be involved in fibrosis. CsA is reported to alter the chemokine ligand-receptor axis. Investigation of potential pro-fibrotic chemokine receptor expression reveals significant upregulation (p<0.05) of CXCR1 (IL8RA) and CXCR4 reported in table II.

Table II
Relative Expression of Chemokine Receptors

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>CsA NO GO</th>
<th>CsA GO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expression</td>
<td>Fold Δ</td>
<td>Expression</td>
</tr>
<tr>
<td>CXCR1</td>
<td>0.065±0.029</td>
<td>1</td>
<td>0.071±0.014</td>
</tr>
<tr>
<td>CXCR4</td>
<td>0.199±0.017</td>
<td>1</td>
<td>0.491±0.286</td>
</tr>
<tr>
<td>CXCR6</td>
<td>0.280±0.035</td>
<td>1</td>
<td>0.259±0.024</td>
</tr>
</tbody>
</table>

Table II. Relative expression of chemokine receptors reported in literature to be involved in fibrosis. Results from Cyclosporine treatment patients without gingival overgrowth (CsA NO GO) and patients with gingival overgrowth (CsA GO). *P< 0.05.

Figure 11. Differential expression of CXCR1 (Interleukin 8 receptor alpha, IL8RA). CXCR1 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of CXCR1. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Figure 12. Differential expression of CXCR4.
CXCR4 is significantly upregulated in CsA GO. Intensity = mean-normalized quantitative expression of CXCR4. CsA NO GO = Cyclosporine without gingival overgrowth, CsA GO = Cyclosporine with gingival overgrowth.

Figure 13. Differential expression of CXCR6.
CXCR6 is significantly upregulated in CsA GO. Intensity = mean-normalized quantitative expression of CXCR6. CsA NO GO = Cyclosporine without gingival overgrowth, CsA GO = Cyclosporine with gingival overgrowth.
5.6 **Chemokine Ligand-Receptor Axis**

The human genome has increased the amount of chemokine ligands, but reduced the amount of variable receptors available to bind, which leads to receptor promiscuity. CsA modifies the chemokine ligand-receptor axis by altering gene expression in GO. Figure 14 displays the ligands and receptors significantly upregulated by CsA in patients with GO. Interestingly, the CXCR1 (IL8RA) receptor gene and its 2 ligands, CXCL6 and CXCL8 (IL8) are upregulated. Other chemokine ligand-receptors that are upregulated along their axis are CCL20/CCR6, CXCL16/CXCR6, and XCL1/XCR1.
Figure 14. Chemokine ligands and receptors in Cyclosporine induced gingival overgrowth (CsA GO).

- = significant difference in gene expression. P<0.5.
- = ligand-receptor axis upregulation.
5.7 **Histological Analysis**

Tissue samples from two patients, under CsA treatment with GO, were selected for histological evaluation with haematoxylin and eosin (H&E) staining. The samples were viewed by light microscopy with magnification up to 50X.

Figure 15a,b. H&E staining of Cyclosporine induced gingival overgrowth (CsA GO) biopsies.  

a (left). Parakeratinized, stratified epithelium with dyskeratosis is noted with parabasal and basal region disruption with slight hyperchromatism. Presence of inflammatory histiocyte and lymphocyte infiltration. Submucosa with a poorly defined epithelial basement membrane is noted. Extensive venule and arteriolar angiogenesis with lymphocytosis and inflammatory histiocytes, lymphocytes (some B lymphocytes), and few eosinophils.  

b (right). Parakeratinized stratified squamous epithelium with dyskeratosis is seen with extension of rete pegs with epithelial hyperplasia. Chronic inflammatory infiltration with localized perivascular lymphocytosis—lymphocytes (B and T lymphocytes) and histiocytes, a few eosinophils and mast cells—is present in loose connective tissue with extracellular matrix venules and arteriolar angiogenesis.
6. DISCUSSION

CsA induced gingival overgrowth is a complex disorder involving a combination of biologic mechanisms. The formation of GO is an end-product of fibrosis, which involves the activation of myofibroblasts to increase the connective tissue matrix and volume. The signaling cascade that leads to this activation may involve the interaction of membrane bound TLRs, EMT events, inflammatory mediators (e.g. chemokines), and other cellular events. The results from our study indicate that CsA influences the expression of many chemokines, as well as cytokines, TLRs, and growth factors. The focus of our study was to examine the role of CsA on chemokines, particularly those that are reported to be profibrotic.

6.1 Chemokines

Out of the 57 chemokine ligand and receptor genes available in the arrays, 25 were noted to be significantly increased in CsA GO patients. The majority of the chemokines showed increased expressions patterns. Our understanding of chemokines has substantially increased since their discovery two decades ago in the field of inflammation. Although chemokines are central to inflammation and immunology, other functions such as angiogenesis and fibrosis have been reported. The multifunctional role of chemokines has led to an increased interest and focus on the topic.
Chemokines are potential mediators of tissue fibrosis. Fibrosis can be a part of normal physiology or pathology; it is described as the end product of inflammation. In wound healing, chemokines function to recruit macrophages, plasma cells and T lymphocytes to the site of injury.\textsuperscript{133} These cells can promote a pro-fibrotic response through the EMT or secretion of chemokines in an autocrine manner. Chemokines have the ability to activate/recruit fibroblasts and promote angiogenesis, which are crucial for tissue fibrosis.\textsuperscript{100} CCL2, CCL3, and CCL5 are noted to be upregulated in most forms of tissue fibrosis.\textsuperscript{100} The results of our investigation show significant upregulation of these three chemokines as well, indicating the chemokine-mediated fibrosis pathway of gingival overgrowth is similar to tissue fibrosis of the liver, lung, skin, and kidneys.

In liver tissues, chemokines recruit leukocytes to areas of liver injury during hepatic fibrogenesis. Hepatic stellate cells (HSCs) are responsible for the initiation and progression of liver fibrosis through the production of extracellular matrix proteins.\textsuperscript{165} HSCs are considered pericytes, pre-cursors to myofibroblasts, which differentiate into collagen-producing cells.\textsuperscript{142} HSCs express CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10 and CXCL12. The CC motif chemokines are more related to fibrogenesis, while the CXC motif chemokines are considered to be responsible for angiogenesis preceding fibrosis. Our data shows that the CC2, CCL3, CCL5, and CXCL8 are significantly upregulated in CsA GO, while CXCL9, CXCL10, and CXCL12 are not altered to a significant degree. Studies show the absence of CCL3 and CCL5 ligands leads to reduced hepatic fibrosis.\textsuperscript{140} While, increased CCL2 expression in patients is directly related to chronic hepatitis.\textsuperscript{166}
In renal fibrosis, interstitial fibrosis is initiated by activation tubular epithelial cells, vascular endothelial cells and fibroblasts. This is followed by recruitment of leukocytes and an increase in the interstitial matrix by activated renal fibroblasts.\textsuperscript{100} CCL2 is considered to be the chief mediator of renal fibrosis; while, CCL5 and CXCL10 are also noted to be increased.\textsuperscript{100} Increased expression of CCL2 is seen in kidney patients with excess macrophage infiltration. Knockout studies on mice show CCL2 deficient mice having less interstitial fibrosis.\textsuperscript{167} In patients with lupus nephritis, CCL2 levels were correlated with disease severity and macrophage infiltration.\textsuperscript{146} With the exception of CXCL10, our data shows similarities with chemokine expression reported for renal fibrosis.

In pulmonary tissues, alveolar epithelial cells induce pulmonary fibrosis through interaction with pro-inflammatory cells (monocytes, neutrophils, mast cells and leukocytes). Human studies have shown increased protein expression of CCL2 in lung epithelial cells and higher levels of CCL2, CCL3, and CCL4 in bronchoalveolar lavage fluid in idiopathic pulmonary fibrosis.\textsuperscript{137} Alveolar epithelial cells express CCL2, CCL3, CCL5, CCL11, CXCL2, CXCL5, CXCL8 and CXCL12.\textsuperscript{100} Our data shows that CCL2, CCL3, CCL5, CXCL2, and CXCL8 are significantly upregulated in CsA GO, while CCL4, CCL11, CXCL5, and CXCL12 are increased, but not altered to a significant degree. Since chemokines function in a tissue specific manner, it is possible that certain chemokines are upregulated, while others are unchanged.

In different fibrotic diseases of the skin (e.g. scleroderma) chemokines are involved in angiogenesis, fibrogenesis and cell differentiation.\textsuperscript{168} Components of the epithelial mesenchymal transition are thought to be involved in the process of dermal
fibrosis as well. Chemokines that have been reported to be involved in fibrotic skin disorders process are CCL2, CCL3, CCL5, CCL7, CCL17, CCL22, CCL27, CXCL1, CXCL8, and CXCL16. Similar to comparisons of our data with other tissues, not all chemokines in GO are upregulated. Our data suggests that only CCL2, CCL3, CCL5, CXCL1, and CXCL8 are significantly upregulated in CsA GO. We attribute this to tissue specificity of chemokines in different fibrotic diseases.

Chemokine receptor expression changes have also been noted in the fibrosis processes. In liver disease, CCR1, CCR2, CCR5, and CXCR3 overexpression is thought to contribute to fibrosis. In renal fibrosis and pulmonary fibrosis, CCR1, CCR2, CCR5, and CXCR3 demonstrate increased protein expression. CXCR3 is the predominant receptor studied in fibrotic diseases of the skin. Our data does not show increased expression of the CXCR3 receptor. However, the data from this study do show an increase in expression of the CXCR1, CXCR4, CXCR6, CCR6 and XCR1 receptors. These results support a role for the chemokine-ligand receptor axis in the development of CsA GO.

6.2 Chemokine Ligand-Receptor Axis

Promiscuity represents the ability for ligands to have more than one binding receptor, and the ability for receptors to have more than one ligand interactions. For example, the CXCL8 (IL8) ligand has two high affinity receptors: CXCR1 (IL8RA) and CXCR2 (IL8RB). We find that CXCL8 is significantly upregulated in CsA GO (>50 fold); however, it is also upregulated in CsA NO GO (>30 fold). This indicates that CsA therapy alone may contribute to an increase in CXCL8 regardless of the GO phenotype. Upon
examination of the CXCL8 receptors, we note that CXCR1 is only upregulated in CsA GO.

The susceptibility to CsA GO may be greatly dependent upon the chemokine ligand-receptor axis. Our examination of global chemokine expression reveals many chemokines are upregulated in CsA GO. Although, some of these have been reported to be pro-fibrotic, other chemokines seen in CsA GO are novel in pro-fibrotic literature. An examination of the chemokine ligand-receptor axis (figure 14) reveals increased expression of certain chemokines without upregulation of the appropriate receptors. There are a total of four chemokine ligand-receptor interactions demonstrating combined upregulation in CsA GO.

The CCL20/CCR6 axis is altered in CsA GO. The binding of CCL20 with CCR6 is responsible for chemotaxis of immature dendritic cells, effector/memory T-cells and B-cells. Additionally, the CCL20/CCR6 axis participates in homeostatic and inflammatory conditions of the mucosa and skin, such as cancer and rheumatoid arthritis. The XC1/XCR1, but not the XCL2/XCR1 axis is altered in CsA GO. XCL1, is produced by T cells and NK cells to assist in infections and inflammatory responses. XCR1 is mostly expressed by dendritic cells. The XCL1/XCR1 axis is important in the cytotoxic immune response mediated by dendritic cells; additionally, it may regulate self-tolerance and the production $T_{\text{reg}}$ cells.

The CXCL16/CXCR6 axis has been implicated in the fibrosis of different organs. CXCL16 is unlike other chemokines in structure and function. CXCL16 is expressed on the cell surface as a membrane-bound molecule, and is present on dendritic cells, macrophages, lymphocytes, smooth muscle cells, and endothelial cells.
CXCL16 induces adhesion through binding to its receptor CXCR6. CXCL16 is cleaved when activated, which promotes migration of activated T cells. The role of the CXCL16/CXCR6 interaction has been studied extensively in inflammatory diseases and fibrosis. Nanki et al. reported that CXCL16 plays an important role in T cell aggregation and activation in the synovium of rheumatoid arthritis.\textsuperscript{172} Chen et al. reported that in renal fibrosis, tubular epithelial cells demonstrated increased protein expression of CXCL16 and the circulating fibroblast precursors expressed CXCR6. In mice, deletion of CXCL16 inhibited myofibroblast activation, decreased collagen deposition, and inhibited collagen I and fibronectin.\textsuperscript{136} Overall, it is believed that CXCL16 functions to recruit fibroblast precursors leading to fibrosis.

The CXCL8/CXCR1 axis has been investigated in chronic liver disease and fibrosis. In patients with chronic liver disease, CXCL8 serum levels were significantly increased. Moreover, as the level of fibrosis was increased, there was increased expression of hepatic CXCR1. Similar to our results, CXCR1, but not CXCR2 or CXCR3, expression was increased on circulating monocytes.\textsuperscript{147} The susceptibility to CsA GO may be dependent upon expression of the chemokine ligand-receptor axis. Accordingly, susceptible individuals may possess the ability to increase CXCR1 or CXCR6 expression with CsA treatment or naturally produce more of these receptors leading to GO. Alternatively, subjects on CsA without GO have increased chemokine ligand production, but not the paired receptor. It is possible that CsA NO GO individuals have a more protected immune response, shielding the tissues from producing receptors to CsA induced pro-fibrotic chemokines.
Fibrocytes, pericytes, and epithelial/endothelial cells undergoing EMT can facilitate fibroblast proliferation and differentiation. Ultimately, fibroblasts differentiate into myofibroblasts, which are responsible for fibrosis in tissues. Fibrocytes are present in peripheral blood and migrate to tissues based on the presence of chemokine receptors. CCR2, CCR5, CCR7, and CXCR4, CXCR6 are responsible for fibrocyte migration into tissues of the lung, liver, heart, kidneys, and skin. Pericytes are of mesenchymal origin and are present around the microvasculature where they act as contractile cells. An important function of pericytes is to provide stability and integrity to the microvasculature. In liver tissues, hepatic stellate cells (HSCs) are considered pericytes, pre-cursors to myofibroblasts, which differentiate into collagen-producing cells.

The epithelial mesenchymal transition (EMT) has been implicated in CsA GO. The EMT is a tissue specific, complex mechanism involving cell-cell and cell-extracellular matrix interactions. This allows for the release of epithelial cells from the basal layer, the reorganization of the cytoskeleton, and alteration of the transcriptional regulation of the cell so that it transforms from a epithelial to a mesenchymal phenotype. The EMT pathway is important in the progression of many epithelial tumors. Chronic inflammation or conditions that encourage tissue disruption may stimulate fibrosis, where altered EMT disrupts tissue integrity and function. Sume et al. reported that epithelial cells experiencing EMT can alter the basement membrane and allow for pro-fibrotic signaling markers to promote gingival overgrowth. Studies have reported a positive correlation between chemokine (CXCL8) expression and carcinoma cells undergoing EMT through induction of the CXCL8/CXCR1 axis. The results of our study indicate that
upregulation of CXCL8 and its receptor CXCR1 may promote an environment favoring EMT and fibrosis through trans-differentiation of epithelial cells to pro-fibrotic myofibroblast cells.

6.4 Other Findings (Collagen, MMPs, TIMPs, etc.)

Data from our study shows a significant increase in expression of type IV collagen, primarily located in the basal lamina, and a minor (not significant) increase in type I collagen, the most abundant collagen, in CsA GO patients. These findings are consistent with the observations of the immunohistochemistry (IHC) samples. In the IHC specimens, cellular level alterations were seen at the level of the epithelium, basement membrane, and extracellular matrix (figure 15).

In vivo studies examining the role of certain MMPs in CsA GO have noted increases in expression patterns. The increase in MMP expression may be directly related to CsA treatment or secondary to inflammation seen with CsA GO. In our study population, the level of inflammation was kept at a minimum with dental prophylaxis every other week. The results from this study reveal that expression of MMP-1 is increased in patients with CsA GO, while CsA therapy alone increases TIMP-1 levels. The increase in MMPs seen in CsA GO may contribute to the discontinuity in the basement membrane seen in the IHC samples.

CsA GO has been reported as a disorder of increased fibroblast proliferation and/or decreased fibroblast apoptosis. Previously studied markers associated with fibroblast proliferation and apoptosis reported in CsA GO literature are p53, caspase-3, FOXO-1, and bcl-2. In comparison with our data, caspase-3 was significantly increased
in CsA GO subjects, while expression levels of p53, FOXO-1, and bcl-2 were not significantly different. Other cytokines and proteins have also been reported in CsA GO literature (Interleukins, TNFα, PDGF, TGF-β, Smad7).5,82,106,107,176

6.5 **Toll-like receptors**

Toll-like receptors (TLR) are proteins that are crucial for the innate immune system. TLRs bind to specific ligands to initiate an immune response, such as lipopolysaccharide (LPS), peptidoglycan, lipoproteins, and bacterial RNA and DNA. In periodontal disease, oral pathogens stimulate the TLRs through binding of bacterial byproducts. This initiates signal transduction and downstream events. The binding of LPS from gram-negative bacteria to TLR stimulates protein kinases that amplify signals for inflammatory mediators (e.g. cytokines, chemokines) or transcription factors.152 In CsA GO, TLR4 expression was noted to be upregulated compared to control, healthy gingiva. TLR alteration by CsA, possibly through its interaction with calcineurin, may initiate a signaling cascade leading to increased cytokine and chemokine productions.154 Our review of TRL expression also revealed significant upregulation of TLR1 and TLR2. TLR1 is known to recognize pathogen-associated molecular pattern (P-AMP) for gram-positive bacteria. While TLR2 is known to recognize many microbial products, including peptidoglycans, lipoteichoic acid, lipoarabinomannan, lipoproteins and lipopeptides. Additionally, TRL2 may recognize gram-positive bacteria, spirochetes, mycobacteria, and mycoplasmas.177 Increased expression of TLR1 and TLR2 may be indicative of baseline inflammation or alterations in CsA GO patients. Since TLRs may cause upstream signaling events for chemokine release, they may drive chemokine expression.
6.6 **Study Strengths**

Based on limitations from previous investigations on CsA GO, this study was designed to minimize confounding risks factors. One of the major limitations of pasts research includes the variability in patients' oral hygiene, blood levels of CsA, and baseline level of inflammation. For the purposes of this study, all patients selected had optimal oral hygiene and frequent recalls at 2 week intervals for dental prophylaxis. This allowed the collected tissue samples to be at low levels of inflammation and periodontal disease causing bacteria. A well maintained patient allowed us to evaluate TLR and chemokines levels while minimizing confounding increases due to an inflammatory response. Additionally, all patients (n=14) were selected from a population of 125 CsA treated individuals based on age, drug dosage, therapy initiation time, lack of periodontal disease, and severity of the GO. Previous studies have also been limited in how they reported the severity of the GO phenotype due to differences in measurement techniques and providers. Consequently, CsA GO prevalence has a wide range of reporting in literature. To control for disease severity amongst subjects, an objective gingival hyperplastic index was utilized.

The use of the “Whole Human Genome” microarray allowed for investigation of >54,000 probes of the gingival biopsies. The ability to examine all available human gene sets in the three groups allowed for investigation into a wide range of biologic pathways and mechanisms. The application of GeneSifter software increased our ability to quantify and compare expression of specific genes or gene products previously reported CsA GO literature. Furthermore, the program allowed for statistical analysis showing a significant
critical value (p < 0.05). Selected genes could then be applied to the Kyoto Encyclopedia of Genes and Genomics (KEGG) pathways to elicit a biological significance.

6.7 Study Weaknesses

The predominant weakness of this study was the limited samples populations of control tissues (n=3) and CsA NO GO tissues (n=3). The ability to collect gingival biopsies from healthy individuals and CsA NO GO subjects proved to be an ethical and clinical challenge. The biopsies obtained were collected during routine periodontal procedures needing gingival tissue removal (e.g. crown lengthening). Other periodontal procedures for gingival tissue collection, such as gingivectomy or pocket reduction surgery, completed on non-GO patients with periodontal disease, excluding them from this study. An increase in the two group sizes would have increased the power of the study and the statistical significance reporting. It may have allowed us to make more appropriate assumptions and conclusions from the data.

Furthermore, oligonucleotide sequences were used for detection of gene expression. Consequently, if the microarray did not contain the corresponding oligonucleotide sequences to different isoforms, the expression levels may be underreported or excluded. Although, the use of a stringent hyperplastic index was a strength of this study, the inclusion of varying severities of GO may contribute to expression differences within the CsA GO group. Future studies using this data may subdivide the CsA GO group into severity of the GO phenotype for more accurate reporting of gene expression.
The selection criteria for inclusion in this study was strict, with subjects not having concomitant medications, low levels of plaque, and adequate oral hygiene. Our selection criteria may not be realistic when compared to patients in a non-controlled environment. CsA GO severity is known to be associated with plaque presence. Although the results of the study may show inherent expression level differences in a well-controlled subgroup, there may be difficulty in applying these results to a broader population.
7. CONCLUSION

Susceptibility to Cyclosporine (CsA) induced gingival overgrowth (GO) may occur at many levels along different biologic processes. CsA is a widely used immunosuppressant with beneficial effects including increased 5-year success rates of organ transplantation above 90%. However, in 25-30% of all CsA treated subjects, CsA can induce GO. Since the first reports of CsA GO, studies have attempted to determine the etiologic genes or biologic mechanisms involved. Authors have proposed a variety of individual genes, proteins, single nucleotide polymorphisms (SNPs), and complex biological pathways to elicit the primary etiology for CsA GO. Nevertheless, there seems to be a lack of integration of all the aforementioned etiologies. The CsA GO phenotype appears to by multifactorial involving more than a single gene interaction.

The objective of our project was to analyze differentially expressed genes of chemokine ligands and receptors that may potentiate CsA GO. In the process of our investigation, the role of chemokines in fibrosis of a variety of tissues became evident. This broadened the scope of the project to include upstream events, such as toll-like receptor (TLR) signaling, and downstream events such as chemokine mediated fibrosis.

Our study found genes for 25 chemokine ligands (n=20) and receptors (n=5) that appear to show significantly altered expression in CsA GO patients. The majority of the chemokines showed increased expressions patterns. Although chemokines are central to inflammation and immunology, a role in other functions such as angiogenesis and fibrosis have been established. Chemokines are noted mediators of tissue fibrosis. Fibrosis can be a part of normal physiology or pathology; it is described as the end
product of inflammation. In wound healing, chemokines function to recruit macrophages, plasma cells and T lymphocytes to the site of injury. These cells can promote a pro-fibrotic response through the EMT or secretion of chemokines in an autocrine manner. Chemokines have the ability to activate/recruit fibroblasts and promote angiogenesis, which are crucial for tissue fibrosis. The results of our investigation show significant upregulation of CCL2, CCL3, and CCL5, which are increased in most forms of tissue fibrosis. Other tissue specific pro-fibrotic chemokines are also increased in CsA treatment.

Additionally, our results indicate that there is an increase in the chemokine ligand-receptor axis of CXCL8/CXCR1 and CXCL16/CXCR6. Both of these chemokine ligand-receptor interactions have been noted in fibrosis of the liver, kidney, lung, and skin. Our study shows that this is also true of CsA GO. It is possible that CsA treatment in certain individuals may promote a pro-fibrotic response leading to GO. Examination of upstream TLRs indicate CsA increases expression of TLR1 and TLR2, which may potentiate chemokine activity and fibrosis.

The process of CsA GO involves the transformation or recruitment of pluripotent cells capable of activation, stimulation, and proliferation of fibroblasts. The sources of these active fibroblasts may be from pericytes, fibrocytes, or the epithelial mesenchymal transition. Chemokine are intimately involved in the proliferation and differentiation of the aforementioned cells and events. Therefore, CsA treatment may function through some or all the biological pathways mentioned to cause GO in certain individuals. The cause of CsA GO remains unknown, but is plausible that CsA mediated alterations of chemokine ligands and receptors may contribute to this phenomenon.
CITED LITERATURE


APPENDICIES

Appendix A.

Differential gene expression heat map of chemokine ligands and receptors, in control, CsA no GO, and CsA GO gingival biopsy samples.
Appendix B.

Relative expression of previously reported genes involved in CsA GO

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>CsA no GO</th>
<th>CsA GO</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>1.25 ± 0.15</td>
<td>2.50 ± 0.13*</td>
<td>6.06 ± 1.63*</td>
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<tr>
<td>IL-1α</td>
<td>0.71 ± 0.21</td>
<td>2.31 ± 0.58*</td>
<td>1.86 ± 0.30*</td>
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<tr>
<td>IL-6</td>
<td>0.81 ± 0.03</td>
<td>0.85 ± 0.13</td>
<td>1.58 ± 0.33*</td>
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<tr>
<td>TGF-β1</td>
<td>0.36 ± 0.07</td>
<td>0.38 ± 0.02</td>
<td>0.58 ± 0.06</td>
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<td>PDGF-B</td>
<td>0.53 ± 0.11</td>
<td>0.57 ± 0.08</td>
<td>0.58 ± 0.02</td>
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<tr>
<td>Collagen Type I</td>
<td>0.84 ± 0.03</td>
<td>0.87 ± 0.09</td>
<td>1.15 ± 0.09*</td>
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<td>Collagen Type IV</td>
<td>0.87 ± 0.14</td>
<td>1.60 ± 0.55</td>
<td>3.13 ± 0.64*</td>
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<td>MMP-1</td>
<td>0.29 ± 0.07</td>
<td>0.38 ± 0.10</td>
<td>1.12 ± 0.22*</td>
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<tr>
<td>MMP-2</td>
<td>0.30 ± 0.11</td>
<td>0.21 ± 0.06</td>
<td>0.64 ± 0.10</td>
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<tr>
<td>MMP-8</td>
<td>0.09 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>0.10 ± 0.01</td>
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<td>MMP-10</td>
<td>6.56 ± 0.56</td>
<td>6.63 ± 0.31</td>
<td>6.31 ± 0.21</td>
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<td>TIMP-1</td>
<td>3.84 ± 1.13</td>
<td>5.24 ± 1.24</td>
<td>8.55 ± 0.85*</td>
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<td>TIMP-2</td>
<td>0.92 ± 0.21</td>
<td>1.00 ± 0.20</td>
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<td>Caspase-3</td>
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<td>0.16 ± 0.03</td>
<td>0.22 ± 0.01*</td>
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<td>FOXO-1</td>
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<td>TLR1</td>
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<td>TLR4</td>
<td>0.03 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.08 ± 0.01*</td>
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</table>

Results from Cyclosporine treatment patients without gingival overgrowth (CsA no GO) and Cyclosporine treatment patients with gingival overgrowth (CsA GO) compared to control, normal gingiva. *P< 0.05.

Appendix C.

Differential expression of IL-1β. IL-1β is significantly upregulated in CsA NO GO and CsA GO. Intensity= mean-normalized quantitative expression of IL-1β. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Appendix D.

Differential expression of IL-1α. IL-1α is significantly upregulated in CsA NO GO and CsA GO. Intensity= mean-normalized quantitative expression of IL-1α. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Appendix E.

Differential expression of IL-6. IL-6 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of IL-6. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Appendix F.

Differential expression of Collagen Type-I. Collagen Type-I is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of Collagen Type-I. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Appendix G.

Differential expression of Collagen Type-IV. Collagen Type-IV is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of Collagen Type-IV. CsA NO GO = Cyclosporine without gingival overgrowth, CsA GO = Cyclosporine with gingival overgrowth.

Appendix H.

Differential expression of MMP-1. MMP-1 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of MMP-1. CsA NO GO = Cyclosporine without gingival overgrowth, CsA GO = Cyclosporine with gingival overgrowth.

Appendix I.

Differential expression of TIMP-1. TIMP-1 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of TIMP-1. CsA NO GO = Cyclosporine without gingival overgrowth, CsA GO = Cyclosporine with gingival overgrowth.
Appendix J.

Differential expression of Caspase-3. Caspase-3 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of Caspase-3. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Appendix K.

Differential expression of TLR-1. TLR-1 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of TLR-1. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.

Appendix L.

Differential expression of TLR-2. TLR-2 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of TLR-2. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Appendix M.

Differential expression of TLR-4. TLR-4 is significantly upregulated in CsA GO. Intensity= mean-normalized quantitative expression of TLR-4. CsA NO GO= Cyclosporine without gingival overgrowth, CsA GO= Cyclosporine with gingival overgrowth.
Appendix N.

University of Illinois at Chicago
Research Information and Consent for Participation in Biomedical Research
Gene and Protein Expression in Gingival Tissue

You are being asked to participate in a research study. Researchers are required to provide a consent form such as this one to tell you about the research, to explain that taking part is voluntary, to describe the risks and benefits of participation, and to help you to make an informed decision. You should feel free to ask the researchers any questions you may have.

Principal Investigator Name and Title: Thomas Hart, DDS, PhD
Department: Periodontics M/C 859
Address and Contact Information: 801 S. Paulina St. Chicago, Illinois, 60612
Emergency Contact Name and Information: Dr. Thomas Hart, 312-413-4467

Why am I being asked?

You are being asked to be a subject in a research study of gum disease. We will be looking for differences in the gum tissues of patients that have gum disease and those that do not. The study will utilize small pieces of gum tissue that are usually discarded during gum/oral surgery. We will study gene and protein expression in gum tissues from patients with and without gum disease.

You have been asked to participate in the research because your dental care includes surgical treatment where small pieces of gum tissue are normally discarded as part of the procedure.

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future dealings with the University of Illinois at Chicago. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

Approximately 100 subjects may be involved in this research at UIC.
Appendix N.

What other options are there?

You have the option not to participate in this study.

What about privacy and confidentiality?

The people who will know that you are a research subject are members of the research team, and if appropriate, your periodontist and dental assistant. No information about you, or provided by you, during the research, will be disclosed to others without your written permission, except if necessary to protect your rights or welfare (for example, if you are injured and need emergency care or when the UIC Office for the Protection of Research Subjects monitors the research or consent process) or if required by law.

The consent form signed by you will be looked at and/or copied for examining the research by: Dr. Thomas Hart.

A possible risk of the research is that your participation in the research or information about you and your health might become known to individuals outside the research. This is unlikely as once the tissue is collected the only information that will be associated with it will be an arbitrary number and your diagnosis [i.e. periodontitis (gum disease) or periodontally healthy (healthy gums)]. No information about you that identifies you will be associated with the tissue sample. The only reference to your personal information will be the consent form and a note in your dental chart that you agreed to participate in the study.

When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity.

What if I am injured as a result of my participation?

Injury is not anticipated as part of this study as you are only being asked to allow us to study small pieces of gum tissue removed during your gum surgery that would otherwise be discarded.

What are the costs for participating in this research?

There are no costs to you for participating in this research.

Will I be reimbursed for any of my expenses or paid for my participation in this research?

You will not be offered payment for being in this study.

Will my cells, tissues, blood, or other biological materials be used to develop commercial products?

Your gum tissue will not be used to develop commercial products.
Appendix N.

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future relations with the University. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

**Signature of Subject or Legally Authorized Representative**

I have read (or someone has read to me) the above information. I have been given an opportunity to ask questions and my questions have been answered to my satisfaction. I agree to participate in this research. I will be given a copy of this signed and dated form.

Signature

Date

Printed Name

Signature of Person Obtaining Consent

Date (must be same as subject’s)

Printed Name of Person Obtaining Consent
# VITA

## ANKUR S PATEL

### EDUCATION

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<th>Address</th>
<th>Degree/Program</th>
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<tr>
<td>2013–2016</td>
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<td>801 S. Paulina Street, Chicago, IL 60612</td>
<td>Specialty Certificate: Periodontics</td>
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<tr>
<td>2009–2013</td>
<td>University of Illinois at Chicago – College of Dentistry</td>
<td>801 S. Paulina Street, Chicago, IL 60612</td>
<td>Doctor of Dental Surgery</td>
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<tr>
<td>2006–2009</td>
<td>University of Illinois at Chicago – College of Liberal Arts &amp; Sciences</td>
<td>1200 W. Harrison Street, Chicago, IL 60607</td>
<td>Bachelor of Science in Biological Sciences</td>
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<td>Honors: <em>Summa Cum Laude; Highest Distinction in Biological Sciences</em></td>
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### EXPERIENCE

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<tr>
<td>2016–Present</td>
<td>Periodontist</td>
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<tr>
<td>2013–2016</td>
<td>Resident – Post-Graduate Periodontics</td>
<td>Department of Periodontics: University of Illinois at Chicago</td>
<td>801 S. Paulina Street, Chicago, IL 60612</td>
</tr>
<tr>
<td>2013</td>
<td>Pre-Clinical Dental Instructor</td>
<td>Oral Medicine &amp; Diagnostic Sciences: University of Illinois at Chicago</td>
<td>1200 W. Harrison Street, Chicago, IL 60607</td>
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<td></td>
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<td>Department of Dentistry, Chicago</td>
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<tr>
<td>2012–2013</td>
<td>Teaching Assistant</td>
<td>Department of Periodontics: University of Illinois at Chicago</td>
<td>801 S. Paulina Street, Chicago, IL 60612</td>
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<td>College of Dentistry, Chicago, IL</td>
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### RESEARCH

Department of Periodontics: University of Illinois at Chicago – College of Dentistry, Chicago, IL  
Mentor: Thomas C. Hart, DDS PhD.,  
Focus: Bioinformatics & Genetics
Projects/Abstracts:

2011 – 2016  “Role of Chemokines in Cyclosporine Induced Gingival Overgrowth.”

Department of Biological Sciences: University of Illinois at Chicago – College of Liberal Arts and Sciences, Chicago, IL
Mentors: Joel Brown, PhD, Lauren Pintor, PhD


PROFESSIONAL AFFILIATIONS

2013 – Present  American Academy of Periodontology (AAP)
2009 – Present  American Dental Association (ADA)
2009 – Present  Chicago Dental Society (CDS)
2009 – Present  Illinois State Dental Society (ISDS)
2009 – Present  Delta Sigma Delta Fraternity (DSD)

HONORS & AWARDS

2014  Graduate Student Research Award: Basic Science Research Project: “Epidermal Differentiation Complex Gene Expression In Hereditary Gingival Fibromatosis and Cyclosporine Induced Gingival Overgrowth.”

2013  American Academy of Periodontology: Dental Student Award
2013  Dr. Harry Saxon Scholarship Award
2013  Dr. Kamlesh Amin Scholarship Award
