An investigation of Crohn's Disease in the Oral Cavity

by

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ABSTRACT

Crohn's disease (CD) is one of the chronic inflammatory bowel diseases (IBD) with a complex etiology involving genetic factors, priming by enteric microflora, environmental factors and a change in the immune-mediated response, the other IBD is ulcerative colitis. The diagnosis of CD has continuously been challenging for physicians and medical practitioners as there is no single ''gold standard'' test or examination. Instead, physicians apply a combination of symptoms, clinical examination, laboratory indices, radiology, and endoscopy with histology to diagnose the disease. The techniques used for diagnostic decision are considered invasive, expensive, and time-consuming; therefore, an ideal non-invasive test is increasingly expected for initial diagnosis and identification of disease activity and the early determination of diagnosis and detection of disease activity are essential for tailoring therapy. Noninvasive specimen collection and analysis to help physicians distinguish CD would allow more rapid and appropriate treatment, as well as the potential to improve quality patient care while reducing both direct and indirect associated cost through the elimination of unnecessary procedures and more efficient medical treatment.

It is well accepted that CD patients have an impaired intestinal epithelial barrier function. This allows the luminal microbiota to position themselves within close proximity of the intestinal epithelium inside the mucous layers. Furthermore, the gaps within the epithelial cell layer permit microbes to invade the intestinal tissues. This invasion triggers the dysregulated immune response characteristic of CD. Recent identification of elevated levels of caspase-1, an integral component of the inflammasome involved in pyroptosis, has been reported. In turn, this has been associated with the increased number of epithelial gaps in the epithelial layer relative to healthy controls.

A systematic literature review was conducted to assess the scope and incidence of oral conditions associated with CD relative to the general population. Although hampered by a range in study designs and oral findings that were and were not reported intentionally, the results indicate that indeed CD patients have an increase in oral ulcerations, particularly aphthous ulcers. There was a loose association between the presence of these ulcers and intestinal disease activity.

Next, a series of experiments were conducted to identify caspases within the oral cavity and quantify them using three study groups: healthy controls, healthy controls who had localized inflammation around the tooth scheduled for extraction, and patients with biopsy-confirmed CD who were in remission. Histology findings indicated that the CD group had more inflammation in both the lamina propria and epithelial layers compared to the two control groups. Results showed a statistically significant difference in caspase-1 densitometry between CD patients and each set of controls ($p<0.01$, for each). The levels of caspase-3 (apoptosis levels) were also detected and quantified to confirm that the inflammatory process in CD patients is more likely to be caspase-1 mediated. CD patients had lower caspase-3 levels in comparison with both control groups ($p<0.01$, for each). In conclusion, our results provide evidence that caspase-1 is likely to play a critical role in the process of cell death in CD patients.

The oral mucosal tissues have similar clinical and immune characteristics of those in the intestinal tract. These findings, although preliminary, suggest that dentists can play a critical role in the diagnosis and monitoring of CD activity in conjunction with gastroenterologists. The oral cavity is readily accessible and mimics key events previously believed to be restricted to intestinal tissues.

PREFACE

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, in 2013, number: Pro00024154.

Some of the research conducted for this thesis forms part of an international research collaboration, involving Dr. J Liu and Dr. R Talwar-Povoledo at the University of Alberta. The systematic literature review presented in chapter 2 was designed and conducted by myself with the assistance of Dr. R Talwar-Povoledo and KP Ismond. The experimental procedures presented in chapter 3 were performed by myself or in conjunction with other team members. I completed the data analysis.

Chapter 2 of this thesis is intended to be submitted to the journal *Community Dentistry and Oral Epidemiology* for publication.

Chapter 3 is intended to be submitted to the journal *Gastroenterology*.

Dr. R Talwar-Povoledo was the supervisory author for both manuscripts and was involved in the study conceptualization and composition.

DEDICATION

This thesis is dedicated to my parents and family.

To my amazing father, Ramadan Elchames, and my exceptional mother, Kiria Naas, who have been a continuous source of support and encouragement. Thank you for always believing in me.

To my wonderful husband, Jamaleddin Hejaji. Thanks for your love, patience, and unconditional support.

To my beautiful boys; Mohamed, Maher, and Awab. You were a constant source of joy and happiness.

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Lastly, I want to thank all of my friends and mentors who have been with me throughout my graduate career. Some of the lessons were about science, but most of the lessons were about life. I am completing my graduate school journey as a stronger and wiser person because of you.

Take benefit of five before five:

Your youth before your old age,

Your health before your sickness,

Your wealth before your poverty,

Your free-time before your preoccupation and

Your life before your death.

Prophet Mohamed (peace and blessings of Allāh be upon him)

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LIST OF ABBREVIATIONS

IBD	inflammatory bowel disease
CD	Crohn's disease
UC	ulcerative colitis
OCD	oral Crohn's disease
CRP	C-reactive protein
NSAIDs	non-steroidal anti-inflammatory drugs
CT	computed tomography
MRI	magnetic resonance imaging
IL	interleukin
TNF-α	tumor necrosis factor-alfa
CARD	caspase-recruitment domain protein
dH_2O	distilled water
H_2O_2	hydrogen peroxide
PBS	phosphate buffered saline
SDS	sodium dodecyl sulphate
M-PER	mammalian protein extraction reagent
IR-Dye	infrared dye
kDa	kilodalton
SD	standard deviation

Chapter 1: Introduction

1.1 What is Crohn's Disease?

Crohn's disease (CD) is a chronic, debilitating condition occurring anywhere along the length of the gastrointestinal tract with common reference to it as a 'gums to bums' disease. CD is considered part of the inflammatory bowel diseases (IBD) which also include; ulcerative colitis and indeterminate colitis. The discriminating feature of CD is that it is a transmural disease wherein cellular inflammation and damage occurs in the mucosal layers closest to the luminal contents, similar to colitis, and then progresses deep into the submucosa and muscle tissue layers[1]. Uncontrolled inflammation causes fistulas, fibrosis, and strictures all of which can impair the movement of luminal contents leading to varying degrees of bowel obstruction that may require immediate surgical intervention. Tissue damage may lead to bowel perforation and leakage of luminal contents including bacteria into the abdominal cavity.

Although it is believed that the Italian physician, Giovanni Battista Morgagni, provided the first clinical description of CD in 1769, the disease acquired a name only after a 1932 publication by Crohn, Ginzburg, and Oppenheimer describing 14 cases of regional ileitis[2, 3]. Although considered to be a 'modern' disease, an evolutionary genomic study of Neanderthals and Denisovans found that they also had several gene deletions associated with CD[4]. The study authors suggest that the genetic basis of CD may have originated with a common ancestor who lived approximately 1 million years ago.

1.1.1. Etiology and pathogenesis

The exact causes of IBD in general are complex and remain unclear. A combination of host genetics, a dysregulated immune response of the host, environmental triggers (e.g., body weight, smoking, medications history), and intestinal microbiota have been implicated in IBD[1]. Risks for CD increase in situations where immediate family members have the disease[1].

In consideration of the complex interplay of etiologic components, it is not surprising that the pathogenesis of CD varies between patients. Generally, CD is diagnosed during adolescence and early adulthood. A second, small peak of incidence occurs later in life, typically between 50 and 70 years of age[1]. However, this does not mean children younger than 15 years are protected from developing CD. In fact, recent clinical practice guidelines have been developed specific for this

young patient population as uncontrolled symptoms or overprescribing of steroids to manage inflammation have been associated with growth impairments and health complications[5].

CD oscillates between states of quiescence, when the disease appears to be in remission, and exacerbation characterized by symptoms such as abdominal pain, diarrhea, rectal bleeding, fistulas, and abscesses. What causes the onset of either of these states is unknown. During exacerbation, patients are categorized based upon the severity of their symptoms (e.g., bowel movements per week, diarrhea, and general wellbeing) using validated tools such as the Harvey Bradshaw Index or the CD Activity Index. In addition, a patient may undergo endoscopy with biopsy for direct visualization and scoring of their intestinal tract as a measure of disease activity. As well, patient samples will be routinely assessed for levels of CD-related inflammatory markers, such as C-reactive protein (CRP) or fecal calprotectin. Based upon one or any combination of these primarily objective measures, patients can be classified as in remission or having active disease graded as mild, moderate, or severe.

Due to its chronicity and young age of onset, the health-related quality of life of patients with CD has been assessed with validated tools, such as the Inflammatory Bowel Disease Questionnaire and Gastrointestinal Quality of Life Index as reviewed by Floyd and Langham[6]. Regardless of assessment tool used or country, CD patients of all ages have consistently indicated a poor health related quality of life relative to the general population. Even during periods of remission, the scores of CD patients improved but they are still below those of the general population. In particular, physical, emotional, and mental states are most affected[6].

1.1.2 Diagnosis

As summarized in the guideline authored by the British Society of Gastroenterology and the European Crohn's and Colitis Organization (ECCO), diagnosis of CD is an involved process[7]. Individuals presenting with symptoms indicative of CD, such as abdominal pain, diarrhea, and weight loss, should undergo a full medical history to rule out other conditions with overlapping symptoms (e.g., traveler's diarrhea and medication side effects) and potentially identify established CD risk factors, such as family history and recent bouts of gastroenteritis. A complete medical examination should be performed in addition to laboratory investigations, including full blood count, urea and electrolytes, liver function tests, C-reactive protein, ferritin, transferrin saturation, vitamin B12, and folate. Additional tests for standard infectious organisms,

such as *Clostridium difficile*, should also be conducted. Colonoscopy with a minimum of 2 biopsies from the 5 major sites of the large intestinal tract represents the gold standard for diagnosing CD. It also facilitates classification of disease severity, location, and characterization of histological features, information needed to inform the treatment strategy.

As reviewed by Mowat and colleagues, imaging tools other than colonoscopy can be used in the management of CD[7]. The value of these imaging tools for the initial diagnosis of CD is limited as they cannot collect biopsies. For instance, ultrasound is emerging as a practical bedside tool for observational purposes in acute cases with abscesses or fistula. Magnetic resonance imaging can be used for surveillance but evidence is still lacking for its use in initial diagnosis. Video capsule endoscopy continues to improve although the capsule itself can become lodged within inflamed tissue, structures, or fistulas requiring steroid therapy or surgical retrieval. Thus comprehensive imaging is required in advance before use of the video capsule endoscopy. Computed tomography scanning is the 'gold standard' for detecting extraluminal complications in CD such as abscesses. Due to the significant radiation exposure to the patient, repeat imaging is not advised and therefore its use is limited to identification of sepsis or obstruction. Similarly, barium fluoroscopy exposes the patient to radiation and should be avoided except in a situation to detect early mucosal disease.

1.1.3 Treatment

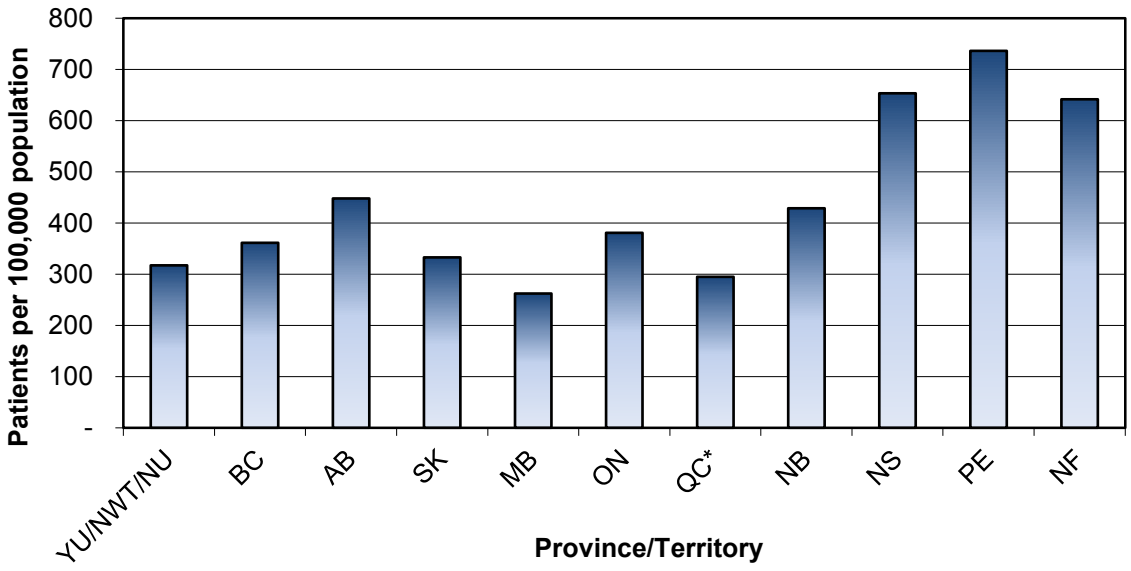
Without knowing the cause(s) of CD, clinical management of CD is challenging. The treatment goals are continuously evolving in response to research findings and availability of new medications. In the past, the aim was to simply help the individual manage their acute symptoms by prescribing medications for pain relief, loperamide for relief of diarrhea, and surgery as needed. Now, the approach is to achieve mucosal healing of the intestinal tract as this has been associated with both the prevention of the frequency and severity of disease flares[8]. Treatments involve the strategic use of one or multiple tiers of medications presented in increasing potency: non-steroidal anti-inflammatories, immunomodulators (e.g., azathioprine, methotrexate, 6-mercaptopurine), short courses of corticosteroids, and biologic agents (e.g., infliximab or adalimumab) directed against a cell signaling protein or cytokine called tumor necrosis factor-α (TNF)[9, 10]. During exacerbation, treatments may be escalated and then reduced during remission. Regardless of disease activity, the evidence-base and practice recommendations indicate that CD

patients should continue medication regimens even after protracted periods of remission or during pregnancy for effective disease management[11]. Surgery to remove the diseased part of the intestinal tract is not recommended except in cases where medications (conservative management) are ineffective or the situation is life threatening. This is because the anastomotic sites (where the tract is reconnected) for strictureplasty or resection have 5 year surgical recurrence rates of ~45% and 24%, respectively[12]. Successive surgical resections of the intestinal tract can result in short bowel syndrome[13].

1.1.4 Incidence and prevalence

Canada's rates for CD are among the highest in the world according to Crohn's and Colitis of Canada, with 0.4% of Canadians affected[14]. Incidence rates are also increasing especially in children aged 10 years and younger. Due to the early age of onset, prevalence rates are continuously increasing across Canada. The distribution by population of CD illustrates a west to east gradient (Fig. 1-1)[15]. Although medical treatment has improved since 2000 with the advent of biologics, patients with CD have a 50% increased risk of dying prematurely relative to the general population[14].

Figure 1-1. Provincial distribution of CD patients in Canada for years 2007-2008



1.1.5 Economic burden

As reviewed by Fedorak and colleagues, the direct costs associated with CD are not excessive. However, as this is a chronic condition typically diagnosed at a young age, the cumulative

lifetime costs become impressive. A patient with CD requires additional clinic appointments, hospitalizations, and routine lab work compared to the general population. With the advent of biologics, medication costs per patient have increased from $809 (US) per annum for mesalamine, and steroids to >$35,000 (US) per annum[16, 17]. When considering the quality adjusted life years, biologic therapies for CD are cost effective[18]. This is because the acute care costs and overall burden on the health care system is decreased while improving the quality of life for the patients.

Indirect costs associated with CD are those incurred by society or the individual and were estimated to be nearly $600 million in 2008[16]. This does not include income loss due to increased employee absenteeism nor loss of annual salary increases, estimated to be ~$3,145 (US) per year, due to the increased health burden of patients with CD[19].

1.2 Extraintestinal manifestations of CD

CD is a systemic disease that is not restricted to the gastrointestinal tract. Individuals may experience extraintestinal manifestations of CD at one or more time points which involve the joints (i.e., peripheral arthritis, axial arthropathies, ankylosing spondylitis, and sacroileitis), skin (i.e., pyoderma gangrenosum, erythema nodosum, Sweet's syndrome, and aphthous ulcers), liver (i.e., primary sclerosing cholangitis), and eyes (i.e., uveitis and episcleritis)[20]. Although the prevalence varies, the range of patients with CD who experience 1 to 5 of these manifestations is 6% to 47% of patients, regardless of age or gender[20]. The initial onset of comorbid conditions occurs before a diagnosis of intestinal CD in one quarter of cases[21]. The delay in presentation and diagnosis of intestinal disease, the underlying cause of the comorbid condition, makes treatment challenging.

If the symptoms of CD are well-controlled, the comorbidities will resolve as well. Frequently, medications for CD are also beneficial for ameliorating comorbidities[22-24].

1.2.1 Specific manifestations involving the oral cavity

The features of the oral mucosal tissues are similar in composition to those forming the intestinal walls[25]. The role of the outermost mucosal layer in both the oral cavity and the intestinal tract is to protect the deeper tissues from invasion of pathogens and loss of moisture through the

secretion of mucus. The epithelium is comprised of cells that are continually produced by progenitors and sloughed off after they mature. Below the epithelium is the lamina propria which is a matrix of loose connective tissue providing support.

As the oral cavity is at the extreme end of the gastrointestinal tract, it is expected that CD manifests in these tissues. Indeed, case reports dating from 1969 describe the presence of oral lesions similar in appearance both macroscopically and histologically to those seen along the intestinal tract in classic CD[26, 27]. Oral lesions with granulomas appear as indurated tag-like lesions, cobblestones, or mucogingivitis[28]. Lip swelling with vertical fissures and deep linear ulcerations may also be present. Lesions without histological evidence of granulomatosis include aphthous stomatitis, pyostomatitis vegetans, diffuse pustules, diffuse labial, and buccal or gingival swellings.

1.3 The altered immune response in CD

Patients with inflammatory bowel diseases have two types of immune responses that are initiated when microorganisms within their intestinal lumen breach the epithelial barrier and gain access to the intestinal wall tissues. Furthermore, these patients have a loss of tolerance to any commensal organisms that may adhere to or breach the epithelium[29].

The first type of immune response is coined “activated” innate response as it involves macrophages and neutrophils actively recruiting other immune cells and releasing mediators that attack the unwanted microbes. Macrophages originate in the blood and their purpose is to engulf and neutralize these foreign bodies. Neutrophils are potent antimicrobial agents releasing reactive oxygen species and toxic molecules (e.g., myeloperoxidases, hydrolytic enzymes, and proteases) that attack microbes upon contact[30]. The second type of immune response is termed “acquired” as it involves the T and B cell immune responses[29]. In Crohn’s, the immune response recruits more effector cells and for a longer duration at the site of “invasion”. When activated, they release proinflammatory cytokines (e.g., interferon-γ and interleukin-17 or -22)[29]. Blocking effector entry or activation, or interrupting the activity of either the proinflammatory cytokines and /or their receptors are therapeutic targets.

1.3.1 How permeable is the intestinal tract to micro-organisms in CD?

Increased intestinal permeability has been demonstrated in CD patients[31-34] and in 10% to 20% of their first-degree relatives[35-37]. In addition, intestinal permeability has been associated with the pathogenesis of CD and has been described as a "leaky gut". Controversies are ongoing over the significance of barrier dysfunction in Crohn's patients and their relatives, because evidence has been reported both for inflammation as the cause of permeability defects[38, 39] and (in animal models) for disruption of epithelial barrier function as the cause of intestinal inflammation.[40]

Confocal endomicroscopy (CEM) study found that the mean epithelial gap density on the surface of villi was 0.01770 ± 0.00564 gaps/cells for healthy controls. However, for CD patients, this was an order of magnitude higher at 0.11730 ± 0.03290 gaps/cells ($p = 0.001$)[41]. The increased presence of gaps within the epithelium of the villi allows the invasion of microbes into the host tissues thereby triggering a cascading immune response.

1.3.2 Types of cell deaths that may give rise to the epithelial gaps

Cell death can occur through a variety of mechanisms, such as apoptosis, pyroptosis, and necroptosis. Both apoptosis and necroptosis are programmed versions of cell death. Apoptosis is a tightly controlled event typically occurring during development and in situations of physiological cellular turnover in mature tissues[42]. Apoptosis does not give rise to inflammation and the mechanistic components are caspase-3 and cytochrome-c release. Whereas necroptosis can be induced by trauma or any process affecting the patency of the cell membrane thereby releasing the damage-associated molecular pattern molecules which activate death mediating factors resulting in inflammation[42]. As an aside, necrosis is uncontrolled cell death in response to acute injury and occurs in the absence of regulators or known physiological inhibitors[42]. Pyroptosis ('fiery death') is a controlled death that gives rise to inflammation. It has been associated with caspase-1, a critical component of the inflammasome assembly which induces pyroptosis[43-45]. As such, caspase-1 is regarded as a critical regulator of innate immunity[43].

Follow-on studies by the Liu team, using both animal models of disease and patients with CD, have associated elevated levels of caspase-1 with the epithelial gaps in the intestinal villi[46]. This indicates that as epithelial cells mature, cellular programming can be occasionally diverted from apoptosis to pyroptosis. Not only does this initiate a small scale immune response and

inflammation, the breach in the epithelial barrier allows the translocation of microbes thereby further enhancing the response and escalating the inflammatory cascade.

1.3.3 Do abnormal epithelial cell deaths in Crohn's occur along the length of the gastrointestinal tract?

There have been a number of case reports and series indicating that CD symptoms are present in the oral cavity in both adults and pediatric patients[47, 48]. Unlike the intestinal tract, the oral cavity is a region that is highly accessible for disease surveillance. Indeed a 2010 article described the potential of monitoring oral manifestations in children as a means of stratifying those at risk for developing CD[49]. However, the authors acknowledged that the oral manifestations are frequently subclinical and poorly described due to the overlap with other non-CD oral conditions.

In spite of these barriers, it may be possible to identify a biomarker in the oral tissues associated with CD that will permit ready diagnosis and/or disease monitoring. Indirect monitoring using fecal calprotectin as a biomarker has quickly been adopted[50] although it is unpleasant for the patients to collect the stool samples. An example of such an attempt to identify an oral biomarker was reported by a Swedish study where they found an increase of interleukin-8 in saliva of patients with inflammatory bowel diseases[51]. Another study associated changes in the oral microbiota with inflammatory cytokines, immunoglobulin A, and lysozymes in a similar patient group[52].

In light of the reported inflammation within the mouths of patients with bowel diseases, it is likely that there are changes in the cell turnover rate within the oral mucosal tissues similar to those seen in the intestinal tract.

1.4 Hypotheses

Based upon the research presented regarding CD, I developed two hypotheses:

H1: The prevalence of oral lesions and inflammation in patients with CD are increased relative to healthy counterparts.

Aim: Conduct a systematic literature review of observational studies comparing oral findings in patients with CD relative to the general population.

H2: The pyroptotic activity in the intestinal epithelial cells also occurs within the cells of the oral mucosal.

Aim 1: Determine if caspase-1 activity was present in the oral cavity and identify in which specific tissues the caspase-1 differed between patients with CD and non-CD controls.

Aim 2: Quantify the caspase-1 activity in patients with CD relative to non-CD controls.

Regarding H1, the prevalence of oral CD manifestations is likely underreported or minimized in preference of treating the clinically and potentially life threatening symptoms affecting the intestinal tract resulting in ulceration, bleeding, fistulas, and strictures. To date most of the literature on CD has been generated by gastroenterologists who are unfamiliar with oral anatomy and oral pathology. This may influence the reporting of the incidence and prevalence of oral manifestations of CD.

To prove this hypothesis, my aim was to conduct a systematic literature review adhering to the Meta-analysis of Observational Studies in Epidemiology (MOOSE) guidelines (2008). The review focused on assessing reports of the prevalence of oral findings associated with CD in unselected cohorts (n>50) with or without comparator groups. Analysis of each study involved assessing the background, design and methods (including practitioners involved), and reporting of results, and identification of potential sources of bias. Results are presented in Chapter 3 and represent a standalone manuscript for publication in a peer-reviewed journal.

For H2, my first aim was to conduct a clinical study to identify the location of caspase-1 in the oral cavity tissue layers. My second aim was to quantify the caspase-1 activity. For both objectives, I would use two groups of negative controls.

Briefly, I conducted a prospective, case-control clinical trial that was approved by the Ethics Review Board at the University of Alberta. Buccal mucosa biopsies were collected from 2 non-CD control groups and 1 CD group and assessed via immunohistochemistry and Western blots for evidence of pyroptosis in the form of caspase-1.

Immunohistochemistry describes the process of detecting specific proteins, called antigens, within a thin section of tissue using antibodies that bind specifically to them. Binding is detected by a colorimetric enzymatic reaction indicating presence of the protein of interest. Within the tissue section, one can locate the position of the proteins and the cells involved.

Western blotting is a similar approach although it uses the total protein extracted from lysed tissue samples. Here the bound antibodies can be quantified relative to the total protein of the sample and then compared to other samples or groups. Quantification is based upon fluorescence emitted by bound antigens.

The results of the pilot study are presented in Chapter 3 and represent a standalone manuscript for publication in a peer-reviewed journal.

Chapter 2: Systematic literature review of the oral mucosal lesions associated with Crohn's disease

Review of the oral mucosal lesions associated with Crohn's disease

Running head: Oral lesions in Crohn's disease

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2.1 ABSTRACT

Objectives:

Oral manifestations have long been reported for patients with Crohn's disease, ranging from buccal hyperplasia and angular cheilitis to aphthous ulcers. The aim of this review was to summarize what is known about the incidence and prevalence of oral lesions associated with Crohn's disease to inform dental practitioners and other frontline health care providers.

Methods:

A systematic literature search string was designed to retrieve all articles concerning oral manifestations of Crohn's disease written in English involving humans. Articles were retrieved from PubMed, Medline, Scopus, Web of Science, and Embase literature databases for all years available. The articles were independently reviewed and selected for full text analysis. Data from large-scale studies that reported on consecutively recruited pre-diagnosed Crohn's patients (n≥50) from a single or multiple centers with or without a comparator group. Studies that did not provide oral findings specific for the Crohn's groups were excluded from analysis.

Results:

From 837 retrieved articles, we selected 6 that fulfilled our selection criteria (n=1328 Crohn's, n=737 ulcerative colitis; n=271 non-IBD controls). The most common oral manifestation of was aphthous ulcers; there were no reports of orofacial granulomatosis. The types of oral lesions selected for reporting varied by study. For Crohn's, the incidence of oral lesions ranged from 4% to 34.8%, with aphthous ulcers being the most common. In comparison, the incidence of oral lesions in ulcerative colitis was similar (2-50%) while healthy controls were much lower (1-25%). The study designs, histological findings, and control for potential confounding factors other than smoking (e.g., disease activity, oral fixtures, medications, disease duration, nutritional intake) varied considerably.

Conclusions:

Oral lesions contribute to the low health-related quality of life of patients with Crohn's. This review has identified opportunities to improve the reporting of the oral lesions in unselected

cohorts. This information is best provided by a dental practitioner who will assist in the management of patients with CD.

2.2 INTRODUCTION

In 1969, the first report of oral Crohn's disease was published. As Crohn's disease is known to affect the entire length of the gastrointestinal tract and is commonly termed the 'gums to bums disease', this is not surprising. However, lesions in the oral cavity are many with frequently overlapping macroscopic appearances and histological findings. Many case reports of orofacial granulomatosis have been published since the term was coined by Wiesenfeld in 1985[53]. In 1991, a review of 79 case reports by Plauth *et al.* of patients presenting with oral Crohn's disease reported that granulomas in oral lesions ranged from 67 to 77%. Such lesions were resolved in response to systemic Crohn's treatments, such as steroids and/or azathioprine, an immunosuppressant.

Since Plauth *et al.*, orofacial granulomatosis is now considered a separate disease that may or may not lead to the development of intestinal Crohn's disease[53, 54]. Other potentially overlapping diseases have also been discriminated, such as Behçet's syndrome, HIV AIDS-related infection, and Wegener's granulomatosis[55].

The aim of this review was to summarize what is known about the incidence and prevalence of oral lesions associated with Crohn's disease to inform dental practitioners. This is important as the incidence and prevalence of Crohn's in developed nations is increasing, while other countries adopting a westernized lifestyle have begun reporting incidents of this disease[16].

2.3 METHODS

A search string was designed using terms from the Medical Subject Headings and keywords to search the databases to identify papers examining patients with Crohn's disease ("Crohn" and "Pediatric Crohn's disease") and terms to describe oral lesions: mucogingivitis, cobblestone*, lip swelling, oral mucosal lesions, oral (lesion*, finding*, ulcer*, manifestation*, mucosa, presentation, disease, fistula*), stomatitis, aphthous stomatitis, angular cheilitis, and mouth mucosa. The following databases were searched for all years available on July 10, 2016: PubMed, Medline, Scopus, Web of Science, and Embase.

Two authors independently reviewed the titles and abstracts of the retrieved articles to identify those that might fulfill the selection criteria: original research articles, oral lesion incidence and/or prevalence presented for Crohn's patients (n≥50), and consecutive recruitment of unselected patients diagnosed previously with Crohn's disease (biopsy and/or endoscopy confirmation). Studies that combined oral lesion data for Crohn's with ulcerative colitis patients were excluded. There was no disagreement regarding the selection of eligible studies for inclusion. Data abstraction was completed adhering to the guidelines for meta-analysis of observational studies in epidemiology[56]. Selected articles were assessed for the quality of reporting according to the Newcastle-Ottawa Scale[57]. In light of the variability in the study designs and reporting, a meta-analysis was not possible.

2.4 RESULTS

The search strategy retrieved a total of 837 articles of which 6 were selected and abstracted for analysis (Fig. 3-1). A moderator was not required to resolve any disagreements regarding article selection at any point throughout the process. The characteristics of the selected studies are presented in Table 3-1. In total, the studies included 1,328 patients with Crohn's disease and two comparator groups: ulcerative colitis (n=737) and health controls (n=271). It should be noted that none of the healthy controls underwent colonoscopy with biopsy to confirm that they were free of gastrointestinal disease.

Figure 2-1. Systematic literature search and selection process overview

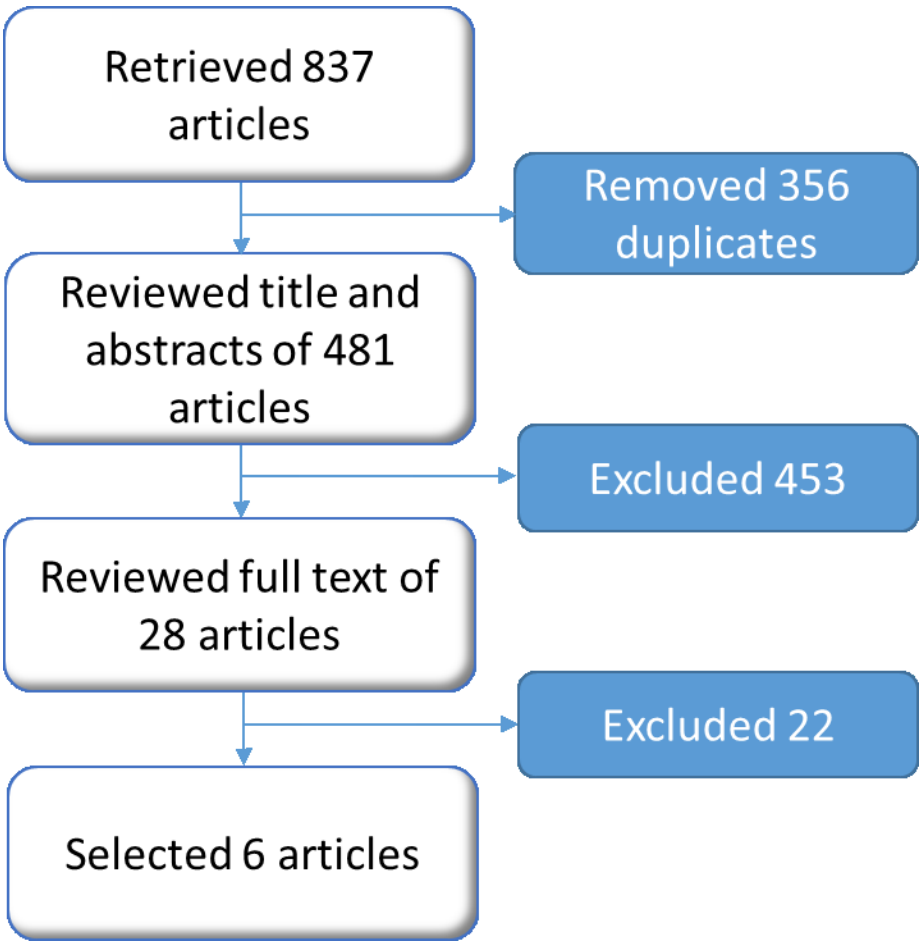

Table 2-1. Study characteristics

Author Country Year	CD (n) Mean age (SD) in yrs	UC (n) Mean age (SD) in yrs	Controls Mean age (SD) in yrs	Design	NOS
Basu[26] England 1975	N=100 Age-matched Male 38%	N=100 Age-matched Male: 42%	N=100 Age-matched 38%	P	***
Greenstein[58] United States	N=498 - -	N=202 - -	- - -	R 1964-1973	**

Author Country Year	CD (n) Mean age (SD) in yrs	UC (n) Mean age (SD) in yrs	Controls Mean age (SD) in yrs	Design	NOS
1976					
Veloso[59] Portugal 1996	N=449 29.4 yrs (10-76) Male 43.9%	N=343 36.4 yrs (11-71) Male: 49.3%	- - -	P 1975- 1994♦	***
Stein[60] Germany 2010	N=147 36.6 yrs (9.9) Male 23% Smoker 37.4%	- - -	- - -	P	**
Varicka[61] Switzerland 2013	N=69 39.6 yrs (12.1) Male 53.6% Smoker 30.4%	N=44 42.3 yrs (14.9) Male 28% Smoker 4.5%	N=113 41.7 yrs (16.0) Male 51.3% Smoker 18.6%	P	***
Laranjeira[62] Portugal 2015	N=65 41.1 yrs (15.2) Male 50% Smoker 20%	N=48 49.2 yrs (18.4) Male 52.1% Smoker 12.5%	N=58 47.4 yrs (16.3) Male 48.3% Smoker 10.3%	P	**

Abbreviations: P, prospective study; R, retrospective study; SD, standard deviation

♦ Mean (range) follow-up of each patient was 4.5 yrs (0.5-20).

The most common reason for the exclusion of large cohort studies was because the data for patients with Crohn's disease was combined with ulcerative colitis and/or indeterminate colitis. Although both of these latter diseases fall within the inflammatory bowel diseases umbrella, the focus of this review was specifically on Crohn's. The second most common reason for excluding studies was due to the sample size falling below 50. The rationale behind excluding studies recruiting less that 50 patients was because it was believed that they were not representative of the larger Crohn's community of patients in consideration of the prevalence of the disease[14].

Variability in the reporting study characteristics, study design, disease duration, and disease activity was evident. The mean age range of those with Crohn's was 29.4 years to 41.1 years which is consistent with the early age of onset, typically in late adolescence and early adulthood. The origin of these studies, Europe and North America, is also consistent with global incidence and prevalence data[14]. Smoking rates were similar among the studies for Crohn's patients and the rates were much higher than reported for either ulcerative colitis or controls. Only one study provided prevalence data over a period of 20 years[59], while 4 studies provided incidence data of oral manifestations based upon a single oral exam[26, 60-62], and one reported incidence data collected over 10 years[58].

There was much variation regarding the reporting of disease activity in the Crohn's patients and pharmacotherapy. Only Vavricka and colleagues provided an indicator of disease activity at the time of oral exam using a standard clinical evaluation tool, the Harvey-Bradshaw Index[61]. Laranjeira *et al.* stated that they assessed Crohn's disease activity but did not provide outcomes nor indicate how activity was measured[62]. None of the studies conducted nor reported endoscopic findings at or near the time of oral exam as the gold standard method of assessing disease activity in Crohn's. Only two studies indicated pharmacotherapy data and illustrated that the majority of patients with Crohn's are taking IBD-related medications on a routine basis. Of note, is that only one study controlled for dentures as these and other oral devices like bracers, retainers, and bruxism appliances have the potential to cause oral lesions.

Table 2-2. Characteristics of the study groups

Author **Country** **Year**	**CD (n)** **Disease activity** **Pharmacotherapy**	**UC (n)** **Disease activity** **Pharmacotherapy**	**Control (n)** **Pharmacotherapy**
Stein[60] Germany 2010	N=147 Steroids 42.2% Immuno 47.6% 5ASA 32.7%	- - -	- - -
Varicka[61]	N=69	N=44	N=113

Author Country Year	CD (n) Disease activity Pharmacotherapy	UC (n) Disease activity Pharmacotherapy	Control (n) Pharmacotherapy
Switzerland 2013	**HBI 4.0±4.3** Steroids 17.4% 5ASA 11.6% Immuno 24.6% Cic/Tacr 0% Biologics 52.2% NSAIDs 4.3%	**CAI 3.7±3.1** Steroids 27.3% 5ASA 65.9% Immuno 29.5% Cic/Tacr 6.8% Biologics 20.5% NSAIDs 0.0%	 Steroids 0% 5ASA 0% Immuno 0% Cic/Tacr 0% Biologics 0% NSAIDs 3.5%
Laranjeira[62] Portugal 2015	N=65 13.8% active disease	N=48 16.7% active disease	N=58

Abbreviations: HBI, Harvey-Bradshaw Index; CAI, colitis activity index; steroids, corticosteroids; 5ASA, aminosalicylates; Immuno, immunosuppressants including 6-mercaptopurine, azathioprine, and methotrexate; Cic/Tacr, cyclosporin and tacrolimus; Biologics, biologic agents including infliximab and adalimumab; NSAIDs, nonsteroidal anti-inflammatories

The most common oral manifestation in Crohn's was aphthous ulcers (Table 3-3); however, Basu *et al.* specifically excluded reporting this data[26]. Comparing Crohn's with ulcerative colitis, there was no significant difference in the incidence or prevalence of oral lesions. Patients with Crohn's disease have a much higher incidence and prevalence of oral lesions relative to control groups. Only one study provided histology results[26] while another study was unclear if a surgeon or dental practitioner completed the oral exam[58].

Smoking rates, pharmacotherapy, and disease duration were not significantly associated with oral lesions. However patients with active Crohn's disease were found to have a higher incidence of oral lesions, in particular, aphthous lesions.

Table 2-3. Results from selected studies

Author Year	Oral mucosal lesions & type			Macroscopic findings	Histology results	Comments
	CD	UC	Control			
Basu[26] 1975	9%	2%	1%	Cobblestone, ulcers were deep & linear	Inflammation in epithelium & lamina propria	*Excluded aphthous ulcers Oral lesions associated with active disease
Greenstein[58] 1976	4%	4%	-	Aphthous stomatitis; no granulomatous lesions	-	NSD between oral lesions & variables
Veloso[59] 1996	11.1% Aphthous ulcers	9.9% Aphthous ulcers	-	Aphthous ulcers, 2 CD pts also had fissural mouth ulcers	-	NSD between oral lesions & variables
Stein[60] 2010	36.7%	-	-	Buccal hyperplasia 20.4%; gingival hyperplasia 27.2%; aphthous ulcers 4.1%; leukoplakia 2.0%; lichen planus oralis 2.7%; candidiasis 3.4%	-	Active CD & oral lesions in only 10.2% of patients. NSD with pharmaco-therapy or smoking

Author	Oral mucosal lesions & type			Macroscopic	Histology	Comments
Vavricka[61] 2013	34.8%	50.0%	24.8%	Aphthous lesions, leukoplakias, mucosal tags, angular cheilitis, glossitis	-	NSD between oral lesions & smoking, pharmaco-therapy or disease activity
Laranjeira[62] 2015	10.8%	11.4%	3.4% Aphthous ulcer, gingival swelling n=1 each	Aphthous ulcers, gingival swelling, angular cheilitis	-	Active IBD inc. oral lesions 35.3% vs 4.2%, P<0.001. NSD b/w oral lesions & pharmaco-therapy, smoking, or disease duration

Abbreviations: NSD, no significant difference

2.5 DISCUSSION

The incidence of oral lesions in patients with Crohn's disease is elevated relative to healthy controls, but is similar to those with ulcerative colitis. The most commonly found oral lesion is the aphthous ulcer. Gingival swelling was the second most common lesion. Active disease was associated with increased incidence of aphthous ulcers. Orofacial granulomatosis was not reported in any of these studies.

The development of aphthous ulcers is primarily attributed to nutrient and/or mineral dietary deficiencies. During periods of active Crohn's disease, achieving adequate daily nutrition is often overlooked by patients and this may be the underlying cause for aphthous ulcers. However, Basu *et al.* determined that the lesions were not attributed to deficiencies in folate, vitamin B12, or iron based upon serum levels[63].

The review informed what oral lesions are most commonly encountered in Crohn's as well as rare manifestations. However, due to concerns with study design, identification of potential confounders, and assessment of Crohn's disease activity, there is still opportunity to investigate this topic further and make use of improved study designs with collaboration between gastroenterology and dentistry and oral pathology.

Identifying an oral biomarker to monitor CD activity would be of great benefit to both the patients and the medical communities. Dental practitioner offices are located within communities unlike gastroenterologists which are primarily found in major city centers or academic institutes. The widespread community-oriented dental offices can accommodate frequent patient visits to perform oral cavity brush biopsies or less frequent buccal mucosal biopsies. Dental practitioners are skilled in performing oral exams and identifying abnormal mucosal lesions. Linkage between the dental offices and the gastroenterologists can be easily facilitated for routine reporting of oral features and oral biomarker levels. In this way, the dental practitioner will be able to participate in disease surveillance and monitoring of disease activity and provide assistance in identification of early loss of drug response. Routine oral examinations are likely appreciated more than providing routine stool samples by patients.

Chapter 3: Intraoral caspase levels – an experimental study

Title (120 characters with spaces, currently 91):

Caspase-1 levels in the oral mucosa are elevated in patients with quiescent Crohn's disease

Short title (45 characters with spaces, currently 40)**:**

Intraoral caspase-1 activity in Crohn's

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Abbreviations: CD, Crohn's disease; CRP, C-reactive protein; DAB, 3,3'-diaminobenzedine; LOI, localized oral inflammation; SES-CD, Simple Endoscopic Score – Crohn's disease

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Disclosures: The authors have nothing to disclose.

Author contributions: JL and RT-P were responsible for the concept and design for this study. All authors were involved in the acquisition and analysis of the data. Statistical analyses were completed by ME. KI drafted the manuscript and all were involved in critical revisions and review prior to submission.

3.1 ABSTRACT

Background & Aims:

It is well documented that Crohn's disease (CD) manifests throughout the length of the digestive tract including the oral cavity. The aim was to determine if there were increased levels of caspase-1 in the oral mucosa of patients with CD to corroborate similar findings in intestinal epithelial cells of patients with inflammatory bowel diseases.

Methods:

We prospectively recruited adults who attended the School of Dentistry clinic and categorized them based upon their clinical status: healthy controls with no evidence of oral abnormalities; those with localized inflammation adjacent to a tooth receiving treatment; and patients with histology-proven CD in remission who had no macroscopic oral mucosal abnormalities. A medical chart review retrieved health data including medications, smoking status, and demographics. A 0.5 cm^3 biopsy was taken from healthy buccal mucosa and processed for immunohistochemistry and western blot analysis to identify caspase-1 and caspase-3. Tissue sections were scored by two independent, blinded reviewers.

Results:

Caspase-1 binding was higher in the lamina propria in CD group compared to either of the control groups. From the western blot, the mean activated caspase-1 densitometry values were significantly higher in the CD group [0.498 ± 0.238 (SD)] versus the control [0.199 ± 0.177] and LOI [0.159 ± 0.134], with *P*-values of 0.004 and 0.001, respectively. No adverse events were reported following the biopsy.

Conclusions:

Caspase-1, but not caspase-3 levels, are increased in healthy looking buccal mucosal tissues in patients with quiescent CD, specifically within the lamina propria. Future work will assess the feasibility of monitoring CD activity in the oral tissues and association of caspase-1 levels with fecal calprotectin and endoscopy reports.

Keywords (3-4)**:** Pyroptosis; Inflammatory Bowel Diseases; Inflammation.

3.2 Introduction

Crohn's disease (CD) is a member of the autoimmune disease family that causes debilitating gastrointestinal symptoms and is associated with dysregulated immune functions leading to chronic inflammation. Recent investigations by Liu and colleagues have identified abnormal lifecycle turnover rates in the epithelial cells of villi located in the small intestine thereby increasing the number of "gaps" at any one time across the epithelial barrier[41, 64, 65]. Such gaps are believed to be access routes whereby contents of the intestinal lumen (e.g., microbiota) and elements characteristically found adherent to the epithelial/mucosal barrier can travel through arriving within the intestinal tissues. Once inside, the immune system is triggered creating a cascade of localized inflammation, including tissue necrosis[65]. During periods of active disease, damage can be clinically seen as ulcerations, bleeding, and/or fistulas. The findings of Liu provide support the hypothesis of the "altered mucosal barrier" whereby the contents of the intestinal lumen are able to cross into the host's body and activate the immune system giving rise to chronic inflammation[41, 46, 64].

The rapid turnover of the epithelial cells has been associated with an unusual mode of cell death, pyroptosis in contrast to the typical controlled process of apoptosis[43, 45, 66]. Further, it has been demonstrated that caspase-1 is critical to initiate the fiery, uncontrolled pyroptosis events[43, 45, 66].

As the gastrointestinal tract is continuous from 'gums-to-bums' and CD symptoms have been reported in the oral cavity, we sought to determine if the elevated levels of caspase-1 were also present within the oral mucosal epithelial cells. Previous studies reported increased levels of inflammatory cytokines in the oral tissues of patients with inflammatory bowel diseases[67, 68]. However, there was no investigation into the levels of the caspase enzymes which are known to

be associated with the signaling cascade leading to cell death. This also provided an opportunity to assess the feasibility of using oral tissues for monitoring CD activity in an effort to reduce the procedural burden of endoscopy on patients as well as reduce the economic burden on health care systems.

3.3 METHODS

3.3.1 Study Patients

Adults (18-75 years), attending the School of Dentistry's clinic at the University of Alberta, Edmonton, AB, indicated as Class I or II according to the Anesthesia Physical Classification System (ASA), for dental extraction due to non-restorable teeth were prospectively enrolled. Additionally, the clinic's dental records were searched to identify individuals with biopsy-proven CD who were invited to participate in the study providing that their CD was in a quiescent phase (registration with ClinicalTrials.gov is in process; Human Ethics Research Review Board at the University of Alberta approval: Pro00024154). Exclusion criteria were any oral conditions other than the dental extraction, presence or history of oral lesions in the past 12 months, previous history of heart disease, active CD, pregnant or nursing women, or inability to provide informed written consent. Enrolled participants were grouped according to their health status: CD, a previous diagnosis of Crohn's disease; C, healthy controls with unremarkable oral findings; and LOI, healthy controls with localized oral inflammation, such as pericornitis around the tooth scheduled for extraction. Demographic information was collected for each participant and smoking status. For those with CD, a medical chart review was conducted to identify the disease location and severity from endoscopy reports, disease duration, medications, and C-reactive protein (CRP) levels near to the date of the oral biopsy.

3.3.2 Oral Biopsies

An oral surgeon (RT-P) obtained one oral biopsy (0.5 cm^3) comprised of the stratified squamous epithelium and the lamina propria from healthy looking buccal mucosa of each participant. Local anesthesia was administered for pain control and the surgical site was rinsed with disinfectant prior to and after the biopsy procedure. The biopsy was immediately transferred to a 1.5 ml Eppendorf, containing 1X phosphate-buffered saline solution (PBS) at pH 7.4 (Thermo Fisher Scientific Inc., Toronto, ON) and stored on ice. In the lab, the biopsy was rinsed carefully then processed for future protein work (tissue flash frozen in liquid nitrogen and stored at -80^oC) and histology (fixed in 10% formalin for 48 hours for paraffin embedment beginning with 70% ethanol). Embedded tissue blocks were sectioned (5 μm) and ribbons were transferred onto glass slides then outlined with a hydrophobic barrier Immedge pen (Vector Laboratories, Burlingame, CA).

3.3.3 Immunohistochemistry

This was performed according to manufacturer's protocol (Cell Signaling Technology, Inc., Danvers, MA). Briefly, following rehydration, the sections were immersed in 1X SignalStain Citrate Unmasking Solution (#14746) for 10 min at 95-98^oC then rinsed. Blocking of endogenous peroxidases was done over 10 min with 3% hydrogen peroxide. The 1X Animal-Free Blocking Solution (#15019) was added for 1 hour to block non-specific proteins. The primary antibody, caspase-1 (#2225), was diluted in SignalStain Antibody Diluent (#8112). Slides were incubated overnight at 4^oC, washed in 1X Tris Buffered Saline with Tween 20 (#9997), then incubated at room temperature for 30 min with SignalStain Boost Detection Reagent (HRP, Rabbit #8114). After washing in buffer, the chromogen substrate (3,3'-diaminobenzidine, DAB) was added (SignalStain® DAB Substrate Kit, #8059). Sections were counterstained with hematoxylin (#14166) followed by Scott's Bluing Agent (Thermo Fisher

Scientific). Sections were mounted in Paramount (mounting medium, Dako) with coverslips. Light microscopy scoring for caspase-1 was performed in a blinded fashion (ME, EP) according to the color intensity of DAB: 0-none; 1-mild; and 2-intense. Sections were also scored for the presence of inflammatory cells in the epithelium and lamina propria: 0-absent or 1-present. Negative controls were sections that had been similarly processed but without exposure to the primary antibody and were scored in parallel.

3.3.4 Western Blotting

Frozen tissues were thawed on ice then lysed with mammalian protein extraction kit (m-PER) according to the manufacturer's protocol (Thermo Fisher Scientific). Protein quantification was completed with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and plate reader (Tecan US, Inc, Morrisville, NC). Total lysates (50 µg normalized for each sample) were separated on a 15% sodium dodecyl sulfate-polyacrylamide gel. Following transfer, the nitrocellulose membranes were blocked with Odyssey Blocking Buffer (#927-40000 by LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature. The membranes were incubated overnight at 4^{o}C with either the caspase-1 or caspase-3 antibody (#2225 and #9662, respectively, Cell Signaling Technology, Inc.) diluted in Odyssey Blocking Buffer. The ß-actin antibody (#4967) served as the normalization control. After washing, membranes were incubated with the fluorescent secondary antibodies (IRDye 800CW donkey anti-rabbit and IRDye 680 goat anti-mouse) for 1 hour at room temperature and analyzed by the LI-COR Odyssey (InfraRed Imaging System). The signals were quantified by the ImageJ software.

3.3.5 Sample Size

As there was no data on caspase staining in the buccal mucosa, the sample size was based upon the study completion rate. To allow for a statistical power of at least 0.9 and an alpha of 0.05, a

total of 30 patients (n=10 per group) were needed. The 90% completion rate estimate was to accommodate instances where a participant requested to be removed from the study after the biopsy was collected.

3.3.6 Statistical Analysis

Results are presented as mean ± standard deviation (SD). For light microscopy LOI scoring only, inter-rater reliability was calculated by Cohen's Kappa with the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL). For densitometry values, one-way ANOVA was used to compare the groups. Indication of significance was set at ≤0.05 for the two-sided *P* values.

3.4 RESULTS

A total of 45 participants were prospectively enrolled in the study. Biopsies and supporting information were collected for 27 participants as 18 were subsequently excluded due to other oral findings at the time of biopsy or decision not to undergo the biopsy (Table 3-1). The mean age of the CD group was significantly older than the two control groups. No adverse events occurred following the biopsy procedure. Participants in the CD group were in remission defined by a Simple Endoscopic Score-Crohn's Disease (SES-CD) of 0 to 2 based upon their endoscopic reports near the time of biopsy. The CRP levels also indicated that their disease was in remission.

Table 3-1: Participant characteristics at or near time of oral mucosal biopsy

Characteristics	**Controls**	**Localized oral inflammation (LOI)**	**Crohn's disease (CD)**
Number of participants	9	9	9
Age (yrs), mean ± SD	39.3±12.7	34.8±13.9	53.9±15.3
Male (%)	4 (44.4)	5 (55.5)	4 (44.4)
Smoker (%)	3 (33.3)	2 (22.2)	1 (11.1)
CD duration in yrs, median (IQR)			15 (14.5-22)
Ileal disease (%)			5 (55.5)

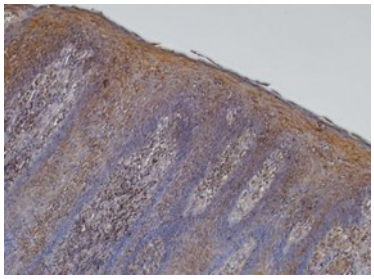
Ileo-colonic disease (%)		3 (33.3)
Colonic disease (%)		1 (11.1)
Remission SES-CD 0-2 (%)		9 (100)
No medications (%)		4 (44.4)
Aminosalicylate (%)		3 (33.3)
Azathioprine (%)		1 (11.1)
Biologic agents (%)		2 (22.2)
CRP as mg/l, median (IQR)		3.3 (1.9-11.3)

3.4.1 Immunohistochemistry

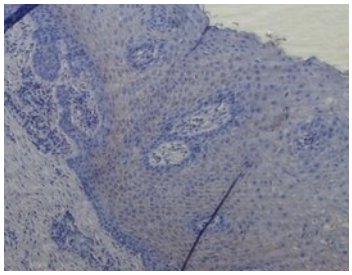
Results of the immunohistochemistry procedure shows the presence of caspase-1 in the stratified squamous epithelial cell layers of the oral mucosa in the healthy controls (Figure 3-1). Staining was more prevalent for those with localized oral inflammation as anticipated. All mucosal layers (epithelium and lamina propria) in Crohn's disease tissue sections stained positively for caspase-1.

Figure 3-1. Caspase-1 presence in oral mucosal layers

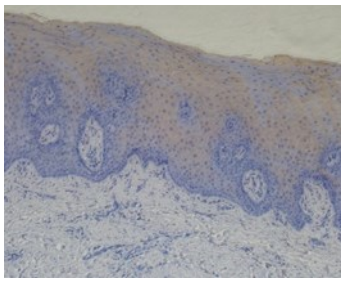
All images at 40X magnification.



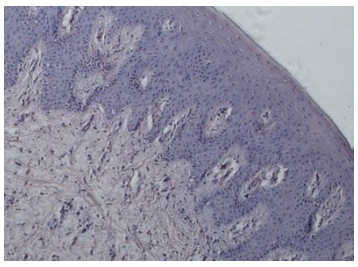
Crohn's Disease



Healthy control



Localized oral inflammation (LOI) (Controls)



Secondary antibody-only control (Negative Control)

Within the stained sections, evidence of cellular inflammation was investigated by both of the blinded scorers independently. Figure 3-2 shows an inflamed cell (arrow) surrounded by infiltrate. This section was from a patient with CD.

Figure 3-2. Inflamed cell (presence of inflammatory cellular infiltrate)

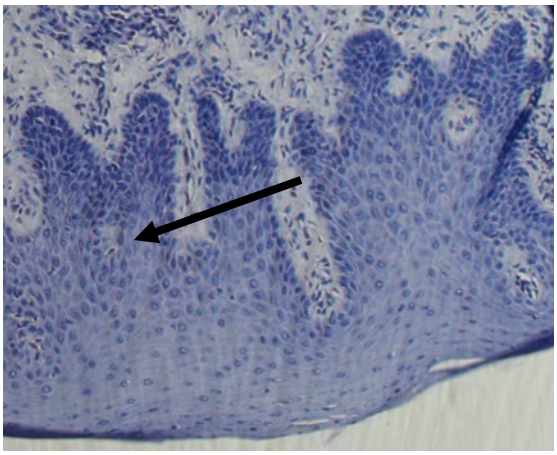


Table 3-2 presents the results from the blinded independent scoring of caspase-1 tissues sections for both inflammation and caspase-1 intensity. The inter-rater reliability agreement of the scoring of the stained sections was κ 0.835. This indicates that there was substantial agreement between the two raters. Inflammation indicated by the presence of inflammatory cellular infiltrate was found through the lamina propria and epithelial cell layers in patients with CD. Surprisingly, the LOI group did not have prevalent inflammation in the epithelial layer and only a moderate amount in the lamina propria. Caspase-1 activity was evident in the epithelial layers of all groups. However, only moderate activity was identified in the lamina propria tissues of the CD group this corresponds to the prevalence of inflammation in this cell layer.

Table 3-2. Presence of inflamed cells and caspase-1 staining

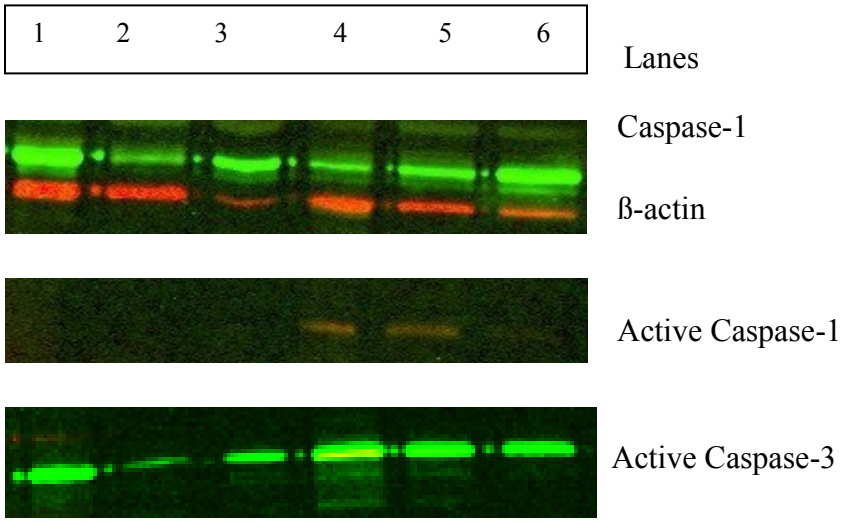
Study group	Lamina propria		Epithelium	
	None	Inflammation	None	Inflammation
Crohn's disease	0.0%	100.0%	22.20%	77.8%
Controls	66.7%	33.3%	77.80%	22.2%
Localized oral inflammation	88.9%	11.1%	77.80%	22.2%

Cell layer • Study group	Caspase-1 staining results		
	None (0)	**Moderate (1)**	**Extensive (2)**
Lamina Propria			
• Crohn's disease	66.7%	33.3%	0%
• Control	100.0%	0%	0%
• Localized oral inflammation	100.0%	0%	0%
Epithelium			
• Crohn's disease	44.4%	33.3%	22.2%
• Control	22.2%	66.7%	11.1%
• Localized oral inflammation	33.3%	66.7%	0%

3.4.2 Westerns

Western blot analysis of caspase protein expression in human oral mucosa tissue (Figure 3-3). After quantification and normalization with b-actin, between and within group comparisons for caspase-1 found that there were significant differences (P=0.002) but not for caspase-3 (P=0.216) (Figure 3-4). Our findings indicate that caspase-1 but not caspase-3 activity is elevated during quiescent CD. Local oral inflammation does not increase caspase-1 activity in comparison with healthy controls. Caspase-1 but not caspase-3 activity can discriminate between patients with Crohn's disease and controls.

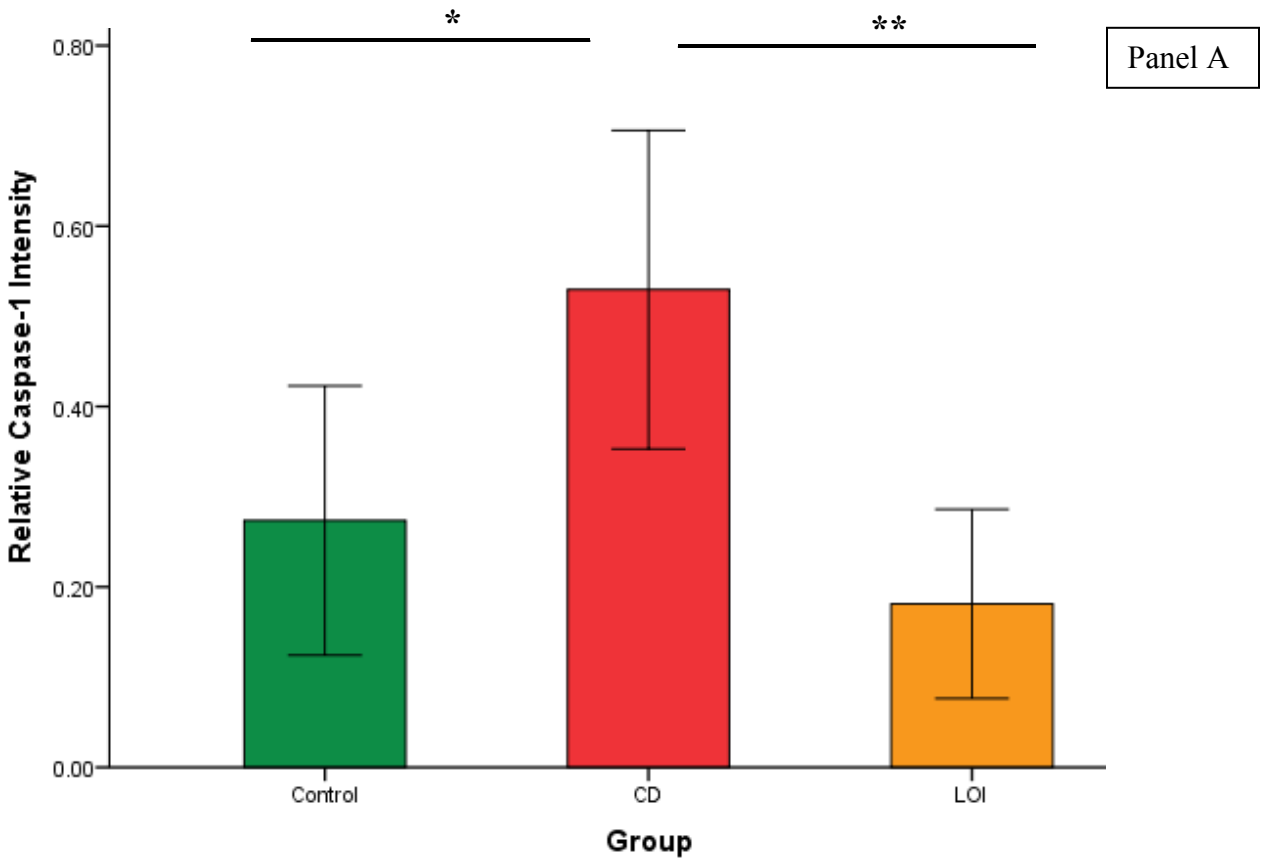
Figure 3-3. Western blot results for caspases-1 and -3

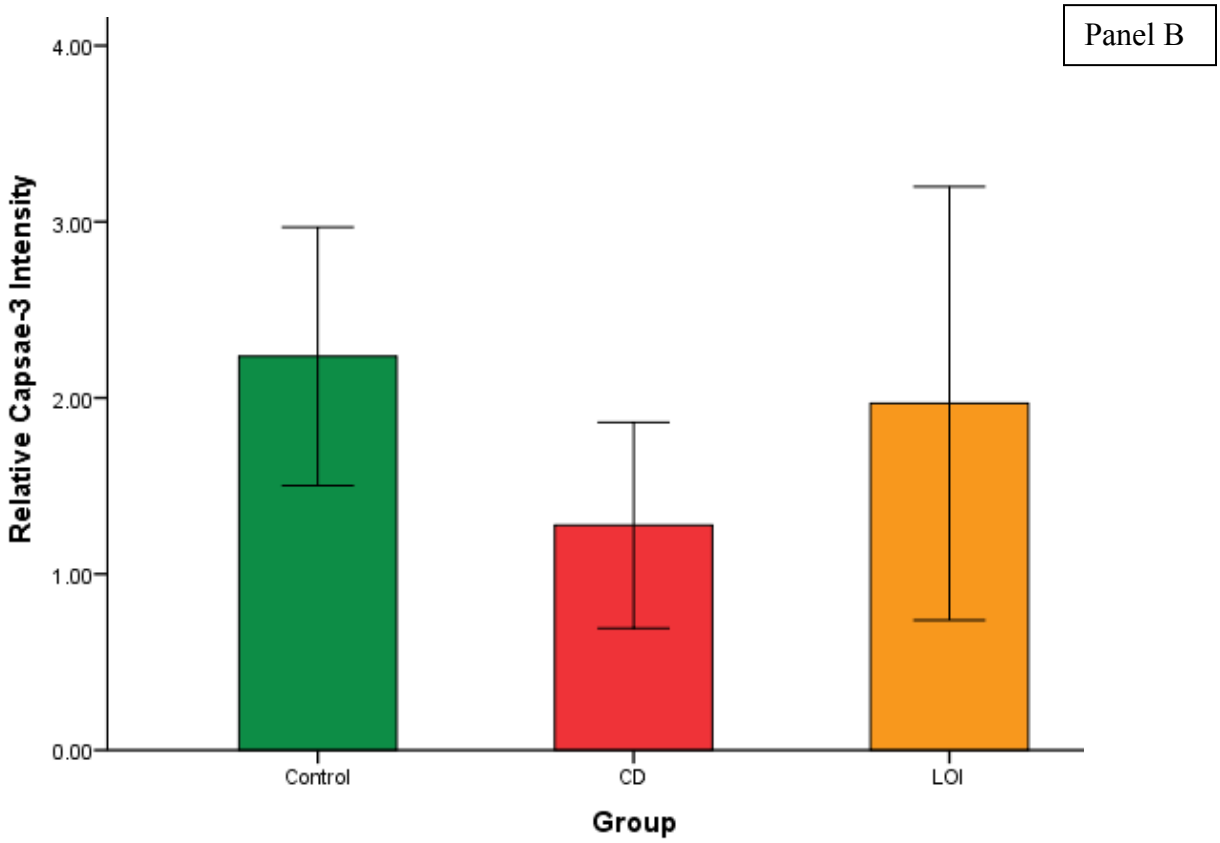


Legend: Lanes 1-3, cell lysates from LOI, lanes 4 and 5 from CD, and lane 6 control.

Figure 3-4. Quantification of caspase-1 and caspase-3

Panel A: Caspase-1 activity in each study group. Panel B: Caspase-3 per study group. * $P<0.05$; ** $P<0.001$.

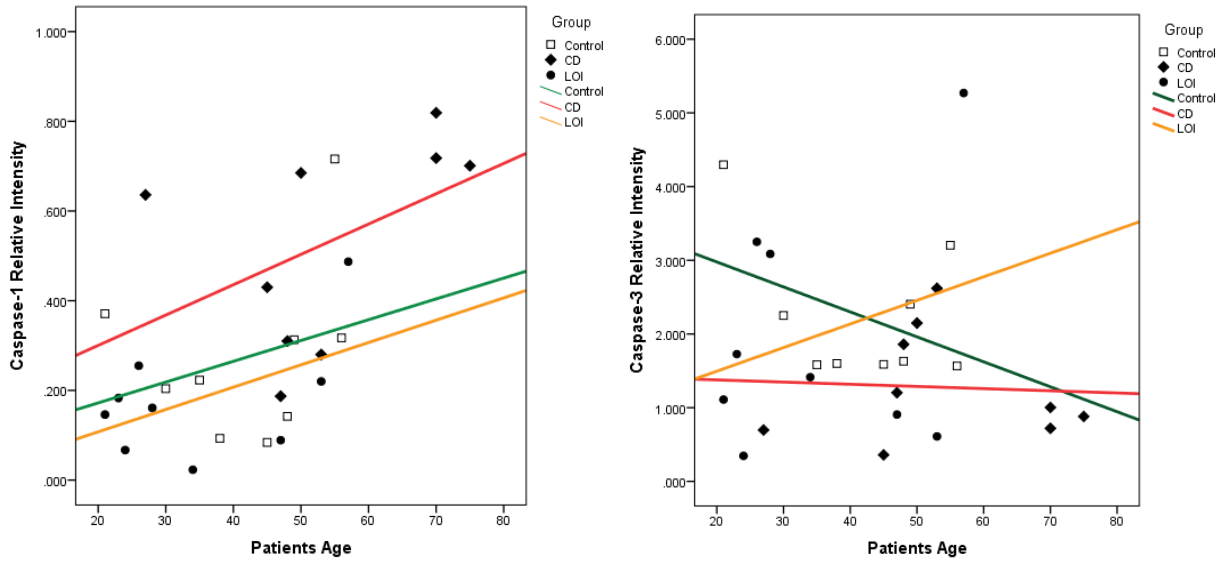




3.4.3 Modeling caspase activity

Given the difference in mean age of the three study groups, regression analyses were completed. The correlation coefficient values for caspases-1 and -3 vary with age and can be used to discriminate between CD and the two control groups (Figure 3-5). For caspases-1 and -3 the coefficient values for: CD: 0.450 and -0.059; controls: 0.281 and -0.418; and LOI 0.507 and 0.278.

Figure 3-5. Correlations between age and caspase activity



3.5 DISCUSSION

For this study we investigated the levels of caspase-1 enzyme in the oral mucosa of CD patients in comparison with two control groups. Our selection criteria for patients was one of the major strengths of our study. The CD patients selected to participate in the study were in remission and were without oral lesions, enabling the biopsy of healthy appearing tissues. The ability to detect cellular changes in CD patients with no presenting symptoms or macroscopic lesions sets the stage for both dental and medical practitioners to readily monitor the disease and its response to IBD-related therapy without endoscopy. Our results also revealed several important findings regarding the use of the oral mucosa as a potential biomarker for CD.

The experiments performed in this study showed that caspase-1-mediated cell shedding could be observed in the oral mucosa of CD, control, and LOI groups, consistent with the previous analysis of intestinal tissue of IBD patients[46] , as well as another article which has reported that caspase-1 activity is increased in cells isolated from inflammatory lesions in CD compared with immune cells from normal intestinal tissue[41, 64]. Evidence of increased caspase-1 activity was identified in the intestinal tissues and in macrophages from patients with both CD and ulcerative colitis[67, 68].

Macroscopic examination is suitable only for the detection of oral lesions such as aphthous ulcers are a measure of intestinal disease activity in CD. This is evidence by its inclusion in the Harvey-Bradshaw Index, commonly used as a tool to assess CD activity based on subjective, clinical parameters. CD is a transmural disease, so it is without surprise that we noted that the CD group had a higher percentage of presence of inflammatory cells in both lamina propria (100%) and epithelial layer (50%) compared to the control and LOI groups.

From the western blot analysis, the mean activated caspase-1 densitometry values were significantly higher in the CD group [0.498 ± 0.238 (SD)] versus the control [0.199 ± 0.177] and LOI [0.159 ± 0.134], with *P*-values of 0.004 and 0.001, respectively. This demonstrates that the cell death in the oral cavity of CD patients is pyroptosis-mediated, not apoptosis.

In addition, quantitative analysis of the caspase-3 data revealed a dramatic loss of caspase-3 protein expression in the CD group [1.219 ± 0.739 (SD)] compared with control [2.133 ± 0.738] and LOI [2.581 ± 1.483] groups, with a statistically significant difference (p-value 0.006 and 0.003, respectively). This provides further evidence pointing to the role of pyroptosis as a prominent cell death mechanism in the oral mucosa.

Surprisingly, oral epithelium staining scores for caspase-1 in CD slides were similar to the control and LOI slides, being in contrast to the histological findings in IBD intestinal mucosa previously described[20]. Such discrepancies may be due to differences in the histological structure of oral and intestinal epithelium. Meanwhile, the presence of staining of caspase-1 in the lamina propria of CD slides only and not in the control and LOI slides is still indicative of a difference of staining distribution between CD and non-CD slides. This distribution of staining throughout the mucosa could be due to the fact that it is believed that in IBD patients there is an increased barrier dysfunction and compromised epithelial integrity as previously reported[46, 69].

The potential use of the oral cavity as a possible adjunctive diagnostic tool for CD is very promising. Used in combination with traditional measures, such as blood work and comprehensive medical exam with family history, could improve who the diagnostic process with only those individuals who have elevated oral caspase-1 levels referred for endoscopy. This would improve patient care by quickly ruling out a potential diagnosis of CD and potentially

reduce the number of colonoscopies with biopsies thereby providing a reduction on health care resources.

With the advent of biologics, gastroenterologists are seeking alternatives to endoscopy for monitoring response to treatment in consideration of the cost burden of the treatments. Although endoscopy is useful for direct visualization and confirmation of mucosal healing via biopsy, it is an invasive procedure associated with health risks, expense, and burden on the patient[16]. Disease surveillance using a biomarker readily accessible in the oral cavity and obtainable by dentists is highly desirable, cost effective, and reduces the resource use of endoscopy suites and staff.

Chapter 4: Concluding Remarks

4.1 Future directions

Although small in scale, the literature review regarding oral mucosal lesions common in CD and the experiments illustrating the presence of pyroptotic events occurring in the oral tissues advances our understanding of the potential for oral diagnostics.

Long recognized as being involved in CD symptoms, the oral cavity has been largely ignored by gastroenterologists in preference for direct visualization of the large bowel via endoscopy. With the recent introduction of double balloon enteroscopy for visualization of the small bowel, the true scope of diseased tissue is only now being appreciated. However, enteroscopy is not without its risks to patients, such as intestinal wall perforation and is not recommended for all patients. Similarly capsule endoscopy, swallowing a pill containing a camera, provides an alternative means to visualize the entire gastrointestinal system but has limited value as the capsule does get trapped in tissues and strictures requiring surgical intervention for retrieval. Other imaging modalities (e.g., ultrasound and computed tomography) have yet to be accepted as a means for monitoring disease activity.

Companion diagnostics, tests that assess a health status in response to a treatment, are very attractive for the management of CD patients. If a patient is in the process of becoming unresponsive to therapy and is at risk of having a disease flare, the practitioner can enhance the therapy by increasing the frequency or dosing of the medication or even prescribing a more potent medication. The therapeutic goal is to avoid the exacerbation of the disease or at least minimize its duration and severity.

Switching from direct monitoring of the intestinal tract for evidence of mucosal healing to assessing the oral mucosa is very attractive from a cost perspective ($750 per endoscopy) and the patient's perspective as the bowel cleansing regiment is unpleasant and requires a full day to complete. Here, we relied upon assessing buccal mucosa biopsies of a considerable size (0.5 cm^3) requiring the participation of a dental surgeon, we believe that there will be other options by which to assess caspase-1 activity and quantify pyroptotic events in the oral tissues.

Here, we focused on CD patients with endoscopy-confirmed quiescent disease. Future work will need to be completed to compare against other disease states and disease activity

indices (e.g., Crohn's Disease Activity Index, CDAI) for universal adoption. Correlation with biomarkers currently being assessed for monitoring disease activity will also have to be undertaken. Fecal calprotectin requires weekly collection of stool samples by the patient and shipment to medical diagnostic laboratories.

Further investigation will provide additional insights on the inflammatory process occurring in regards to the layers of the mucosa as well as the cells undergoing the inflammatory process, which can be accomplished by looking at specific layers using salt split technique, which has been described in evaluation of subepidermal blistering disorders, and identifying which layer of the tissue is undergoing inflammation.

The use of a less invasive technique, such as brush biopsy, cheek swabs, and saliva samples would definitely be more favorable to the patients. Ultimately, predictive markers should be accessible early on, and should be generally available as well as easy and quick to analyze.

Nevertheless, data available of histological data about activity of CD are limited. Several clinical trials have shown that treatment with different drugs can alter the histology of the mucosa, promoting healing and normalization of the mucosa[1-6].

4.2 Limitations

There are several potential limitations to our study. Given the relative small sample size in the three groups, these results should be interpreted with caution. Furthermore, our study included only patients with known CD; therefore, it does not answer the question whether caspase-1 levels should be included in the diagnostic workup of patients complaining of abdominal symptoms (diarrhea, bloating, and lower abdominal pain). It is also important to point out that while CD patients have a higher level of caspase-1 densitometry levels, they certainly cannot replace endoscopic procedures in patients with no previous diagnosis. Another limitation was the absence of positive controls, due to the fact that we could not obtain them for the immunohistochemical staining, which restrained our ability to make solid conclusions regarding the histology scoring results.

Furthermore, quantification of the stained slides by visual scoring is sometimes controversial, as different raters have different interpretations.

4.3 Role of dental practitioners

Patients with CD are at increased risk for developing dental caries, oral infections, and oral mucosal conditions, including lesions and ulcerations[61]. As with other autoimmune diseases, oral manifestations can be specific or non-specific and may precede diagnosis of the underlying, systemic condition[70]. Importantly, there is no distinction between the different types of oral manifestations and the systemic disease. The most common oral condition reported in CD patients (Chapter 2) were aphthous ulcers which also appear in the general population. Diagnosis of diseases based upon oral manifestations is not recommended, but the dental practitioner has the opportunity to actively participate alongside gastroenterologists in the provision of quality patient care.

The systematic literature review indicates a weak association between oral manifestations and active intestinal disease. The experimental results (Chapter 3) suggest that patients with CD can be discriminated from non-IBD patients with or without localized oral mucosal inflammation based upon elevated caspase-1 levels irrespective of IBD-related medication use. Ease of biopsy, prevalence of dental practices in urban and rural communities, and accessibility of the oral tissues clearly demonstrates the potential for dental practitioners to become actively engaged in patient care and disease monitoring activities reporting findings to the gastroenterologist on a regular basis.

This thesis focused on CD but the systematic review also highlighted the prevalence of oral lesions in comparator groups comprised of patients with ulcerative colitis. Localized to the large intestine, this inflammatory bowel disease is associated with increased oral alterations similar to CD. Other systemic autoimmune conditions, including rheumatic conditions, would also benefit from the active involvement of dental practitioners providing an oral biomarker associated with disease activity was identified[70].

The patient with CD is taking continuous medications as recommended by national guidelines to lengthen the duration of remission or to control disease flares. The IBD-related

medications (e.g., 5-ASA, immunosuppressants, corticosteroids, and biologics) are known to ameliorate extraintestinal manifestations of CD as well as the intestinal. The timing of the oral manifestations varies from patient to patient. The dental practitioner in concert with the gastroenterologist can offer oral therapies to resolve annoying, yet not debilitating, oral concerns. Mouth rinses can be administered without risk to the patients' usual medication regimen to treat oral ulcerations. A healthy mouth may improve the poor quality of life reported by patients even during periods of remission while not restricting their ability to achieve adequate daily nutrition and break the cycle of oral lesions due to diet deficiencies[28].

In conclusion, investigating the collaborative nature of dental practitioners and other health care specialists is an exciting future that will change the scope of dentistry and role of dental practitioners.

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