

From the Department of Dental Medicine
Karolinska Institutet, Stockholm, Sweden

GATEWAY TO THE GUT

ALTERATIONS IN SALIVA

IN INFLAMMATORY BOWEL DISEASE

Mirjam Majster



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Gateway to the gut

Alterations in saliva in inflammatory bowel disease

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by

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Master of Science of Dental Surgery

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to the ones who ensured me the most beautiful of lives
mamici, makani, dedaruci i dajdži

POPULAR SCIENTIFIC SUMMARY

Inflammatory bowel disease (IBD), which consists of Crohn's disease and ulcerative colitis, is a cyclical chronic inflammatory disease of the gastrointestinal tract that affects approximately 6.8 million of the world's population, many of them in Scandinavia. These diseases are often difficult to diagnose and patients can suffer from painful and psychosocially inconvenient symptoms such as diarrhea and stomachache for many years before they are diagnosed and treated. To complicate matters further, in a large proportion of IBD patients, the disease spreads to other organs – such as the mouth, where it can cause mucosal changes similar to those in the intestines, but also painful ulcers and worse dental health. Unfortunately, there is currently no cure for IBD as its cause is still not fully understood. However, it is known that the immune system of IBD patients reacts improperly to inflammation, which results in a self-sustaining cycle of chronic inflammation.

This thesis investigated different mediators of inflammation as a reflection of the defective immune system in IBD. The results showed that inflammatory mediators are elevated throughout the body in IBD – in the intestines, in blood, and more interestingly – in saliva. More specifically, we were among the first to describe the expression of a newly discovered protein, IL-34, in healthy intestines and showed that it is increased in inflamed intestines of IBD patients, adding yet another mediator to the complicated image of the defective immune system in IBD.

The remaining part of the thesis investigated known inflammatory mediators in the saliva of IBD patients, and their potential to reflect intestinal inflammation. We analyzed calprotectin, the most known marker of IBD which is analyzed in stool, and showed for the first time that calprotectin can be measured in the saliva of IBD patients and that it is significantly elevated in IBD patients compared to healthy individuals, regardless of their oral health. This increase in salivary calprotectin concentration was particularly pronounced in patients with new-onset Crohn's disease, in whom the concentration decreased after medical treatment.

Intrigued by these findings, we investigated the expression of 92 known inflammatory markers in saliva from IBD patients and compared them to their expression in blood – the hallmark for assessment of health. Most of the inflammatory proteins could be detected in saliva and their salivary expression reflected clinical parameters of intestinal inflammation more prevalently than blood. Furthermore, IL-6 and MMP-10 – both proteins implicated in the development of IBD and its spread to other organs – were elevated in the saliva of IBD patients, meaning that saliva might contain clues that explain the mechanisms behind the spread of IBD to other organs.

Would salivary markers prove to be sufficiently reliable and specific for IBD, patients could potentially provide a simple saliva sample instead of blood or stool samples in the future. This would not only be a simple and cost-effective screening method for IBD but possibly also enable the patients themselves to follow up their disease activity, in order to be able to predict flare-ups and adjust treatment in time, thus avoiding serious complications of their disease, including hospitalization. However, regardless of the future clinical utility of these investigated inflammatory mediators, our findings provide added knowledge which may increase the understanding of the cause and sustention of IBD and its oral manifestations.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Inflammatorisk tarmsjukdom (IBD), som innefattar Crohns sjukdom och ulcerös kolit, är en cyklisk kronisk inflammatorisk sjukdom i mag-tarmkanalen som drabbar cirka 6.8 miljoner människor i världen, många av dem i Skandinavien. Dessa sjukdomar kan vara svårdiagnostiserade och patienter kan drabbas av smärtsamma och psykosocialt besvärliga symtom såsom diarré och magont i många år innan de diagnostiseras och behandlas. För att komplicera saker och ting ytterligare, sprids sjukdomen till andra organ hos en stor del av IBD-patienterna – såsom munnen, där det förekommer slemhinneförändringar som liknar dem i tarmen vid IBD, men även smärtsamma sår och en försämrad tandhälsa. Tyvärr finns det för närvarande inget botemedel mot IBD eftersom sjukdomsorsaken fortfarande inte är helt klarlagd. Det är emellertid känt att immunsystemet hos IBD-patienter reagerar felaktigt på inflammation, vilket resulterar i en ond cirkel av kronisk inflammation.

Denna avhandling undersökte olika inflammatoriska mediatorer som en reflektion av det defekta immunsystemet vid IBD. Resultaten visade att inflammatoriska mediatorer är förhöjda i hela kroppen vid IBD – i tarmarna, i blodet och mest intressant – i saliven. Mer specifikt var vi bland de första som beskrev uttrycket av ett nyupptäckt protein, IL-34, i friska tarmar och visade att det var förhöjt i inflammerade tarmar hos IBD-patienter, vilket tillför ännu en mediator till den komplicerade bilden av det defekta immunförsvaret vid IBD.

Den återstående delen av avhandlingen undersökte kända inflammatoriska mediatorer i saliv hos IBD-patienter och deras potential att återspegla tarminflammation. Vi analyserade kalprotektin, den mest kända markören för IBD som vanligtvis analyseras i avföringen, och visade för första gången att kalprotektin kan mätas i saliv hos IBD-patienter, där den är signifikant förhöjd hos IBD-patienter jämfört med friska individer, oberoende av deras munhälsa. Denna ökade salivkoncentration var särskilt uttalad hos patienter med nydebuterad Crohns sjukdom, hos vilka koncentrationen minskade efter behandling.

Utifrån dessa resultat undersökte vi vidare uttrycket av 92 inflammatoriska markörer i saliv hos IBD-patienter och jämförde dem med deras uttryck i blod – den gyllene standarden för hälsobedömning. De flesta av de inflammatoriska proteinerna kunde mätas i saliv och deras salivnivåer återspeglade kliniska parametrar för tarminflammation i större utsträckning än blod. Vidare kunde vi påvisa att IL-6 och MMP-10 – båda två proteiner som är inblandade i utvecklingen av IBD och dess spridning till andra organ – var förhöjda i saliven hos IBD-patienter. Detta innebär att saliven kan innehålla proteiner som i sin tur kan förklara mekanismerna bakom spridningen av IBD till andra organ, särskilt munnen.

Skulle salivmarkörer visa sig vara tillräckligt tillförlitliga och specifika för IBD, kan framtida patienter potentiellt komma att lämna ett enkelt salivprov istället för blod- eller avföringsprover. Detta skulle inte bara vara en enkel och kostnadseffektiv screeningmetod för IBD utan också göra det möjligt för patienterna att själva följa upp sin sjukdomsaktivitet för att kunna förutsäga skov och justera behandlingen i god tid för att undvika allvarliga sjukdomskomplikationer, vilket inkluderar sjukhusinläggningar. Oavsett vad den framtida kliniska nyttan av dessa undersökta inflammatoriska mediatorer visar sig vara, ger våra resultat en ökad kunskap som kan bidra till en bättre förståelse av orsaken och upprätthållandet av IBD och dess orala manifestationer.

ABSTRACT

Inflammatory bowel disease (IBD), which consists of Crohn's disease and ulcerative colitis, is a chronic immune-mediated disease thought to result from genetic and environmental interaction which influence the commensal flora to trigger an inappropriate mucosal immune response. IBD primarily affects the intestines but is not restricted to them. Extraintestinal manifestations are frequently observed within the oral cavity. This thesis aimed to investigate different mediators of inflammation within the intestines and oral cavity as a reflection of defective immune responses in IBD.

In our first study, we investigated the intestinal localization of macrophage growth factors IL-34 and CSF-1 and their involvement in IBD. IL-34 and CSF-1 demonstrated distinct expression patterns in the human intestine and were significantly elevated in human and experimental IBD. Infiltrating cells of the lamina propria and intestinal epithelial cells expressed IL-34, regulated by TNF- α through the NF- κ B pathway. As a result, the newly discovered growth factor IL-34 was proposed as a new modulator of IBD.

The remaining part of this thesis investigated the expression of inflammatory proteins in saliva in relation to IBD. The second study aimed to analyze calprotectin, an established fecal marker of IBD, for the first time in saliva of IBD patients. We found that calprotectin was significantly elevated in saliva of IBD patients, particularly in CD and most prominently in newly diagnosed CD patients. This opened up for new hypotheses in the oral-gut connection in IBD and supported the notion that the oral cavity may contain early evidence of intestinal inflammation.

In a third study, we compared the profile of 92 known inflammatory proteins in saliva and serum of IBD patients. The salivary and circulatory inflammatory profiles were similar but reflected different aspects of IBD activity. Several serum proteins were significantly altered in IBD patients compared to controls, whereas IL-6 and MMP-10 – proteins involved in the pathogenesis of IBD and its extraintestinal manifestations – were significantly elevated in stimulated saliva of IBD patients, providing additional proof of subclinical inflammatory mimicry of intestinal disease by the oral cavity.

In the final study, we confirmed our previous findings related to elevated salivary calprotectin in IBD and showed that the concentrations were not significantly affected by oral disease. Moreover, we investigated potential sources of salivary calprotectin and showed that neutrophils isolated from saliva express calprotectin, demonstrate reduced CD11b expression in IBD patients, but share a similar ability to secrete calprotectin.

In conclusion, the work presented in this thesis highlights the aberrant immune responses associated to IBD and provides proof that such mechanisms can be reflected by the oral cavity.

Key words: inflammatory bowel disease, macrophage growth factors, oral manifestations, saliva, biomarkers, calprotectin, innate immunity.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles and a manuscript, which are referred to in the text by their Roman numerals (I-IV).

- I. Interleukin 34: a new modulator of human and experimental inflammatory bowel disease.
Stephanie Zwicker, Gisele L Martinez, Madeleen Bosma, Marco Gerling, Reuben Clark, **Mirjam Majster**, Jan Söderman, Sven Almer, Elisabeth A Boström.
Clinical science. 2015; 129(3): 281-290.
- II. Salivary calprotectin is elevated in patients with active inflammatory bowel disease.
Mirjam Majster, Sven Almer, Elisabeth A Boström.
Archives of oral biology. 2019;107: 104528.
- III. Salivary and serum inflammatory profiles reflect different aspects of inflammatory bowel disease activity.
Mirjam Majster, Ronaldo Lira-Junior, Charlotte M Höög, Sven Almer, Elisabeth A Boström.
Inflammatory bowel diseases. 2020; 26(10): 1588-1596.
- IV. Interplay between salivary calprotectin and oral neutrophils in patients with inflammatory bowel disease and their relation to oral disease.
Mirjam Majster, Sven Almer, Sebastian Malmqvist, Annsöfi Johannsen, Ronaldo Lira-Junior, Elisabeth A Boström.
Manuscript.

Scientific papers mentioned, but not part of thesis:

Salivary colony stimulating factor-1 and interleukin-34 in periodontal disease.
Gisele L Martinez, **Mirjam Majster**, Nadja Bjurshammar, Annsöfi Johannsen, Carlos Marcelo Figueredo, Elisabeth A. Boström.
Journal of Periodontology. 2017; 88(8): e140-e149.

MMP-12 and S100s in saliva reflect different aspects of periodontal inflammation.
Sofia Björnrot Holmström, Ronaldo Lira-Junior, Stephanie Zwicker, **Mirjam Majster**, Anders Gustafsson, Sigvard Åkerman, Björn Klinge, Mattias Svensson, Elisabeth A. Boström.
Cytokine. 2019; 113: 155-16.

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LIST OF ABBREVIATIONS

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AUC-ROC	Area under the receiver-operating characteristic curve
BIOMARKAPD	Biomarkers for Alzheimer's and Parkinson's disease
BMI	Body mass index
BOP	Bleeding on probing
BSA	Bovine serum albumin
CCL	C-C motif ligand
CD	Crohn's disease
CD 14/15/16	Cluster of differentiation 14/15/16
cDNA	Complementary deoxyribonucleic acid
CRP	C-reactive protein
CSF-1	Colony stimulating factor 1
CSF-1R	Colony stimulating factor 1 receptor
CXCL	C-X-C motif ligand
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMFT	Decayed, missing, filled teeth
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
EIM	Extraintestinal manifestation
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GCF	Gingival crevicular fluid
GDPR	General Data Protection Regulation
GI	Gastrointestinal
GLM	General linear model
HBI	Harvey Bradshaw Index
IBD	Inflammatory bowel disease
IBD-U	Inflammatory bowel disease unclassified
Ig	Immunoglobulin

IL	Interleukin
JAK	Janus kinase
LPS	Lipopolysaccharide
LSD	Least significant difference
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MUC5	Mucin 5
NET	Neutrophil extracellular trap
NF- κ B	Nuclear factor- κ B
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PD	Probing depth
PEA	Proximity extension assay
PGA	Physician global assessment
PI	Plaque index
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROC	Receiver-operating characteristic
S100 A8/A9	Calprotectin
SARS-CoV 2	Severe acute respiratory syndrome coronavirus 2
SCCAI	Simple Clinical Colitis Activity Index
SES-CD	Simple Endoscopic Score for Crohn's disease
sIg	Secretory immunoglobulin
SLE	Systemic lupus erythematosus
T _H cell	T helper cell
TLR-4	Toll-like receptor-4
TNF	Tumor necrosis factor
UC	Ulcerative colitis
UCEIS	Ulcerative Colitis Endoscopic Index of Severity
USD	United States dollar

1 INTRODUCTION

1.1 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) – a hypernym of Crohn’s disease (CD) and ulcerative colitis (UC) – is a chronic immune-mediated inflammatory disease of the gut which affects over 6.8 million people worldwide.¹ Although both considered as IBD, CD and UC are two distinct diseases. UC mainly affects the mucosa of the colon, while CD is characterized by transmural lesions which can occur throughout the whole intestinal tract. The disease course is progressive in nature and is characterized by episodes of remission and relapse, which exerts a major toll on patients in burden of medication, hospitalizations, surgery, health-related physical and mental quality of life, economic productivity, and social functioning.^{2,3} The following section will describe the clinical presentation of these diseases, treatment strategies, their pathogenesis, and their effect on other organs – specifically the oral cavity.

1.1.1 Clinical symptoms and disease diagnosis

Symptoms of IBD normally debut in early adulthood around 15-30 years of age, but may also occur later in life, and is slightly more prevalent in females than males.^{4,5} Classical symptoms include prolonged periods of diarrhea, abdominal pain, weight loss, and fatigue. Patients with UC may also present with more acute symptoms such as bloody stools, rectal urgency, or passage of pus or/and mucus during defecation.⁶ Due to the variability of symptoms and their unspecific occurrence in many gastrointestinal conditions, there is no single method for diagnosing IBD. Instead, diagnosis is based on patient anamnesis, family disease history, biochemical analyses of blood and stool, exclusion of gastrointestinal infections, endoscopy, histologic assessment of intestinal mucosa, and radiological and/or ultrasound imaging.⁷

Biochemical analysis of blood includes a full blood count, C-reactive protein (CRP), electrolytes, and liver enzymes.^{8,9} The blood analysis may reveal thrombocytosis, anaemia, leukocytosis, and raised CRP, reflecting the ongoing inflammatory processes. Evidence of malnutrition and hypoalbuminaemia is also common. However, none of these findings are specific to IBD, and only serve as a complement to further tests which are required for proper diagnosis.

Stool samples are required for microbiological analysis to exclude infectious colitis caused by pathogens such as *Clostridium difficile*. Furthermore, stool is often analyzed for calprotectin, also known as S100 A8/A9, an acute-phase antimicrobial protein most abundantly found in neutrophil cytosol and granules, but also in monocytes, activated macrophages, and keratinocytes.¹⁰⁻¹² Calprotectin has both intra- and extracellular functions, such as homeostatic regulation of neutrophils and the cytoskeleton-plasma membrane interactions, but can also promote inflammation through endogenous activation of toll-like receptor-4 (TLR-4) and the receptor for advanced glycation end products (RAGE).¹³⁻¹⁵

Fecal levels of calprotectin correlate to neutrophil recruitment to the inflamed intestine and are thus believed to reflect inflammation in IBD. As part of the immune response, neutrophils undergo cell disruption and apoptosis, resulting in the release of intracellular components – including calprotectin – into the gut lumen, which is reflected in feces.¹⁶⁻¹⁹ Fecal determination of calprotectin has proven to be useful in several clinical situations, such as during the diagnostic work-up of patients with suspected IBD, and in the differential diagnosis of irritable bowel syndrome versus inflammatory and infectious bowel disorders.^{18, 20} Once IBD is diagnosed, fecal calprotectin is used to monitor disease activity over time, for early prognosis of flare in patients in remission, as a surrogate marker for presence of endoscopic inflammation, and as a selection criteria for enrolling patients into clinical trials of anti-inflammatory medication.²¹⁻²⁴ Fecal calprotectin is thus the most readily used marker for diagnosing and monitoring IBD, and is available as a tool for patient self-testing through digital applications.

Despite the specificity of fecal calprotectin, ileocolonoscopy with biopsy is required for a definite diagnosis. Endoscopically, CD is characterized by discontinuous transmural lesions, presence of strictures and fistulae, and perianal involvement, while UC is characterized by continuous and confluent colonic involvement at various length, with clear demarcation to non-inflamed bowel.⁷ Histologically, biopsies from CD patients contain granulomas and focal crypt architectural abnormalities, in conjunction with focal or patchy chronic inflammation extending through all histological layers. The histopathological findings in UC biopsies are less defined in nature, and include early focal or diffuse basal plasmacytosis, mucosal and crypt distortion, mucosal atrophy, and an irregular or villous mucosal surface.²⁵

Despite these extensive diagnostic measures, IBD can still be difficult to diagnose. The average time from onset of first symptoms to IBD diagnosis in Europe, based on data from Switzerland, Austria, and Italy, is 7 months for CD and 3 months for UC.²⁶⁻²⁸ While this could be considered as acceptable, there is a non-negligible portion of patients (25-30% for CD, 10-15% for UC) who receive their diagnosis > 24 months after the first disease symptoms.^{26,27} Recent preliminary data from the U.S. show even greater delays in diagnosis, with 64% of IBD patients reporting that they received their diagnosis after > 1 year, and seeing a mean of 3.5 physicians before establishing an IBD diagnosis.²⁹ The reason for diagnostic delays is complex and highly dependent on the individual case, particularly with respect to socio-economic predispositions, however one reason could be the overall vagueness of symptoms associated with IBD.^{26, 27} Moreover, it can be challenging to distinguish CD from UC, and thus a third diagnosis has emerged – IBD unclassified (IBD-U) – for patients exhibiting clinical and endoscopic signs of chronic colitis, but with subtle features of both CD and UC.³⁰ Altogether, this proves the need for more effective tools and/or workups for the diagnosis of IBD, particularly since diagnostic delays are negatively correlated to overall disease prognosis.^{29,31}

1.1.2 Global disease burden

Over 6.8 million people worldwide currently suffer from IBD, out of which about 60 thousands in Sweden alone, with the highest prevalence in Europe and North America (0.5%).^{5, 32, 33} However, the adaptation of a “western-like” lifestyle by newly industrialized countries in Africa, Asia, and South America, in combination with migration, has contributed to a growing incidence of IBD, in particular UC, in these regions.³² By 2025, the absolute number of IBD patients in areas of high incidence such as North America, is expected to increase more than 70% compared to ten years earlier, resulting in an increase in already high societal costs, besides personal suffering and decreased quality of life.³⁴ In 2017, 1 in 100 000 deaths in western Europe were due to IBD, and IBD patients are estimated to lose on average 10.7 years of their life due to the disease.⁵

In a systematic literature review, the estimated indirect annual cost per patient in 2013 ranged between 1159-14135 USD for CD, and from 926-6583 USD for UC.³⁵ Furthermore, the growing use of biological therapy in IBD alone accounts for an annual cost of over 25 000 USD per patient.³⁶ In Sweden, the average personal monthly cost in 2007 due to CD was €721 per patient, possibly due to twice the need for sick-leave compared to matched controls, and by the end of 2007 the total economic burden of the disease summed up to €184 million due to hospitalization, medication, and sick leave.^{37, 38} Considering the increasing prevalence of IBD in Sweden and the wide use of costly biological treatment, the economic burden is most likely substantially increased today.

1.1.3 Etiology and pathogenesis

To this date, no single cause of IBD has been identified. Instead, the disease is believed to be caused by genetic susceptibility and triggering environmental factors that lead to an increased bowel permeability, microbial dysbiosis and translocation, causing inflammation which is perpetuated by an abnormal immune response (summarized in Fig. 1).

1.1.3.1 Susceptibility and triggering factors

Risk factors for developing IBD include genetic predisposition; improved sanitary conditions causing inadequate exposure to environmental antigen, and increasing consumption of antibiotics; environmental factors such as smoking, appendectomy, and vitamin D deficiency; and lifestyle habits such as insufficient diet, poor sleep, physical inactivity, and excessive stress.³⁹

At present, there are 163 identified genetic loci associated to IBD, out of which 110 are associated with both CD and UC, 30 solely to CD, and 23 to UC.⁴⁰ These loci are enriched for genes involved in primary immunodeficiency, T-cell function, modulation of cytokine and chemokine production, and mycobacterial diseases. Strong genetic associations include nucleotide-binding oligomerization domain-containing protein 2 (*NOD2*) – a bacterial sensing gene, *ATG16L1* and *IRGM* – genes involved in autophagy, and *IL23R* – encoding the interleukin (IL-) 23 receptor.⁴⁰ *NOD2* has, besides serving as a bacterial sensor, also been associated with barrier dysfunction, which is believed to be a primary causative step in IBD pathogenesis.^{41, 42}

1.1.3.2 “The leaky-gut”

The intestinal mucosal barrier is an important physical boundary between the external and internal environment, and comprises of the intestinal epithelium, an overlaying protective mucus layer, and commensal bacteria. In IBD, all three components show evidence of an impaired barrier function. The intestinal epithelium of IBD patients reveals increased permeability via both transcellular and paracellular routes, while the mRNA expression of several mucins is reduced in the ileum of CD patients, and colonic mucus of UC patients is increasingly permeable to microbes.⁴³⁻⁴⁶ Concurrently, the microbial diversity within the gut of IBD patients is decreased – displaying an increased abundance in Bacteroidetes and Proteobacteria, while Firmicutes, in particular *Faecalibacterium prausnitzii*, are decreased – which is hypothesized to contribute to the pathogenesis of the disease.⁴⁷⁻⁴⁹

Altogether, these anomalies allow for transmucosal penetration (commonly referred to as leakage) of exogenous substances such as dietary antigens, pathobionts, or xenobiotics, which subsequently triggers an immune response, initiating mucosal inflammation.

1.1.3.3 Aberrant immune response

The role of the immune system is to protect the host against harmful bodily and foreign substances. The innate immune system, consisting of dendritic cells, macrophages, innate lymphoid cells, and neutrophils, is the first line of defense which is activated when the mucosal barrier integrity is challenged. In the event of successful antigen breach of the mucus barrier, the antigen will be taken up by antigen presenting cells such as dendritic cells or macrophages, and transported to lymphoid tissues to initiate a secondary immune response by lymphocytes (mainly T-cells), which orchestrate the clearance of the antigen.⁵⁰

In IBD, these pathways are aberrant and lead to an unproportioned inflammatory response which prevents the cessation of inflammation, eventually resulting in a self-sustaining cycle of chronic inflammation.⁵¹ Immunologically, IBD is strongly T-cell mediated, as the balance between immunosuppressive T regulatory cells and inflammation inducing T helper (T_H) cells is disrupted.⁵² IBD is nowadays considered to be driven by T_H1 and T_H17 cells, which are enriched in the mucosa of IBD patients and more responsive to pro-inflammatory stimulation by IL-23, which promotes a more pathogenic phenotype of these cells.^{53, 54} However, various cell types other than T_H cells show impaired function in conjunction to IBD – neutrophils and macrophages have both been implicated in the disease pathogenesis.⁵¹

Neutrophil infiltration is among the earliest signs of intestinal inflammation, as neutrophils account for the front-line defense against invading pathogens by means of phagocytosis, cellular degranulation, and release of neutrophils extracellular traps (NETs). In IBD, their presence leads to impaired epithelial barrier function and tissue destruction through oxidative and proteolytic damage caused by excessive defense mechanisms.^{55, 56} Microscopically, this is apparent as an initial lesion is caused by an inflammatory infiltrate surrounding intestinal crypts followed by superficial ulceration in the mucosa.²⁵ Moreover, neutrophils have been shown to perpetuate intestinal inflammation through the release of multiple inflammatory

mediators.⁵⁷ As mentioned, they are the major source of calprotectin, but have also been shown to represent the main source of IL-23 in the colon of IBD patients, which further demonstrates the complicated interplay between cells of the immune system in the pathogenesis of IBD.^{11, 58}

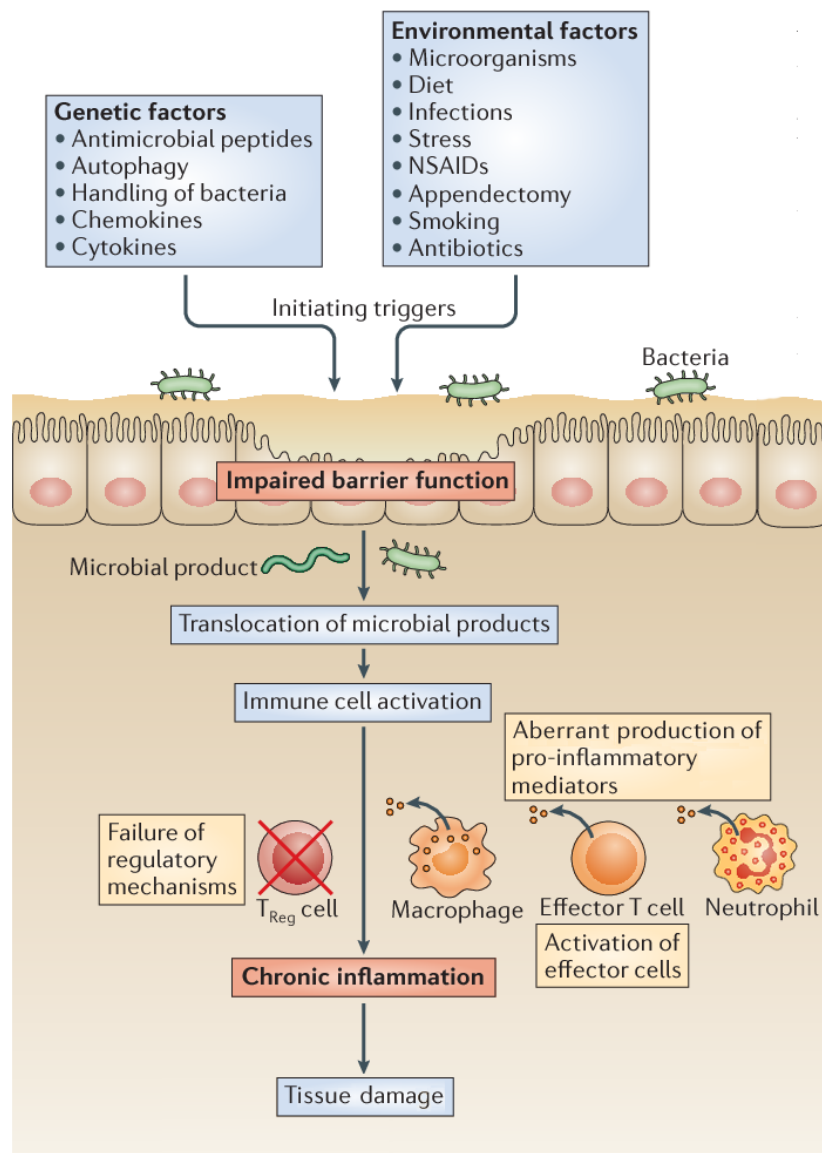


Figure 1. Schematic summary of the pathogenesis of IBD.
Adapted from Neurath M. F.⁵⁹ with permission from the publisher.

The second wave of response to intestinal inflammation is mediated by macrophages, which are highly heterogeneous phagocytic tissue resident or monocyte-derived cells that demonstrate a continuum of activation states. Their survival, proliferation, and differentiation is regulated by the colony stimulating factor 1 receptor (CSF-1R) and its ligands colony stimulating factor 1 (CSF-1, also known as M-CSF; macrophage colony stimulating factor) and IL-34.⁶⁰ Macrophages are among the most abundant immune cells in intestinal mucosa, in which they are present in a hypoactive state, clearing pathogens without stimulation of inflammation.⁶¹ Yet, in IBD, these cells participate in disease development through inappropriate response to and inefficient clearance of microbes, and impaired transition from pro-inflammatory to anti-inflammatory responses.⁶²⁻⁶⁴ Microscopically, they are responsible for the formation of granulomas, which are the histological hallmark of CD.²⁵ Macrophages with a pro-inflammatory phenotype, expressing CD14, dominate inflamed mucosa of CD patients and contribute to disease pathogenesis through the release of proinflammatory cytokines, such as tumor necrosis factor (TNF-) α , IL-6, and IL-23.^{65, 66} Moreover, both CSF-1 and its receptor CSF-1R have been shown exacerbate colonic damage in experimental mouse models of IBD.^{67, 68} However, at the time when this thesis was initiated, there was no insight on the involvement of the then recently discovered macrophage growth factor IL-34 in IBD.⁶⁹

It still remains to understand whether these observed immune cell phenotypes are a cause or consequence of intestinal inflammation. Namely, a large number of proinflammatory cytokines and chemokines, neuropeptides, reactive oxygen species, and danger-associated molecular patterns are excessively secreted in IBD, and their role to initiate, mediate and perpetuate mucosal inflammation has been intensely investigated.⁵⁹ Essentially all chemokines that have been studied are elevated in IBD mucosa compared to controls, including chemokine C-C motif ligand (CCL) 2-5 and 7, chemokine C-X-C motif ligand (CXCL) 5, and CXCL10.^{70, 71} Similarly, several proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α are increased in intestinal mucosa of IBD patients.⁷²⁻⁷⁴

TNF- α , one of the major cytokines involved in IBD, induces hypervascularization and angiogenesis, enhances pro-inflammatory cytokine secretion by macrophages and T cells, and promotes tissue damage by augmenting production of matrix metalloproteinases (MMPs).⁷⁵⁻⁷⁷ Furthermore, activation of the transcription factor nuclear factor- κ B (NF- κ B) by TNF- α and its receptor is shown to induce T cell resistance to apoptosis in IBD.⁷⁸ IL-6 is a more recent addition to cytokines implicated in the pathogenesis of IBD, here through its trans-signaling effects via soluble IL-6 receptor, resulting in immune cell activation, inhibition of apoptosis, and induction of T_H17 differentiation.⁷³ Other notable cytokines that are elevated in IBD include members of the IL-12 family, such as IL-12 and IL-23, due to their capacity to perpetuate T_H responses and suppresses regulatory T cell activity.^{79, 80} However, despite therapeutic success by drugs targeting said cytokines and receptors, a full comprehension of the cause and mechanisms of IBD is still out of reach, and much is left to be investigated regarding the interplay between different cell types and their mediators.

1.1.4 Treatment

The treatment strategy of IBD is pathophysiology-oriented and based on targeting the aberrant immune response. Once diagnosed, IBD patients receive systemic treatment and are regularly clinically and biochemically monitored for early detection of flare or progression of disease.⁷ In general, CD and UC patients are initially treated by corticosteroids, which are later de-escalated in transit towards maintenance therapy. UC patients are typically treated with 5-aminosalicylic acid (mesalazine), while both CD and UC patients may receive immunomodulators such as thiopurines and methotrexate. In case of no effect, inhibitors targeting TNF- α (adalimumab, infliximab, or golimumab), IL-12/23 (ustekinumab), or integrins (vedolizumab) are introduced and administered intravenously or subcutaneously.⁸ ⁹ More recently, yet another drug has been added to the arsenal of biological treatment of IBD, and specifically for UC - namely tofacitinib, an inhibitor of the intracellular signal mediators janus kinases (JAK) which in turn blocks the downstream effects of numerous proinflammatory cytokines.⁸¹ Besides pharmacological treatment, patients suffering from malnutrition often receive dietary support as well.

In case of more severe and complex disease phenotypes of CD and UC, including occurrence of abscesses, strictures and fistulas, bowel resection (partial colectomy and/or anastomosis or ileo-/colostomy) may be needed. Nonetheless, surgery should be postponed for as long as possible due to the risk of post-surgical complications and high likelihood of recurrence of the disease.⁸²

Although corticosteroids are highly effective in quickly suppressing symptoms of acute inflammation, they have shown poor success in maintaining remission or in inducing mucosal healing, as patients often develop steroid dependency.⁸³ Immunomodulators do quench the aberrant immune system of IBD patients, but are less specific compared to biologic treatment which target cytokines involved in inflammation and T-cell differentiation. Despite effective symptomatic alleviation, as little as 30% of IBD patients reach mucosal healing with the current treatment strategies.⁸⁴⁻⁸⁶ Moreover, these treatment regimens seem to require life-long compliance, as discontinuation of immunomodulatory or biologic treatment is associated with high rates of relapse.⁸⁷ Therefore, new treatment strategies are constantly under development, such as inhibitors of IL-6, fecal microbiota transplantations, and stem cell transplantations.⁸⁸⁻⁹⁰ However, a cure for IBD is still far from reach until there is a more comprehensive understanding of the pathogenesis of these complex diseases.

1.1.5 Extraintestinal manifestations

Despite the fact that IBD is a disorder that primarily affects the gut, it is not restricted to the intestinal tract and may affect other organs. Extraintestinal manifestations (EIMs) of IBD most commonly involve cutaneous (pyoderma gangrenosum and erythema nodosum), ocular (episcleritis and uveitis), pulmonary (bronchitis and pneumonia), biliary (primary sclerosing cholangitis) or osseous tissues (arthritis and spondylarthropathy).⁹¹ It is estimated that up to

50% of IBD patients experience at least one EIM, both coincidentally with intestinal manifestations or independently, which considerably contributes to morbidity and mortality, and a decreased quality of life.⁹²⁻⁹⁶ EIMs are more common in CD patients than in UC, can precede IBD diagnosis, and can reflect symptomless intestinal inflammation.⁹⁷⁻⁹⁹

The mechanisms by which EIMs occur are not clear. Certain manifestations, such as primary sclerosing cholangitis or pyoderma gangrenosum, can be defined as an inflammatory entity caused by the same processes that drive inflammation in the gut, such as impaired lymphocyte function and homing, and a cytokine imbalance in favor of inflammation.¹⁰⁰ In addition, a strong genetic background is increasingly recognized as a predisposing factor for development of EIMs.¹⁰¹ However, the manifestations can also develop as a result of IBD treatment, as in the case of pulmonary symptoms that risk to develop due to immunosuppressive therapy.¹⁰²

One group of EIMs of stomatological importance are orofacial manifestations, whose prevalence ranges from 20 to 50% in most publications, and about which the greater part of this thesis will focus on.^{103, 104} Prior to presenting the oral manifestations of IBD, the components of the oral cavity and their function will be briefly described.

1.2 THE ORAL CAVITY

The gastrointestinal tract can be regarded as a tubular organ that spans from the mouth to the anus, with the purpose of nutrient ingestion, digestion, absorption, and defecation. The oral cavity (or mouth) is a complex anatomical compartment, which initiates digestive processes, plays an important role in the mucosal immune system, and reflects both local secretory and systemic status. It consists of maxillary bones, teeth, the tongue, salivary glands, and oral mucosa. Together, these components enable sensory perceptions such as taste, temperature, and structure, subsequently resulting in mastication and deglutition, as well as initiation of potential immune responses.

1.2.1 Composition and function in homeostasis

1.2.1.1 *Oral mucosa*

The oral mucosa is another one of the body's mucous membranes, whose primary function is to maintain a barrier against mechanical stress and trauma, ingested pathogens and biochemical irritants. In addition, it also sustains immune tolerance towards commensal microbes and food antigens to maintain homeostasis. The oral mucosa is in direct contact with the outside world and is under constant burden, which is apparent from the constant cell renewal within its epithelium.¹⁰⁵

Oral mucosa lines the entire oral cavity, excluding teeth. Similar to the gut, the oral mucosal epithelium is covered by a layer of mucus, mixed with saliva, which acts as an additional barrier against microorganisms and other foreign molecules.¹⁰⁶ The epithelial cells are supported by the basement membrane, under which the lamina propria lays, comprising of fibroblasts, and immune cells (predominantly lymphocytes, but also macrophages, recruited monocytes, neutrophils, and innate lymphoid cells), as well as nerve tissue, blood and lymphatic vessels.^{107, 108} Other than that, oral mucosa can contain salivary glands and other specialized structures such as papillae of the tongue.

The host–antigen interplay within oral mucosa is much less well understood than that of intestinal mucosa. The two mucosal compartments resemble histologically and physiologically, but have distinct functional dissimilarities. Oral mucosa is thicker and denser in order to provide a sturdy mechanical barrier against harmful substances, while intestinal mucosa is thinner in order to enable absorption of nutrients and electrolytes, and contains muscular layers that are needed for bolus/fecal propulsion through the gut.¹⁰⁹ Interestingly, both tissues share a comparable microbial burden, based on the diversity of the microbiome, which requires constant and diverse immune response. Yet, the oral microbiome is distinct from that in the gut, and is mainly dominated by Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes.¹¹⁰

1.2.1.2 Teeth

The oral cavity contains some among the most unique and strong structures of the body – namely teeth. Their function is to allow for mastication of ingested food in order to facilitate digestion, and to aid in pronunciation of sounds during speech. The tooth consists of an enamel covered crown which is exposed to the oral cavity, and a dentin root which is anchored to the alveolar bone. Dentin encloses the pulp, which maintains tooth vitality through immunologic and regenerative functions via its blood and nerve supply.¹¹¹

The teeth and their supporting structures are daily challenged by microbial and biochemical attacks which cause demineralization of its hard tissue components. This is counterbalanced by salivary buffering and regular dental hygiene practices which help to remove the excessive accumulation of microbial biofilm on tooth surfaces.¹¹² The microbial biofilm also exerts a major toll on the tissues that support and anchor the tooth – the periodontium – which comprises of cementum, the mineralized tissue that covers the root dentin; the periodontal ligaments that anchor the tooth to the alveolar bone; and the gingival sulcus, the unique interface between the teeth and the gingiva, and only place in the body where hard tissue breaches soft tissue, that executes constant physiological immune surveillance.¹¹¹

Disturbance of the equilibrium between the microbial/biochemical challenge and tissue regeneration may lead to a breach of the tissue barrier and disease. Two of the most common dental diseases are caries and periodontitis. Dental caries implicates the gradual destruction of the dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates. If left untreated, the caries lesion may penetrate beyond enamel and dentin, into the tooth pulp, causing painful infection and pulp necrosis.¹¹³ Clinically, caries lesions are assessed by visual examination and probing, which may be complemented by radiographic exams, and graded based on the extent of decay (D1-D3) and its overall prevalence in an individual (amount of decayed, filled, and missing teeth/surfaces).^{114, 115}

Periodontal disease is a biofilm-induced chronic inflammatory condition that gradually affects the tooth-supporting structures: gingiva, periodontal ligament, and alveolar bone.¹¹⁶ Gingivitis, the initial stage, is a reversible inflammation confined to the soft tissue, and is clinically characterized by gingival redness, edema, and bleeding upon gingival probing (BOP).¹¹⁷ If left untreated, gingivitis may progress into periodontitis, leading to the spread of inflammation to the periodontal ligament and alveolar bone, resulting in progressive loss of tooth attachment.¹¹⁸ Periodontitis is classified in stages (I-IV) defined by severity, complexity, and extent of the disease. The classification is based on a composite evaluation of the periodontal probing depth of the gingival sulcus, clinical attachment/tooth loss, secondary complications such as masticatory dysfunction, and can be complemented by radiographic assessment of the alveolar bone resorption.¹¹⁸

1.2.1.3 Saliva

Saliva is an oral biofluid secreted from the contra-lateral parotid-, submandibular-, sublingual-, and minor salivary glands that reside in the oral mucosa. The salivary glands are exocrine glands composed of acini (small bud-like structures), consisting of serous-, mucous epithelial cells, or a mixture of the two, which secrete macromolecular and fluid components of saliva into the acinar lumen.¹⁰⁷ Secretions by the serous glands are watery in their consistency, while mucous glands produce viscous mucin-containing secretions that make up and enable retention of the protective mucous layer covering oral mucosal surfaces.¹¹⁹ The parotid gland consists of few mucus glands and mainly serous glands, while the submandibular and lingual glands have an increasing mucous gland content, and a majority of the minor glands are mucous.¹²⁰ The constituents are moved from the acinus through ductal systems by contractile support from myoepithelial cells, in which ductal cells further contribute to the secretion, to then exit into the oral cavity through an excretory duct. Once secreted into the oral cavity, the salivary secretion mixes with endo- and exogenous components derived from gingival crevicular fluid (GCF, exudate from the gingival sulcus), mucosal-, and tooth surfaces, and is then commonly referred to as ‘whole saliva’ (illustrated in Fig. 2).¹⁰⁷

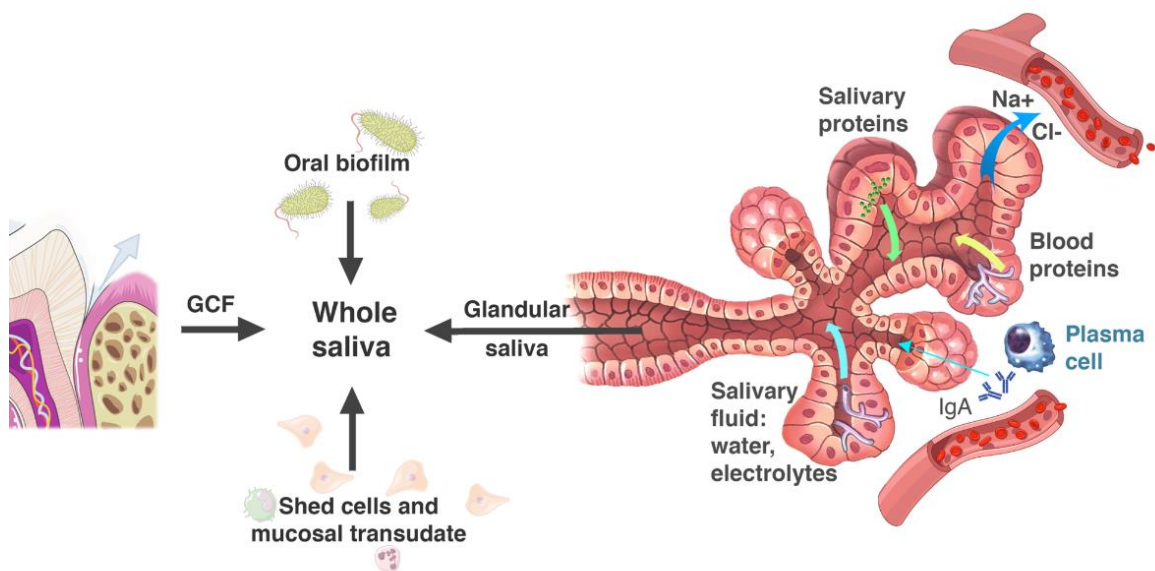


Figure 2. The constituents of whole saliva. Salivary fluid is primarily derived from salivary gland secretion, which contains proteins secreted by salivary acinar cells, secreted immunoglobulins from plasma cells, and protein transudate from circulation. Once secreted into the oral cavity, the salivary fluid mixes with endo- and exogenous components derived from gingival crevicular fluid (GCF, exudate from the gingival sulcus of teeth), oral microorganisms, and shed epithelial and immune cells. *The figure was created by the author using images licensed under creative commons and from Servier Medical Art by Servier.*

Salivary secretion is dependent on a rich circulatory and neuronal supply to the acinar and ductal cells. The salivary glands are tightly associated with the autonomic nervous system, with both parasympathetic and sympathetic nerves regulating salivary content and secretion.^{121, 122} The parotid and submandibular glands mainly receive sympathetic innervation, whereas the sublingual and minor salivary glands receive less sympathetic

stimulation.¹²³ Sympathetic nerve stimulation is shown to induce protein secretion in parotid and submandibular glands, while parasympathetic stimulation induces salivary flow rates resulting in an increase in salivary volume, and stimulates mucin secretion from the mucous glands.^{124, 125} Protein synthesis in the salivary glands during mastication or gustatory stimulation is mainly regulated by the major-regulated pathway which is induced by cholinergic stimulation and increasing intracellular cyclic adenosine monophosphate which activates the protein kinase system. This in turn stimulates exocytosis of protein-containing granules in the apical portions of the cells of a salivary gland acinus.^{126, 127} The continuous constitutive-like pathway and the minor-regulated pathway activated by low cholinergic stimulation instead both contribute to the resting salivary protein secretion during parasympathetic activity.^{127, 128} Influenced by these differences in salivary composition due to stimulation source, saliva is often referred to as either 'stimulated' or 'unstimulated'. Unstimulated saliva, or resting saliva, is present in a state with no exogenous stimuli and consists mainly of secretions from the submandibular and sublingual glands with a high mucin component. Stimulated saliva, which is either obtained by masticatory or gustatory stimulation, is mainly comprised of parotid secretions and is thus less viscous, and more abundant in volume than unstimulated saliva (approx. 2 ml/min compared to 0.4 ml/min).^{129, 130}

Saliva is important for maintaining homeostatic functions in the oral cavity. It lubricates food, facilitating bolus formation, swallowing, and digestion, but also contributes to tooth development and integrity through clearance of food and non-adherent bacteria. It does so by containing a vast array of molecules including water, electrolytes, enzymes, immunoglobulins, glycoproteins, antimicrobial proteins, growth factors, regulatory peptides, and desquamated keratinocytes and neutrophils.^{131, 132} For example, the buffering capacity of saliva helps to maintain a neutral pH throughout the oral cavity, which not only prevents bacterial growth but also hinders acid assaults on the teeth.¹³³ The tooth enamel integrity is further strengthened by remineralization through ions present in saliva, such as calcium, phosphate, and fluoride.¹³⁴ The enzyme α -amylase aids in digestion through breakdown of starch, while lactoferrin and lysozyme elicit antimicrobial properties.¹⁰⁷

Moreover, saliva contributes to mucous membrane integrity and protects from dryness, ulcer formation and enhances mucosal repair, and also serves important immunoregulatory functions including expression of secretory immunoglobulin A (sIgA) and production of immune-suppressive cytokines such as transforming growth factor (TGF-) β and IL-10 along with mucins.¹³⁵⁻¹³⁸ Furthermore, several studies indicate that the protein contents in saliva induce oral keratinocyte proliferation, suggesting its role in maintaining regenerative capacity of oral keratinocytes. Wound healing models incubated in saliva show significant increase in keratinocyte proliferation, and mouse models depleted of saliva have shown impaired wound healing.¹³⁹⁻¹⁴² The importance of saliva in maintaining oral integrity is further strengthened in studies of patients suffering from hyposalivation, as these patients have increased susceptibility towards oral candidiasis, and are more prone to develop caries.^{143, 144}

Saliva also contains blood constituents derived from fluids of the gingival capillaries through GCF.¹⁴⁵ Proteomic studies have identified more than 1000 different proteins in saliva of the three major glands, and about 40% of proteins detected in circulation are also detected in saliva.^{146, 147} Each salivary gland is enveloped by capillaries which allow for diffusion of blood-based molecules through the permeable acinar cells. These molecules enter the salivary gland either by transcellular or paracellular transport.¹⁴⁸⁻¹⁵⁰ In addition, it has been proposed that the secretory apparatus of the gland allows for leakage from the blood stream, and/or that specialized receptors in the gland facilitate the transcellular diffusion.¹⁵¹⁻¹⁵³ The fact that identified salivary proteins also can be found in circulation and in other body fluids supports a combined local and systemic synthesis of salivary proteins and favors the potential use of saliva to reflect not only local but also systemic conditions.¹⁴⁶

1.2.2 Saliva as a mirror of systemic status

In addition to the above mentioned components, saliva contains a great variety of biomolecules, including DNA, mRNA, microRNA (miRNA), proteins, metabolites and microbiota, whose dynamics very well might identify early establishment of local and systemic diseases, and provide evaluation of disease progress and prognosis.¹⁵⁴ Recent revelations have proven the hypothesis correct and henceforth contributed to establishment of the field of salivary diagnostics.¹⁵⁵ As a result, saliva has been redefined from being a glandular and mucosal secretion contributing to digestion, and anatomical barrier for infectious agents, to also being an important site of markers with potential to reflect disease (biomarkers). This, in conjunction with the growing interest in rapid and non-invasive diagnostics tests, has led the development of saliva-based analysis (mainly protein based) used in forensic medicine investigations, and in the diagnosis or to monitor cardiovascular disease, renal disease, human immunodeficiency virus, celiac disease, dental caries, periodontal disease, and oral cancer, to name some.¹⁵⁶⁻¹⁶³ Saliva is also readily used for accurate quantification of circulating levels of the steroid hormones cortisol and progesterone, and can be repeatedly collected to establish patterns over time.^{164, 165}

It is obvious that saliva is a candidate milieu for biomarkers of oral diseases, as it is a reservoir for the products of the affected tissues. One of the most accepted links between salivary markers and local disease lie within the salivary microbiome, as salivary dysbiosis contributes to the development of periodontal disease and dental caries, but can also reflect local and systemic cancers, psychiatric disorders, and muscle and joint disorders.¹⁶⁶⁻¹⁷¹ Oral cancers, such as oral squamous cell carcinoma, are known to be reflected in various components of saliva. Significant differences in DNA-methylation patterns are apparent in saliva of oral carcinoma patients, and can be detected using salivary mRNA and miRNA.¹⁷²⁻¹⁷⁴ The salivary transcriptome also shows promising utility in diagnosing lung-, breast-, and ovarian cancer.¹⁷⁵⁻¹⁷⁷ The mechanism by which peripheral cancers alter salivary composition is still unclear, but it has been suggested that the molecules detected in fact are diffused cancer-derived exosomal vesicles.^{178, 179}

The salivary metabolome can also reflect disease, as metabolomic assays are able to discriminate healthy controls from patients with periodontal disease, oral leucoplakia, oral-, pancreatic-, and breast cancer.^{180, 181} Yet, the salivary proteome remains the predominantly preferred medium for discovery and establishment of salivary biomarkers, even though its applicability has been questioned due to protein degradation. Nevertheless, the field of salivary omics and diagnostics will most probably expand drastically, as saliva nowadays is more commonly included in national medical biobanks which will entitle more powered studies in the future.¹⁸²

ORAL MANIFESTATIONS OF IBD

1.2.3 Clinical manifestations

A non-negligible proportion of IBD patients develop oral mucosal manifestations. Oral mucosal manifestations are more extensively investigated and described in CD, but have recently been recognized as important manifestations of UC as well. The mechanism behind occurrence of oral manifestations in IBD is yet to be understood, however, a recent study was able to refute the link between poor oral health and development of oral manifestations in IBD patients, suggesting the role of genetic and/or immunological mechanisms instead.¹⁸³

The prevalence of oral EIMs in CD patients ranges from 20 to 50%.^{103, 104} These are mucocutaneous manifestations most commonly located on the lips and buccal mucosa and can be divided into non-specific and distinct disease-specific lesions. Disease-specific lesions include cobblestoning, orofacial granulomatosis (lip swelling and muco-gingivitis), mucosal tags, and deep linear ulcerations in buccal sulci (Fig. 3).¹⁸⁴⁻¹⁸⁹ Development of oral abscesses has also been reported (Fig. 3).¹⁹⁰ The disease-specific lesions resemble intestinal lesions in appearance and behavior, as they fluctuate between flare and remission, resemble histologically, and respond to IBD therapy.^{184, 191} This might very well lead to unrecorded numbers of oral manifestations in IBD, as patients seldom seek dental care during a flare. Non-specific lesions include aphthous ulcerations, stomatitis and glossitis, all which can be seen in conditions not linked to IBD. Nevertheless, IBD patients are described to suffer from such manifestations to a larger extent compared to healthy controls.¹⁹²⁻¹⁹⁴ It is argued that they may result from host deficiencies of, for instance, vitamins and electrolytes, caused by malnutrition or treatment.^{185, 195}



Figure 3. Oral manifestations of IBD. Clinical pictures of A) buccal cobblestoning and mucosal tags, B) angular cheilitis and lip swelling, and C) an aphthous ulcer in IBD patients.

Images A and B courtesy of Sven Almer, C from the author.

Oral mucosal manifestations have been classified as important primary markers of CD.¹⁹⁶ A prospective study showed that oral manifestations predate the development/detection of CD by up to 4 years in 30% of the patients.¹⁸⁹ Moreover, 37% of the patients with IBD-associated oral manifestations, but no intestinal symptoms, demonstrate intestinal disease upon rectal biopsy or bowel radiology, supporting the theory that oral mucosal manifestations might be part of the primary phase of CD.⁹⁹ Furthermore, disease-specific oral manifestations are

more common in pediatric CD, and the occurrence of oral manifestations at diagnosis of pediatric IBD is associated to more severe forms of CD.^{104, 187, 197} This is particularly clear in the case of orofacial granulomatosis, where its concurrence in pediatric CD precedes intestinal symptoms and is associated to a more severe disease phenotype, possibly regulated by genotypic differences in *NOD2*.^{198, 199}

UC patients are believed to have less, but distinctly different, oral manifestations compared to CD patients.¹⁹¹ A reported specific oral manifestation of UC is pyostomatitis vegetans, which presents with topical oral ulcers surrounded by mucosal “snail-tracks”.^{200, 201} Similar to CD, specific oral manifestations of UC resemble intestinal ones in appearance, both macro- and microscopically, characterized by intra- and subepithelial microabscesses.^{191, 200} Yet, unlike specific CD manifestations, pyostomatitis vegetans seems to be more resistant to systemic IBD treatment.¹⁰³ UC patients may also suffer from non-specific oral manifestations such as aphthous ulcers, but to a lesser extent compared to CD patients.²⁰² The occurrence of aphthous stomatitis during intestinal disease flare-ups in UC patients is suggested to be associated with disease activity in UC, and non-specific lesions are present in about a third of pediatric UC patients.^{185, 203} However, far more work remains to be done to identify and characterize oral manifestations of UC as extensively as it has been done for CD.

1.2.4 Subclinical manifestations

1.2.4.1 Cellular aggregation in oral mucosa

Despite established clinical recognition of the importance of oral mucosal manifestations in IBD, little is known about the underlying mechanisms that lead to the formation of said manifestations. Yet, several immunological findings have been described within oral mucosa of IBD patients. For example, granulomas are present in 60% of macroscopically healthy buccal mucosa of CD patients, independent of disease activity, location, and duration, and of oral microbial content.²⁰⁴ Granulomas have also been identified in minor salivary glands of IBD patients, and lip biopsies from CD patients demonstrate an increase in infiltrated submucosal lymphocytes and sIgA.^{205, 206}

To this date, there are only few studies investigating possible alterations of oral mucosal cells in IBD patients compared to controls – and if so, with focus on epithelial cells.^{207, 208} Oral epithelial cells obtained from clinically healthy oral mucosa of pediatric CD patients mirror the aberrant immune response that is evident in the gut, demonstrating hyperresponsive towards microbial stimuli through spontaneous production of CXCL-8, -9 and -10. This is particularly evident in treatment naïve pediatric patients.²⁰⁷ Oral epithelium of pediatric CD patients might lack the microbial hyporesponsiveness which is acquired at birth, possibly due to improper regulation of the secretory leukocyte protease inhibitor, which directly limits the activation of NF- κ B.²⁰⁸

Altogether, these findings suggest that oral mucosa presents with similar pathogenic mechanisms that foster chronic intestinal inflammation in IBD, which deserves further attention.

1.2.4.2 Altered protein concentrations in saliva

Along with reports of altered appearance of minor salivary glands from lip biopsies, both hyposalivation (reduced salivary secretion/flow) and xerostomia (subjective feeling of dry mouth) have been reported as non-specific oral manifestations of IBD.²⁰⁹ CD patients experience severe xerostomia, possibly as a result of elevated salivary mucin 5 (MUC5) secretion.¹⁹³ Xerostomia is reported among UC patients as well and is worsened by increased disease activity.¹⁹⁴ Yet, studies have been unable to show any significant alteration in salivary secretion flow in IBD patients compared to controls.^{193, 210}

Moreover, several cytokines – some of which are implicated in IBD pathogenesis – have been investigated in saliva of IBD patients. Salivary IL-6 is significantly elevated in CD patients, but not UC, compared to controls.²¹¹ Furthermore, IL-6 concentration increases, together with IL-1 β and TNF- α , in unstimulated saliva during flare compared to remission in CD patients. Interestingly, the salivary levels of these important pro-inflammatory cytokines are comparable between patients in remission and controls, and IL-6 and TNF- α correlate positively with the occurrence of specific oral manifestations in active CD.²¹⁰ Furthermore, the humoral immunity is altered within the saliva of IBD patients: IgA, IgG, and IgM are increased in unstimulated saliva of CD patients compared to controls independent of disease activity, while UC patients present with elevated IgA and IgG concentrations in unstimulated saliva.²¹²⁻²¹⁵

Biochemically, unstimulated saliva of both CD and UC patients contains increased levels of nitric oxide, indicating nitrosative stress in the mouth of IBD patients.²¹⁶ Moreover, saliva of CD patients is oxidatively stressed, containing high levels of lipid peroxide markers and decreased antioxidant capacity, but contains increased levels of the human epidermal growth factor and TGF- β 1.²¹⁶⁻²¹⁸ These findings go well in hand with current evidence on the effect of oxidative stress on the pathogenesis of CD.²¹⁹

Last but not least, saliva has been proposed for genotyping of CD patients, as *CARD15* genes, strongly associated with increased susceptibility towards CD, are detectable in saliva.²²⁰ The applicability of salivary genotyping has further been demonstrated in an attempt to perform salivary DNA collection on a national level in Denmark, and DNA methylation has shown to be equally reflected in intestinal mucosa and saliva.^{221, 222}

1.2.4.3 Increased prevalence of caries and periodontitis

Overall, IBD patients suffer from worsened oral health compared to healthy controls. It has been shown that Swedish CD patients perceive that they have worsened dental status, and Swedish IBD patients seek dental care more often compared to controls.^{223, 224} Furthermore, the IBD-specific health-related quality of life is negatively affected by self-perceived oral health problems in IBD patients.²²⁵

Indeed, it has been proven that both adult and pediatric IBD patients suffer from caries and periodontal disease to a larger extent compared to healthy controls.^{226, 227} Yet, poor dental status remains to be determined as either causality or association to IBD, as a Swedish population based cohort study failed to associate poor oral health with risk of developing IBD.²²⁸ It is important to remember that findings of poor oral health in IBD patients very well might be due to psychosocial changes associated with the disease, such as loss of motivation to maintain proper oral hygiene, dietary change, and poly-medication which in turn can alter the composition and amount of saliva as well as the microbiome. However, there are substantial observations regarding the oral microbiome mediated link between oral and intestinal health.

The oral microbiome of pediatric CD patients is less diverse, and several oral compartments in IBD patients show similarities to the microbial dysbiosis seen in the gut.^{214, 229-231} Furthermore, several putative oral pathogens have been detected in intestinal biopsies of IBD patients, where they are believed to be involved in the modulation of colon motility in IBD.^{232, 233} Moreover, several mechanisms by which pathobionts residing in the oral cavity colonize and induce inflammation in the gut have been proposed; oral *Streptococcus* is able to enter circulation and exacerbate experimental colitis in mice by enhanced secretion of interferon γ (IFN- γ); and *Klebsiella* obtained from saliva of IBD patients is able to exacerbate intestinal inflammation in IL-10 knockout mouse models of experimental colitis through activation of T_H cells.²³⁴⁻²³⁶ Altogether, this shows that the alteration in the oral microbiome might be a contributing factor to the development of IBD, or is merely a reflection of chronic inflammation and exhaustion of the host immune system. Nevertheless, it is adding to the growing evidence of substantial oral mirroring of intestinal pathologies in IBD.

2 RESEARCH AIMS

The overall aim of this thesis was to investigate different mediators of inflammation within the intestines and oral cavity as a reflection of defective immune responses in IBD. Initiating this doctoral thesis, we aimed to explore the intestinal expression of the then recently discovered macrophage growth factor IL-34 during homeostasis, and in human and experimental IBD – which at the time was not known. The remaining part of this thesis aimed to investigate salivary alterations in IBD with focus on the established fecal marker of IBD – calprotectin – and the salivary inflammatory profile of 92 inflammatory proteins.

More specifically, the aims of each study were:

- to assess the intestinal gene expression of macrophage growth factors IL-34 and CSF-1, and their joint receptor CSF-1R during homeostasis, and in human and experimental IBD
 - to histologically assess the expression of IL-34 in the human intestine and investigate the pathways by which colon epithelial cells express *IL-34*
 - to compare the expression of *IL-34* and *CSF-1*, and related cytokines in monocytes from IBD patients and controls, and to investigate the effect of IL-34 and CSF-1 stimulated differentiation of the expression of inflammatory mediators in macrophages
- Study I*
- to validate a methodological protocol for the analysis of calprotectin in saliva
 - to compare the expression of calprotectin in unstimulated and stimulated saliva of IBD patients to controls, and assess its capacity to reflect disease activity before and after treatment
- Study II*
- to broadly explore the salivary and circulatory inflammatory profiles in IBD patients and matched controls, and assess their correlation to clinical parameters of IBD activity
 - to identify protein differences between IBD patients and controls in serum, unstimulated, and stimulated saliva, before and after treatment
- Study III*
- to assess the calprotectin levels in stimulated saliva of IBD patients and controls, in relation to clinical parameters of disease activity and fecal calprotectin
 - to determine the effect of the oral health status on salivary calprotectin levels in IBD patients and controls
- Study IV*
- to assess the phenotype of salivary neutrophils from IBD patients and controls with regards to CD14, CD15, and CD16 expression, together with their expression and secretion of calprotectin

3 MATERIALS AND METHODS

3.1 *IN VIVO* AND *VITRO* EXPRESSION OF IL-34 AND CSF-1 IN IBD

The following section describes the methods used for *study I*, in which we investigated the intestinal expression and involvement of macrophage growth factors and their joint receptor CSF-1R in IBD – with particular emphasis on IL-34.

3.1.1 Biopsies from healthy and inflamed human intestines

In order to investigate the intestinal gene expression of *IL-34*, *CSF-1*, and *CSF-1R* in IBD, ileal and colorectal biopsies from inflamed and non-inflamed sites were obtained from 52 adult IBD patients (21 CD, 29 UC, and 2 IBD-U) during routine endoscopy at the Division of Gastroenterology at the University Hospital, Jönköping, Sweden. Inflammation was determined by means of macroscopic and histological assessment. For investigations regarding homeostatic expression of the growth factors and receptor, intestinal biopsies were obtained from 33 individuals without endoscopic intestinal pathology during the workup of gastrointestinal disorders at the above clinic. Age, gender, IBD diagnosis, and treatment were noted from the study participants.

In total, 183 biopsies were obtained from the 85 individuals. An ileal biopsy was obtained from all individuals, together with ≥ 1 biopsy from the colon. Thus, we were able to obtain biopsies from several intestinal segments, spanning from ileum to rectum, from the same individual (mainly controls). The diversity of the biopsies allowed for assessment of the expression of *IL-34*, *CSF-1*, and *CSF-1R*, and their correlation to *TNF- α* , throughout the whole intestinal tract. However, biopsies with dissent between macroscopic and histological assessment regarding the presence of inflammation were excluded.

3.1.2 *In vitro* cell stimulation

3.1.2.1 *Colon epithelial cells*

The expression of *IL-34* and *CSF-1*, and the regulation thereof by *TNF- α* was assessed in a commercially available cell line of human colon epithelial cells – Caco2 (ATCC, USA). Cells were seeded in 24-well plates in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% FBS, 1% NEEA (Thermo Fisher Scientific) and 1% GlutaMAX (Invitrogen, USA) at 37 °C, 5% CO₂. Following cellular attachment after 48 h, the medium was changed and cells were incubated in the absence/presence of *TNF- α* (1, 10, and 100 ng/ml, BioLegend, USA) for 6 h, or the NF- κ B signaling pathway inhibitors celastrol²³⁷ (Tocris Bioscience, UK) or IMD-0354²³⁸ (Tocris Bioscience) 1 h before stimulation with *TNF- α* . Following incubation, cells were lysed whereupon lysates were subjected to RNA isolation.

3.1.2.2 Macrophages

Next, we investigated the expression of *IL-34*, *CSF-1*, *TNF- α* , and *IL-1 β* in monocytes from IBD patients versus controls. We also assessed the effect of IL-34 and CSF-1 stimulation on the expression of the pro- and anti-inflammatory cytokines *TNF- α* , *IL-1 β* , and *IL-10* in differentiated macrophages.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy-coated blood from 10 IBD patients and 6 controls using Ficoll-Hypaque gradient centrifugation (BD Diagnostics, USA). Monocytes were then isolated using the EasySep Human monocyte enrichment kit without CD16 depletion (StemCell Technologies, Canada) and were plated in six-well plates (5×10^5 monocytes/well) with complete RPMI 1640 medium supplemented with 50 ng/ml of IL-34 or CSF-1 (BioLegend) for 8 days in order to generate macrophages. As for the Caco-2 cells, macrophages were lysed after incubation, and RNA was isolated from lysates.

3.1.3 DSS-mouse model of intestinal inflammation

To determine the translatability of the expression of *IL-34* and *CSF-1* from human to murine intestines, we used a recognized mouse model of intestinal inflammation (colitis) where dextran sodium sulfate (DSS), a chemical agent with anticoagulant properties, is used to induce epithelial damage in the intestines.²³⁹ The DSS colitis model is a widely used murine model within IBD research owing to its rapidity, simplicity, reproducibility, and controllability.

Colitis was induced in mice by administration of 3% DSS (molecular mass 40 kDa, #DB001, TdB Consultancy) with the drinking water, provided ad libitum for 5 days. Untreated control mice received tap water only. The mice, >10 weeks of age, were housed in groups of three to ten at 20–22 °C in a 12 h light-dark cycle and fed with standard chow diet. Body weight was measured daily.

3.1.4 Real-time qPCR

The intestinal and intracellular mRNA expression of *IL-34*, *CSF-1*, their joint receptor *CSF-1R*, and related inflammatory cytokines was assessed in IBD and control biopsies, and in-vitro cultured cells by quantitative polymerase chain reaction (qPCR).

3.1.4.1 RNA isolation and cDNA synthesis

Human intestinal biopsies, maintained in RNAlater RNA stabilization reagent (Qiagen, Germany) prior to sample preparation, were mechanically homogenized using a TissueRuptor and disposable probes (Qiagen). RNA was purified using the AllPrep DNA/RNA mini kit (Qiagen) according to the manufacturer's instructions, either manually or using the automated QIAcube system (Qiagen). Sample degradation was abated by the RNasin plus RNase inhibitor (Promega Corporation, USA). Two sets of 2 μ g of RNA from each biopsy were reverse-transcribed in a total volume of 20 μ l using the High-Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Applied Biosystems, USA) according to the manufacturer's instructions. For each biopsy, the resulting cDNA libraries were pooled.

Total RNA was isolated from Caco-2 cells, monocytes, and macrophages using the Quick-RNA MiniPrep kit (Zymo Research, USA) and reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Of note, RNA was purified from intestines of DSS-mice with lithium chloride due to the inhibition of reverse transcriptase and polymerase by DSS, according to described methods.²⁴⁰

3.1.4.2 SYBR® Green gene expression assay

The mRNA expression of *IL-34*, *CSF-1*, *CSF-1R*, and *TNF-α* in human and mouse intestinal biopsies was analyzed using the iTaq Universal SYBR Green supermix (Life Technologies, USA) on a ViiA7 Real-Time PCR System (Life Technologies). Primer sequences are presented in Table 1. The relative mRNA expression of specific genes was calculated using the comparative threshold ($\Delta\Delta C_t$) method, using human *TBP* and mouse *Tbp* as housekeeping genes.²⁴¹ In the cases where several biopsies were available from the colon of an individual, the average of all inflamed or non-inflamed biopsies were calculated.

SYBR Green (Bio-Rad Laboratories, USA) in the 7500-fast-real-time detection system (Applied Biosystems) was used to detect the mRNA levels of *IL-34*, *CSF-1*, *CSF-1R*, *TNF-α*, *IL-10*, and *IL-1β* (Eurofins, Luxembourg) in Caco-2 and monocytes/macrophages. Primer sequences are presented in Table 1. Target genes were normalized against endogenous controls *ACTB* (encoding β-actin) and *RPL-13a* using the $\Delta\Delta C_t$ method. To rule out the possibility of DNA contamination, samples in which the reverse transcription reaction had been omitted were also subjected to PCR, yielding no amplification.

	Forward	Reverse
hIL34	GCCACCCATCCTGGAAGTA	GACAACACGGATTCCACCTT
hCSF1	GTGGAAGTCCAGTGTAGAGG	TGGAGGGCAGACCACATT
hCFS1R	ATGCTACCACCAAGGACACA	AGCCTCCTGGGTTTCTGG
hTNFA	GACAAGCCTGTAGCCCATGT	TCTCAGCTCCACGCCATT
hIL1B	TACCTGTCCTGCGTGTTGAA	TCTTTGGGTAATTTTGGGATCT
hTBP	CCACTCACAGACTCTCACAAC	CTGCGGTACAATCCCAGAACT
hGAPDH	TCCCACTGGCGTCTTCACC	GGCAGAGATGATGACCCTTTT
hRPL13A	CAAGCGGATGAACACCAAC	TGTGGGGCATACTC
mIl34	TTGCTGTAACAAAGCCCCAT	CCGAGACAAAGGGTACACATTT
mCsf1	GGCTTGGCTTGGGATGATTCT	GAGGGTCTGGCAGGTACTC
mTbp	GCTCTGGAATTGTACCGCAG	CTGGCTCATAGCTCTGGCTC

Table 1. List of primer sequences used for the analysis of mRNA expressions in *study 1*.

3.1.5 Histological assessment

3.1.5.1 Immunohistochemistry

The histological localization of IL-34 in the human colon was depicted in fresh biopsies from the non-inflamed ascending and sigmoid colon of two IBD patients. The biopsies were preserved in Histocon solution (Histolab Products, Sweden) after excision, thereafter embedded in optimal cutting temperature compound (OCT; Histolab Products) and cut into 7 µm sections. Sections were fixed in acetone, followed by blocking of endogenous peroxidase by methanol and H₂O₂, and endogenous alkaline phosphatase by 20% acetic acid. Endogenous avidin and biotin was blocked, whereupon sections were incubated in 5% goat serum prior to overnight incubation at 4 °C with an anti-IL-34 antibody (1:500; Abcam, UK). Sections were incubated with biotinylated secondary goat anti-rabbit (1:500; Vector Laboratories, USA), followed by incubation in Vectastain ABC complex (Vector Laboratories), and development in DAB (diaminobenzidine) solution (Vector Laboratories). Sections were counterstained in hemalaun, dehydrated and mounted in histograde mounting media (Histolab Products). Rabbit IgG control (I-1000; Vector Laboratories) was used as isotype control.

3.1.5.2 Immunofluorescence

The protein level expression of IL-34 by colon epithelial cells was assessed by immunofluorescent staining of Caco-2 cells cultured on chamber slides (Sarstedt, Germany). Cells were fixed in acetone, blocked with 10% normal goat serum in phosphate-buffered saline (PBS), and incubated with anti-IL-34 antibody (same as above) overnight at 4 °C. Chamber slides were incubated with secondary goat anti-rabbit IgG conjugated with Alexa Fluor® 488 (A11034; Invitrogen), diluted in blocking buffer. Sections stained with the secondary antibody alone were used as negative controls. Sections were mounted in ProLong Gold antifade reagent with DAPI (Invitrogen) and scanned on a LSM710 confocal microscope (Zeiss) in single-photon mode.

3.2 SALIVARY ALTERATIONS IN IBD

The following section describes the methods used throughout *study II-IV*, in which we investigated salivary protein alterations with respect to inflammatory bowel disease.

3.2.1 Cohort recruitment

3.2.1.1 *Study II*

For *study II* where we investigated the expression of salivary calprotectin with respect to IBD activity and treatment, 23 IBD patients with ongoing intestinal inflammation were recruited at the Division of Gastroenterology at the Karolinska University hospital in Stockholm. Inclusion criteria were: age ≥ 18 years, a diagnosis of CD or UC according to established routine criteria, and active intestinal inflammation verified by endoscopy. The exclusion criteria were: pregnancy, breast feeding, comorbidities of the gastrointestinal tract (i.e. any diagnosis other than IBD), previous irradiation of the head and neck, and subjective symptoms from the oral cavity. The cohort consisted of 12 patients with established IBD in flare despite treatment (5 CD and 7 UC), and 11 patients which were newly diagnosed and treatment naïve (7 CD and 4 UC). All IBD patients were invited for re-sampling 10–12 weeks after treatment escalation in order to assess the effect of treatment on calprotectin levels, whereof fifteen returned (7 newly diagnosed: 6 CD, 1 UC; 8 with established disease: 3 CD, 5 UC).

Fifteen controls were recruited at the dental clinic of the Department of Dental Medicine at Karolinska Institutet in Stockholm. The inclusion criteria were: age ≥ 18 years, no missing teeth (excluding third molars and orthodontic extractions), available dental records with the last examination performed within one year prior to recruitment with no signs of periodontal disease or caries. The exclusion criteria were: diagnosis of systemic disease, ongoing use of medication excluding contraceptives, use of antibiotics in the previous 3 months, and ongoing or previous smoking or use of Swedish snus. Twelve of the patients were also included in the methodological assessment of the salivary calprotectin analysis.

3.2.1.2 *Study III*

Study III aimed to more extensively investigate salivary protein alterations related to IBD. Thus, we assessed the inflammatory protein profile in saliva and serum of IBD patients with active disease compared to controls, and the effect of treatment on the profiles of IBD patients. For this purpose, we used samples from 20 of the patients recruited for *study II* (Fig. 4). Three patients within *study II* (2 with established IBD, 1 newly diagnosed) were excluded from analysis in *study III* due to the lack of comparative samples of unstimulated and stimulated saliva and/or follow-up samples, in combination with comorbidities which we deemed may affect the readout (namely SLE and Sjögren's syndrome). Instead, one additional newly diagnosed, treatment naïve patient was recruited according to the above described method in *study II*, resulting in a total of 21 IBD patients. Out of these, 11 patients were newly diagnosed and untreated (8 CD, 3 UC), and 10 had established and treated disease (4 CD, 6 UC). Samples from the same 15 IBD patients which returned for re-sampling in *study II* were used in *study III* as well.

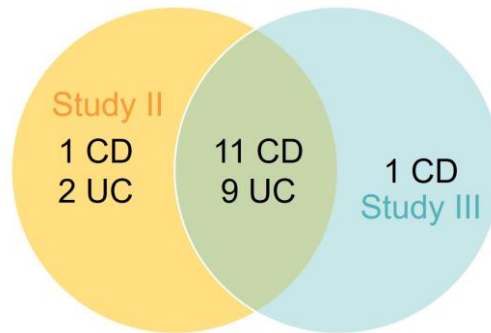


Figure 4. Cohort overlap in study II and III.
Amount of unique and shared IBD patients in study II and III, respectively.

To minimize possible group variances which may affect the results, 22 controls matched for age, sex, body mass index (BMI), and smoking/use of Swedish snuff were recruited at the GHP Stockholm Gastro Center in Stockholm. The exclusion criteria were identical to those for the IBD patients, with the additional exclusion of individuals with an inflammatory disease with ongoing medication and antibiotic treatment ≤ 3 months before sampling. Like the IBD patients, all controls had undergone colonoscopy in the workup of gastrointestinal disorders, and none of them presented macroscopic signs of intestinal inflammation.

3.2.1.3 Study IV

Wishing to further evaluate the salivary expression of calprotectin – now including investigations regarding the possible confounding effect of oral disease and secretory potential by oral neutrophils, we recruited 21 new IBD patients at the Division of Gastroenterology at the Karolinska University hospital in Stockholm. Inclusion criteria were: age ≥ 18 years, and a well-established diagnosis of IBD irrespective of disease activity and ongoing treatment. Exclusion criteria were: other chronic inflammatory conditions (unrelated to IBD), pregnancy, breast feeding, and a history of irradiation of the head and neck. Fourteen of the IBD patients had CD, 6 had UC, and one patient had IBD-U. The majority of the IBD patients (81%) were in remission or had mild disease activity, 4 had moderate disease activity, and none had ongoing severe activity.

Twenty controls were recruited at the GHP Stockholm Gastro Center, the Department of Orofacial Medicine, Folktandvården Stockholms län AB, Danakliniken specialist dental care, and the Department of Dental Medicine, Karolinska Institutet (all in Stockholm) based on the above described exclusion criteria. The original intention was to recruit all controls at the GHP Stockholm Gastro Center, similar to the process used in *study III*. However, due to emergence of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) pandemic and its subsequent strain on the healthcare, patients were instead recruited and sampled in conjunction planned dental visits at collaborative clinics, with the vast majority of the controls (n=15) being recruited at the Department of Dental Medicine at Karolinska Institutet.

	Study II	Study III	Study IV
Cohort	23 IBD ^a (12 CD, 11 UC) 15 controls	21 IBD ^a (12 CD, 9 UC) 22 matched controls	21 IBD (14 CD, 6 UC, 1 IBD-U) 20 controls
Clinical examination	Endoscopy (only IBD)	Endoscopy	Dental examination
Sample type	Saliva (unstim. and stim.) Serum (only IBD)	Saliva (unstim. and stim.) Serum	Stimulated saliva Feces
Timepoints for IBD sampling	2x Active intestinal inflammation 10-12 weeks after treatment (n=15)	2x Active intestinal inflammation 10-12 weeks after treatment (n=15)	1x

Table 2. Summary of the cohorts and samples used in study II-IV.

^a Includes patients with established and treated disease and newly diagnosed, untreated patients.

All IBD patients in study IV had established disease and were treated.

3.2.2 Gastrointestinal disease assessment

As mentioned, all IBD patients included in *study II* and *III* were endoscopically examined at baseline and proven to have an ongoing intestinal inflammation (flare). The endoscopic disease activity was graded according to the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) or the Simple Endoscopic Score for Crohn's disease (SES-CD).^{242, 243} Furthermore, patients' overall disease activity was graded on a 4-grade scale as a physician global assessment (PGA) by an experienced gastroenterologist (SA) based on the following parameters: presence of diarrhea, stool frequency, abdominal pain, fatigue, fever, and weight loss. The grades were 0 = clinical remission, 1 = mild disease activity, 2 = moderate disease activity, and 3 = severe disease activity.

Demographic and clinical data was obtained from the IBD patients, such as current weight and height for BMI calculations, nicotine consumption, the year of IBD diagnosis, surgical and medical treatment, and blood counts (when available from patient records). Moreover, fecal calprotectin values obtained within 3 weeks of baseline were included in the analysis (n=15 at baseline, n=7 at follow-up). Each IBD diagnosis was clinically sub-characterized using the Montreal classification with respect to CD location (upper GI, ileum, ileocolon, or colon) and behavior (inflamed, stricturing, penetrating, or perianal), and disease extent in the case of UC (if left-sided, extensive, proctitis, or undefined).²⁴⁴

Diversely, the IBD patients in *study IV* were not endoscopically examined. Instead, the assessment of the current disease activity was based on PGA, and the Harvey Bradshaw Index (HBI) for CD and Simple Clinical Colitis Activity Index (SCCAI) for UC – both established disease activity indices based on patient-reported outcomes with regards to stool frequency and consistency, urgency to defecate, abdominal pain, general wellbeing, and occurrence of extra-intestinal manifestations.^{245, 246} Similar to *study II* and *III*, demographic and clinical data were obtained with regards to BMI, nicotine consumption, the year of IBD diagnosis, Montreal classifications, and surgical and medical treatment.

3.2.3 Oral disease assessment

No oral examination of study participants was performed in *study II* and *III*. Instead, the participants were enquired regarding their dental anamnesis and potential subjective symptoms from the oral cavity such as pain, discomfort, loosening of teeth, or dental fractures. Declaration of dental symptoms would result in exclusion from recruitment, however, reports of previous or ongoing oral aphthous ulcers were not resulting in exclusion but instead considered as potential IBD manifestation relevant to the study.

In *study IV* we wished to investigate the effect of the oral health status on the levels of salivary calprotectin within our cohort, and thus all participants except 3 controls (excluded from said analyses) were thoroughly examined in the oral cavity. The clinical oral examination included recordings of the number of teeth, plaque index (PI), bleeding on probing (BOP), periodontal probing depth, furcation involvement, number of decayed,

missing and filled teeth (DMFT, excluding 3rd molars), and radiographic examination (lacking from certain controls). A periodontal diagnosis was determined for each participant, based on the most recent disease criteria.^{117, 118} Furthermore, the oral mucosa of all IBD patients was clinically characterized based on the consensus of 3 dentists (EAB, RLJ, and MM) with regards to mucosal appearance and presence of cobblestoned mucosa, pyostomatitis vegetans, angular cheilitis, and aphthous ulcers, to mention some.

3.2.4 Saliva sampling

For protein concentration assessments in saliva, unstimulated and stimulated whole saliva was obtained from the IBD patients (in conjunction to endoscopy at baseline and after 10-12 weeks of treatment escalation) and controls in *study II* and *III*, while only stimulated whole saliva was obtained for the purpose of *study IV*. Unstimulated saliva was collected by instructing the donor to sit in a charioteer position with the body slightly bent forward and head facing the floor, passively drooling in a cup for 10-15 minutes. Stimulated whole saliva was collected through masticatory stimulation by chewing on a 0.5 g paraffin tablet (Ivoclar Vivadent, Liechtenstein) for 5-8 minutes while actively spitting into a cup.

The total volume of unstimulated and stimulated saliva of each participant was measured for salivary flow determination. Samples were immediately transferred to 15 ml Falcon tubes and put on ice, then centrifuged at 1800 x g (for unstimulated saliva, due to handling difficulties related to high sample viscosity) and 600 x g (for stimulated saliva) for 10 min. at +4 °C, whereupon supernatant was aliquoted and stored in -80 °C until analysis.

Of note, unstimulated saliva samples were not possible to obtain from 3 of the IBD patients with established and treated disease included in *study II*, out of which one of the patients returned for re-sampling after treatment escalation and therefore was included in *study III*. Samples were not obtainable due to donor declination or xerostomia, and were excluded from subsequent statistical analyses.

3.2.4.1 Assessment of sample stability with regards to calprotectin

For the purpose of protocol optimization and methodological assessment, both unstimulated and stimulated saliva samples were obtained from 12 of the controls in *study II* during two occasions. The first sampling was carried out in the morning (before 9 AM) when the participants were instructed not to eat or drink >8 h prior to sampling (fasting). The second sampling was carried out on the same day at noon, after the donor had eaten (non-fasting).

Unstimulated saliva was collected for 15 minutes and stimulated saliva for 5 minutes, and thereupon centrifuged, in accordance to the methods above. One aliquot of each sample was stored in -80 °C in order to serve as a control condition, while other aliquots were exposed to different conditions in order to assess the ability of salivary calprotectin to withstand different temperature settings without the presence of protease inhibitors. The conditions consisted of repeated thawing from -80 °C, storage in -20 °C, 3-day storage in +4 °C or room temperature prior to freezing in -80 °C, and a two years long storage in -20 °C or -80 °C.

3.2.5 Serum sampling

Serum samples were obtained in conjunction with saliva sampling from the IBD patients included in *study II* and *III* (from all patients at baseline, and from 15 after 10-12 weeks of treatment), and the controls in *study III* for circulatory assessment of the proteins analyzed in saliva. Blood was drawn into a Z Serum Clot Activator Vacuette tube (Greiner Bio-One, Austria) and centrifuged for 15 minutes at $2000 \times g$, whereupon serum was collected and aliquoted for storage at $-80\text{ }^{\circ}\text{C}$ until analysis.

3.2.6 Fecal sampling

Fresh fecal samples were provided within ≤ 24 h prior to the oral exam and saliva sampling by 15 of the IBD patients in *study IV* for fecal determination of calprotectin. Protein isolation was achieved by mixing a weighed amount of fecal sample ($0.05\text{ g} \pm 0.02\text{ g}$) with 1 ml of fecal protein extraction buffer (BioVendor, Czech Republic). Samples were homogenized by vortexing, whereupon they were centrifuged at $10\ 640 \times g$ for 5 min., and the supernatant was collected for protein analysis. Measured protein concentrations were adjusted for fecal sample mass in grams.

3.2.7 *In vitro* studies of salivary neutrophils

Following salivary determination of calprotectin with respect to intestinal and oral disease, we investigated the phenotype of salivary neutrophils from IBD patients and controls, and whether they could be a potential source of calprotectin in saliva in *study IV*.

3.2.7.1 *Salivary cell isolation*

In order to isolate neutrophils from saliva, samples of uncentrifuged stimulated saliva were diluted with PBS and serially filtered through $70\ \mu\text{m}$, $40\ \mu\text{m}$, and $20\ \mu\text{m}$ filters (Corning, USA, and pluriSelect, Germany), similarly to previously described protocols.²⁴⁷

3.2.7.2 *Flow cytometric analysis*

The cell pellets generated from the salivary filtration of samples from 5 IBD patients and 10 controls were resuspended in FACS buffer (2% FBS, 1 mM EDTA in PBS) and plated for staining. Cells were stained with a viability dye (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain, Invitrogen) in the dark at room temperature, washed in FACS buffer, and incubated with human TruStain FcX (BioLegend) in FACS buffer for 10 minutes. Cells were then incubated with the following fluorochrome-conjugated antibodies (BioLegend) for 30 min. on ice: CD11b (clone ICRF44, PE), CD15 (clone W6D3, BV510), and CD16 (clone 3G8, APC). After incubation, cells were washed, fixed in Cytfix (BD Biosciences, USA), and resuspended in FACS buffer. Cells were analyzed by flow cytometry using the BD FACSVerser (BD Biosciences) and the obtained data was analyzed using FlowJo, version 10 (Tree Star, USA).

3.2.7.3 Immunofluorescent staining

Isolated salivary cells from two controls were seeded in glass chamber slides (Thermo Fisher Scientific) and allowed to adhere for 1 hour. Thereafter, cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Non-specific sites were blocked with 10% goat serum in PBS supplemented with 2% bovine serum albumin (BSA) for 1 h at room temperature. Cells were thereafter incubated with mouse anti-calprotectin (Abcam) or an isotype control for 1.5 h at 37 °C. Specific staining was detected by an Alexa® Fluor 488 goat anti-mouse antibody (Thermo Fisher Scientific). Slides were mounted in VECTASHIELD PLUS antifade mounting media with DAPI (Vector laboratories) and visualized using a Leica DMLA fluorescence microscope equipped with the Leica DFC450 (Leica Microsystems ltd., Switzerland).

3.2.7.4 Salivary neutrophil stimulation

Lastly, we isolated salivary cells from stimulated saliva samples from three IBD patients (2 CD, 1 UC, mean age: 48 ± 9) and age- and gender-matched controls (mean age: 46 ± 15). The cells were manually counted using a Bürker chamber and cultured in 96-well plates in complete RPMI 1640 medium (2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES, and 2% fetal bovine serum), alone for 1 or 3 h, or together with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA; AppliChem, Germany) or *P. gingivalis* lipopolysaccharide (LPS; Invivogen, USA) for 1 h at 37 °C, 5% CO₂, after which supernatants were collected. Additionally, the cells were lysed with lysis buffer (Thermo Fisher Scientific) to assess the intracellular concentrations of calprotectin.

3.2.8 Calprotectin ELISAs

Calprotectin concentrations in unstimulated and stimulated saliva, and serum were assessed using a human enzyme-linked immunosorbent assay (ELISA) precoated with the primary antibody (BioVendor) in *study II*, as it was the only kit which we succeeded to validate for salivary analysis amongst several available kits at the time. By the time that we initiated *study IV*, another human ELISA for the detection of calprotectin was available on the market (R&D Systems, USA), which we validated for salivary analysis and subsequently used in *study IV*.²⁴⁸ We also assessed the agreement between the two kits by comparing measured values of 8 saliva samples and found no significant difference in the measurements, and an overall correlation between the kits ($r=0.83$, $p=0.01$), thus deeming the R&D Systems ELISA as the more cost-effective assay for subsequent calprotectin analyses.

3.2.8.1 Validation for salivary analysis

Due to reports of interference between salivary extracellular matrix components with immunoassay binding during calprotectin analysis, both ELISAs were validated based on spike recoveries and linearity of dilution assessments, which should range between 80-120% according to criteria established by the BIOMARKAPD consortium.^{249, 250}

In short, saliva samples were serially diluted to assess at which dilutions linearity was achieved. A dilution at which the saliva matrix showed no interference with assay linearity and fit the range of the standard curve was used for spike recovery tests. Diluted samples were mixed with known concentrations of recombinant protein (kit standard), referred to as a spike. Recovery, meaning observed concentration in relation to expected concentration, was calculated with reference to the unspiked sample.

3.2.8.2 Assay protocols

The calprotectin ELISAs were conducted according to the instructions provided by the respective manufacturer (BioVendor quantikine for *study II* and R&D Systems duoset for *study IV*) with appropriate dilutions based on the assay validations. Calprotectin was analyzed in unstimulated saliva, stimulated saliva and serum samples in *study II*, while stimulated saliva, feces, and cell culture supernatants and lysates were analyzed in *study IV*.

In *study II*, the saliva samples were thawed on ice, centrifuged 10 640 x g for 5 min. at +4 °C, and diluted 1:300 in provided reagent diluent, while serum samples were thawed, vortexed, and diluted 1:200. Similarly, in *study IV*, stimulated saliva samples were thawed and centrifuged, and thereupon diluted 1:10⁴ in 1% BSA/PBS, while extracted fecal protein samples and cell supernatants/lysates were vortexed and diluted 1:10⁴ and 1:10⁵, respectively.

Readings were made for both assays at 450 nm with wavelength correction at 540 nm using a microplate spectrophotometer (SpectraMAX 340, Molecular Devices, USA), and were interpolated on a 4-parametric curve. The detection range of the BioVendor quantikine assay was 1–64 ng/ml and 0.94–6 ng/ml for the R&D Systems duoset assay.

3.2.8.3 Total protein

The total amount of protein was determined in all samples except serum according to the DC protein assay protocol (Bio-rad Laboratories). In short, 5 µl of centrifuged sample was added to a low-binding 96-well plate (Sarstedt), mixed with 25 µl of Reagent A and 200 µl of Reagent B followed by a 15 min. incubation in the dark. Readings were made at 690 nm with a SpectraMAX 340 microplate spectrophotometer (Molecular Devices). Standards were prepared from BSA and PBS.

3.2.9 Olink inflammatory protein panel

In order to establish the inflammatory protein profile in IBD versus controls, 92 inflammation-related proteins were analyzed in the saliva and serum samples obtained at baseline and after treatment, using the Olink Inflammation panel (Olink Proteomics, Uppsala, Sweden). The analysis was executed in 2018. In short, the method was based on proximity extension assay (PEA) technology: antibody pairs labeled with matching DNA oligonucleotides were bound to their specific target protein, forming a polymerase chain reaction target sequence through proximity-dependent DNA polymerization, which was

detected and quantified using standard real-time polymerase chain reaction (Fig. 5).²⁵¹ Utilizing this assay technology we overcame the specificity issues of multiplexed immunoassays, as each of the 92 oligonucleotide antibody-pairs contained unique DNA sequences which only hybridized to each other.

For this exploratory cohort we set a detection limit of 50%, meaning that analytes detected in < 50% of the serum, unstimulated or stimulated saliva samples were excluded from further analysis and referred to as undetectable. Total protein concentrations in saliva were measured using the Qubit Protein Assay according to the manufacturer's instructions (Life Technologies). Protein interactions and biological functions were investigated using the STRING database.²⁵² Delta values were calculated by subtracting values retrieved from patients with IBD during flare from values obtained after treatment of the same patients.

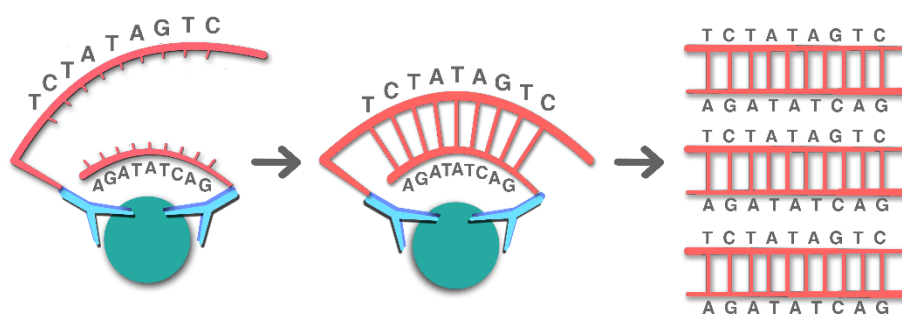


Figure 5. Proximity extension assay technology by Olink Proteomics. Antibody pairs, labelled with matching DNA oligonucleotides, bind to the target antigen in solution. Oligonucleotides brought in close proximity hybridize and are extended by a DNA polymerase, creating a DNA barcode which is amplified by PCR and can be detected and quantified by qPCR. *Illustration by the author.*

ADA	Adenosine Deaminase	IL-15RA	Interleukin-15 receptor subunit alpha
ARTN	Artemin	IL-17A	Interleukin-17A
AXIN	Axin-1	IL-17C	Interleukin-17C
β-NGF	Beta-nerve growth factor	IL-18	Interleukin-18
CASP-8	Caspase-8	IL-18R1	Interleukin-18 receptor 1
CCL3	C-C motif chemokine 3	IL-20	Interleukin-20
CCL4	C-C motif chemokine 4	IL-20RA	Interleukin-20 receptor subunit alpha
CCL19	C-C motif chemokine 19	IL-22RA1	Interleukin-22 receptor subunit alpha-1
CCL20	C-C motif chemokine 20	IL-24	Interleukin-24
CCL23	C-C motif chemokine 23	IL-33	Interleukin-33
CCL25	C-C motif chemokine 25	LAP TGF-β-1	Latency-associated peptide transforming growth factor beta-1
CCL28	C-C motif chemokine 28	LIF	Leukemia inhibitory factor
CD40	CD40L receptor	LIF-R	Leukemia inhibitory factor receptor
CDCP1	CUB domain-containing protein 1	CSF-1	Macrophage colony-stimulating factor 1
CXCL1	C-X-C motif chemokine 1	MMP-1	Matrix metalloproteinase-1
CXCL5	C-X-C motif chemokine 5	MMP-10	Matrix metalloproteinase-10
CXCL6	C-X-C motif chemokine 6	MCP-1	Monocyte chemotactic protein 1
CXCL9	C-X-C motif chemokine 9	MCP-2	Monocyte chemotactic protein 2
CXCL10	C-X-C motif chemokine 10	MCP-3	Monocyte chemotactic protein 3
CXCL11	C-X-C motif chemokine 11	MCP-4	Monocyte chemotactic protein 4
CST5	Cystatin D	CD244	Natural killer cell receptor 2B4
DNER	Delta and Notch-like epidermal growth factor-related receptor	NT-3	Neurotrophin-3
CCL11	Eotaxin	NRTN	Neurturin
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1	OSM	Oncostatin-M
FGF-21	Fibroblast growth factor 21	OPG	Osteoprotegerin
FGF-23	Fibroblast growth factor 23	PD-L1	Programmed cell death 1 ligand 1
FGF-5	Fibroblast growth factor 5	EN-RAGE	Protein S100-A12
FGF-19	Fibroblast growth factor 19	SLAMF1	Signaling lymphocytic activation molecule
Flt3L	Fms-related tyrosine kinase 3 ligand	SIRT2	SIR2-like protein 2
CX3CL1	Fractalkine	STAMBP	STAM-binding protein
GDNF	Glial cell line-derived neurotrophic factor	SCF	Stem cell factor
HGF	Hepatocyte growth factor	ST1A1	Sulfotransferase 1A1
INF-γ	Interferon gamma	CD6	T cell surface glycoprotein CD6 isoform
IL-1α	Interleukin-1 alpha	CD5	T-cell surface glycoprotein CD5
IL-2	Interleukin-2	CD8A	T-cell surface glycoprotein CD8 alpha chain
IL-2RB	Interleukin-2 receptor subunit beta	TSPL	Thymic stromal lymphopoietin
IL-4	Interleukin-4	TNFB	TNF-beta
IL5	Interleukin-5	TRANCE	TNF-related activation-induced cytokine
IL6	Interleukin-6	TRAIL	TNF-related apoptosis-inducing ligand
IL-7	Interleukin-7	TGF-α	Transforming growth factor alpha
IL-8	Interleukin-8	TWEAK	Tumor necrosis factor (Ligand) superfamily, member 12
IL10	Interleukin-10	TNF	Tumor necrosis factor
IL-10RA	Interleukin-10 receptor subunit alpha	TNFSF14	Tumor necrosis factor ligand superfamily member 14
IL-10RB	Interleukin-10 receptor subunit beta	TNFRSF9	Tumor necrosis factor receptor superfamily member 9
IL-12B	Interleukin-12 subunit beta	uPA	Urokinase-type plasminogen activator
IL-13	Interleukin-13	VEGF-A	Vascular endothelial growth factor A

Table 2. Olink inflammatory panel. A list of the 92 proteins analyzed in serum and saliva in *study III*.

3.3 STATISTICAL ANALYSES

Throughout *studies II-IV*, the distribution of gender, nicotine consumption, periodontal diagnoses, and protein detection by the Olink assay were tested using the Pearson χ^2 test or Fisher's Exact test, when applicable. The salivary analyte concentrations in *study III* and *IV* were adjusted to salivary flowrates to counteract confounding effects, whereas calprotectin concentrations from supernatants and lysates resulting from cell experiments in *study IV* were adjusted to the amount of cultured cells. In *study II*, saliva samples obtained from the controls during non-fasting conditions were used for the comparison to salivary samples from IBD patients, to match the sampling conditions.

Differences between groups in continuous variables such as analyte concentrations/expression, clinical parameters, age, and salivary flowrates were tested by the Mann-Whitney U test throughout all four studies, while the Kruskal-Wallis test was used for the assessment of calprotectin concentration differences between periodontal diagnoses in *study IV*. Age and smoking/use of Swedish snus were adjusted between controls and patients in *study II* by the general linear model (GLM) procedure with analysis of covariance (ANCOVA). In *study I*, the protein expression differences between sites of colon were tested by analysis of variance (ANOVA) with an LSD (least significant difference) post-hoc test. The Wilcoxon signed-rank test was used for all paired analyses in *study II-IV*, such as concentration differences of respective analyte before and after IBD treatment, the effect of the sampling strategy and storage on salivary calprotectin, and time-dependent and intra-versus extracellular calprotectin concentration differences in salivary neutrophils. Spearman correlation coefficients were calculated for correlations throughout all four studies. Missing datapoints were excluded from analyses.

In *study III*, sample clustering was visually investigated on score plots resulting from principal component analysis (PCA). Significances in fold differences were adjusted for false discovery through the original false discovery rate method of Benjamini and Hochberg, with the false discovery rate set at 5%. Analyte sensitivity and specificity towards IBD, as assessed in *study II* and *III*, was evaluated by the receiver-operating characteristic (ROC) curve from which areas under the curves were calculated.

SPSS (version 19.0 in *study I* and *II*, 25.0 in *study III* and *IV*; IBM Corporation, USA) and GraphPad Prism (version 6.05 in *study II*, 7.04 in *study III*, and 8.0 in *study IV*; GraphPad Software Inc., USA) were used for statistical analyses and the graphical presentation of results. Statistical significance was set at $p \leq 0.05$. All tests were two-sided.

3.4 ETHICAL CONSIDERATIONS

All four studies were conducted in accordance with the Declaration of Helsinki of the World Medical Association, and all protocols were approved by local ethical boards. *Study I* was approved by the Regional Ethical Review Board in Linköping, Sweden (ref. 2011/201-31), and *studies II-IV* by the Regional Ethical Review Board in Stockholm, Sweden (ref. 2015/17-31, later changed to 2019-01306 after approved protocol amendment). Written informed consent was obtained from all participants. Animal procedures were in compliance with protocols approved by local government authorities (The Board of Agriculture, Experimental Animal Authority, Stockholm, Sweden).

We deemed that the risks involved in sampling within these studies were not greater than those that occur in normal clinical activities in connection with blood tests and endoscopic examination and sampling. Moreover, the participants that were endoscopically examined were done so based on clinical indications, independent of the studies. There was expertise and equipment in place to handle bleeding or perforation, should these occur in connection with the sampling. Advantageously, salivary and fecal sampling is non-invasive, and thus presented no risk to the individual. Serum sampling, on the other hand, is invasive and may cause psycho- and/or physiological discomfort to the participant. To avoid discomfort, serum sampling was predominantly performed using existent catheters placed for routine interventions in conjunction to the participant's regular gastrointestinal examination. Overall, we considered that the benefit of added knowledge within the modulation of IBD pathogenesis and oral inflammatory manifestations of the disease outweigh any risks with these studies.

The emergence of the global pandemic spread of SARS-CoV 2, the causal agent of the infectious coronavirus disease of 2019 (COVID-19),²⁵³ required additional ethical considerations. Namely, the virus was first detected in Sweden during the spring of 2020, when the recruitment of IBD patients and controls for *study IV* was ongoing. As little was known at the time about the virus and its morbidity and mortality in IBD, we concluded the recruitment and sampling of IBD patients for the time being. Furthermore, as mentioned in the description of the cohort recruitment, we took the decision not to recruit the controls from gastroenterological outpatient clinics, but instead recruited the controls among patients visiting the dental clinics at the Department of Dental Medicine and at the specialist center Danakliniken. This way we could minimize the unnecessary societal circulation of individuals which had no other indication to visit dental clinics other than participation in *study IV*. This was crucial since saliva is a reservoir for SARS-CoV 2 and can thus lead to viral transmission²⁵⁴ – a risk which we did not deem ethically justifiable for the sole purpose of this study.

Individuals were informed that participation is voluntary, and their decision did not affect the medical care received. We registered personal data, data from medical journals, and results from the analyzes, and handled them in accordance with the European Union's General Data Protection Regulation (GDPR) and the Swedish Public Access and Secrecy Act.

4 RESULTS AND DISCUSSION

This thesis is based on work presented in papers I-IV (Zwicker et al., 2015; Majster et al., 2019, Majster et al., 2020, and Majster et al. 2021) and addresses the expression of inflammatory mediators in the intestines and oral cavity in relation to IBD. The following section summarizes and discusses the major findings related to these studies. Please refer to the published papers, would the reader wish to have more detailed information.

4.1 IL-34 MODULATES HUMAN AND EXPERIMENTAL IBD

This thesis was initiated investigating the intestinal gene expression of the macrophage growth factors IL-34 and CSF-1, and their shared receptor CSF-1R during homeostasis and intestinal inflammation. Emphasis was placed on IL-34, whose intestinal expression and potential contribution to IBD pathogenesis had not been explored at the time, while experimental mouse models had previously shown that blockade of CSF-1 and CSF-1R ameliorates chemically induced colitis. However, little was known about the expression of these factors throughout the healthy human intestinal tract and in inflamed mucosa of IBD patients.^{67, 68}

We began by assessing the homeostatic expression of the macrophage growth factors and their shared receptor throughout different segments of the intestinal tract, and showed consistent expression of *CSF-1R* throughout normal ileum and colon. Yet, *IL-34* expression was significantly elevated in ileum compared to colon while *CSF-1* followed the opposite location pattern. When further stratifying the *IL-34*, *CSF-1*, and *CSF-1R* expression within the different parts of the colon, we found higher *IL-34* expression in the left colonic segments, most notably in the rectum, whereas *CSF-1R* expression was higher in sigmoid compared with transverse colon, and no differences were observed for *CSF-1R*. This is interesting, as spatiotemporal differences in the expression of IL-34 and CSF-1 have been shown in other organ systems, suggesting that the two growth factors have non-redundant and complementary roles in health and disease.^{60, 255-257}

When investigating the expression of *IL-34*, *CSF-1*, and *CSF-1R* in colonic tissue of IBD patients, *IL-34* expression was shown to be significantly increased in inflamed compared with non-inflamed tissue of both CD and UC patients, while *CSF-1* was significantly increased in inflamed CD colon and *CSF-1R* in UC colon. The same upregulation of *IL-34* and *Csf-1* expression was confirmed during the state of severe intestinal inflammation in mice with DSS-induced colitis. This is in line with findings by others showing that both the gene and protein expression of IL-34 and CSF-1R is increased in inflamed mucosa of IBD patients.^{258, 259} It is however unknown whether this is a mere reflection of ongoing intestinal inflammation in IBD as non-inflamed ileal and colonic mucosa of IBD patients demonstrated similar expression levels of *IL-34* and *CSF-1* compared to healthy intestinal biopsies. This suggests that macrophage growth factors do not reflect an underlying predisposition for the development of intestinal inflammation in IBD. Instead, the expression of *CSF-1R* was significantly lower in the ileum of CD patients and colon of UC patients compared to

controls, which could play a role in the development of IBD. It has in fact been shown that inhibition of CSF-1R reduces the recruitment of proinflammatory macrophages to the intestines and attenuates colitis in experimental mouse models.²⁶⁰

As we were among the first to show the expression of *IL-34* during intestinal homeostasis and inflammation, we investigated its histological localization in order to gain insight into its potential cellular source in the gut. A positive signal for IL-34 was detected in the epithelium and the connective tissue of the colon. Of note, we investigated the expression of IL-34 in non-inflamed biopsies from IBD patients and not in healthy controls, and the epithelial expression of IL-34 was not confirmed by others in healthy intestinal mucosa despite our findings that *IL-34* is equally expressed in non-inflamed intestinal mucosa of IBD patients and healthy intestines.²⁵⁹ However, it was later shown that epithelial cells express CSF-1R which confirms that IL-34 targets these cells.²⁵⁸

As we were the first ones reporting IL-34 expression in the intestinal epithelium, we next compared the *in vitro* *IL-34* expression to the *CSF-1* expression in intestinal epithelial cells (Caco-2 cells) and their regulation by TNF- α – one of the most potent pro-inflammatory mediators in IBD pathogenesis, and the inducer of IL-34 in fibroblasts.²⁶¹ Just as in fibroblasts, TNF- α stimulated *IL-34* expression by Caco-2 cells in a dose-dependent manner,²⁶¹ but stimulated the *CSF-1* expression as well. Yet, the mechanism behind TNF- α -stimulated expression of *IL-34* and *CSF-1* seems to differ, as blocking of the NF- κ B pathway resulted in reduced TNF- α -stimulated *IL-34* expression in colon epithelial cells, but did not affect *CSF-1*. The inductive effect by TNF- α on epithelial expression of *IL-34* and *CSF-1* is interesting, as macrophages are known secretors of TNF- α and closely interact with epithelial cells in IBD. Macrophages promote proliferation and survival of colonic epithelial progenitor cells, and depletion of them in experimental colitis enhances epithelial damage.^{262, 263}

Therefore we hypothesized that the protein expression of the macrophage growth factors within the connective tissue of the colon was accounted for by immune cells such as monocytes and macrophages. Thus, we analyzed the gene expression of *IL-34* and *CSF-1* in monocytes isolated from IBD patients and controls, but saw no difference between the two groups. When differentiating monocytes into macrophages by IL-34 and CSF-1 the cells decreased their expression of *TNF- α* and *IL-10*. Yet, IL-34-differentiated macrophages expressed significantly more *IL-10* compared with macrophages differentiated by CSF-1 which adds to the notion that the two growth factors have different functions. Others have also shown that IL-34 protein expression is significantly elevated in the connective tissue of inflamed IBD mucosa and instead isolated lamina propria mononuclear cells from normal colon for functional studies of IL-34.²⁵⁹ A bilateral induction between IL-34 and TNF- α was shown in these cells, and IL-34 additionally induced the IL-6 production by lamina propria mononuclear cells.²⁵⁹ TNF- α inhibition decreased IL-34 expression in *in vitro* experiments, and treatment by TNF- α inhibitors decreased the expression of *IL-34* in inflamed mucosa – suggesting that current treatment strategies target the gene. Yet, IL-34 neutralization

decreased the TNF- α and IL-6 production in IBD mucosal explants, which indicates therapeutic potential of IL-34 inhibition.²⁵⁹

Altogether, this shows that IL-34 interacts with known mediators of intestinal inflammation and shows potential to play a role in the pathogenesis of IBD. Moreover, epithelial cells, monocytes, and macrophages demonstrate certain similar but also different reactions when exposed to IL-34 or CSF-1, which has been further described in subsequent work from our research group.²⁶⁴ Our findings indicate different roles of the macrophage growth factors in the regulation of cytokines and chemokines, and provide yet another puzzle piece to the complicated interplay between inflammatory mediators and immune cells in IBD. Moreover, since we were able to confirm that our findings regarding *IL-34* and *CSF-1* expression in human intestinal inflammation can be translated to mouse models of DSS-induced colitis, we provided proof that the expression and function of these cytokines can be studied further in such disease models. The roles and interactions of IL-34 and CSF-1 have since been vastly studied, and we are steadily gaining more and more knowledge about their involvement in health and disease.²⁶⁵⁻²⁶⁷ During the mere process of this thesis, our findings have been confirmed by others and antibody neutralization of IL-34 and CSF-1 was shown to reduce TNF- α and IL-6 in the colon of DSS-mice.²⁵⁷

After demonstrating the complex interplay between cells and cytokines in IBD in *study I*, the remainder of this thesis focused on identifying inflammatory mediators in the oral cavity during IBD and investigations of their potential to reflect intestinal inflammation.

4.2 SALIVARY CALPROTECTIN – AN ORAL-GUT LINK

The investigations of oral immunological manifestations by inflammatory mediators in IBD in this thesis were initiated during *study II* through assessment of calprotectin – a clinically established fecal marker of IBD – in the saliva of IBD patients, in relation to disease activity and IBD treatment. Prior to our investigations, it was known that calprotectin is expressed in saliva and increases during periodontal inflammation, however its salivary expression had never been related to IBD.²⁶⁸ After thorough validation of our analytical methods and protocol, we compared calprotectin levels in unstimulated and stimulated saliva from IBD patients with ongoing intestinal inflammation to controls. Calprotectin was significantly increased in saliva of IBD patients compared to controls: CD patients demonstrated higher concentrations of calprotectin in both unstimulated and stimulated saliva, while UC patients only had significantly increased concentrations in stimulated saliva. This suggested that salivary calprotectin is more specific to CD than UC, which was confirmed by statistical analyses of its discriminatory capability through AU-ROC. Crohn's patients are generally more prone to develop oral manifestations, which could explain why the increase of salivary calprotectin was more evident among these patients compared to UC.¹⁹¹

Next, we were interested in comparing the salivary levels during active intestinal inflammation versus after treatment using serum as a comparison, in an attempt to establish whether salivary calprotectin reflects IBD activity. However, calprotectin concentrations

were not altered after treatment in either of the two saliva types. Yet, serendipitously, we noted that newly diagnosed, untreated CD patients had significantly higher calprotectin concentrations in stimulated saliva, and tended to have higher concentrations in unstimulated saliva, compared to CD patients with established disease. Moreover, calprotectin decreased in unstimulated saliva after treatment in newly diagnosed, untreated CD patients. This also goes well in hand with the prevalence of oral manifestations in CD and the fact that oral manifestations are among some of the first disease manifestations in early-onset CD.¹⁹⁷ However, it could also be that patients with established disease received a treatment insufficient in targeting their intestinal inflammation but sufficient enough for maintaining oral homeostasis, resulting in lowered salivary calprotectin concentrations.

Altogether, this was the first time that calprotectin was described in the saliva of IBD patients. It was done so in a small but well-characterized IBD cohort, and included matched samples from IBD patients during an endoscopically confirmed flare and after treatment. However, our findings need to be confirmed in larger cohorts. Moreover, the promising results regarding its possible clinical utility were confronted by doubts of its specificity – it was yet not known how oral disease could affect these observed differences. Namely, if a disease marker is to be deemed as clinically justifiable, it should be specific and not require additional examinations to exclude confounding factors²⁶⁹ – such as oral disease, in this case. In order to assess the confounding effects of the two common oral diseases caries and periodontitis, we recruited a new cohort of IBD patients and controls in *study IV* and orally examined all participants in conjunction with saliva sampling.

Study IV enabled us to confirm our findings from *study II*, as IBD patients had significantly increased calprotectin in stimulated saliva compared to controls. This was yet again particularly pronounced in CD patients. When instead relating salivary calprotectin to oral disease, no significant difference was observed between individuals with or without caries/periodontitis, contrary to previous findings.^{268, 270} Moreover, IBD patients with periodontitis had significantly higher calprotectin concentrations compared to controls with periodontitis, when adjusting to salivary flow. This suggests that the effect of IBD on salivary calprotectin concentrations outweighs that of periodontal inflammation.

Salivary calprotectin concentrations did not correlate to parameters of IBD activity such as CRP, PGA, HBI/SCCAI, nor fecal calprotectin in neither of the two studies. And yet, *study II* showed that it was highly sensitive and specific in distinguishing IBD patients from controls. Furthermore, the IBD patients in *study IV* had low disease activity at the time of sampling, while the patients in *study II* all had ongoing intestinal inflammation confirmed by endoscopy. Still, the mean calprotectin concentrations in saliva were equivalent between these two cohorts. Given that we did not observe a correlation between salivary calprotectin concentrations and PGA, HBI, SCCAI, nor fecal calprotectin, the elevated calprotectin levels in saliva are more probably a reflection of subclinical oral manifestation of IBD rather than ongoing bowel inflammation. The subclinical oral involvement in IBD is thus more probably a ‘trait’ rather than ‘state’ marker of IBD, meaning that it appears early in the course of disease (as shown in

naïve CD patients in *study II*) and remains stable over time, irrespective of disease severity (as shown by the lack of correlation to indexes of intestinal inflammation in *studies II* and *IV*).

Many more studies remain to determine the utility of salivary calprotectin with regards to IBD diagnosis or the reflection of disease activity. Nevertheless, the methodological validation in *study II* has shown that the protein is stable in saliva and that samples can be stored within storage conditions available at most outpatient clinics (+4 °C and -20 °C). Food ingestion prior to sampling did not affect salivary calprotectin concentrations neither. Furthermore, *study IV* indicated that there is no confounding effect related to the oral disease status of the sampled individual, suggesting that oral screening prior to sampling is not needed. However, one must remember that calprotectin is an alarmin that acts as a danger signal for any potential threat to the immune system. Considering that the oral cavity is under constant burden, more possible confounding effects of common oral insults on salivary calprotectin expression must be investigated to determine thresholds between health and disease. Would calprotectin prove to have clinical utility in IBD diagnosis or management, it is most probably going to be well received. When parents of children suffering from IBD were asked regarding their perspectives about potential future non-invasive tests for diagnosing and monitoring their child's disease – where our findings in *study II* were given as an example – salivary sampling was ranked as the most preferred test.²⁷¹

4.3 ABBERANT NEUTROPHILS IN THE ORAL CAVITY OF IBD PATIENTS

We did not aim to assess the salivary origin of calprotectin in *study II*. Nevertheless, we observed greater calprotectin concentrations in stimulated compared to unstimulated saliva which might provide hints regarding the mechanisms behind the secretion of calprotectin in saliva. The great difference between unstimulated and stimulated saliva is the predominant contribution of secretion by the parotid gland in stimulated saliva.¹²⁹ However, this is most unlikely the explanation for the observed concentration differences as some of the lowest calprotectin concentrations in healthy individuals are detected in parotid saliva.²⁷² Instead, the highest concentrations reported are those in GCF and mucosal transudate, which makes them a more probable source of increased calprotectin in stimulated saliva, as GCF leaks from periodontal pockets and mucosal cells are shed during mastication.^{272, 273}

However, experiments assessing the temperature stability of salivary calprotectin in *study II* revealed that concentrations significantly increased when samples were stored in +4 °C for 3 days, which suggested the presence of organic matter with the ability to secrete calprotectin at these temperatures. Given the involvement of neutrophils in intestinal inflammation through calprotectin secretion and their abundance in saliva, *study IV* aimed to assess the phenotype of salivary neutrophils from IBD patients and controls, and whether they could be a potential source of calprotectin in saliva.

We isolated neutrophils from the saliva of IBD patients and controls through serial filtrations and measured the expression of CD15, CD16, and CD11b by flow cytometry. Salivary neutrophils from patients and controls were comparable in their expression of CD15 and

CD16, however CD15+CD16+ neutrophils from IBD patients expressed significantly less CD11b compared to controls. CD11b is a cell adhesion molecule and part of the Mac-1 complex that interacts with ICAM-2 on activated endothelium, orchestrating the transmigration of neutrophils from circulation to tissues.²⁷⁴ Its role in IBD is yet not clear, as blockage of Mac-1 has been shown to both reduce and enhance colonic damage murine models of colitis.^{275, 276} However, CD11b has been shown to be crucial in the neutrophil transepithelial migration and their subsequent contribution in the pathogenesis of intestinal inflammation in UC patients.²⁷⁷⁻²⁷⁹ Conversely, surface expression of CD11b in neutrophils isolated from gingival crevicular fluid is significantly increased in periodontitis patients compared to controls, and oral neutrophils have been shown to have a higher expression of CD11b compared to colonic neutrophils during homeostasis and local inflammation, such as periodontitis.^{280, 281} We did not observe a difference in the prevalence of periodontal disease between the IBD patients and controls whose saliva was used for cellular phenotyping, however the controls tended to have a higher probing depth which could possibly account for the elevated expression of CD11b.

Next, we showed that salivary neutrophils express calprotectin and secrete it in a similar time-dependent manner in IBD patients and controls. There was no difference in the secretion of calprotectin between unstimulated and PMA or LPS stimulated cells from IBD patients or controls. This could be due to the fact that calprotectin is mainly released as a result of neutrophil disruption or death, and not inflammatory stimulation.¹⁹ But it is also possible that there are other neutrophil stimulants *in vivo* which account for the significant increase of salivary calprotectin in IBD patients versus controls, which deserves further investigation.²⁸²⁻²⁸⁴ Yet, connecting back to the +4 °C related findings in *study II* – unpublished observations from our own research group show that salivary neutrophils are able to secrete calprotectin when incubated at +4 °C, even though in small concentrations.

These preliminary findings related to neutrophils and salivary calprotectin urges the analysis of oral mucosa in IBD, in order to better understand if salivary calprotectin concentrations are a reflection of underlying subclinical alterations within the oral cavity which may result through chronic intestinal inflammation.

4.4 THE SALIVARY INFLAMMATORY PROFILE IN IBD

In *study III* we explored and compared the circulatory and salivary inflammatory profiles of IBD patients and matched controls, in relation to IBD activity and treatment, in order to gain a broader understanding of the subclinical inflammatory changes within the oral cavity during IBD. The majority, 67 out of 92, of the proteins were detected in saliva, yet demonstrated a distinctly different expression pattern in saliva compared to serum. It has been shown that about 40% of previously suggested disease markers in plasma can be detected in saliva as well.¹⁴⁷ However, the protein expression correlated scarcely between saliva and serum, while the expression of the majority of the analyzed proteins correlated positively between

unstimulated and stimulated saliva. As with calprotectin, it therefore seems that the proteins detected in saliva are of oral origin and not a transudate of systemic expression.

When we compared the salivary and circulatory protein expressions between IBD patients and controls, we saw that 21 proteins involved in the IL-17 and JAK-STAT signaling pathways were significantly increased in serum of IBD patients, and 4 proteins involved in the positive regulation of proteolysis were significantly decreased. No protein expression differences were observed in unstimulated saliva, while two of the proteins elevated in serum – IL-6 and MMP-10 – were also significantly elevated in stimulated saliva of IBD patients. Both IL-6 and MMP-10 have been implicated in the pathogenesis of IBD and are elevated in serum and in inflamed and non-inflamed intestinal mucosa of patients with IBD.²⁸⁵⁻²⁸⁷ As described in the introduction of this thesis, IL-6 is an important mediator of intestinal inflammation, and has been reported in unstimulated saliva of IBD patients.^{210, 211, 288, 289} MMP-10, on the other hand, is a stromelysin and enzymatic regulator of the extracellular matrix with a hitherto unknown function in IBD. Yet, both proteins are implicated in the pathogenesis of dermatological manifestations of IBD, which makes it even more relevant to further assess these proteins in saliva and oral mucosa in IBD.^{290, 291}

When we related the protein profiles to clinical indexes reflecting IBD activity, the circulatory proteins mainly reflected the biochemical status of disease activity. The salivary proteins, on the other hand, demonstrated more prevalent correlations to clinical parameters and predominantly reflected endoscopic activity. MMP-10 expression in saliva significantly correlated to SES-CD, and IL-6 expression followed a similar but insignificant trend. Thus, unlike calprotectin, these two salivary proteins could be ‘state’ markers of intestinal inflammation in IBD. Altogether, these findings provide additional proof that the oral cavity harbors evidence of aberrant immune responses, similar to those in the intestines of IBD patients.

Interestingly, the protein expression in stimulated saliva correlated negatively to salivary flow, which implies that parotid secretions dilute these proteins. Thus, these proteins most probably do not originate from the parotid gland, but may instead be secreted by the other salivary glands. Another potential source are the mucosal constituents of saliva, such as oral keratinocytes and leukocytes shed through masticatory stimulation, which are present in both saliva types but more abundantly so in stimulated saliva.¹³² If the latter would show to be the case, this is yet another indication to investigate the oral mucosa of IBD patients.

Unfortunately, the TNF- α detection in this Olink panel was shown to be subpar which prompted the company to update its assay some short time after our analysis. The TNF- α assessment in this cohort was thus not optimal – which is a pity as it would be relevant to investigate this potent inflammatory mediator, which is hypothesized to regulate the extraintestinal manifestation of IBD, in relation to IL-6 and MMP-10.²⁹² Furthermore, it would have provided an opportunity to potentially validate previous salivary findings by others in this well-characterized cohort with paired samples before and after treatment. Still, the Olink assay provided with us insight regarding the salivary expression of CSF-1, which we reported to be significantly increased in inflamed compared to non-inflamed mucosa of

IBD patients in *study I*. The macrophage growth factor was detected in both unstimulated and stimulated saliva, however, similar to our findings in *study I*, it was not differentially expressed between IBD patients and controls.

Study II and *III* had overlapping cohorts, and samples from 20 IBD patients were analyzed in both studies (Fig. 4). This allowed for assessment of the correlation between the expressions of the significantly altered salivary proteins – calprotectin, IL-6, and MMP-10. Remarkably, in stimulated saliva, calprotectin concentrations tended to positively correlate to IL-6 and negatively to MMP-10 (Fig. 6). This is interesting, since calprotectin induces IL-6 production in human gingival fibroblasts, but can inhibit MMPs.^{293, 294} What this correlation reflects – or effects – in the oral cavity during IBD remains to be investigated.

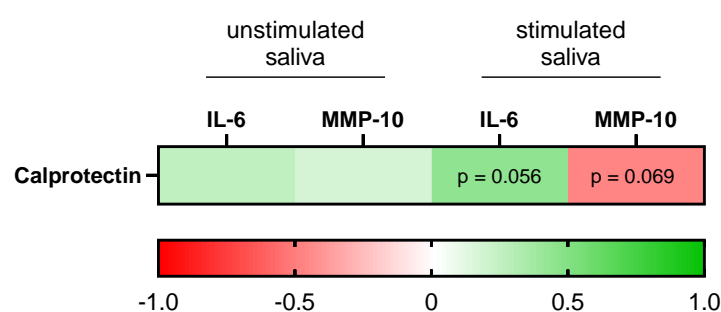


Figure 6. Correlation between altered salivary proteins in study II and III. Heatmap showing the correlation between unstimulated and stimulated concentrations of calprotectin (*study II*), and IL-6 and MMP-10 (*study III*) in 20 IBD patients whose samples were included both studies. Analyzed by Spearman's ρ .

4.5 THE EFFECT OF IBD TREATMENT ON SALIVARY PROTEINS

Except for calprotectin in unstimulated saliva of naïve CD patients, none of the analyzed proteins were significantly affected by IBD treatment at short-term follow-up. Interestingly, neither did the 92 inflammatory proteins in serum in *study III* – only serum calprotectin decreased after treatment in *study II*. In both studies, the PGA decreased in the vast majority of the IBD patients which suggests symptomatic relief but does not reflect endoscopic or microscopic disease activity.²⁹⁵ Unfortunately, the IBD patients in studies *II-III* were not endoscopically examined at follow-up and we did not obtain HBI or SCCAI scores as substitute indexes of the disease activity.

It is reasonable to assume that 3 months of treatment might be too short of a time to evaluate treatment success in IBD as only 35% of IBD patients reach clinical remission after 17 weeks of Vedolizumab treatment.²⁹⁶ Yet another probable explanation to the lack of alteration in salivary and circulatory proteins might lie within IL-6, which is known not to decrease in saliva nor circulation after immunomodulatory treatment of rheumatoid arthritis.^{297, 298}

Given that the STRING-analysis revealed that IL-6 orchestrates the expression of the rest of the significantly altered proteins, it is therefore not surprising that no other proteins were affected by treatment. In the light of this, it would be interesting to assess the effect of IL-6 inhibitors on the levels of these inflammatory mediators.⁸⁸

4.6 OBSERVATIONS REGARDING THE ORAL HEALTH IN IBD

We did not observe differences in oral health status between IBD patients and controls in *study IV*. Others have also not been able to detect an increased prevalence of periodontitis in IBD patients, however, caries findings are consistently reported in the literature.²²⁷ Instead, we observed an increased prevalence of periodontitis in controls. However, this could be explained by our control selection which was affected by the SARS-CoV 2 pandemic (described in the materials and methods), and the small sample size. This resulted in the controls being recruited among patients at dental clinics, which would suggest a greater prevalence of oral disease compared to that of IBD patients outside the dental setting.

The purpose of our study was however not to assess differences in oral health between patients and controls, but to determine potential confounding effects on the determination of salivary calprotectin in IBD, given that salivary calprotectin is known to be affected by caries and periodontitis.^{268,270} However, oral health is an important factor to take into account when analyzing the oral-gut connections in IBD and should always be considered, particularly with regards to periodontitis. Alarming, severe alveolar bone loss has recently been described as an IBD manifestation in a pediatric case of CD.²⁹⁹ Therefore, the pathogenic link between IBD and periodontitis should be further investigated.

Periodontal disease could mask the IBD-related expression of the herein analyzed and many other inflammatory mediators, as the gingival mucosa of IBD patients with periodontitis harbors increased concentrations of inflammatory cytokines compared to the intestine, some of which are significantly increased during IBD flare.^{300,301} The thesis author has analyzed some of the inflammatory mediators presented herein in disease settings other than IBD – namely periodontitis – and has shown that calprotectin increases in correlation to bleeding on probing, and that IL-34 and CSF-1 are present in saliva in which they display complementary roles during periodontal disease.^{248,256} Nevertheless, the salivary expression of IL-34 in IBD would be interesting to assess, since IL-34 is overexpressed in the inflamed salivary glands in patients suffering from another systemic inflammatory disease, namely Sjögren's syndrome.³⁰²

An interesting finding during our oral examination of participants in *study IV* was that more than 70% of the IBD patients presented with at least one oral manifestation. Out of these (n=15), only 2 patients were aware of their current manifestations and 4 patients had no subjective remembrance of a history of oral manifestations. The most common manifestations were mucosal ulcerations, followed by buccal hypertrophy, cobblestoning, angular cheilitis, tongue, and lichenoid lesions. This is among the highest reported prevalence of oral manifestations in an IBD cohort to date and indicates that clinical oral

involvement in IBD is more prevalent than previously thought.^{104, 184, 192} In turn, this could explain why we detect significantly increased concentrations of inflammatory mediators in saliva. Moreover, considering that the cohort in *study IV* mainly consisted of IBD patients in clinical remission or with mild disease activity, this adds to the author's proposed hypothesis that oral manifestations might be a trait marker of IBD, rather than a state marker.

Of note, salivary calprotectin concentrations did not correlate to the occurrence of oral manifestations in this cohort. Nevertheless, the oral mucosa of IBD patients shows signs of aberrant immune responses²⁰⁴⁻²⁰⁸, which may be reflected by other inflammatory proteins in saliva such as IL-6, for example. Moreover, it would be relevant to investigate whether the altered saliva proteins identified in this thesis mediate inflammatory responses by immune cells within the oral mucosa of IBD patients, such as neutrophils and macrophages whose histopathological presence has been confirmed in oral manifestations of IBD.^{199, 303} Furthermore, to the best of the author's knowledge, there are no reports regarding the phenotype of these immune cells nor whether they possess a causative role in the development of oral manifestations in IBD. In sum, the oral mucosa should be more closely investigated in IBD, with the aim to characterize additional aberrant immune responses and mechanisms involved in the generation of oral manifestations of IBD.

5 CONCLUSIONS

This thesis investigated aberrant immune responses associated with IBD, predominantly within the oral cavity. Attention was mainly given to cytokines and chemokines – their local (intestinal or oral) and circulatory expression in relation to intestinal inflammation. As a result, this thesis proposed IL-34 as a new modulator of IBD and showed that several inflammatory mediators are elevated in saliva, suggesting that the oral cavity mirrors intestinal inflammation and might contain clues regarding the mechanism behind oral manifestations of IBD.

The major findings of this thesis are:

- *IL-34* and *CSF-1* demonstrate distinct expression patterns in the human normal intestine, and are significantly elevated in inflamed intestines of IBD patients as well as in a mouse model of colitis. Infiltrating cells of the lamina propria and intestinal epithelial cells express IL-34, and TNF- α regulates *IL-34* expression in intestinal epithelial cells through the NF- κ B pathway.
- Calprotectin is significantly elevated in saliva of IBD patients, particularly in CD patients. Salivary calprotectin differences between patients and controls are most prominent in newly diagnosed untreated CD patients, in which the calprotectin concentration in unstimulated saliva decreases after treatment.
- Saliva and serum inflammatory profiles share a similar composition but reflect different aspects of IBD activity – inflammatory proteins in serum correlate to biochemical status while salivary proteins reflect endoscopic disease activity. The majority of the investigated inflammatory proteins are detected in saliva, and IL-6 and MMP-10 – proteins involved in the pathogenesis of IBD and its EIMs – are significantly elevated in stimulated saliva of IBD patients.
- Calprotectin is significantly elevated in stimulated saliva of IBD patients compared to controls in a second cohort and is not significantly affected by oral disease. Neutrophils isolated from saliva are a source of calprotectin, and demonstrate reduced CD11b expression in IBD patients, but share a similar ability to secrete calprotectin.

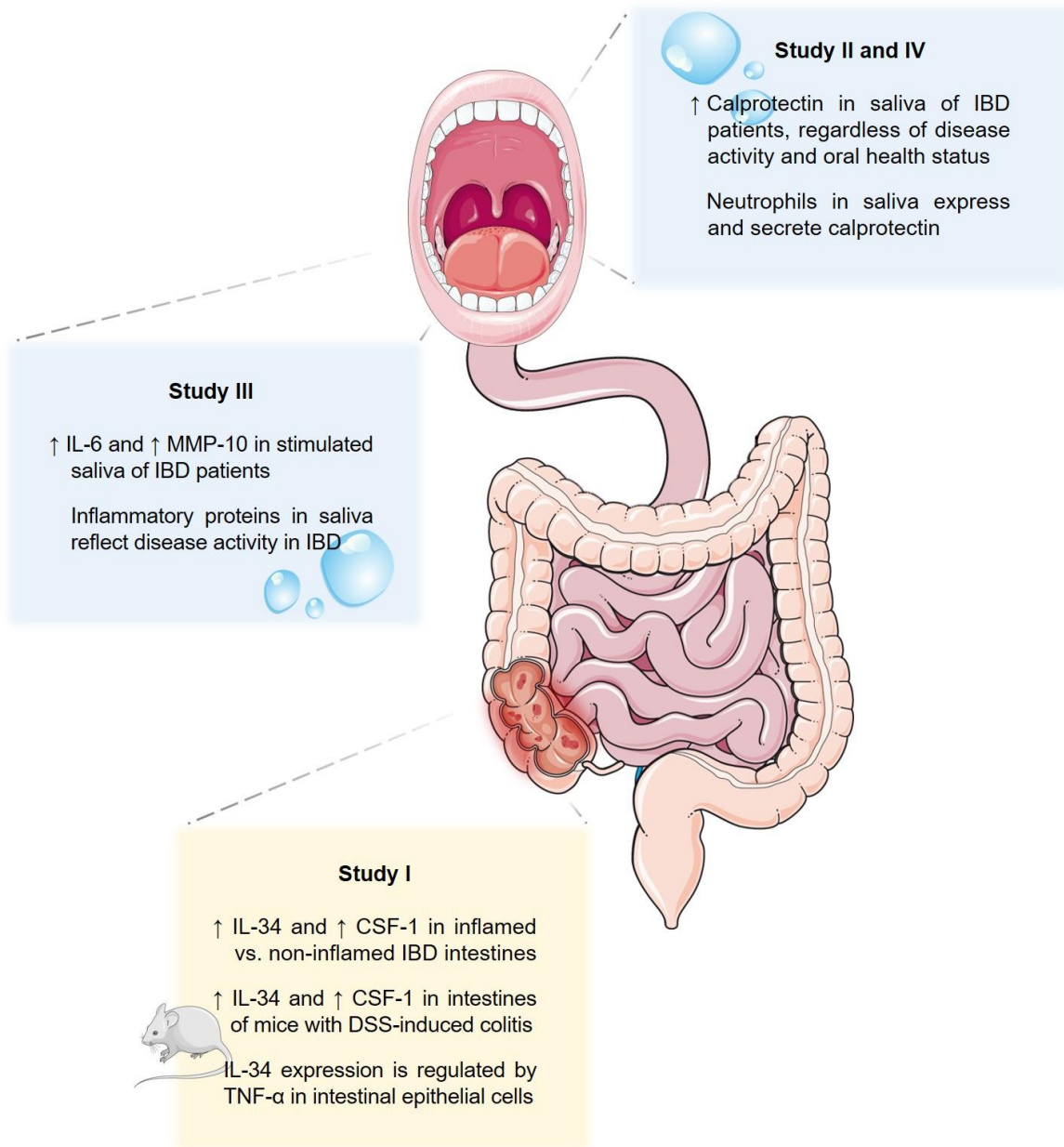


Figure 7. Illustrative summary. The summarized major findings regarding the expression of inflammatory mediators in the intestine and saliva in inflammatory bowel disease. ↑ indicates significant increase. *The figure was created by the author using images licensed under creative commons from Servier Medical Art by Servier and Wikimedia Commons.*

6 POINTS OF PERSPECTIVE

This thesis has shown that inflammatory mediators are elevated in IBD throughout the gastrointestinal tract – from the mouth to the intestines – which could reflect an aberrant immune response. Still, many questions remain unanswered before final conclusions can be drawn regarding which factors are primary or secondary to IBD. This thesis only focused on the immune aspects of IBD, but their role in disease pathogenesis must be considered in light of the other pathogenic components, such as the environment, the genetic make-up, and the microbiome.

The salivary results obtained within this thesis are based on small, yet well-characterized cohorts. Despite being able to confirm our results regarding elevated salivary calprotectin in IBD, these findings need to be validated in large and homogenous cohorts to truly test the clinical applicability of the protein. The same is true for the findings in *study III*, despite the fact that elevated salivary IL-6 has been described in several IBD cohorts.^{210, 211} When analyzing salivary proteins, one should always be aware of the numerous variables which may affect the salivary protein expression (ex. pH, microbiota, proteases, temperature) and exclude these as confounding factors if a protein is to be clinically relevant.³⁰⁴

The field of salivary research in IBD has greatly focused on salivary alterations in relation to the disease, yet there are few studies investigating the origin of these changes. The field should therefore include investigations that characterize the immune responses within oral mucosa which, with the aid of current high-throughput technologies, could generate large insights regarding the link between the oral mucosal immune system and salivary findings in IBD. However, the current SARS-CoV 2 pandemic proposes a non-negligible challenge in future research involving the oral cavity. Studies performed during ongoing spread of the virus must account for the potentially longstanding oral health effects of a SARS-CoV 2 infection, such as impairment of the oral immune system and consequent development of oral lesions, which may mask mechanisms related to IBD, or any oral-systemic link for that matter.³⁰⁵

Nevertheless, upon discovery of sufficiently reliable and specific salivary markers of IBD, salivary samples could potentially substitute blood and fecal samples in the future. This would not only be a simple and cost-effective screening method for IBD in hereditary susceptible individuals, but also enable the patients themselves to follow up their disease activity, in order to be able to predict flare-ups and adjust treatment in time, to decrease the morbidity and subsequent societal costs of IBD. However, regardless of the future clinical utility of these investigated inflammatory mediators, our findings provide added knowledge that could be used for a greater understanding of the cause and sustention of IBD and its oral manifestations, and for the formulation of new treatment strategies. Furthermore, studies and comparisons of the expression of inflammatory mediators in different disease settings may eventually provide insight into the inter-organ spread of defective immune responses during chronic inflammation.

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8 REFERENCES

1. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet*. 2007;369(9573):1627-40.
2. Devlen J, Beusterien K, Yen L, Ahmed A, Cheifetz AS, Moss AC. The burden of inflammatory bowel disease: a patient-reported qualitative analysis and development of a conceptual model. *Inflammatory bowel diseases*. 2014;20(3):545-52.
3. Knowles SR, Graff LA, Wilding H, Hewitt C, Keefer L, Mikocka-Walus A. Quality of Life in Inflammatory Bowel Disease: A Systematic Review and Meta-analyses-Part I. *Inflammatory bowel diseases*. 2018;24(4):742-51.
4. Johnston RD, Logan RFA. What is the peak age for onset of IBD? *Inflamm Bowel Dis*. 2008;14(suppl_2):S4-S5.
5. Alatab S, Sepanlou SG, Ikuta K, Vahedi H, Bisignano C, Safiri S, et al. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Gastroenterology & Hepatology*. 2020;5(1):17-30.
6. Stephan RT, Fergus S, Loren CK. Inflammatory bowel disease: translating basic science into clinical practice. Targan SR, Shanahan F, Karp LC, editors. Oxford: Oxford : Wiley-Blackwell; 2010.
7. Maaser C, Sturm A, Vavricka SR, Kucharzik T, Fiorino G, Annese V, et al. ECCO-ESGAR Guideline for Diagnostic Assessment in IBD Part 1: Initial diagnosis, monitoring of known IBD, detection of complications. *Journal of Crohn's and Colitis*. 2018;13(2):144-64K.
8. Gomollon F, Dignass A, Annese V, Tilg H, Van Assche G, Lindsay JO, et al. 3rd European Evidence-based Consensus on the Diagnosis and Management of Crohn's Disease 2016: Part 1: Diagnosis and Medical Management. *Journal of Crohn's & colitis*. 2017;11(1):3-25.
9. Magro F, Gionchetti P, Eliakim R, Ardizzone S, Armuzzi A, Barreiro-de Acosta M, et al. Third European Evidence-based Consensus on Diagnosis and Management of Ulcerative Colitis. Part 1: Definitions, Diagnosis, Extra-intestinal Manifestations, Pregnancy, Cancer Surveillance, Surgery, and Ileo-anal Pouch Disorders. *Journal of Crohn's & colitis*. 2017;11(6):649-70.
10. Dale I, Brandtzaeg P, Fagerhol MK, Scott H. Distribution of a new myelomonocytic antigen (L1) in human peripheral blood leukocytes. Immunofluorescence and immunoperoxidase staining features in comparison with lysozyme and lactoferrin. *Am J Clin Pathol*. 1985;84(1):24-34.
11. Fagerhol MK, Anderson KB, Naess-Andresen CF, Brandtzaeg P, Dale I. Calprotectin (the L1 leukocyte protein). In: Smith VL, Dedman JR, editors. Stimulus response coupling: the role of intracellular calcium-binding proteins. Boca Raton: CRC Press Inc; 1990. p. 187-210.
12. Stroncek DF, Shankar RA, Skubitz KM. The subcellular distribution of myeloid-related protein 8 (MRP8) and MRP14 in human neutrophils. *J Transl Med*. 2005;3(1):36.
13. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol*. 2003;170(6):3233-42.

14. Sopalla C, Leukert N, Sorg C, Kerkhoff C. Evidence for the involvement of the unique C-tail of S100A9 in the binding of arachidonic acid to the heterocomplex S100A8/A9. *Biol Chem.* 2002;383(12):1895-905.
15. Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. *Carcinogenesis.* 2008;29(10):2035-43.
16. Roseth AG, Schmidt PN, Fagerhol MK. Correlation between faecal excretion of indium-111-labelled granulocytes and calprotectin, a granulocyte marker protein, in patients with inflammatory bowel disease. *Scandinavian journal of gastroenterology.* 1999;34(1):50-4.
17. Bunn SK, Bisset WM, Main MJ, Gray ES, Olson S, Golden BE. Fecal calprotectin: validation as a noninvasive measure of bowel inflammation in childhood inflammatory bowel disease. *J Pediatr Gastroenterol Nutr.* 2001;33(1):14-22.
18. Costa F, Mumolo MG, Bellini M, Romano MR, Ceccarelli L, Arpe P, et al. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. *Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver.* 2003;35(9):642-7.
19. Voganatsi A, Panyutich A, Miyasaki KT, Murthy RK. Mechanism of extracellular release of human neutrophil calprotectin complex. *Journal of leukocyte biology.* 2001;70(1):130-4.
20. Tibble JA, Sigthorsson G, Foster R, Forgacs I, Bjarnason I. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology.* 2002;123(2):450-60.
21. Tibble JA, Sigthorsson G, Bridger S, Fagerhol MK, Bjarnason I. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. *Gastroenterology.* 2000;119(1):15-22.
22. D'Haens G, Ferrante M, Vermeire S, Baert F, Noman M, Moortgat L, et al. Fecal calprotectin is a surrogate marker for endoscopic lesions in inflammatory bowel disease. *Inflammatory bowel diseases.* 2012;18(12):2218-24.
23. Sipponen T, Savilahti E, Karkkainen P, Kolho KL, Nuutinen H, Turunen U, et al. Fecal calprotectin, lactoferrin, and endoscopic disease activity in monitoring anti-TNF-alpha therapy for Crohn's disease. *Inflammatory bowel diseases.* 2008;14(10):1392-8.
24. De Vos M, Dewit O, D'Haens G, Baert F, Fontaine F, Vermeire S, et al. Fast and sharp decrease in calprotectin predicts remission by infliximab in anti-TNF naive patients with ulcerative colitis. *Journal of Crohn's & colitis.* 2012;6(5):557-62.
25. Magro F, Langner C, Driessen A, Ensari A, Geboes K, Mantzaris GJ, et al. European consensus on the histopathology of inflammatory bowel disease. *Journal of Crohn's & colitis.* 2013;7(10):827-51.
26. Vavricka SR, Spigaglia SM, Rogler G, Pittet V, Michetti P, Felley C, et al. Systematic evaluation of risk factors for diagnostic delay in inflammatory bowel disease. *Inflammatory bowel diseases.* 2012;18(3):496-505.
27. Novacek G, Gröchenig HP, Haas T, Wenzl H, Steiner P, Koch R, et al. Diagnostic delay in patients with inflammatory bowel disease in Austria. *Wiener klinische Wochenschrift.* 2019;131(5-6):104-12.
28. Cantoro L, Di Sabatino A, Papi C, Margagnoni G, Ardizzone S, Giuffrida P, et al. The Time Course of Diagnostic Delay in Inflammatory Bowel Disease Over the Last Sixty Years: An Italian Multicentre Study. *Journal of Crohn's and Colitis.* 2017;11(8):975-80.

29. Gallinger Z, Ungaro R, Colombel J-F, Sandler RS, Chen W. P030 delayed diagnosis of crohn's disease is common and associated with an increased risk of disease complications. *Inflamm Bowel Dis*. 2019;25(Supplement_1):S14-S5.
30. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut*. 2006;55(6):749-53.
31. Schoepfer AM, Dehlavi MA, Fournier N, Safroneeva E, Straumann A, Pittet V, et al. Diagnostic delay in Crohn's disease is associated with a complicated disease course and increased operation rate. *The American journal of gastroenterology*. 2013;108(11):1744-53; quiz 54.
32. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet*. 2017;390(10114):2769-78.
33. SWIBREG Årsrapport 2019 [Internet]. 2020.
34. Kaplan GG. The global burden of IBD: from 2015 to 2025. *Nat Rev Gastroenterol Hepatol*. 2015;12(12):720-7.
35. Kawalec P. Indirect costs of inflammatory bowel diseases: Crohn's disease and ulcerative colitis. A systematic review. *Arch Med Sci*. 2016;12(2):295-302.
36. Marchetti M, Liberato NL. Biological therapies in Crohn's disease: are they cost-effective? A critical appraisal of model-based analyses. *Expert Rev Pharmacoecon Outcomes Res*. 2014;14(6):815-24.
37. Mesterton J, Jonsson L, Almer SH, Befrits R, Friis-Liby I, Lindgren S. Resource use and societal costs for Crohn's disease in Sweden. *Inflammatory bowel diseases*. 2009;15(12):1882-90.
38. Everhov Å H, Khalili H, Askling J, Myrelid P, Ludvigsson JF, Halfvarson J, et al. Work Loss Before and After Diagnosis of Crohn's Disease. *Inflammatory bowel diseases*. 2019;25(7):1237-47.
39. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol*. 2015;12(4):205-17.
40. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature*. 2012;491(7422):119-24.
41. Buhner S, Buning C, Genschel J, Kling K, Herrmann D, Dignass A, et al. Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation? *Gut*. 2006;55(3):342-7.
42. Hollander D, Vadheim CM, Brettholz E, Petersen GM, Delahunty T, Rotter JI. Increased intestinal permeability in patients with Crohn's disease and their relatives. A possible etiologic factor. *Ann Intern Med*. 1986;105(6):883-5.
43. Olaison G, Sjö Dahl R, Tagesson C. Abnormal intestinal permeability in Crohn's disease. A possible pathogenic factor. *Scandinavian journal of gastroenterology*. 1990;25(4):321-8.
44. Arslan G, Atasever T, Cindoruk M, Yildirim IS. (51)CrEDTA colonic permeability and therapy response in patients with ulcerative colitis. *Nucl Med Commun*. 2001;22(9):997-1001.
45. Buisine MP, Desreumaux P, Debailleul V, Gambiez L, Geboes K, Ectors N, et al. Abnormalities in mucin gene expression in Crohn's disease. *Inflammatory bowel diseases*. 1999;5(1):24-32.

46. Johansson ME, Gustafsson JK, Holmen-Larsson J, Jabbar KS, Xia L, Xu H, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut*. 2014;63(2):281-91.
47. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, et al. Microbiota of de-novo pediatric IBD: increased *Faecalibacterium prausnitzii* and reduced bacterial diversity in Crohn's but not in ulcerative colitis. *The American journal of gastroenterology*. 2012;107(12):1913-22.
48. Andoh A, Imaeda H, Aomatsu T, Inatomi O, Bamba S, Sasaki M, et al. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. *J Gastroenterol*. 2011;46(4):479-86.
49. Cao Y, Shen J, Ran ZH. Association between *Faecalibacterium prausnitzii* Reduction and Inflammatory Bowel Disease: A Meta-Analysis and Systematic Review of the Literature. *Gastroenterology research and practice*. 2014;2014:872725.
50. Jiri M, Warren S, Michael WR, Hilde C, Bart NL, Brian LK. *Mucosal Immunology*. 4 ed. Mestecky J, Strober W, Russell MW, Cheroutre H, Lambrecht BN, Kelsall BL, editors: Elsevier Science; 2015.
51. de Souza HSP, Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nature Reviews Gastroenterology & Hepatology*. 2015;13:13.
52. Ueno A, Jijon H, Chan R, Ford K, Hirota C, Kaplan GG, et al. Increased Prevalence of Circulating Novel IL-17 Secreting Foxp3 Expressing CD4+ T Cells and Defective Suppressive Function of Circulating Foxp3+ Regulatory Cells Support Plasticity Between Th17 and Regulatory T Cells in Inflammatory Bowel Disease Patients. *Inflamm Bowel Dis*. 2013;19(12):2522-34.
53. Kobayashi T, Okamoto S, Hisamatsu T, Kamada N, Chinen H, Saito R, et al. IL23 differentially regulates the Th1/Th17 balance in ulcerative colitis and Crohn's disease. *Gut*. 2008;57(12):1682.
54. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *The Journal of experimental medicine*. 2005;201(2):233-40.
55. Kucharzik T, Walsh SV, Chen J, Parkos CA, Nusrat A. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. *Am J Pathol*. 2001;159(6):2001-9.
56. Li T, Wang C, Liu Y, Li B, Zhang W, Wang L, et al. Neutrophil Extracellular Traps Induce Intestinal Damage and Thrombotic Tendency in Inflammatory Bowel Disease. *Journal of Crohn's & colitis*. 2020;14(2):240-53.
57. Brazil JC, Louis NA, Parkos CA. The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. *Inflammatory bowel diseases*. 2013;19(7):1556-65.
58. Kvedaraite E, Lourda M, Ideström M, Chen P, Olsson-Åkefeldt S, Forkel M, et al. Tissue-infiltrating neutrophils represent the main source of IL-23 in the colon of patients with IBD. *Gut*. 2016;65(10):1632-41.
59. Neurath MF. Cytokines in inflammatory bowel disease. *Nature Reviews Immunology*. 2014;14(5):329-42.

60. Wei S, Nandi S, Chitu V, Yeung YG, Yu W, Huang M, et al. Functional overlap but differential expression of CSF-1 and IL-34 in their CSF-1 receptor-mediated regulation of myeloid cells. *Journal of leukocyte biology*. 2010;88(3):495-505.
61. Smith PD, Smythies LE, Shen R, Greenwell-Wild T, Gliozzi M, Wahl SM. Intestinal macrophages and response to microbial encroachment. *Mucosal Immunol*. 2011;4(1):31-42.
62. Campos N, Magro F, Castro AR, Cabral J, Rodrigues P, Silva R, et al. Macrophages from IBD patients exhibit defective tumour necrosis factor- α secretion but otherwise normal or augmented pro-inflammatory responses to infection. *Immunobiology*. 2011;216(8):961-70.
63. Smith AM, Rahman FZ, Hayee B, Graham SJ, Marks DJ, Sewell GW, et al. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *The Journal of experimental medicine*. 2009;206(9):1883-97.
64. Steinbach EC, Plevy SE. The role of macrophages and dendritic cells in the initiation of inflammation in IBD. *Inflammatory bowel diseases*. 2014;20(1):166-75.
65. Kamada N, Hisamatsu T, Okamoto S, Chinen H, Kobayashi T, Sato T, et al. Unique CD14 intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-gamma axis. *The Journal of clinical investigation*. 2008;118(6):2269-80.
66. Thiesen S, Janciauskiene S, Uronen-Hansson H, Agace W, Högerkorp C-M, Spee P, et al. CD14^{hi}HLA-DR^{dim} macrophages, with a resemblance to classical blood monocytes, dominate inflamed mucosa in Crohn's disease. *J Leukoc Biol*. 2014;95(3):531-41.
67. Marshall D, Cameron J, Lightwood D, Lawson ADG. Blockade of colony stimulating factor-1 (CSF-1) leads to inhibition of DSS-induced colitis. *Inflamm Bowel Dis*. 2006;13(2):219-24.
68. Huynh D, Akçora D, Malaterre J, Chan CK, Dai X-M, Bertoncello I, et al. CSF-1 Receptor-Dependent Colon Development, Homeostasis and Inflammatory Stress Response. *PloS one*. 2013;8(2):e56951.
69. Lin H, Lee E, Hestir K, Leo C, Huang M, Bosch E, et al. Discovery of a cytokine and its receptor by functional screening of the extracellular proteome. *Science (New York, NY)*. 2008;320(5877):807-11.
70. Dorner BG, Scheffold A, Rolph MS, Huser MB, Kaufmann SH, Radbruch A, et al. MIP-1 α , MIP-1 β , RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(9):6181-6.
71. Uguccioni M, Gionchetti P, Robbiani DF, Rizzello F, Peruzzo S, Campieri M, et al. Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis. *The American journal of pathology*. 1999;155(2):331-6.
72. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, et al. Localization of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. *Gastroenterology*. 1993;104(3):749-58.
73. Mudter J, Neurath MF. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. *Gut*. 2007;56(2):293-303.
74. Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet*. 1992;339(8785):89-91.
75. Atreya R, Zimmer M, Bartsch B, Waldner MJ, Atreya I, Neumann H, et al. Antibodies against tumor necrosis factor (TNF) induce T-cell apoptosis in patients with inflammatory bowel diseases via TNF receptor 2 and intestinal CD14⁺ macrophages. *Gastroenterology*. 2011;141(6):2026-38.

76. Di Sabatino A, Pender SL, Jackson CL, Prothero JD, Gordon JN, Picariello L, et al. Functional modulation of Crohn's disease myofibroblasts by anti-tumor necrosis factor antibodies. *Gastroenterology*. 2007;133(1):137-49.
77. Su L, Nalle SC, Shen L, Turner ES, Singh G, Breskin LA, et al. TNFR2 activates MLCK-dependent tight junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis. *Gastroenterology*. 2013;145(2):407-15.
78. Holtmann MH, Douni E, Schütz M, Zeller G, Mudter J, Lehr H-A, et al. Tumor necrosis factor-receptor 2 is up-regulated on lamina propria T cells in Crohn's disease and promotes experimental colitis in vivo. *Eur J Immunol*. 2002;32(11):3142-51.
79. Monteleone G, Biancone L, Marasco R, Morrone G, Marasco O, Luzzza F, et al. Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells. *Gastroenterology*. 1997;112(4):1169-78.
80. Liu Z, Yadav PK, Xu X, Su J, Chen C, Tang M, et al. The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. *Journal of leukocyte biology*. 2011;89(4):597-606.
81. Sandborn WJ, Su C, Sands BE, D'Haens GR, Vermeire S, Schreiber S, et al. Tofacitinib as Induction and Maintenance Therapy for Ulcerative Colitis. *The New England journal of medicine*. 2017;376(18):1723-36.
82. Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology*. 1990;99(4):956-63.
83. Rutgeerts PJ. The limitations of corticosteroid therapy in Crohn's disease. *Aliment Pharmacol Ther*. 2001;15(10):1515-25.
84. Colombel JF, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, et al. Infliximab, azathioprine, or combination therapy for Crohn's disease. *The New England journal of medicine*. 2010;362(15):1383-95.
85. Sandborn WJ, Kamm MA, Lichtenstein GR, Lyne A, Butler T, Joseph RE. MMX Multi Matrix System mesalazine for the induction of remission in patients with mild-to-moderate ulcerative colitis: a combined analysis of two randomized, double-blind, placebo-controlled trials. *Aliment Pharmacol Ther*. 2007;26(2):205-15.
86. Modigliani R, Mary JY, Simon JF, Cortot A, Soule JC, Gendre JP, et al. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. *Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives*. *Gastroenterology*. 1990;98(4):811-8.
87. Torres J, Boyapati RK, Kennedy NA, Louis E, Colombel JF, Satsangi J. Systematic Review of Effects of Withdrawal of Immunomodulators or Biologic Agents From Patients With Inflammatory Bowel Disease. *Gastroenterology*. 2015;149(7):1716-30.
88. Danese S, Vermeire S, Hellstern P, Panaccione R, Rogler G, Fraser G, et al. Randomised trial and open-label extension study of an anti-interleukin-6 antibody in Crohn's disease (ANDANTE I and II). *Gut*. 2019;68(1):40-8.
89. Paramsothy S, Paramsothy R, Rubin DT, Kamm MA, Kaakoush NO, Mitchell HM, et al. Faecal Microbiota Transplantation for Inflammatory Bowel Disease: A Systematic Review and Meta-analysis. *Journal of Crohn's & colitis*. 2017;11(10):1180-99.
90. Panés J, García-Olmo D, Van Assche G, Colombel JF, Reinisch W, Baumgart DC, et al. Expanded allogeneic adipose-derived mesenchymal stem cells (Cx601) for complex perianal fistulas in Crohn's disease: a phase 3 randomised, double-blind controlled trial. *Lancet*. 2016;388(10051):1281-90.

91. Harbord M, Annese V, Vavricka SR, Allez M, Barreiro-de Acosta M, Boberg KM, et al. The First European Evidence-based Consensus on Extra-intestinal Manifestations in Inflammatory Bowel Disease. *Journal of Crohn's & colitis*. 2016;10(3):239-54.
92. Das KM. Relationship of extraintestinal involvements in inflammatory bowel disease: new insights into autoimmune pathogenesis. *Dig Dis Sci*. 1999;44(1):1-13.
93. Hiller A, Biedermann L, Fournier N, Butter M, Vavricka SR, Ciurea A, et al. The appearance of joint manifestations in the Swiss inflammatory bowel disease cohort. *PLoS one*. 2019;14(4):e0211554.
94. Keller R, Mazurak N, Fantasia L, Fusco S, Malek NP, Wehkamp J, et al. Quality of life in inflammatory bowel diseases: it is not all about the bowel. *Intestinal research*. 2021;19(1):45-52.
95. Juliao-Baños F, Arrubla M, Osorio L, Camargo J, Londoño J, Cáceres C, et al. Characterization and prevalence of extraintestinal manifestations in a cohort of patients with inflammatory intestinal disease in Medellín, Colombia. *Gastroenterol Hepatol*. 2020.
96. Jansson S, Malham M, Paerregaard A, Jakobsen C, Wewer V. Extraintestinal Manifestations Are Associated With Disease Severity in Pediatric Onset Inflammatory Bowel Disease. *J Pediatr Gastroenterol Nutr*. 2020;71(1):40-5.
97. Veloso FT, Carvalho J, Magro F. Immune-related systemic manifestations of inflammatory bowel disease. A prospective study of 792 patients. *Journal of clinical gastroenterology*. 1996;23(1):29-34.
98. Vavricka SR, Rogler G, Gantenbein C, Spoerri M, Prinz Vavricka M, Navarini AA, et al. Chronological Order of Appearance of Extraintestinal Manifestations Relative to the Time of IBD Diagnosis in the Swiss Inflammatory Bowel Disease Cohort. *Inflammatory bowel diseases*. 2015;21(8):1794-800.
99. Scully C, Cochran KM, Russell RI, Ferguson MM, Ghouri MA, Lee FD, et al. Crohn's disease of the mouth: an indicator of intestinal involvement. *Gut*. 1982;23(3):198-201.
100. Vavricka SR, Galvan JA, Dawson H, Soltermann A, Biedermann L, Scharl M, et al. Expression Patterns of TNFalpha, MAdCAM1, and STAT3 in Intestinal and Skin Manifestations of Inflammatory Bowel Disease. *Journal of Crohn's & colitis*. 2018;12(3):347-54.
101. van Sommeren S, Janse M, Karjalainen J, Fehrmann R, Franke L, Fu J, et al. Extraintestinal manifestations and complications in inflammatory bowel disease: from shared genetics to shared biological pathways. *Inflammatory bowel diseases*. 2014;20(6):987-94.
102. Storch I, Sachar D, Katz S. Pulmonary manifestations of inflammatory bowel disease. *Inflammatory bowel diseases*. 2003;9(2):104-15.
103. Lourenco SV, Hussein TP, Bologna SB, Sipahi AM, Nico MM. Oral manifestations of inflammatory bowel disease: a review based on the observation of six cases. *Journal of the European Academy of Dermatology and Venereology : JEADV*. 2010;24(2):204-7.
104. Pittock S, Drumm B, Fleming P, McDermott M, Imrie C, Flint S, et al. The oral cavity in Crohn's disease. *The Journal of pediatrics*. 2001;138(5):767-71.
105. Dawes C. Estimates, from salivary analyses, of the turnover time of the oral mucosal epithelium in humans and the number of bacteria in an edentulous mouth. *Arch Oral Biol*. 2003;48(5):329-36.
106. Adams D. The mucus barrier and absorption through the oral mucosa. *Journal of dental research*. 1975;54 Spec No B:B19-26.

107. Ten Cate AR. Oral histology : development, structure, and function. 4. ed. St. Louis, London: Mosby; 1994.
108. Dutzan N, Konkel JE, Greenwell-Wild T, Moutsopoulos NM. Characterization of the human immune cell network at the gingival barrier. *Mucosal Immunol.* 2016;9:1163.
109. Martini F. Fundamentals of anatomy and physiology. 11. edition ed. Essex: Pearson Education; 2017.
110. Huttenhower C, Gevers D, Knight R, Abubucker S, Badger JH, Chinwalla AT, et al. Structure, function and diversity of the healthy human microbiome. *Nature.* 2012;486:207.
111. Hand AR, Frank ME. Fundamentals of Oral Histology and Physiology. 1st ed. ed. Somerset, USA: John Wiley & Sons, Incorporated; 2015.
112. Abou Neel EA, Aljabo A, Strange A, Ibrahim S, Coathup M, Young AM, et al. Demineralization-remineralization dynamics in teeth and bone. *International journal of nanomedicine.* 2016;11:4743-63.
113. Selwitz RH, Ismail AI, Pitts NB. Dental caries. *The Lancet.* 2007;369(9555):51-9.
114. Bader JD, Shugars DA, Bonito AJ. Systematic reviews of selected dental caries diagnostic and management methods. *J Dent Educ.* 2001;65(10):960-8.
115. Petersen PE, Baez RJ, World Health O. Oral health surveys: basic methods. 5th ed ed. Geneva: World Health Organization; 2013 2013.
116. Kinane DF, Stathopoulou PG, Papapanou PN. Periodontal diseases. *Nature Reviews Disease Primers.* 2017;3(1):17038.
117. Trombelli L, Farina R, Silva CO, Tatakis DN. Plaque-induced gingivitis: Case definition and diagnostic considerations. *Journal of periodontology.* 2018;89 Suppl 1:S46-S73.
118. Tonetti MS, Greenwell H, Kornman KS. Staging and grading of periodontitis: Framework and proposal of a new classification and case definition. *Journal of periodontology.* 2018;89 Suppl 1:S159-S72.
119. Yakubov G, Gibbins H, Proctor G, Carpenter G. Oral mucosa: physiological and physicochemical aspect. In: Khutoryanskiy V, editor. Mucoadhesive materials and drug delivery systems. 36. Chichester, UK: Wiley; 2014.
120. Tandler B. Introduction to mammalian salivary glands. *Microsc Res Tech.* 1993;26(1):1-4.
121. Garrett JR. The proper role of nerves in salivary secretion: a review. *Journal of dental research.* 1987;66(2):387-97.
122. Garrett JR, Kidd A. The innervation of salivary glands as revealed by morphological methods. *Microsc Res Tech.* 1993;26(1):75-91.
123. Rossoni RB, Machado AB, Machado CR. A histochemical study of catecholamines and cholinesterases in the autonomic nerves of the human minor salivary glands. *Histochem J.* 1979;11(6):661-8.
124. Matsuo R, Garrett JR, Proctor GB, Carpenter GH. Reflex secretion of proteins into submandibular saliva in conscious rats, before and after preganglionic sympathectomy. *J Physiol.* 2000;527 Pt 1:175-84.
125. Culp DJ, Graham LA, Latchney LR, Hand AR. Rat sublingual gland as a model to study glandular mucous cell secretion. *Am J Physiol.* 1991;260(6 Pt 1):C1233-44.

126. Moller K, Benz D, Perrin D, Soling HD. The role of protein kinase C in carbachol-induced and of cAMP-dependent protein kinase in isoproterenol-induced secretion in primary cultured guinea pig parotid acinar cells. *Biochem J.* 1996;314 (Pt 1):181-7.
127. Huang AY, Castle AM, Hinton BT, Castle JD. Resting (basal) secretion of proteins is provided by the minor regulated and constitutive-like pathways and not granule exocytosis in parotid acinar cells. *The Journal of biological chemistry.* 2001;276(25):22296-306.
128. Castle AM, Huang AY, Castle JD. The minor regulated pathway, a rapid component of salivary secretion, may provide docking/fusion sites for granule exocytosis at the apical surface of acinar cells. *Journal of cell science.* 2002;115(Pt 14):2963-73.
129. Edgar WM. Saliva and dental health. Clinical implications of saliva: report of a consensus meeting. *British dental journal.* 1990;169(3-4):96-8.
130. Pijpe J, Kalk WW, Bootsma H, Spijkervet FK, Kallenberg CG, Vissink A. Progression of salivary gland dysfunction in patients with Sjogren's syndrome. *Ann Rheum Dis.* 2007;66(1):107-12.
131. Segal A, Wong DT. Salivary diagnostics: enhancing disease detection and making medicine better. *European journal of dental education : official journal of the Association for Dental Education in Europe.* 2008;12 Suppl 1:22-9.
132. Theda C, Hwang SH, Czajko A, Loke YJ, Leong P, Craig JM. Quantitation of the cellular content of saliva and buccal swab samples. *Sci Rep.* 2018;8(1):6944.
133. Mandel ID. The role of saliva in maintaining oral homeostasis. *Journal of the American Dental Association (1939).* 1989;119(2):298-304.
134. ten Cate JM, Featherstone JD. Mechanistic aspects of the interactions between fluoride and dental enamel. *Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists.* 1991;2(3):283-96.
135. Brennan MT, Fox PC. Cytokine mRNA expression in the labial salivary glands of healthy volunteers. *Oral diseases.* 2000;6(4):222-6.
136. Brogden KA, Johnson GK, Vincent SD, Abbasi T, Vali S. Oral inflammation, a role for antimicrobial peptide modulation of cytokine and chemokine responses. *Expert review of anti-infective therapy.* 2013;11(10):1097-113.
137. Mason GI, Hamburger J, Bowman S, Matthews JB. Salivary gland expression of transforming growth factor beta isoforms in Sjogren's syndrome and benign lymphoepithelial lesions. *Molecular pathology : MP.* 2003;56(1):52-9.
138. Tabak LA, Levine MJ, Mandel ID, Ellison SA. Role of salivary mucins in the protection of the oral cavity. *Journal of oral pathology.* 1982;11(1):1-17.
139. Gabriel MO, Grunheid T, Zentner A. Glycosylation pattern and cell attachment-inhibiting property of human salivary mucins. *Journal of periodontology.* 2005;76(7):1175-81.
140. Groschl M, Topf HG, Bohlender J, Zenk J, Klussmann S, Dotsch J, et al. Identification of ghrelin in human saliva: production by the salivary glands and potential role in proliferation of oral keratinocytes. *Clinical chemistry.* 2005;51(6):997-1006.
141. Groschl M, Topf HG, Kratzsch J, Dotsch J, Rascher W, Rauh M. Salivary leptin induces increased expression of growth factors in oral keratinocytes. *Journal of molecular endocrinology.* 2005;34(2):353-66.
142. Grossman N, Binyamin LA, Bodner L. Effect of rat salivary glands extracts on the proliferation of cultured skin cells--a wound healing model. *Cell and tissue banking.* 2004;5(4):205-12.

143. Mathews SA, Kurien BT, Scofield RH. Oral Manifestations of Sjögren's Syndrome. *J Dent Res.* 2008;87(4):308-18.
144. Papas AS, Joshi A, MacDonald SL, Maravelis-Splagounias L, Pretara-Spanedda P, Curro FA. Caries prevalence in xerostomic individuals. *J Can Dent Assoc.* 1993;59(2):171-4, 7-9.
145. Brill N, Björn H. Passage of Tissue Fluid Into Human Gingival Pockets. *Acta Odontologica Scandinavica.* 1959;17(1):11-21.
146. Denny P, Hagen FK, Hardt M, Liao L, Yan W, Arellanno M, et al. The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *Journal of proteome research.* 2008;7(5):1994-2006.
147. Loo JA, Yan W, Ramachandran P, Wong DT. Comparative human salivary and plasma proteomes. *Journal of dental research.* 2010;89(10):1016-23.
148. Drobitch RK, Svensson CK. Therapeutic drug monitoring in saliva. An update. *Clinical pharmacokinetics.* 1992;23(5):365-79.
149. Haeckel R, Hanecke P. The application of saliva, sweat and tear fluid for diagnostic purposes. *Annales de biologie clinique.* 1993;51(10-11):903-10.
150. Jusko WJ, Milsap RL. Pharmacokinetic principles of drug distribution in saliva. *Annals of the New York Academy of Sciences.* 1993;694:36-47.
151. Humphrey SP, Williamson RT. A review of saliva: normal composition, flow, and function. *The Journal of prosthetic dentistry.* 2001;85(2):162-9.
152. Nater UM, Rohleder N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: current state of research. *Psychoneuroendocrinology.* 2009;34(4):486-96.
153. Papacosta E, Nassis GP. Saliva as a tool for monitoring steroid, peptide and immune markers in sport and exercise science. *Journal of science and medicine in sport / Sports Medicine Australia.* 2011;14(5):424-34.
154. Spielmann N, Wong DT. Saliva: diagnostics and therapeutic perspectives. *Oral diseases.* 2011;17(4):345-54.
155. Zhang Y, Sun J, Lin C-C, Abemayor E, Wang MB, Wong DTW. The emerging landscape of salivary diagnostics. *Periodontol 2000.* 2016;70(1):38-52.
156. Cardoso EM, Arregger AL, Tumilasci OR, Elbert A, Contreras LN. Assessment of salivary urea as a less invasive alternative to serum determinations. *Scandinavian journal of clinical and laboratory investigation.* 2009;69(3):330-4.
157. Marley G, Kang D, Wilson EC, Huang T, Qian Y, Li X, et al. Introducing rapid oral-fluid HIV testing among high risk populations in Shandong, China: feasibility and challenges. *BMC public health.* 2014;14:422.
158. Soukup M, Biesiada I, Henderson A, Idowu B, Rodeback D, Ridpath L, et al. Salivary uric acid as a noninvasive biomarker of metabolic syndrome. *Diabetology & metabolic syndrome.* 2012;4(1):14.
159. al-Bayaty HF, Aldred MJ, Walker DM, Newcombe RG, Swift G, Smith PM, et al. Salivary and serum antibodies to gliadin in the diagnosis of celiac disease. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology.* 1989;18(10):578-81.
160. Bonne NJ, Wong DT. Salivary biomarker development using genomic, proteomic and metabolomic approaches. *Genome medicine.* 2012;4(10):82.

161. Zhang L, Farrell JJ, Zhou H, Elashoff D, Akin D, Park NH, et al. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology*. 2010;138(3):949-57.e1-7.
162. Sorsa T, Tjaderhane L, Konttinen YT, Lauhio A, Salo T, Lee HM, et al. Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation. *Ann Med*. 2006;38(5):306-21.
163. Krasse B. Caries risk : a practical guide for assessment and control. Chicago: Chicago : Quintessence Pub. Co.; 1985.
164. Riadfahmy D, Read GF, Walker RF, Griffiths K. Steroids in saliva for assessing endocrine function. *Endocr Rev*. 1982;3(4):367-95.
165. Blair J, Adaway J, Keevil B, Ross R. Salivary cortisol and cortisone in the clinical setting. *Curr Opin Endocrinol Diabetes Obes*. 2017;24(3):161-8.
166. Ge X, Rodriguez R, Trinh M, Gunsolley J, Xu P. Oral microbiome of deep and shallow dental pockets in chronic periodontitis. *PloS one*. 2013;8(6):e65520.
167. Burne RA, Zeng L, Ahn SJ, Palmer SR, Liu Y, Lefebure T, et al. Progress dissecting the oral microbiome in caries and health. *Adv Dent Res*. 2012;24(2):77-80.
168. Ahn J, Chen CY, Hayes RB. Oral microbiome and oral and gastrointestinal cancer risk. *Cancer Causes Control*. 2012;23(3):399-404.
169. Mager DL, Haffajee AD, Devlin PM, Norris CM, Posner MR, Goodson JM. The salivary microbiota as a diagnostic indicator of oral cancer: a descriptive, non-randomized study of cancer-free and oral squamous cell carcinoma subjects. *J Transl Med*. 2005;3:27.
170. Farrell JJ, Zhang L, Zhou H, Chia D, Elashoff D, Akin D, et al. Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer. *Gut*. 2012;61(4):582-8.
171. Lira R, Akerman S, Klinge B, Bostrom EA, Gustafsson A. Salivary microbial profiles in relation to age, periodontal, and systemic diseases. *PloS one*. 2018;13(3):14.
172. Viet CT, Schmidt BL. Methylation array analysis of preoperative and postoperative saliva DNA in oral cancer patients. *Cancer Epidemiol Biomarkers Prev*. 2008;17(12):3603-11.
173. Li Y, St John MA, Zhou X, Kim Y, Sinha U, Jordan RC, et al. Salivary transcriptome diagnostics for oral cancer detection. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2004;10(24):8442-50.
174. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009;15(17):5473-7.
175. Zhang L, Xiao H, Zhou H, Santiago S, Lee JM, Garon EB, et al. Development of transcriptomic biomarker signature in human saliva to detect lung cancer. *Cell Mol Life Sci*. 2012;69(19):3341-50.
176. Zhang L, Xiao H, Karlan S, Zhou H, Gross J, Elashoff D, et al. Discovery and preclinical validation of salivary transcriptomic and proteomic biomarkers for the non-invasive detection of breast cancer. *PloS one*. 2010;5(12):e15573.
177. Lee YH, Kim JH, Zhou H, Kim BW, Wong DT. Salivary transcriptomic biomarkers for detection of ovarian cancer: for serous papillary adenocarcinoma. *J Mol Med (Berl)*. 2012;90(4):427-34.

178. Lau CS, Wong DT. Breast cancer exosome-like microvesicles and salivary gland cells interplay alters salivary gland cell-derived exosome-like microvesicles in vitro. *PloS one*. 2012;7(3):e33037.
179. Lau C, Kim Y, Chia D, Spielmann N, Eibl G, Elashoff D, et al. Role of pancreatic cancer-derived exosomes in salivary biomarker development. *The Journal of biological chemistry*. 2013;288(37):26888-97.
180. Sugimoto M, Wong DT, Hirayama A, Soga T, Tomita M. Capillary electrophoresis mass spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles. *Metabolomics*. 2010;6(1):78-95.
181. Wei J, Xie G, Zhou Z, Shi P, Qiu Y, Zheng X, et al. Salivary metabolite signatures of oral cancer and leukoplakia. *Int J Cancer*. 2011;129(9):2207-17.
182. Galloway JW, Keijser BJB, Williams DM. Saliva in studies of epidemiology of human disease: the UK Biobank project. *Periodontol 2000*. 2016;70(1):184-95.
183. Laranjeira N, Fonseca J, Meira T, Freitas J, Valido S, Leitao J. Oral mucosa lesions and oral symptoms in inflammatory bowel disease patients. *Arq Gastroenterol*. 2015;52(2):105-10.
184. Plauth M, Jenss H, Meyle J. Oral manifestations of Crohn's disease. An analysis of 79 cases. *Journal of clinical gastroenterology*. 1991;13(1):29-37.
185. Fatahzadeh M. Inflammatory bowel disease. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2009;108(5):e1-10.
186. Scheper HJ, Brand HS. Oral aspects of Crohn's disease. *International dental journal*. 2002;52(3):163-72.
187. Rowland M, Fleming P, Bourke B. Looking in the mouth for Crohn's disease. *Inflammatory bowel diseases*. 2010;16(2):332-7.
188. Lankarani KB, Sivandzadeh GR, Hassanpour S. Oral manifestation in inflammatory bowel disease: a review. *World journal of gastroenterology : WJG*. 2013;19(46):8571-9.
189. Williams AJ, Wray D, Ferguson A. The clinical entity of orofacial Crohn's disease. *The Quarterly journal of medicine*. 1991;79(289):451-8.
190. Malins TJ, Wilson A, Ward-Booth RP. Recurrent buccal space abscesses: a complication of Crohn's disease. *Oral surgery, oral medicine, and oral pathology*. 1991;72(1):19-21.
191. Asquith P, Thompson RA, Cooke WT. Oral manifestations of Crohn's disease. *Gut*. 1975;16(4):249-54.
192. Szczeklik K, Pytko-Polonczyk J, Cibor D, Owczarek D, Mach T. Oral mucosa lesions in patients with active Crohn's disease - a prospective study. *Przegl Lek*. 2017;74(2):57-61.
193. de Vries SAG, Tan CXW, Bouma G, Forouzanfar T, Brand HS, de Boer NK. Salivary Function and Oral Health Problems in Crohn's Disease Patients. *Inflammatory bowel diseases*. 2018;24(6):1361-7.
194. Goldinova A, Tan CX, Bouma G, Duijvestein M, Brand HS, de Boer NK. Oral health and salivary function in ulcerative colitis patients. *United European gastroenterology journal*. 2020;8(9):1067-75.
195. Judge TA LG. Inflammatory bowel disease. *Current Diagnosis and Treatment in Gastroenterology*. 2nd ed. New York: Lange Medical Books/Mc Graw-Hill; 2003. p. 108-30.
196. Wolters FL, Russel MG, Sijbrandij J, Schouten LJ, Odes S, Riis L, et al. Crohn's disease: increased mortality 10 years after diagnosis in a Europe-wide population based cohort. *Gut*. 2006;55(4):510-8.

197. Harty S, Fleming P, Rowland M, Crushell E, McDermott M, Drumm B, et al. A prospective study of the oral manifestations of Crohn's disease. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2005;3(9):886-91.
198. Saalman R, Mattsson U, Jontell M. Orofacial granulomatosis in childhood-a clinical entity that may indicate Crohn's disease as well as food allergy. *Acta Paediatr*. 2009;98(7):1162-7.
199. Gale G, Sigurdsson GV, Ostman S, Malmborg P, Hogkil K, Hasseus B, et al. Does Crohn's Disease with Concomitant Orofacial Granulomatosis Represent a Distinctive Disease Subtype? *Inflammatory bowel diseases*. 2016;22(5):1071-7.
200. Storwick GS, Prihoda MB, Fulton RJ, Wood WS. Pyodermatitis-pyostomatitis vegetans: a specific marker for inflammatory bowel disease. *J Am Acad Dermatol*. 1994;31(2 Pt 2):336-41.
201. Markiewicz M, Suresh L, Margarone J, 3rd, Aguirre A, Brass C. Pyostomatitis vegetans: A clinical marker of silent ulcerative colitis. *J Oral Maxillofac Surg*. 2007;65(2):346-8.
202. Greenstein AJ, Janowitz HD, Sachar DB. The extra-intestinal complications of Crohn's disease and ulcerative colitis: a study of 700 patients. *Medicine (Baltimore)*. 1976;55(5):401-12.
203. Boirivant M, Cossu A. Inflammatory bowel disease. *Oral diseases*. 2012;18(1):1-15.
204. Sinčić BM, Tomaš MI, Gobić MB, Juretić M, Kovač D, Lekić A, et al. Clinical Relevance of CD-68 Positive Cells in Normal Buccal Mucosa in Patients with Inflammatory Bowel Disease. *Croat Chem Acta*. 2012;85(2):171-6.
205. Schnitt SJ, Antonioli DA, Jaffe B, Peppercorn MA. Granulomatous inflammation of minor salivary gland ducts: a new oral manifestation of Crohn's disease. *Hum Pathol*. 1987;18(4):405-7.
206. Crama-Bohbouth G, Bosman FT, Vermeer BJ, van der Wal AM, Biemond I, Weterman IT, et al. Immunohistological findings in lip biopsy specimens from patients with Crohn's disease and healthy subjects. *Gut*. 1983;24(3):202-5.
207. Damen GM, Hol J, de Ruiter L, Bouquet J, Sinaasappel M, van der Woude J, et al. Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease. *J Pediatr Gastroenterol Nutr*. 2006;42(2):142-9.
208. Menckeberg CL, Hol J, Simons-Oosterhuis Y, Raatgeep HR, de Ruiter LF, Lindenbergh-Kortleve DJ, et al. Human buccal epithelium acquires microbial hyporesponsiveness at birth, a role for secretory leukocyte protease inhibitor. *Gut*. 2015;64(6):884-93.
209. Katz J, Shenkman A, Stavropoulos F, Melzer E. Oral signs and symptoms in relation to disease activity and site of involvement in patients with inflammatory bowel disease. *Oral diseases*. 2003;9(1):34-40.
210. Szczeklik K, Owczarek D, Pytko-Polonczyk J, Kesek B, Mach TH. Proinflammatory cytokines in the saliva of patients with active and non-active Crohn's disease. *Polskie Archiwum Medycyny Wewnętrznej*. 2012;122(5):200-8.
211. Nielsen AA, Nielsen JN, Schmedes A, Brandslund I, Hey H. Saliva Interleukin-6 in patients with inflammatory bowel disease. *Scandinavian journal of gastroenterology*. 2005;40(12):1444-8.
212. Crama-Bohbouth G, Lems-van Kan P, Weterman IT, Biemond I, Pena AS. Immunological findings in whole and parotid saliva of patients with Crohn's disease and healthy controls. *Dig Dis Sci*. 1984;29(12):1089-92.

213. Crama-Bohbouth G, Pena AS, Verspaget HW, vd Zon A, Biemond I, Weterman IT, et al. Immunological findings in whole and parotid saliva of patients with ulcerative colitis and healthy controls. *Hepatogastroenterology*. 1989;36(4):185-7.
214. Said HS, Suda W, Nakagome S, Chinen H, Oshima K, Kim S, et al. Dysbiosis of Salivary Microbiota in Inflammatory Bowel Disease and Its Association With Oral Immunological Biomarkers. *DNA Res*. 2014;21(1):15-25.
215. Savage NW, Barnard K, Shirlaw PJ, Rahman D, Mistry M, Escudier MP, et al. Serum and salivary IgA antibody responses to *Saccharomyces cerevisiae*, *Candida albicans* and *Streptococcus mutans* in orofacial granulomatosis and Crohn's disease. *Clinical and experimental immunology*. 2004;135(3):483-9.
216. Jahanshahi G, Motavasel V, Rezaie A, Hashtroudi AA, Daryani NE, Abdollahi M. Alterations in antioxidant power and levels of epidermal growth factor and nitric oxide in saliva of patients with inflammatory bowel diseases. *Dig Dis Sci*. 2004;49(11-12):1752-7.
217. Rezaie A, Ghorbani F, Eshghtork A, Zamani MJ, Dehghan G, Taghavi B, et al. Alterations in salivary antioxidants, nitric oxide, and transforming growth factor-beta 1 in relation to disease activity in Crohn's disease patients. *Annals of the New York Academy of Sciences*. 2006;1091:110-22.
218. Szczeklik K, Krzysciak W, Cibor D, Domagala-Rodacka R, Pytko-Polonczyk J, Mach T, et al. Markers of lipid peroxidation and antioxidant status in the serum and saliva of patients with active Crohn disease. *Polish archives of internal medicine*. 2018;128(6):362-70.
219. Zhu H, Li YR. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. *Exp Biol Med*. 2012;237(5):474-80.
220. Hong J, Leung E, Fraser A, Krissansen GW. Nucleic acid from saliva and salivary cells for noninvasive genotyping of Crohn's disease patients. *Genetic testing*. 2008;12(4):587-9.
221. Hearn NL, Coleman AS, Ho V, Chiu CL, Lind JM. Comparing DNA methylation profiles in saliva and intestinal mucosa. *BMC Genomics*. 2019;20:9.
222. Kappelman MD, Lange A, Randell RL, Basta PV, Sandler RS, Laugesen K, et al. 1 Feasibility of salivary DNA collection in a population-based case-control study: a pilot study of pediatric Crohn's disease. *Clin Epidemiol*. 2018;10:215-22.
223. Rikardsson S, Jonsson J, Hultin M, Gustafsson A, Johannsen A. Perceived oral health in patients with Crohn's disease. *Oral health & preventive dentistry*. 2009;7(3):277-82.
224. Johannsen A, Fored MC, Hakansson J, Ekbohm A, Gustafsson A. Consumption of Dental Treatment in Patients with Inflammatory Bowel Disease, a Register Study. *PloS one*. 2015;10(8):8.
225. Tan CXW, Brand HS, Iqbal S, De Boer NKH, Forouzanfar T, de Visscher J. A self-reported survey on oral health problems in patients with inflammatory bowel disease with a stoma. *Oral surgery, oral medicine, oral pathology and oral radiology*. 2020;130(3):e80-e6.
226. Papageorgiou SN, Hagner M, Nogueira AVB, Franke A, Jager A, Deschner J. Inflammatory bowel disease and oral health: systematic review and a meta-analysis. *J Clin Periodontol*. 2017;44(4):382-93.
227. Tan CXW, Brand HS, Kalender B, De Boer NKH, Forouzanfar T, de Visscher J. Dental and periodontal disease in patients with inflammatory bowel disease. *Clinical oral investigations*. 2021.
228. Yin WY, Ludvigsson JF, Liu ZW, Roosaar A, Axell T, Ye WM. Inverse Association Between Poor Oral Health and Inflammatory Bowel Diseases. *Clin Gastroenterol Hepatol*. 2017;15(4):525-31.

229. Docktor MJ, Paster BJ, Abramowicz S, Ingram J, Wang YE, Correll M, et al. Alterations in diversity of the oral microbiome in pediatric inflammatory bowel disease. *Inflammatory bowel diseases*. 2012;18(5):935-42.
230. Kelsen J, Bittinger K, Pauly-Hubbard H, Posivak L, Grunberg S, Baldassano R, et al. Alterations of the Subgingival Microbiota in Pediatric Crohn's Disease Studied Longitudinally in Discovery and Validation Cohorts. *Inflamm Bowel Dis*. 2015;21(12):2797-805.
231. Xun Z, Zhang Q, Xu T, Chen N, Chen F. Dysbiosis and Ecotypes of the Salivary Microbiome Associated With Inflammatory Bowel Diseases and the Assistance in Diagnosis of Diseases Using Oral Bacterial Profiles. *Front Microbiol*. 2018;9:17.
232. Dinakaran V, Mandape SN, Shuba K, Pratap S, Sakhare SS, Tabatabai MA, et al. Identification of Specific Oral and Gut Pathogens in Full Thickness Colon of Colitis Patients: Implications for Colon Motility. *Front Microbiol*. 2019;9:23.
233. Gevers D, Kugathasan S, Denson Lee A, Vázquez-Baeza Y, Van Treuren W, Ren B, et al. The Treatment-Naive Microbiome in New-Onset Crohn's Disease. *Cell Host & Microbe*. 2014;15(3):382-92.
234. Kojima A, Nakano K, Wada K, Takahashi H, Katayama K, Yoneda M, et al. Infection of specific strains of *Streptococcus mutans*, oral bacteria, confers a risk of ulcerative colitis. *Sci Rep*. 2012;2:332.
235. Kojima A, Nomura R, Naka S, Okawa R, Ooshima T, Nakano K. Aggravation of inflammatory bowel diseases by oral streptococci. *Oral Dis*. 2014;20(4):359-66.
236. Atarashi K, Suda W, Luo C, Kawaguchi T, Motoo I, Narushima S, et al. Ectopic colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science (New York, NY)*. 2017;358(6361):359.
237. Lee J-H, Koo TH, Yoon H, Jung HS, Jin HZ, Lee K, et al. Inhibition of NF- κ B activation through targeting I κ B kinase by celastrol, a quinone methide triterpenoid. *Biochem Pharmacol*. 2006;72(10):1311-21.
238. Tanaka A, Konno M, Muto S, Kambe N, Morii E, Nakahata T, et al. A novel NF- κ B inhibitor, IMD-0354, suppresses neoplastic proliferation of human mast cells with constitutively activated c-kit receptors. *Blood*. 2005;105(6):2324-31.
239. Chassaing B, Aitken JD, Malleshappa M, Vijay-Kumar M. Dextran sulfate sodium (DSS)-induced colitis in mice. *Curr Protoc Immunol*. 2014;104:15.25.1-15.25.14.
240. Viennois E, Chen F, Laroui H, Baker MT, Merlin D. Dextran sodium sulfate inhibits the activities of both polymerase and reverse transcriptase: lithium chloride purification, a rapid and efficient technique to purify RNA. *BMC Res Notes*. 2013;6(1):360.
241. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods*. 2001;25(4):402-8.
242. Travis SP, Schnell D, Krzeski P, Abreu MT, Altman DG, Colombel JF, et al. Developing an instrument to assess the endoscopic severity of ulcerative colitis: the Ulcerative Colitis Endoscopic Index of Severity (UCEIS). *Gut*. 2012;61(4):535-42.
243. Daperno M, D'Haens G, Van Assche G, Baert F, Bulois P, Maunoury V, et al. Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc*. 2004;60(4):505-12.

244. Silverberg MS, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Canadian journal of gastroenterology = Journal canadien de gastroenterologie*. 2005;19 Suppl A:5a-36a.
245. Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet*. 1980;1(8167):514.
246. Walmsley RS, Ayres RC, Pounder RE, Allan RN. A simple clinical colitis activity index. *Gut*. 1998;43(1):29-32.
247. Lakschevitz FS, Aboodi GM, Glogauer M. Oral Neutrophils Display a Site-Specific Phenotype Characterized by Expression of T-Cell Receptors. *J Periodontol*. 2013;84(10):1493-503.
248. Holmstrom SB, Lira-Junior R, Zwicker S, Majster M, Gustafsson A, Akerman S, et al. MMP-12 and S100s in saliva reflect different aspects of periodontal inflammation. *Cytokine*. 2018;113:155-61.
249. Sweet SP, Denbury AN, Challacombe SJ. Salivary calprotectin levels are raised in patients with oral candidiasis or Sjogren's syndrome but decreased by HIV infection. *Oral microbiology and immunology*. 2001;16(2):119-23.
250. Mitchell JS, Lowe TE. Matrix effects on an antigen immobilized format for competitive enzyme immunoassay of salivary testosterone. *Journal of immunological methods*. 2009;349(1-2):61-6.
251. Assarsson E, Lundberg M, Holmquist G, Bjorkesten J, Thorsen SB, Ekman D, et al. Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. *PloS one*. 2014;9(4):e95192.
252. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res*. 2019;47(D1):D607-d13.
253. Wu F, Zhao S, Yu B, Chen Y-M, Wang W, Song Z-G, et al. A new coronavirus associated with human respiratory disease in China. *Nature*. 2020;579(7798):265-9.
254. Huang N, Pérez P, Kato T, Mikami Y, Okuda K, Gilmore RC, et al. SARS-CoV-2 infection of the oral cavity and saliva. *Nat Med*. 2021.
255. Easley-Neal C, Foreman O, Sharma N, Zarrin AA, Weimer RM. CSF1R Ligands IL-34 and CSF1 Are Differentially Required for Microglia Development and Maintenance in White and Gray Matter Brain Regions. *Front Immunol*. 2019;10:2199.
256. Martinez GL, Majster M, Bjurshammar N, Johannsen A, Figueredo CM, Bostrom EA. Salivary Colony Stimulating Factor-1 and Interleukin-34 in Periodontal Disease. *Journal of periodontology*. 2017;88(8):e140-e9.
257. Lin W, Xu D, Austin CD, Caplazi P, Senger K, Sun Y, et al. Function of CSF1 and IL34 in Macrophage Homeostasis, Inflammation, and Cancer. *Front Immunol*. 2019;10(2019).
258. Franzè E, Marafini I, De Simone V, Monteleone I, Caprioli F, Colantoni A, et al. Interleukin-34 Induces Cc-chemokine Ligand 20 in Gut Epithelial Cells. *Journal of Crohn's and Colitis*. 2015;10(1):87-94.
259. Franze E, Monteleone I, Cupi ML, Mancina P, Caprioli F, Marafini I, et al. Interleukin-34 sustains inflammatory pathways in the gut. *Clinical science (London, England : 1979)*. 2015;129(3):271-80.

260. Manthey CL, Moore BA, Chen Y, Loza MJ, Yao X, Liu H, et al. The CSF-1-receptor inhibitor, JNJ-40346527 (PRV-6527), reduced inflammatory macrophage recruitment to the intestinal mucosa and suppressed murine T cell mediated colitis. *PloS one*. 2019;14(11):e0223918.
261. Chemel M, Le Goff B, Brion R, Cozic C, Berreur M, Amiaud J, et al. Interleukin 34 expression is associated with synovitis severity in rheumatoid arthritis patients. *Ann Rheum Dis*. 2012;71(1):150.
262. Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS. Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. *Proc Natl Acad Sci U S A*. 2005;102(1):99.
263. Qualls JE, Kaplan AM, Van Rooijen N, Cohen DA. Suppression of experimental colitis by intestinal mononuclear phagocytes. *J Leukoc Biol*. 2006;80(4):802-15.
264. Zwicker S, Bureik D, Bosma M, Martinez GL, Almer S, Boström EA. Receptor-Type Protein-Tyrosine Phosphatase ζ and Colony Stimulating Factor-1 Receptor in the Intestine: Cellular Expression and Cytokine- and Chemokine Responses by Interleukin-34 and Colony Stimulating Factor-1. *PloS one*. 2016;11(11):e0167324.
265. Nieto JC, Zamora C, Cantó E, Garcia-Planella E, Gordillo J, Ortiz MA, et al. CSF-1 regulates the function of monocytes in Crohn's disease patients in remission. *Sci Rep*. 2017;7(1):92.
266. Boulakirba S, Pfeifer A, Mhaidly R, Obba S, Goulard M, Schmitt T, et al. IL-34 and CSF-1 display an equivalent macrophage differentiation ability but a different polarization potential. *Sci Rep*. 2018;8(1):256.
267. Lelios I, Cansever D, Utz SG, Mildenerberger W, Stifter SA, Greter M. Emerging roles of IL-34 in health and disease. *J Exp Med*. 2020;217(3).
268. Haririan H, Andrukhov O, Pablik E, Neuhofer M, Moritz A, Rausch-Fan X. Comparative Analysis of Calcium-Binding Myeloid-Related Protein-8/14 in Saliva and Serum of Patients With Periodontitis and Healthy Individuals. *Journal of periodontology*. 2016;87(2):184-92.
269. Strimbu K, Tavel JA. What are biomarkers? *Current opinion in HIV and AIDS*. 2010;5(6):463-6.
270. McLachlan JL, Sloan AJ, Smith AJ, Landini G, Cooper PR. S100 and Cytokine Expression in Caries. *Infect Immun*. 2004;72(7):4102-8.
271. Ho SSC, Keenan JI, Day AS. Parent Perspectives of Diagnostic and Monitoring Tests Undertaken by Their Child with Inflammatory Bowel Disease. *Pediatric gastroenterology, hepatology & nutrition*. 2021;24(1):19-29.
272. Cuida M, Brun JG, Tynning T, Jonsson R. Calprotectin levels in oral fluids: the importance of collection site. *European journal of oral sciences*. 1995;103(1):8-10.
273. Kido J, Nakamura T, Kido R, Ohishi K, Yamauchi N, Kataoka M, et al. Calprotectin, a leukocyte protein related to inflammation, in gingival crevicular fluid. *J Periodontal Res*. 1998;33(7):434-7.
274. Pick R, Brechtefeld D, Walzog B. Intraluminal crawling versus interstitial neutrophil migration during inflammation. *Mol Immunol*. 2013;55(1):70-5.
275. Palmen MJHJ, Dijkstra CD, Van Der Ende MB, Pena AS, Van Rees EP. Anti-CD11b/CD18 antibodies reduce inflammation in acute colitis in rats. *Clin Exp Immunol*. 1995;101(2):351-6.
276. Abdelbaqi M, Chidlow JH, Matthews KM, Pavlick KP, Barlow SC, Linscott AJ, et al. Regulation of dextran sodium sulfate induced colitis by leukocyte beta 2 integrins. *Lab Invest*. 2006;86(4):380-90.

277. Fournier BM, Parkos CA. The role of neutrophils during intestinal inflammation. *Mucosal Immunol.* 2012;5(4):354-66.
278. Parkos CA, Delp C, Arnaout MA, Madara JL. Neutrophil migration across a cultured intestinal epithelium. Dependence on a CD11b/CD18-mediated event and enhanced efficiency in physiological direction. *The Journal of clinical investigation.* 1991;88(5):1605-12.
279. Vainer B, Nielsen OH, Horn T. Comparative Studies of the Colonic In Situ Expression of Intercellular Adhesion Molecules (ICAM-1, -2, and -3), β 2 Integrins (LFA-1, Mac-1, and p150,95), and PECAM-1 in Ulcerative Colitis and Crohn's Disease. *The American Journal of Surgical Pathology.* 2000;24(8).
280. Watanabe K, Blew B, Scherer M, Burke J, Koh G, Block C, et al. CD11b mRNA expression in neutrophils isolated from peripheral blood and gingival crevicular fluid. *Journal of clinical periodontology.* 1997;24(11):814-22.
281. Chadwick JW, Fine N, Khoury W, Tasevski N, Sun C-X, Boroumand P, et al. Tissue-specific murine neutrophil activation states in health and inflammation. *J Leukoc Biol.* 2020; 1- 9.
282. Ryckman C, Gilbert C, de Médicis R, Lussier A, Vandal K, Tessier PA. Monosodium urate monohydrate crystals induce the release of the proinflammatory protein S100A8/A9 from neutrophils. *J Leukoc Biol.* 2004;76(2):433-40.
283. Kido J, Kido R, Suryono, Kataoka M, Fagerhol MK, Nagata T. Calprotectin release from human neutrophils is induced by *Porphyromonas gingivalis* lipopolysaccharide via the CD-14-Toll-like receptor-nuclear factor kappaB pathway. *Journal of periodontal research.* 2003;38(6):557-63.
284. Tardif MR, Chapeton-Montes JA, Posvandzic A, Pagé N, Gilbert C, Tessier PA. Secretion of S100A8, S100A9, and S100A12 by Neutrophils Involves Reactive Oxygen Species and Potassium Efflux. *Journal of immunology research.* 2015;2015:296149.
285. Andersson E, Bergemalm D, Kruse R, Neumann G, D'Amato M, Repsilber D, et al. Subphenotypes of inflammatory bowel disease are characterized by specific serum protein profiles. *PloS one.* 2017;12(10):e0186142.
286. León AJ, Gómez E, Garrote JA, Bernardo D, Barrera A, Marcos JL, et al. High Levels of Proinflammatory Cytokines, but Not Markers of Tissue Injury, in Unaffected Intestinal Areas from Patients with IBD. *Mediators Inflamm.* 2009;2009:10.
287. Dobre M, Milanesi E, Manuc TE, Arsene DE, Tieranu CG, Maj C, et al. Differential Intestinal Mucosa Transcriptomic Biomarkers for Crohn's Disease and Ulcerative Colitis. *Journal of immunology research.* 2018:10.
288. Stevens C, Walz G, Singaram C, Lipman ML, Zanker B, Muggia A, et al. Tumor necrosis factor- α , interleukin-1 β , and interleukin-6 expression in inflammatory bowel disease. *Dig Dis Sci.* 1992;37(6):818-26.
289. Atreya R, Mudter J, Finotto S, Mullberg J, Jostock T, Wirtz S, et al. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med.* 2000;6(5):583-8.
290. Bister V, Makitalo L, Jeskanen L, Saarialho-Kere U. Expression of MMP-9, MMP-10 and TNF-alpha and lack of epithelial MMP-1 and MMP-26 characterize pyoderma gangrenosum. *J Cutan Pathol.* 2007;34(12):889-98.

291. Bernardo D, Vallejo-Diez S, Mann ER, Al-Hassi HO, Martinez-Abad B, Montalvillo E, et al. IL-6 promotes immune responses in human ulcerative colitis and induces a skin-homing phenotype in the dendritic cells and Tcells they stimulate. *European journal of immunology*. 2012;42(5):1337-53.
292. Greuter T, Vavricka SR. Extraintestinal manifestations in inflammatory bowel disease - epidemiology, genetics, and pathogenesis. *Expert review of gastroenterology & hepatology*. 2019;13(4):307-17.
293. Nishikawa Y, Kajiura Y, Lew JH, Kido J, Nagata T, Naruishi K. Calprotectin Induces IL-6 and MCP-1 Production via Toll-Like Receptor 4 Signaling in Human Gingival Fibroblasts. *J Cell Physiol*. 2017;232(7):1862-71.
294. Isaksen B, Fagerhol MK. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. *Molecular pathology : MP*. 2001;54(5):289-92.
295. Zallot C, Peyrin-Biroulet L. Deep remission in inflammatory bowel disease: looking beyond symptoms. *Current gastroenterology reports*. 2013;15(3):315.
296. Mühl L, Becker E, Müller TM, Atreya R, Atreya I, Neurath MF, et al. Clinical experiences and predictors of success of treatment with vedolizumab in IBD patients: a cohort study. *BMC Gastroenterol*. 2021;21(1):33.
297. Sikorska D, Orzechowska Z, Rutkowski R, Prymas A, Mrall-Wechta M, Bednarek-Hatlinska D, et al. Diagnostic value of salivary CRP and IL-6 in patients undergoing anti-TNF-alpha therapy for rheumatic disease. *Inflammopharmacology*. 2018;26(5):1183-8.
298. Eng GP, Bouchelouche P, Bartels EM, Bliddal H, Bendtzen K, Stoltenberg M. Anti-Drug Antibodies, Drug Levels, Interleukin-6 and Soluble TNF Receptors in Rheumatoid Arthritis Patients during the First 6 Months of Treatment with Adalimumab or Infliximab: A Descriptive Cohort Study. *PloS one*. 2016;11(9):e0162316-e.
299. Huang M-L, Wu Y-Q, Ruan W-H. A rare case of pediatric Crohn's disease and alveolar bone loss: a report and review. *Translational Pediatrics*. 2020;9(5):720-5.
300. Menegat JSB, Lira-Junior R, Siqueira MA, Brito F, Carvalho AT, Fischer RG, et al. Cytokine expression in gingival and intestinal tissues of patients with periodontitis and inflammatory bowel disease: An exploratory study. *Arch Oral Biol*. 2016;66:141-6.
301. Figueredo CM, Martins AP, Lira-Junior R, Menegat JB, Carvalho AT, Fischer RG, et al. Activity of inflammatory bowel disease influences the expression of cytokines in gingival tissue. *Cytokine*. 2017;95:1-6.
302. Ciccia F, Alessandro R, Rodolico V, Guggino G, Raimondo S, Guarnotta C, et al. IL-34 is overexpressed in the inflamed salivary glands of patients with Sjogren's syndrome and is associated with the local expansion of pro-inflammatory CD14(bright)CD16+ monocytes. *Rheumatology (Oxford, England)*. 2013;52(6):1009-17.
303. Woo VL. Oral Manifestations of Crohn's Disease: A Case Report and Review of the Literature. *Case Reports in Dentistry*. 2015;2015:830472.
304. Raittio E, Farmer J. Methodological Gaps in Studying the Oral-Systemic Disease Connection. *J Dent Res*. 2021;100(5):445-7.
305. La Rosa GRM, Libra M, De Pasquale R, Ferlito S, Pedullà E. Association of Viral Infections With Oral Cavity Lesions: Role of SARS-CoV-2 Infection. *Frontiers in Medicine*. 2021;7(1059).