# CHROMOFUNGIN AMELIORATES COLITIS AND REDUCES ENDOPLASMIC RETICULUM STRESS AND P53-APOPTIC PATHWAYS

BY

ABDOULAYE DIARRA

A Thesis Submitted to the Faculty of Graduate Studies of

The University of Manitoba

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Immunology

University of Manitoba

Winnipeg, Manitoba

# Abstract |

**Background**: Chromogranin-A produced by enterochromaffin cells is implicated in the development of ulcerative colitis (UC). In an animal model of UC, the lack of CHGA regulates the plasticity of macrophages (MØs) and p/53 caspase activation in colitis. In UC and macrophages, the transcription factor X-linked binding protein (XBP1) is a crucial component of the endoplasmic reticulum (ER) stress response, which induces intestinal mucosal injury and epithelial apoptosis. Intestinal epithelial apoptosis is mediated by p53-apoptotic pathway through the activation of p53-upregulated modulator of apoptosis. Previously, we showed that chromofungin (CHGA47-66; CHR) protects against acute colitis by regulating macrophage-cytokines production. However, the effects of CHR on ER stress/p53-dependent apoptosis in colitis are still unknown.

**Aims:** We aimed to investigate whether CHR suppress inflammation-induced epithelial apoptosis through ER stress/ p53-apoptosis

**Methods:** Naïve peritoneal MØs were plated, incubated, with rhodamine-CHR at different time points and detected using confocal microscopy. In vitro, MØs were also exposed to CHR or PBS and then to LPS to promote classically activated MØs. XBP1 and modulator of apoptosis, PUMA, BAD, BAX, BAK1, Trail, Casp3, and 8 were quantified. In vivo, UC-related colitis was induced in C57BL/6 mice by administering dextran sulphate sodium DSS. Mice were treated with CHR

started for 5-days. The above markers were quantified using whole colonic sections, q-RT-PCR, and western blot. Mucosal biopsies from control and UC patients were collected, and gene expression of the above markers was determined.

**Results:** We found MØs displaying rhodamine within the intracellular compartment. LPS-stimulated MØs treated with CHR demonstrated a significant decrease of Xbp1, Puma, Bad, Bax, and Bak1 when compared to LPS-stimulated MØs treated with PBS.

In colitic conditions, CHR treatment ameliorated the onset and severity of colitis, which was associated with a significant reduction in ER stress/p53-dependent apoptosis genes and proteins we found an increase in ER stress marker (XBP1), apoptotic markers within the colonic mucosa of active UC patients

**Conclusions:** CHR decreases the severity of colitis and the inflammatory process via the suppression of mucosal and proinflammatory MØs-related ER stress and p53-dependent apoptosis. Functional analysis of CHGA may lead to a novel therapy for IBD.

# Acknowledgments |

My first acknowledgment goes to the Almighty for giving the strength to accomplish this research. Secondly, I'm grateful to my supervisor Dr. Jean-Eric Ghia for taking me to this research group. I'm also honoured to express my respect to him. His expert guidance, effort, patience, constructive feedback, willingness, and cooperation have led to the completion of this research work. It is hard to find a supervisor willing to let a student design their research and work with no pressure and at their own pace. I want to thank you for giving me a chance to work under his excellent supervision and guidance.

I would like to sincerely thank Dr. Jude Uzonna and Dr. Suresh Mishra for being part of my committee and guiding me at every step of this MSc program. I'm indebted to them for spending their precious time advising me with great ideas.

I would also like to thank Dr. Murooka's lab and Dr. Marshall for letting me use their lab and teaching me imaging and western blot technics.

I want to thank Dr. Charles N Bernstein, for being a great support in providing biopsies from his patients.

I have been in many laboratories during my undergrad studies, but Ghia's Lab top the list. The support Dr. Nour Eissa, Dr. Laetitia Kermarrec, Mr. Kunal Kapoor, gave me was unmatched. I want to thank them for creating a great work environment in this laboratory.

I cannot forget to be grateful to the past, and present department head Dr. Xi Yang and Dr. Marshall, for their incredible help, support, and guidance whenever required. I sincerely thank all the professors, technicians, and immunology coworkers and friends for their support and for taking me to the immunology family.

I want to thank William Stefura from Dr. Hayglass' lab for teaching all the lab rules. I can't finish without thanking Susan Ness, Karen Morrow, and Silvia Panameno for their administrative support.

I want to acknowledge all the funding agencies: Research Manitoba, Mitacs, Mindel and Tom Olenick and Crohn's and Colitis Canada; without them, anything would have been possible. I am also thankful to the Center for Animal Care Services (CACS), the veterinary and the University of Manitoba.

Finally, I am asking for forgiveness for sacrificing those innocent mice for this study, and their participation is acknowledged with a heavy heart.

**Dedication**

I dedicate this work to my family back in Mali and who have believed in me and supported me throughout these last two years and to all the patients who have endured a lot with the IBD disease.

# Table of contents

# List of Tables & Figures |

*For all the figures presented, copyright permission was obtained.*

# Abbreviations |

ATF6: Activating transplant factor 6

ANOVA: Analysis of variance

ATG16L1: Autophagy related 16 like 1

BAD: Bcl associated against cell death

BAK-1: Bcl-2 antagonist killer 1

BAX: Apoptosis regulator BCL-2 like protein 4

BIP: Binding immunoglobin protein

CASP: Caspsase

CST: Catestatin

CCL20: Chemokine (C-C motif) ligand 20

CHGA: Chromogranin-A

CHR: Chromofungin

CD: Crohn's disease

DNA: Deoxyribonucleic acid

DC: Dendritic cells

DSS: Dextran sulphate sodium

ELISA: Enzyme-linked immunosorbent assay

ERAD: Endoplasmic reticulum association protein degradation

ER Stress: endoplasmic reticulum stress

EC: Enterochromaffin cells

FADD:  FAS association death domain

FMT: Fecal microbial transplant

FBS: Fetal bovine serum

GWAS: Genome-wide association studies

GALT: Gut association lymphoid tissue

IRE1-alpha: Inositol requiring transmembrane kinase1 alpha

IL: Interleukin

IBD: Inflammatory bowel disease

MIRNA: Micro RNA

MO: Macrophage

NOD2: Nucleotide organization domain containing

PUMA: p53 upregulated modulator of apoptosis

PERK: Pancreatic ER elf2 kinase

PCR: Polymerase chain reaction

PAMPs: Pathogen associated molecular patterns

RNA: Ribonucleic acid

(SO2): Sulphur dioxide

STAT3: Signal transducer and activator of transcription 3

SOCS3: Suppressor of signalling 3

SRB: Sulphate reproducing bacteria

TNF-alpha: Tumour necrosis alpha

TREG: Regulation cells

TSLP: Thymic stromal lymphopoietin

TLR: Toll-like receptor

TNBS: Trinitrobenzene sulphonic acid

UC: Ulcerative colitis

VS: Vasostatin

XBP1: X-binding protein-1

# Chapter 1 | Synopsis

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic idiopathic, relapsing inflammatory conditions of the gastrointestinal tract [1]. It is characterized by chronic inflammation at the various sites of the intestine, which leads to diarrhea, stomach pain, fever, and tiredness. This chronic condition has a tremendous impact on the quality of life due to devasting symptoms and substantial personal burden.

CD and UC have distinct pathologic and clinical characteristics, and despite the amount of research conducted they are still poorly understood. In general, IBD is believed to result from the dysregulation of mucosal response to environmental factors in genetically susceptible hosts [2]. The incidence of IBD began to rise in the early twentieth century, and in 1932 UC and CD have been recognized by the medical community as two distinct forms of IBD. In the late twentieth century, UC and CD have increased in the Western world, including Europe, Australia, and New Zealand [3]. Recent epidemiological studies have shown that IBD is a common disease in North America; its incidence in Canada is approximately is 15/100000 population. In 2012, the best estimate of the prevalence of IBD was 233,000 Canadians, including 129,000 people living with CD and 104,000 people living with UC. In 2018, this number rose to 270,000 Canadians living with IBD [3-5]. The direct annual cost of caring for Canadians living with IBD is estimated at $2.8 billion, and mainly IBD patients are subjected to pay over $11,900 per year [3]. Despite many years of research, there is no cure for IBD. The current expensive therapies are directed at reducing the inflammation that triggers symptoms and helping patients achieve periods of remission.

Therefore, considering the substantial cost and the poor quality of life of IBD patients, it is in our interest to do more extensive research to find and test new therapeutics options.

# Chapter 2 | Literature review

## 1 Inflammatory bowel disease

### 1.1 Definition

Inflammatory bowel disease is an umbrella term used to describe a group of chronic, progressive inflammatory disorders of the digestive tract [1, 6]. Two main conditions are included under the heading of IBD; these are UC and CD. Several rare circumstances may be classified as IBD, such as IBD (U) and Intermediate Colitis [7]. The critical distinction between UC and CD is the location of inflammation and the extent of the inflammation. UC only affects the colon or large intestine, while CD can affect the entire digestive system from the mouth to anus [2, 8].

#### 1.1.1 Crohn's Disease & Ulcerative Colitis

UC was first described by Samuel Wilks in 1859 [9]. It is a condition in which parts of the lining of the rectum and the colon become inflamed and ulcerated. Ulcers present on the surface of colon's or rectum's linings can induce bleeding and production of mucus [10, 11] **(Figure 1)**. UC is most common in industrialized countries such as countries in Asia, the USA, and Canada. At some exceptions, several studies have reported that the higher incidence in certain ethnic or racial groups is related to environmental influences, food habits, and lifestyle rather than the actual genetic differences [3]. The distribution incidence for UC is bimodal with a first peak in the second decades of life, followed by a second peak between 50 and 80 years of age [10, 12].

Population-based studies have shown no significant gender differences in UC [10, 12]. UC's hallmark symptoms include bloody diarrhea with rectal urgency, and abdominal cramps and pain, which can get intense during the bowel movement. Roughly 50% of diagnosed UC patients showed mild symptoms associated to many stools a day with or without blood, some pain, cramping and a constant need for emptying their bowel. Severe cases, that are less frequent, consist of severe bleeding (more than six bloody stools a day) and anemia. In some cases, children with UC may fail to develop or grow properly. The symptoms of UC, along with other complications, can vary depending on the extent of the inflammation in the rectum and the colon [3].

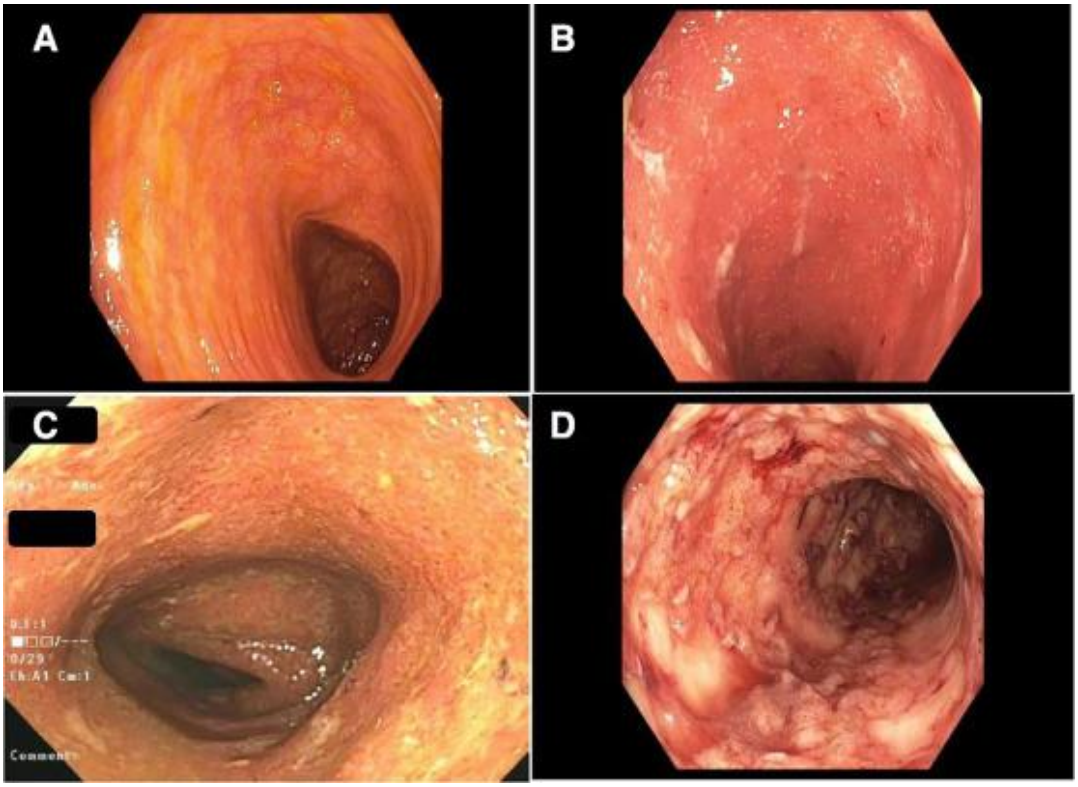



**FIGURE 1. IMAGE SHOWING A) HEALTHY COLON, B) MILD, C) MODERATE AND D) SEVERE ULCERATIVE COLITIS** [10]**.**

**COPYRIGHT PERMISSION WAS OBTAINED WITH ELSEVIER SUPPORT SERVICE.**

Less famous than UC, CD usually involves the terminal ileum, cecum perianal, and colon, but it can affect any intestine region in an irregular pattern [13]. In contrast to UC, which affects the colon and the rectum's inner lining, CD extends into the deeper layers of the intestinal wall. Histologically CD describes a thickened submucosa, transmural inflammation, granulomas, and fissuring ulceration **(Figure 2)** [13]. The distribution of CD is characterized by 25% of patients with colitis, 25% with ileitis, and 50% with ileocolitis [5].

The incidence of CD has stabilized in North America compared to the last century, while that rate was increasing in the '80s. But there is still a lot of variation in the incidence and the prevalence based on geographic region, environment, and ethnic group. Persistent diarrhea, cramping abdominal pain, fever, and rectal bleeding are the hallmarks symptoms of CD, but they vary from patient to another [13, 14]. The heterogeneous manifestation and the overlapping characteristics with other inflammatory disorders make the prognosis and the management of this disease extremely challenging.

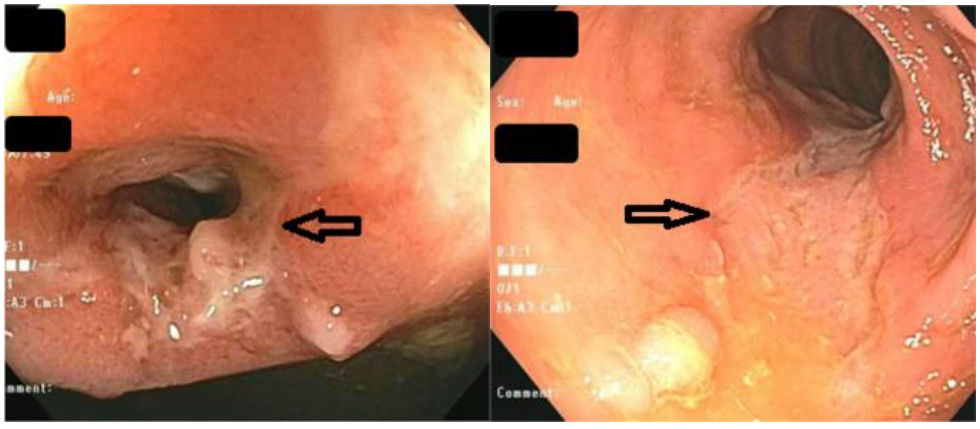


**FIGURE 2. COLONOSCOPY IMAGES SHOWING ILEOCECAL COLITIS AND CROHN'S DISEASE (CD), THE ARROWS ARE POINTING TOWARD AN ULCER PRESENTING DUODENAL CD** [15]**. COPYRIGHT PERMISSION WAS OBTAINED WITH ELSEVIER SUPPORT SERVICE.**

## 1.2 Prevalence of Incidence

During the 20th century, IBD was considered a disease of the Western world or high-income countries: North America, Europe, Australia, and New Zealand [3, 16]. From 2008 to 2009, a nationwide study in the USA reported a 0.49% prevalence of IBD [3]. Also, epidemiological studies from Europe have shown considerable geographic variability with the highest incidence of IBD in western Europe and Scandinavia and lower IBD rates in countries alongside the Mediterranean Sea. At the turn of the 21st century, this trend has changed. A shift in the epidemiological pattern has been demonstrated, indicating a stabilization the incidence in Western word countries with a high load or burden and prevalence and a rapid rise in newly industrialized countries in South America, Eastern Europe, Asia, and Africa [16].

In 2017, the presence of IBD was reported in 195 countries and territories, demonstrating its worldwide presence. Therefore, IBD was considered a global disease that can manifest in any geographic region and within any race or ethnicity. A comprehensive age-standardized prevalence study of IBD has revealed an increased rate from 79.5 per 100,000 people in 1990 to 84.3 per 100,000 people in 2017. In the same survey, females seem to show a higher number of prevalence cases and an age-standardized prevalence rate [16]. Similarly, in 2017 several studies demonstrated an increase of 67% in the total number of IBD-related deaths worldwide, and the Western world countries, including Canada, had a massive contribution to these numbers [3, 5].

Canada has among the highest rate of inflammatory bowel disease in the world, with a prevalence of nearly 0.7% of Canadians [3]. In 2018, over 270,000 Canadians were believed to be afflicted with IBD, and disease surveillance systems have tracked the incidence of IBD in provinces

across Canada. Rate varies between regions; the highest incidence of IBD is reported in Nova Scotia at 54.6 per 100,000 people [5]. Alberta, Manitoba, Ontario, Quebec, and Saskatchewan were in the same range from 18, 7 to 28.3. Males are less likely to have CD as compared to women in Canada, and there are no differences for UC [3, 5]. Looking at ethnicity's incidence, it was first described that IBD was a Caucasian disease that descended from Western Europe; however, this notion has faded away over the generation. Several studies demonstrated that individuals who immigrated from South Asia and Africa, where IBD is uncommon, were also found to develop IBD likely [5, 16]. In other words, the risk of developing IBD is striking among the first- and second-generation offspring of these immigrants. This trend suggests that Westernization and the environment may have a high impact on the risk of developing IBD.

## 1.3 Etiopathology

The etiopathology of IBD is complex and is believed to be a mix of genetic, enteric neutral dysregulation, environmental, gut microbiota, immune and endocrine dysregulation (**Figure 3**)

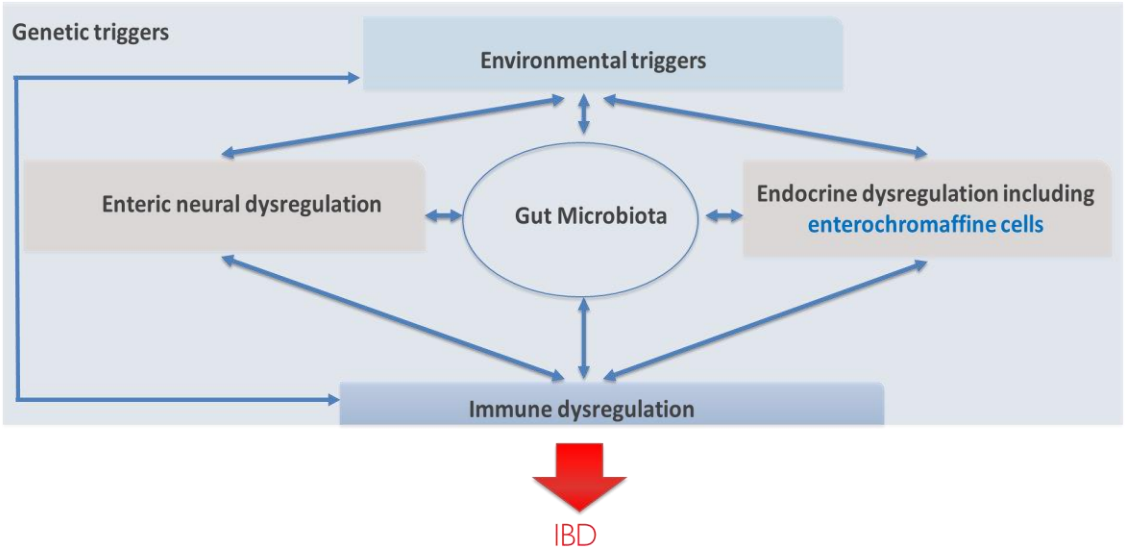



**FIGURE 3. CURRENT SUSPECTED ETIOPATHOLOGY OF INFLAMMATORY BOWEL DISEASE (IBD).**

### 1.3.1 Genetic Background Susceptibility

The genetic foundation of IBD has long been recognized. CD and UC are believed to occur in genetically predisposed individuals who have an abnormal immune response to microorganisms in the gut. Epidemiological evidence for genetic contributions has revealed that 15% of patients with CD have an affected family member with IBD, and twin studies for CD have shown 50% concordance in monozygotic twins compared to dizygotic [17]. Within the past years, there have been significant advances in our understanding of genetic contributions to IBD. Technological progress in genetic and DNA sequencing have permitted many genome-wide association studies (GWAS) [18]. GWAS in IBD has increased the number of genetic loci that are associated with risk for IBD to 163 [19]. The majority of GWAS-identified risk variants are located in non-protein-coding regions of the genome [19]. Nucleotide-binding oligomerization domain containing 2 (NOD2) was the first gene linked to CD, discovered in 2001 [5]. It encodes for a

protein that acts as an intracellular receptor for bacterial products in monocytes and transduces signals leading to NF-KB activation [17]. In one study, Conney et al. showed that activation of NOD2 with muramyl dipeptide induces autophagy in dendritic cells and those cells from patients with a NOD2 gene variant are deficient in autophagy induction with reduced localization of bacterial autophagosome [20]. Another study has linked the presence of multiple NOD2 options to increased levels of *Enterobacteriaceae* [21]. Genetic analyses reported two other autophagy-related genes, IRGM, and ATG16L1; these two autophagy genes play an essential role in the immune response. Single nucleotide polymorphism in ATG16L1 (T300A) is linked with an increased risk of CD and Similar defects were also noted in CD patients who were homozygous for the ATG16L1 T300A variant. This T300A variant exhibits enhanced degradation via caspase 3, which leads to a reduction in the levels of ATG16L1 proteins and a reduced level in the flux of autophagy [21]. ATG16L1 usually helps in sequestration of intracellular bacteria during autophagy, but ATG16L1 hypomorphic results in defects in Paneth cell and their antimicrobial peptide secretion.

Moreover, ATG16L1 deficient MØs have shown an increase in proinflammatory cytokines [21]. Overall, most of these data suggest that alteration in the makeup of gut microbial communication may arise from the presence of specific risk alleles. Micro RNAs (miRNAs) also play a role in the pathogenesis of many diseases, including IBD, and they are essential in the development and regulation of the immune system [17]. These endogenous small non-coding single stranded RNAs are 18-24 nucleotides long and found in the genomic DNA. It is estimated that miRNAs regulate more than 60% of protein-coding mRNAs, and there are growing evidence of their implication in inflammatory disease [17]. Jensen et al. have shown that patients with IBD have shown higher

expression of miRNAs in tissue samples and blood than healthy patients [22]. Other studies have revealed a unique miRNA expression profiles in CD and UC and functional analyses of the deregulated miRNAs associated them with IBD pathogenesis [17]. Lastly, GWAS identified IBD susceptibility loci associated with several genes involved in immune regulation and signaling [19]. For example, genes encoding for IL-10 cytokines and their receptors are implicated in both CD and UC. The IL-10 gene usually encodes for anti-inflammatory cytokines and stimulates DC, and variant IL-10 genes are associated with an increase of 35% in the odds of developing UC [19]. CD risk SNPs seem to be able to disrupt ER stress balance, which can lead to intestinal inflammation [19, 23].

### 1.3.2 Environmental Factors

Genetic susceptibility does not explain all the variance of the disease recommending a role for environmental factors. Many environmental factors such as smoking, infection, drugs, air pollution, diet or nutrition, and gut microbiome have been investigated in IBD [23]. In 1989, Strachan established the hygiene hypothesis with the central principle that abnormal immune responses such as autoimmunity and allergy are the results of improvements in personal hygiene [24, 25]. In other words, children growing up in relatively sterile environments without adequate exposure to microbes insufficiently educate their system for handling organisms [5]. Recent evidence suggested that the hygiene hypothesis might not work for all the global populations, but it may be relevant in countries following migration from inadequate resources to affluent countries [23].

#### *1.3.2.1 Nutrition*

Diet has been heavily linked with IBD, and dietary factors have a strong influence on the intestinal microbiome [5]. For instance, studies have shown that refined sugars and reduced consumption of dietary fibers profoundly affect the microbiome diversity [23, 25]. Intensive work using mice models have described an increased risk of developing UC and CD with high intake polyunsaturated fatty acid omega-6 fatty acid and omega-6 fatty acid saturated fats, respectively [25]. It is believed that the consumption of a high-fat diet may increase colonic epithelial natural killer T cells and reduce circulating T-regulatory cells [25]. In IL-10 deficient mice, high fat can change the bile acid composition, which will increase expansion if sulphate-reducing bacteria. These bacteria can induce colitis by expressing a large amount of toxic hydrogen sulphide [26]. But animal models' studies have yet to be translated into humans.

#### *1.3.2.2 Smoking*

Cigarette smoking is one of the first environmental risk factors that has been consistently associated with IBD [23]. Cigarette smoking increases the risk of developing CD in adults, but the mechanism by which smoking is inducing this effect is poorly understood [5, 23]. In UC, Johnson et al. explained that smoking modulates the immune system and may involve the reduction of tumor necrosis factor-alpha (TNF-alpha) production through the action of the nicotinic acetylcholine receptor alpha-7 subunit [27]. The carbon monoxide from cigarette smoking in UC can increase IL-10 production, increase mucin synthesis, and decrease IL-8 expression and cause hyperfusion of the rectum leading to damage to the colonic tissue [27]. The implication of smoking with IBD is tricky because cigarette smoking increases the risk of developing CD, but adults who quit smoking are at increased risk of UC [4, 5].

#### *1.3.2.3 Air Pollution*

Air pollution, one of the leading causes of IBD incidence increase, has recently increased in Asia's developing countries that are experiencing rapid industrialization [23]. Air pollution may increase the risk of developing CD in children and young adults due to the alteration in the intestinal microbiome [5]. Kaplan et al. were able to show that exposures to sulfur dioxide (SO2) and nitric acid may increase the risk of early-onset UC and CD [28].

### 1.3.3 Dysfunctional Immune Response

IBD is an idiopathic disorder caused by chronic and excessive inflammation of the gastrointestinal tract [29]. Generally, in the absence of intestinal inflammation, gut homeostasis is maintained by suppressing extreme immune responses to foreign antigens [29]. During inflammation state, immune cells secrete products that are actively involved in the initiation and preservation of inflammation, leading to gut tissue damage [29]. One of the characteristics of acute inflammation in IBD is an enhanced infiltration of mast cells, lymphocytes, MØs, and activated neutrophils at the mucosal lining of the intestine [29, 30]. Furthermore, dysfunction of the intestinal immune system and cross-reactivity against host cells are essential mechanisms by which inflammation occurs [30]. Also, in IBD damage to the epithelium mucosa has been linked to elevated concentration of cytokines, chemokines, and effectors cells such as activated $CD4^+$ T cells, $CD8^+$ cytotoxic cells, intraepithelial cells and perforin granzymes containing T cells [29, 30].

#### *1.3.3.1 Cytokines & Chemokines*

Many inflammatory cytokines are involved in the pathogenesis of IBD, for example, the IL-1 family cytokines. In UC, IL-1-beta induces inflammation, and it originates from monocytes and MØs, which expresses active IL-1-beta in the colonic mucosa [30, 32]. IL-18, another IL-1 family member, is present at a high level in the mucosa of CD patients. It has been proposed that IL-18 augments Th1 responses and reduces IL-10 releases by Treg cells [29, 32]. Both IL-6 and IL-16 receptor are increased in either UC and CD. TNF-alpha has an essential regulatory role in IBD because it can increase IL-1-beta, IL-33, and IL-6 expression [29, 33]. On the contrary to proinflammatory cytokines, many immunosuppressive or anti-inflammatory cytokines are produced, including IL-10. According to several studies, inconsistencies in IL-10 concentration has been demonstrated in IBD [30, 35]. Some research has shown that IL-10 expression level was higher in IBD patients than in healthy controls [34], and another investigation demonstrated that IL-10 levels in the serum of patients with UC and CD are similar to controls subjects [35]. IL-17 is critical in the pathogenesis of IBD; research from Fujino et al. has shown that the IL-17 mRNA level was enhanced in the mucosa of patients with IBD [29]. The pathogenic role of IL-17 is controversial because of IL-17 has multiple isoforms. Il-17A can inhibit through phosphorylated STAT3 suppression, decreasing inflammation, and progression of acute colitis [36]. It has also been reported that IL-17 can increase T cells' recruitment of during the inflammatory response [38].

Some chemokines like IL-8, a neutrophil attractant, are found at increased levels in UC patients' tissue compared with healthy controls [29]. Moreover, various reports have shown upregulation

of chemokine ligand CCL2, CCL3, CCL4, CCL20, and CX-C motif chemokines in IBD tissues from patients [38, 39].

#### *1.3.3.2 Dendritic Cells*

In IBD patients, DCs are attracted by chemokines such as CCL20 and accumulate in the site of inflammation. This accumulation correlates with a large number of DCs in the intestine with plasmocytes DCs and myeloid DCs downregulated in the peripheral blood [40]. All these DCs are identified in the mesenteric lymph node of UC and CD patients, and myeloid DCs of CD patients tend to produce high levels of IL-23 and low level of IL-10 [42]. Dysregulated intestinal epithelial barrier might also affect intestinal DCs. According to this study, intraepithelial cells isolated from patients with CD do not produce thymic stromal lymphopoietin, which usually maintains local DC in a quiescent stage [13]. DC plays a significant role in the pathogenesis of IBD by modulating the tolerance to the commensal microflora and dietary antigens and affecting immune response [13].

#### *1.3.3.3 Macrophages*

The gut-associated lymphoid tissue GALT includes a collection of immune cells within the gastrointestinal tract. The proximity of these immune cells is essential for a variety of reasons: clearing of microbes and sampling from the extracellular to gain access to the laminar propria to maintain immune tolerance [43]. MØs are a heterogeneous population that shows a broad spectrum of activation states. This broad-spectrum MØs is simplified into two groups: “inflammatory” M1 and “wound healing or tissue repair” M2 MØs [44]. MØs polarization happens through specific combinations of cytokines within the microenvironment, suggesting plasticity between activation states of MØs [43].

M1 polarization occurs through IFN-gamma, TNF-alpha stimulation, and engagement of PRRs by T helper, granulocytes, and PAMPS, respectively, to activate suppressor of signalling 3 (SOCS3) which induce M1 phenotype [45]. M1 MØs, in turn, produce inflammatory cytokines such as TNF-alpha, IL-12, IL-6, and reactive oxygen species. These mediators promote the differentiation of th17 and th1 cells [45]. M1 is essential for eradicating of intracellular infection, but in the context of IBD, they produce proinflammatory cytokine that contributes to IBD pathogenesis. Moreover, an unregulated M1 activity can cause tissue damage [46].

On the other side, there are M2 MØs; their polarization usually happens through IL-4 produced by granulocytes or Th2 cells in response to tissue injury [44]. Activated M2 MØs produce growth factors, metalloproteases, and even initiate phagocytosis of debris without providing any proinflammatory cytokines [43]. Indeed, several studies have proven that downregulation of M2 function can enhance the host's chances of developing specific infection [47]. Aside from M2 microbicidal features, they are also implicated in the recruitment of $Foxp3^{+}$ T regulatory (Treg) cells, one of the significant components regulating the local immune responses. Lastly, similarly, M1 unregulated M2 MØs activity can promote fibrotic lesions and enhance allergic reactions [48, 49].

Macrophages play a critical role in maintaining intestinal homeostasis, but they can also drive the development of IBD [13]. In IBD patients, MØs increases at the site of inflammation; those MØs can initiate a rapid response to antigens [50]. Furthermore, aberrant CD14-expressing MØs isolated from the IBD mucosa produce high-level IL-12 and IL-23 [51]. Animal models support this concept of dysregulated MØs; for example, a murine model study showed increased infiltration

of CCL-2 or MCP-1 monocytes and immature MØs in the gut mucosa. Those MØs produce a large number of proinflammatory molecules such as TNF-alpha and IL-6 and nitric oxide [52].

#### *1.3.3.4 Th1 Cells*

Many evidences have confirmed that overactivation of Th1 immune response can induce CD. Th1 cell activation is found to be associated with the excessive activation of the IL-23 pathway in genetically predisposed individuals [15, 53]. T cells isolated from the colonic lamina propria of CD patients produce a large amount of IFN-gamma and have an increased expression of IL-12RB2, T-bet [54]. Early in CD, mucosal T cells launch a Th1 response that resembles an acute infection and disappears with the disease's progression. Furthermore, the animal colitis model using T-bet deficient $CD4^{+}CD45^{+}$ cells cannot induce Colitis in $Rag^{-/-}$ recipients [55].

#### *1.3.3.5 Th2 Cells*

The type 2 immune responses in the gastrointestinal tract are commonly known as protective mechanisms against injury caused by intracellular invaders such as helminths [56]. Th2 cells have a core signature response that includes the secretion of cytokines IL-4, IL-15, and IL-13 by lymphocytes that express the transcription factor GATA-3 [57]. This protective function of Th2 cells includes smooth muscle hypercontractility, enhanced mucus secretion, induction of intestinal mastocytosis, and wound healing by Th2 mediators [58]. These functions classify Th2 cells to become a critical regulator of intestinal homeostasis [59, p. 2]. However, when it becomes dysregulated, Th2 immunity may lead to harmful consequences. First, the consistent activation of Th2 effector pathways can worsen inflammation through different mechanisms; for example, a tissue repair process may cause excessive collagen deposition, fibrosis, and structuring complications.

Moreover, thymic stromal lymphopoietin, IL-25, and IL-23 are typically released after epithelium injury to alert the immune system of a breach [59]. IL-33, a member of the IL-1 cytokine family, acts as an alarming factor and triggers a wound healing response at the level of the mucosa [60]. This anti-inflammatory factor has also been characterized to induce inflammation. In one study, Bessa et al. were able to show that proinflammatory effects during inflammation were dependent upon loss of nuclear sequestration of IL-33 [61]. They proved their finding by using mouse strain with an abolished signal for nuclear localization of IL-33, where all the mice developed multiorgan inflammation. In other words, the Th2 responses mediator IL-33 has two functions as it can exert either a protective and proinflammatory effects depending on its cellular compartmentalization [59].

#### *1.3.3.6 Th17 Cells*

Th-17 cells are believed to be one of the main pathogenic factors in IBD [31]. Studies have reported a vast infiltration of Th17 cells in the inflamed intestinal mucosa of patients' cells, where secreted IL-17 was increased [30]. Some reports have suggested that cytokine such as IL-1-beta, IL-6, IL-23, and TGF-beta can induce Th17 polarization [55]. Th17 plastic property allows them to play a relevant in IBD pathogenesis [30]. In IBD, Th17 cells release IL-17, which is a robust proinflammatory factor. IL-17 and its different isoforms are abundant in patients with UC, and their expression in the peripheral blood cells is correlated with UC severity [30].

#### *1.3.3.7 T Regulatory Cells*

T regulatory cells mainly protect against autoimmunity by inhibiting immune responses against environmental and food antigens in the gut [13]. Several studies have reported low

numbers of Treg in peripheral blood but an increase in inflamed colons; additionally, the ratio of Th17 in peripheral blood is reduced in IBD patients compared with controls [62]. It is believed that this low number of Treg can be an explanation about why the system cannot control intestinal inflammation [63]. However, studies have proven that Treg cells in IBD are not defective, as isolated Tregs from the inflamed colon or peripheral can still maintain normal cell-contact dependent and cytokine independent suppressive capacity [64].

### 1.3.4 Microbial Pathogens

#### *1.3.4.1 Gut Microbiome*

A vast number of microorganisms colonize our bodies' epithelial surfaces, including the skin and mucosae. They represent the so-called normal microflora, the microbiota [65]. Trillions of commensal microbes reside in the intestinal tract: ten times more cells than the number of cells constituting the human body [65, 66]. These bacteria are present at a different location of our body that provides suitable conditions for their growth and proliferation. The most significant number of bacteria cells is in the large intestine, with an estimated $10^{14}$ Bacteria [65]. With the technology advancement in high throughput sequencing and culturing techniques, rapid progress has been made in the enumeration, characterization, and classification of the human microbiota. The adult human gut contains more than 1000 different bacterial species with four main phyla that dominate: *Firmicutes, Bactoriodetes, Actinobacteria, and Proteobacteria* [67, 68]. But comparative studies of human fecal microbiota also have considerable differences between the composition of the microbiota of individuals [65, 69]. Some of the well-characterized roles of

commensal bacteria include efficient host nutrient absorption and protection from pathogen colonization.

Furthermore, they are also critical in developing the host immune system and maintaining immune cell homeostasis [66]. The microbiota has often been linked with a commensal relationship to the host immune in which the bacteria thrive in the productive environment of the gut. In General, the host benefits from multiple functions provided by the bacteria [67].

#### *1.3.4.2 Dysbiosis*

The physical closeness of these bacteria to the host represents a challenge to the immune system, as it must be able to discriminate between self or non-self. In contrast to their beneficial properties, dysregulated interactions between commensal bacteria and the host can cause inflammatory bowel disease (IBD) **(Figure 4)** [66]. In IBD, intestinal microflora has been analyzed thoroughly, and studies using conventional fecal-based or mucosal Isolation and culture techniques have shown increased concentrations of anaerobes, mainly Gram-negative anaerobes including *Bacteroides, Clostridium perfringens, Enterobacteriaceae and reduction of beneficial bacteria such as bifidobacterial* [70, 72]. By using quantitative PCR and fluorescent in situ hybridization analysis, Schultz et al. and Swidsnki et al., have demonstrated increased bacteria such as *E.coli*, *Bacteroides* in species in the rectal mucus layer, the ileum and the colon [73, 74]. *Bacteroides, Clostridia* species can produce enterotoxins and proteolytic properties that enhance mucosal permeability [75].

Furthermore, *Clostridium difficile*, a virulent strain of the clostridium species, has been described to reactivate quiescent IBD and induce acute experimental epithelial injury by Fas-mediated

apoptosis [76]. *Bacteroides fragilis* was shown to cause experimental colitis and IL-17 production [76, 77], and CD patients have been reported with altered intestinal microbiota and enhanced mucin degradation leading to epithelial permeability. CD is characterized by an increase in *Ruminococus gravis* and a decrease in *Faecalibacterium prausnitzii, Bifidobacterium adolescentis,* and Clostridium. One mechanism that describes these observations is the decrease of butyrate-producing bacteria such as *F. prausnitzii* along with an increase in sulphate-reducing bacteria (SRBs). SRBs metabolize sulphate into hydrogen sulphide, which is a toxic molecule that can block butyrate utilization and inhibit phagocytosis [67, 76]. This chemical compound can also prevent epithelial cell utilization of short-chain fatty acids [72]. Individuals with genetic predisposition who have mutations in IBD susceptibility genes also have an increased bacterial translocation, which leads to excessive toll-like receptor stimulation, proinflammatory cytokine secretion, and activation of the acquired immune response followed by an inflammatory response [76, 77].

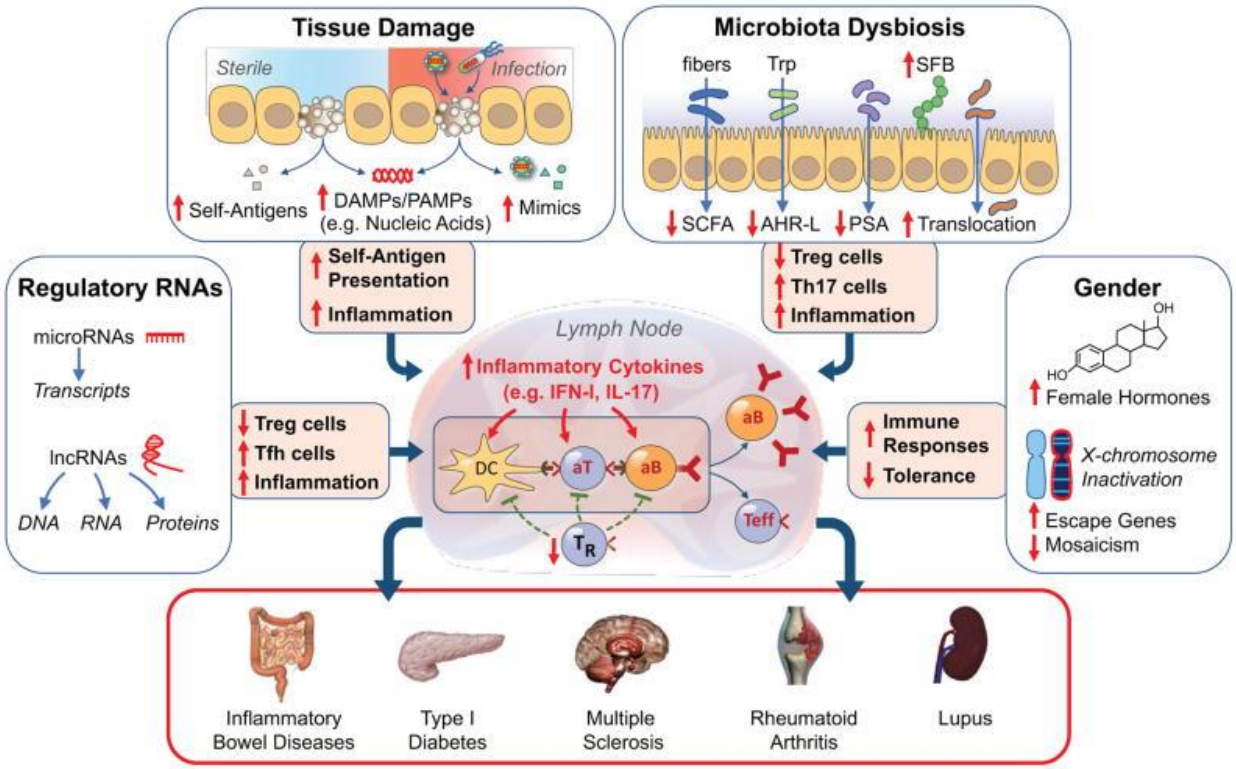



**FIGURE 4. MICROBIOTA DYSBIOSIS CAN LEAD TO THE DISPLACEMENT OF BENEFICIAL BACTERIA AND TO THE EXPANSION OF ADHERENT BACTERIA, CAUSING TISSUE DAMAGE** [76]**. COPYRIGHT PERMISSION WAS OBTAINED WITH DR. BACCALA.**

## 1.4 Treatments

### 1.4.1 Immunomodulatory, anti-inflammatory, and antibiotic treatments

There is currently no cure for IBD, and the purpose of the current treatments is to reduce the symptoms, achieve, maintain remission, and prevent complications [78]. Health care professionals have set two different types of goals for medical treatments of CD and UC. On the one hand, there is the short-term goal of therapy, which brings down uncontrolled symptoms by suppressing the inflammatory response to induce remission. On the other hand, the long-term

goal is to maintain remission, healing, and decrease flares [79]. Patients with CD and UC are treated with different approaches depending on the characteristics of their diseases. A typical approach to treat those patients is the use of corticosteroids during periods of flares to reduce symptoms [5]. But corticosteroids can have a lot of side effects and have weak potency to maintain remission; they are not usually prescribed in the long term [5]. For long-term treatment, clinicians opt for immune modifiers or biological therapies [80]. In some cases, or during severe illnesses and patients with fistulizing diseases, biological therapies might start even before steroids [5]. Here is some treatment that is used nowadays:

- Corticosteroids: steroids can be prednisone and budesonide and others, these medications are nonspecific, can suppress the immune system and are used to treat moderate to severely active CD [81].
- Immune modifiers: also called immunomodulators; these drugs can help decrease corticosteroid dependency and help maintain disease remission. Immune modifiers can be azathioprine and cyclosporine [82].
- Some antibiotics, such as metronidazole and ciprofloxacin, are used to treat abscesses and perianal fistulas.
- Anti-inflammatory drugs have also been used to treat CD; for example, five aminosalicylates are used to treat proctocolitis [5].
- Biological therapies are usually monoclonal antibody that blocks a specific molecule or pathway. Canada has approved infliximab, adalimumab, and ustekinumab against IBD. Infliximab and adalimumab are anti-TNF. They work by blocking the immune system production of tumor necrosis factor [83]. Ustekinumab is an anti-IL-12 and IL-23, it was

first to treat psoriasis, and now it is accepted for use CD. This monoclonal antibody targets the p40 subunit of IL-12 and IL-23 works, thereby blocking their effect. In UC severe cases, Vedolizumab is given to patients who do not achieve remission through conventional therapy [84]. Vedolizumab is a monoclonal IgG-1 that inhibits the interaction between α 4B7 and the mucosal addressin cell adhesion molecule-1 (MAdCAM-1). This antibody inhibits inflammation by blocking lymphocyte translocation from the blood into the inflamed gut tissue [85].

Treatments options for UC are similar to CD, but with several exceptions, for example, patients with UC are first treated with 5-aminosalicylates instead of corticosteroids.

### 1.4.2 Alternative therapeutic interventions targeting the intestinal microbiota dysbiosis in IBD

Dysbiosis plays a massive role in IBD pathogenesis, an alternative treatment of IBD will be to treat IBDs by correcting the dysbiosis [86]. In early 1989 a surgeon from Kansas City came up with the idea of fecal microbial transplant (FMT) [87]. Fecal microbial transplant is a procedure in which unfractionated fecal microbiota from a healthy donor is injected into the intestinal tract of a sick individual to cure disease. FMT has been proven to be highly effective against recurrent *C. difficile* colitis and is thought to work by correcting the depleted intestinal microbiota that facilitated the outgrowth of *C. difficile* organisms [86, 88]. A prospective trial of FMT in China with 30 patients with CD showed clinical remission for half of the patients one year after treatment. In that study, FMT was delivered in jejunum during endoscopy, and the results revealed that

specific subsets of IBD patients might benefit from FMT [89]. In another study, 61 adults with UC were given FMT and placebo as controls; thirty-three percent of patients in active treatment felt better and were in remission after seven weeks. Additionally, in response to FMT treatment, patients showed increased in feces producing bacteria such as *Ruminococcus*, *Blautia*, and *lachnospiraceae* [90].

FMT corrects the microbial dysbiosis by introducing bacteria into the intestine, in contrary to that, there are antibiotics. The antibiotics perturb the gut microbiota by removing large communities; they are frequently used as primary therapy of CD. Metronidazole, ciprofloxacin and rifaximin have reasonably good support for treating CD. In one controlled study, patients with active colonic and ileocolonic CD treated with metronidazole for four months improved significantly, similar trials with ciprofloxacin for six weeks showed the same results. In a two-year study, Selby et al. showed that the combination of three antibiotics (clarithromycin, rifabutin, and clofazimine) designed to treat *Mycobacterium avuim paratuberculosis* could be beneficial against CD [91]. Lastly, probiotics can also be an attractive alternative therapy. Probiotics are microorganisms with beneficial effects on the host. Unfortunately, very little is known on this subject, and few designed clinical trials have been conducted with commercial probiotics [86]. But one study was performed by Pascarella et al., where a combination of probiotics VSL#3 was tested. This VSL#3 induced remission of active UC in several placebo-controlled studies [92, 93].

## 1.5 Models of IBD: Animal Models

What is the right animal model for IBD study? The intestine with a diverse cell population is highly complex organs necessitating comprehensive animal models. Human samples can be used to study IBD, but they come with some restrictions. Limitations include the ethical use of collected tissue, the small sample size, the genetic variability between tissue from one individual to another [94]. In that case, animal models are necessary and are needed to investigate or provide information representative of intestinal disease in people [94]. Appropriate use of animal models can help us to elucidate the process involved in the disease's onset and its progression. Different organisms are used to study intestinal disorders depending on the characteristic of each body. For example, rats are used because of their capacity to give a large number of samples. Drosophila has also been used to investigate the mechanism involved in innate immunity [95]. Pigs, non-human primates, and dogs have all developed IBD and exhibit similar gene dysfunction to people with CD [82, 96]. However, mice are the most commonly used animal model for intestinal studies, and genetically modified murine models are particularly crucial in deciphering the underlying pathogenesis of intestinal inflammation [94]. In IBD animal models, chemicals are the preferred method to induce tissue injury; two main chemicals are used to cause inflammation: the dextran sulphate sodium and the 2,4,6-trinitrobenzene sulphonic acid.

### 1.5.1 Dextran Sulphate Sodium

DSS is commonly used to induce inflammation, and its treatment can cause both acute and chronic inflammation by adjusting concentration and duration [94]. For example, the

administration of DSS in mice for two months in a cycle rotation of one week of DSS treatment followed by two weeks of rest [97]. Acute inflammation is obtained by administering DSS at a concentration of 1-5% for a week [98]. DSS treatment usually disrupts the epithelial barrier, causing vascular and mucosal injury through exposure to lamina propria to luminal contents and foreign antigens [99]. This exposure will cause bacterial influx, which will activate inflammatory pathways resulting in an increased production of the inflammatory cytokines [99].

Furthermore, DSS treatment affects innate immunity. It changes the expression of MYD88, TLR4, and TLR9; small changes in these factors of innate immunity can contribute to epithelial cells damage and subsequent intestinal inflammation [100]. DSS is an effective inducer of intestinal inflammation, but there are some weaknesses to this treatment that cannot be denied. For example, there can be a significant difference in the severity of tissue injury after DSS treatment in mice and depending on the sanitary status of the facility. Inconsistency in the amount of mucosal damage can be observed after DSS treatment [100]. But those factors can be controlled; therefore, DSS is still considered as the best chemical model to induce UC-like intestinal injury.

### 1.5.2 Trinitrobenzene Sulphonic Acid

TNBS can be used to induce both acute and chronic inflammation in animal models [94], [100, 101]. TNBS independently cannot cause inflammation; it needs to be solubilized in ethanol. This mixture can cause intestinal inflammation by altering host proteins [102]. TNBS stimulates immune-mediated response by producing hapten-self-antigens that are recognized and can contribute to acute intestinal inflammation [102]. Furthermore, the ethanol presents in the

mixture can cause epithelial barrier damage; it was also shown that rectal administration of TNBS in 40% ethanol leads to epithelial necrosis causing crypt architecture destruction [98]. The primary immune response during acute inflammation is a proinflammatory Th1 response increasing IL-12, IFN-gamma, and TNF-alpha. But due to the instability of the TNBS over the last decade, laboratories have started to use a more stable component represented by Di-nitrobenzene sulphonic acid (DNBS).

There are several other agents used to induce colitis are, oxazolone, azoxymethane. All these chemicals have the goal to cause tissue injury in a specific way [94]. For this work, I used the DSS model because of its lower cost and ease of induction.

# 2 Endoplasmic reticulum stress, apoptosis, and IBD

## 2.1 Endoplasmic reticulum stress and IBD

### 2.1.1 ER stress

The endoplasmic reticulum (ER) is the primary site for the synthesis and the folding of membrane and secretory proteins. Additionally, ER is also associated with lipid biosynthesis, energy metabolism, and homeostasis of intracellular $Ca^{2+}$ [103]. The ER stress arises when misfolded or toxic protein accumulates in the ER [104]. For ER homeostasis to be restored, mammalian cells came up with signal transduction pathways to eliminate the toxic proteins. These cells can also increase the elimination of misfolded proteins by ER association protein degradation (ERAD) signalling pathway and modify the capacity for protein folding and

modification [104, 105]. Elimination of deleterious proteins by signalling transduction pathways are called unfolded protein response (UPR). Three main protein sensors on the ER membrane constitute the UPR-signalling pathways: Inositol-requiring transmembrane kinase/endonuclease (IRE1), pancreatic ER elF2 kinase (PERK) and activating transcription factor 6 (ATF6) [106]. Under normal condition, all these sensors are attached to a chaperone binding immunoglobin protein (Bip) are maintained inactive **(Figure 5)**; but dissociation of Bip form the different sensors cause and activation of the UPR signalling [107, 108]. IRE1 is a conserved ER stress and exists under two isoforms. IRE1-alpha, which is ubiquitous and IRE1-beta [108]. Dissociation of Bip upon sensing of the unfolded protein causes activation of IRE1-alpha through dimerization and transphosphorylation. The endoribonuclease activity of IRE1-alpha removes 26-BP pair from the unspliced mRNA encoding the transcriptional factor X-binding protein 1 (XBP1u); this unspliced protein, in turn, can generate the functional spliced protein XBP1s via a shift in the reading frame [109, p. 1]. XBP1s is a transcription factor that can induce the expression of genes involved in protein folding, protein secretion, maturation, and the ERAD signalling [104, 108].

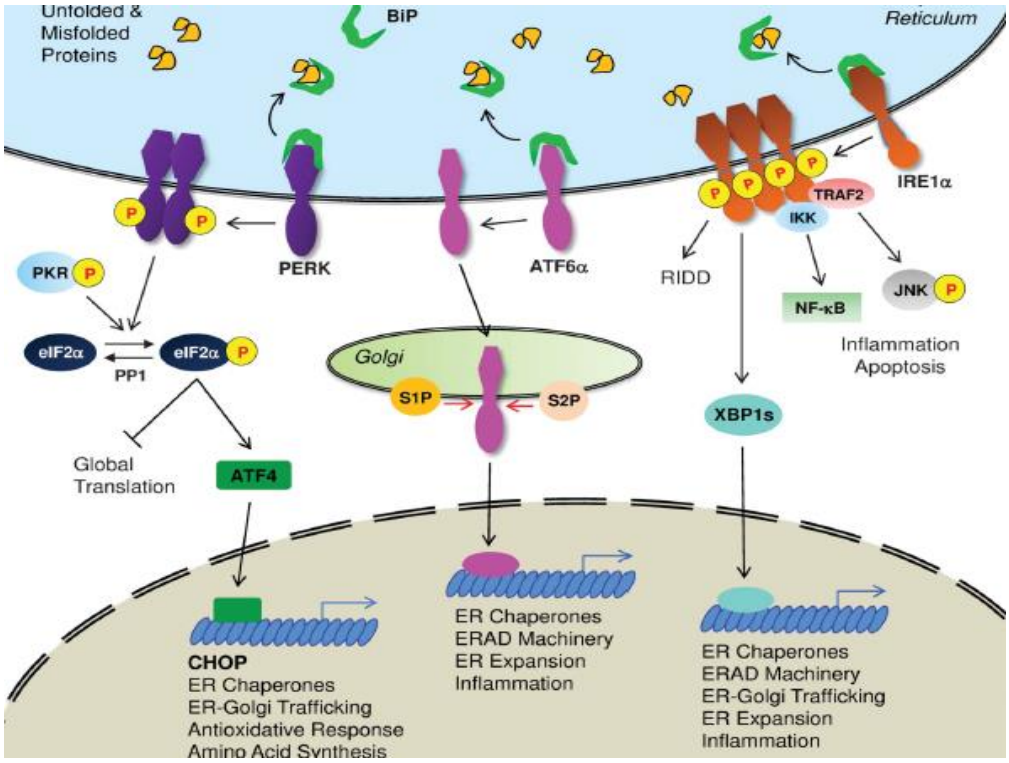



**FIGURE 5. THE THREE BRANCHES OF THE UNFOLDED PROTEIN RESPONSE (UPR). STRESS SENSOR PROTEINS ACTIVATING TRANSCRIPTION FACTOR (ATF 6), INOSITOL REQUIRING ENZYME-1 (IRE1) AND RNA-DEPENDENT PERK PATHWAYS [**104**].**

### 2.1.2 Endoplasmic Reticulum Stress & IBD

Recent studies have shown that ER and the UPR are critical factors associated with IBD's susceptibility [105]. The implication of ER stress in the pathogenesis of IBD is mediated by the impaired mucosal barrier function, regulating the innate or adaptive immune response. An initial study of IRE1/XBP1 signalling showed that the genetic deletion of IRE1-beta increases the protein level of BIP in the colonic mucosa [110]. Further studies have shown that IRE1-alpha in IECs leads to spontaneous colitis accompanied by loss of Goblets cells and dysregulation of epithelial barrier

[111, p. 1]. IRE1-alpha $^{-/-}$ deficient mice are more susceptible to DSS-induced colitis and ER stress apoptosis [104]. XBP1, which is a transcriptional factor of IRE1 signalling in response to ER stress, has been heavily linked to IBD **(Figure 6)**. Deep sequencing of the XBP1 gene and promoter has revealed nucleotide polymorphism SNPs in both UC and CD patients compared to healthy patients [112]. These SNPs in XBP1 were also found to be associated with decreased XBP1-upregulated UPR target genes [112]. Genetic factors are not the only factors that influence ER stress signalling leading to intestinal inflammation; environmental factors also play an essential role [108]. As examples, the anti-inflammatory cytokines can block ER stress by modulating ATF6 recruitment of Bip promoter. TNF-alpha can enhance ER stress and UPR signalling by inducing ROS production and its accumulation in the ER [113].

Moreover, the intestinal microbiota is another environmental factor that is correlated with IBD. The microbiota produces an inflammatory molecule, which in turn can trigger ER stress [104]. As an example, the trierixin derived from streptomycin sp has been described to be an inhibitor of XBP1 splicing [114].

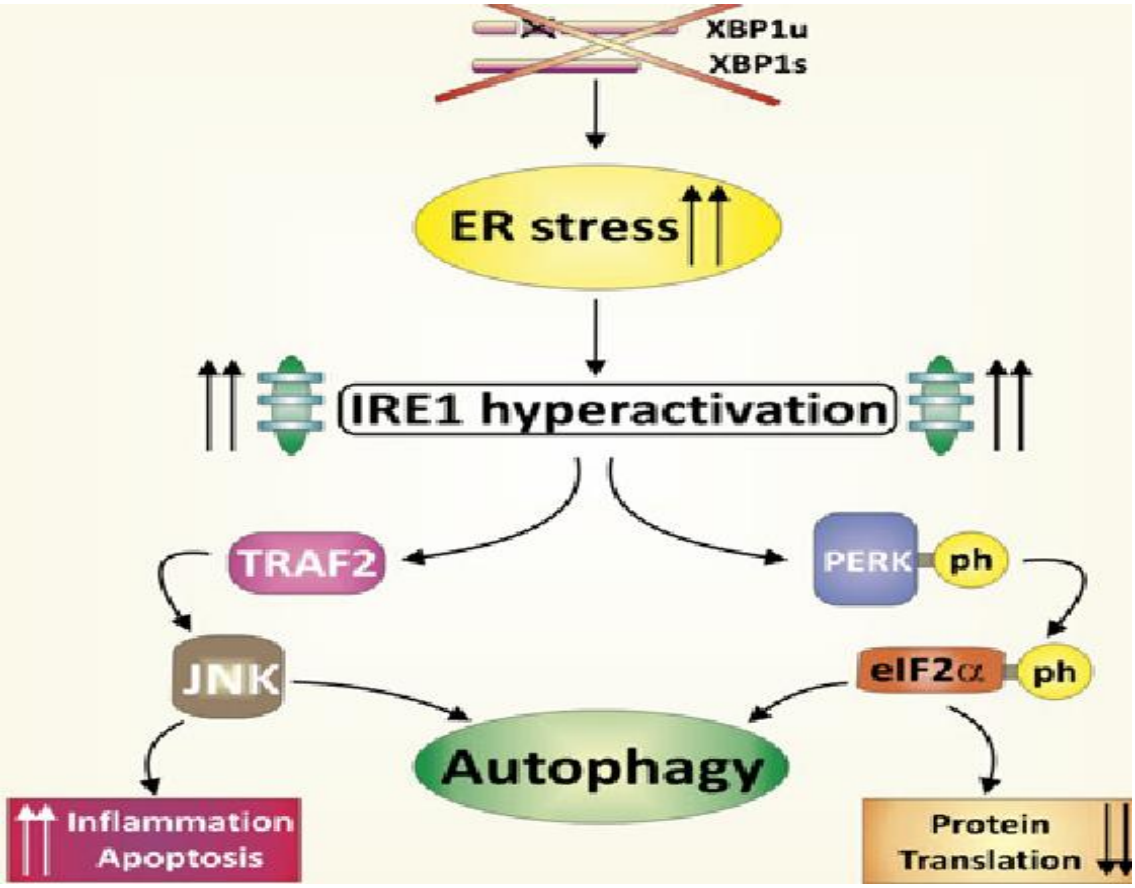



FIGURE 6. HYPOMORPHIC XBP1 FUNCTION LEADS TO ER STRESS AND CONSECUTIVE MASSIVE ACTIVATION OF INOSITOL REQUIRING ENZYME-1 (IRE1), THE KINASE END ENDO-RIBONUCLEASE UPSTREAM OF XBP1, ALONG WITH ACTIVATION OF OTHER BRANCHES OF THE UNFOLDED PROTEIN RESPONSE (UPR) [115, p. 2].

## 2.2 Apoptosis and IBD

### 2.2.1 Apoptosis

Apoptosis was first discovered in 1972 by Kerr, Wyllie, and Currie. In 1990, Horvitz described the mechanism involved in the apoptosis in mammalian cells [116]. Apoptosis is a form of cell death through which the elimination of cells happens without releasing harmful

substances into the surrounding [116]. This programmed cell death involves the genetically determined removal of cell death. Conserved from worms to mammals, apoptosis occurs typically during development and ageing as homeostatic mechanisms to maintain cell populations in tissue [116]. It also happens as a defence mechanism when cells are damaged by disease. In other words, apoptosis is necessary to purge the body of pathogens invaded cells and eliminate activated or autoimmune cells [117]. Morphologically, apoptotic bodies appear as round masses with a condensed cytoplasm and fragmented nuclei [116]. The mechanism of apoptosis is complex and tightly regulated and involves an energy-dependent cascade of molecular events. So far, two main-apoptotic pathways have been reported: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway **(Figure7)**.

The intrinsic and the extrinsic pathway all converge at the same terminal, where the cleavage of caspase 3 results in DNA fragmentation, cross-linking of proteins, and formation of apoptotic bodies [118]. One crucial factor that initiates apoptosis is the activation of caspases. Caspases are proenzyme or specific proteases which contains a cysteine on the active site that cleaves at one particular aspartic acid on targets [119]. Caspases can be divided into three subclasses: Initiation (caspase-8), intermediate, and terminal (caspase-3) caspases.

In the extrinsic pathway, the interaction between the ligand-receptor initiates the destruction complexes through the recruitment of intracellular FAS associated death domain (FADD) or TNF-alpha receptor (TRADD) that enable the catalytic activity of caspase-8 [120]. On the other hand, the intrinsic pathway is usually observed when a repair mechanism failure happens, and the intracellular machinery stimulates the transcription of P 53 [121]. This P 53 gene is the caretaker of the genome, and it stimulates other proteins such as P 53 upregulated modulatory of apoptosis

(PUMA), Bcl-2 family proteins [122, 123]. The Bcl-2 family includes anti-apoptotic and pro-apoptotic members, for example, Bcl-2 associated agonist of cell death (Bad), apoptosis regulator Bcl-2 like protein4 (Bax) and Bcl-2 antagonist killer1 (BAK1) are all pro-apoptotic members [123].

### 2.2.2 Apoptosis in IBD

In IBD, the intestinal epithelium of patients showed high levels of apoptosis. In one study, Souza et al. investigated apoptosis by examining the expansion of FAS/FAS ligands in intestinal mucosa for inflamed and control patients. The tunnel assay analyzed apoptotic cell density and distribution. Their study showed that epithelium from patients with UC has higher rates of apoptosis than controls. No differences were observed with CD patients [124]. Generally, in the small intestine epithelium, all cells except Paneth and intestinal stem cells roam from the crypt base to the villus tips to shed. Some of those cells usually undergo apoptosis before shedding. Iwamoto et al. discovered that increased apoptotic features were found in the crypts of active UC compared to healthy conditions [125].

Moreover, proteomic studies comparing CD and UC have shown 47% of all changes in the epithelial cell proteome were associated with signal transduction pathways that included pro-apoptotic mechanisms [126]. In the same study, the programmed cell death protein 8, which is related to an independent apoptosis pathway, was upregulated in inflamed UC compared to non-inflamed controls. To verify the implication of T-helper type 2 immune response in enhancing the apoptotic ratio, Rosen et al. checked the level of IL-13 in UC. Increased levels of IL-13 in UC was

found and was associated with epithelial cell apoptosis [127]. Some studies have focused their studies on confirming the role of apoptosis in IBD mechanisms. For example, knockout mice for XBP1 have been shown to develop enteritis and subsequent apoptotic cell death. Other studies have demonstrated that NF-KappaB deficiency leads to apoptosis of colonic epithelial cells with defective expression of antimicrobial peptides and translocation of bacteria [128, 129]. The exact role of apoptosis in IBD pathogenesis is still unknown, and further investigation is yet required.

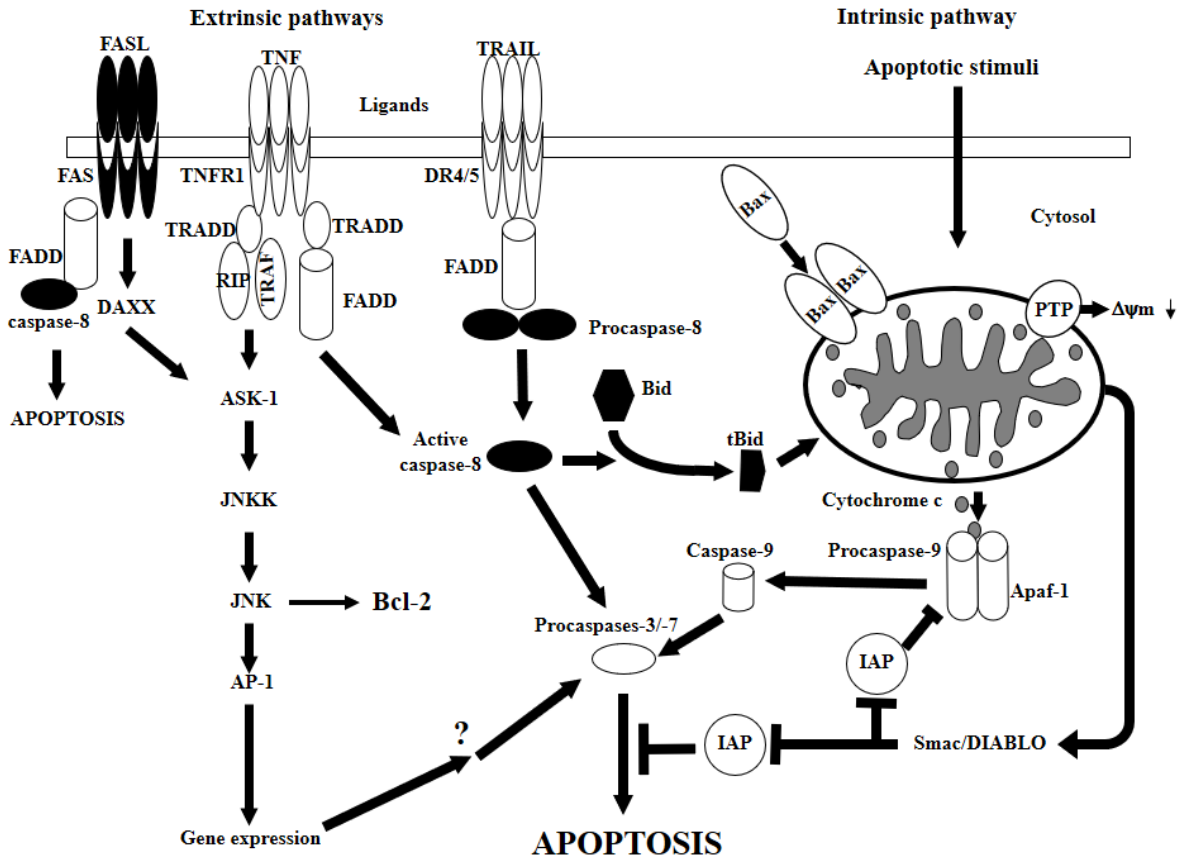



**FIGURE 7. OVERVIEW OF THE APOPTOTIC PATHWAYS. ON THE LEFT THE EXTRINSIC OR DEATH RECEPTOR PATHWAY AND ON THE RIGHT, THE INTRINSIC OR MITOCHONDRIAL PATHWAY (COPYRIGHT PERMISSION OBTAINED FROM DR. SPENCER GIBSON)**

# 3 Chromogranin & IBD

Granins belong to the family of proteins that constitute the major component of secretory granules of various endocrine and neuroendocrine cells [130]. They are produced as pre-proteins in ribosomes and undergo post-translational modifications in the endoplasmic reticulum and the Golgi apparatus [131]. The granin family consists of eight proteins: Chromogranin (CHG) A, B, C, secretogranin III, IV, V, VI, and VEGF. Structurally, granins are composed of single-polypeptide chains of approximately 180 to 700 amino acids [132]. Many proteolytic sites with enzymatic activity characterize these granins, where appropriate cleavage at those sites will lead to several biologically active peptides [130]. CHGA was the first one to be identified from the Granin family [133]. CHGA is encoded by the gene CHGA/ChgA located on chromosome 14, with 49 kDa; its expression correlates with the number of secretory vesicles in neuroendocrine cells [134]. CHG can also be found in many other cells, including immune cells, epithelial cells, and peripheral neurons. CHGA arises several biological peptides that exert a broad range of biological functions by influencing the endocrine, neuroendocrine, cardiovascular, and immune systems **(Figure 8)** [135]. In the context of IBD, a study demonstrated that degradation of intestinal homeostasis is associated with intestinal cell differentiation, including changes in the level of CHGA [136]. Moreover, high levels of CHGA have been demonstrated in IBD patients, proposing that CHGA might play a role in the intestinal immune response by maintaining the intestinal inflammation under control [137].

## 3.1 Intracellular Function of CHGA

Calcium homeostasis is a function that is regulated by CHG as it has the high-binding capacity and low affinity for $Ca^{2+}$ [138]. Additionally, CHG helps in the activation of calcium channels, which increases the exchange of free bond calcium in the secretory granules and calcium mobilization in the cytoplasm [135]. In IBD, it was indicated that the colon has persistent, abnormal motility and reduced segmented contraction. These observations are likely related to the altered smooth muscle contractions. The smooth muscle depends on $Ca^{2+}$ release from the intracellular compartment during inflammation [139]. The reduced $Ca^{2+}$ influx and altered $Ca^{2+}$ release lead to dysmotility during the progression of intestinal inflammation [139]. As $Ca^{2+}$ signalling activates multiple pathways in IBD, regulating the $Ca^{2+}$ may be an exciting approach to control the excessive immune response [140]. Neuropeptides and hormones are usually stored in neuroendocrine cell secretory granules [141]. Studies have shown that CHGA upregulates the biogenesis of granules and also inhibits their degradation. CHGA drives secretory granules as they aggregate in the acidic environment of immature vesicles and promote the building of trans-Golgi membranes causing granules formation [142, 143].

## 3.2 Extracellular Functions of CHGA

The sources of CHGA are adrenal medulla, adrenergic nerve, and neuroendocrine cells [140, 144]. It is proved that CHGA has a clear role in maintaining gut homeostasis; it also modulates the peptide secretion of pituitary glands in the central nervous system level. Moreover, CHG induces neural apoptosis indirectly by leading microglial cells to generate

neurotoxic agents and TNF-alpha [145]. Angiogenesis is the process of new blood vessels formation pre-existing vessels, and it is a mechanism that is commonly observed in IBD patients. The presence of this angiogenesis suggests that the local microvasculature plays a role in the progression of the inflammation [146, 147]. Various evidence indicates that CHGA regulates angiogenesis via influencing different factors like vascular endothelial growth factor, hypoxia-inducible factor-1, and fibroblast factor [148, 149]. The regulation of these angiogenic factors happens through proteolysis of thrombin-induced mechanisms. CHGA may maintain intestinal homeostasis through angiogenesis.

In human, data suggest variation in the CHGA gene are associated with autonomic function, which also describes the association between plasma CHG levels and release of catecholamine neurotransmitter [150]. To further understand the role of CHGA, knockout mouse models were developed. Studies with these mice showed elevated blood pressure and alteration of adrenal chromaffin granules [149]. In another study, these mice showed enhanced release of catecholamines with no abnormality on the development and regular chromaffin pellets in the adrenal gland [142]. More research is still needed to elucidate the role of CHGA in various diseases. But since CHGA is considered as pro-protein, its derived peptides play also an important role in regulating gut function.

## 3.3 Chromogranin-Derived Peptides

CHGA belongs to the granin family of proteins that are stored in chromaffin granules in the adrenal medulla. In the gastrointestinal tract, enterochromaffin cells constitute the most

extensive endocrine population, and the release of CHGA is then subjected to a cell and tissue-specific processing [140, 151, 152]. Serving as a prohormone, it undergoes cleavage by convertases 1-3 and other proteases to produce bioactive fragments **(Figure 8)**, including chromofungin (CHR, aa47-66), catestatin (CST, aa352-372), pancreastatin (PST, aa273-301) and vasostatin-I and II **(Figure 8)** [153, 154]. These peptides lack receptors, and their sequence similarity with cell-penetrating peptides suggests that they may enter cells to exert their actions in normal or harmful conditions [137].

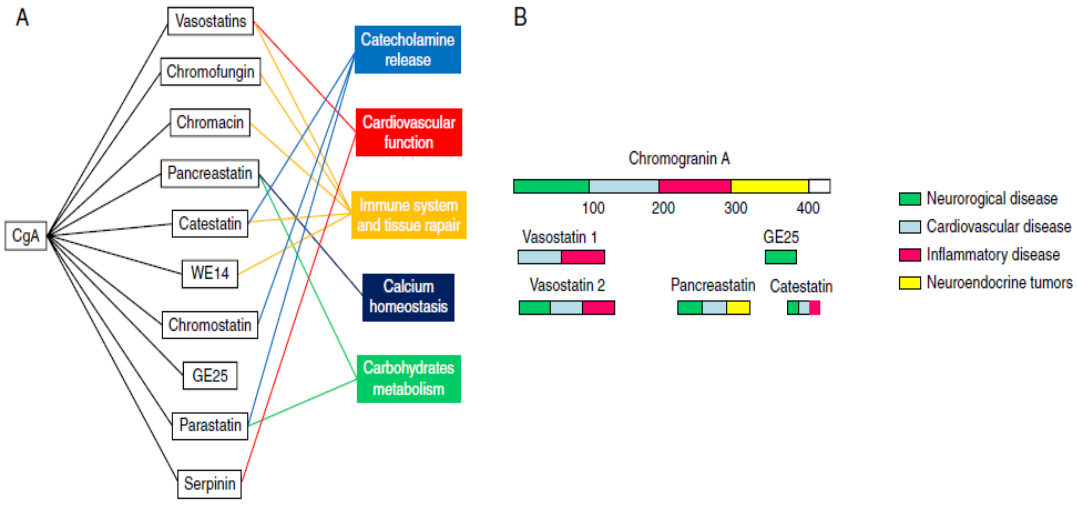



**FIGURE 8. CELLULAR PROCESSING OF CHROMOGRANIN-A (CHGA) GENERATES SEVERAL BIOLOGICALLY ACTIVE PEPTIDES, THAT REGULATE VARIOUS ENDOCRINE, CARDIOVASCULAR AND IMMUNE RESPONSES** [152].

### 3.3.1 Chromofungin (CHR)

CHR ($CHGA_{47-66}$) is a 19 amino acid peptide and consists of RILSILRHQNLLKELQDLA. This active short peptide has antimicrobial and antifungal activity [140]. Antimicrobial peptides usually regulate the maintenance of intestinal barrier function and immune homeostasis; mucosal studies of IBD patients have reported an increased dysregulation of the antimicrobial peptides [155]. Additionally, CHR may activate other immune cells, such as neutrophils, critical in the development of the immune response [156]. It also plays facilitates communication between the endocrine and immune system [140]. CHR can have a paracrine effect. The release of cationic and amphipathic CHR from the activated polymorphonuclear neutrophils can lead to a regulation of calcium influx, leading to the activation of unstimulated and release of critical immune regulatory molecules [157]. Furthermore, studies have shown that CHR reduces colitis in DSS-induced models through the modulation of MØs and epithelial plasticity [152]. In one study, Lugardon, and Metz-Boutigue examined the structure of the CHR using H-NMR spectroscopy in water and trifluoroethanol. Their analysis indicated an alpha-helical structure in solution [158]. The COOH terminal moiety of the CHR peptide has been massively associated with the amphipathic and antifungal activity. The structural representation of CHR shows the amphipathic helical character of the C-terminal part, followed by hydrophobic leucine residues in position 57,58,61,64 and 67, and a hydrophilic characteristic at the N-terminal [158].

To verify CHR's protective potential in the context of inflammation, scientist Filice et al. demonstrated that CHR acts as a postconditioning agent for ischemia or reperfusion injury that decreases lactate dehydrogenase activity, reduces infarct size, and protect against myocardial injuries by activation of specific kinases [159]. Lugardon et al. tested the penetration of CHR in a

lipid monolayer and concluded that CHR could enter the cell through interaction with lecithin monolayers and calmodulin, where $Ca^{2+}$ plays a significant role in that interaction [158].

### 3.3.2 Other CHGA-derived Peptides

#### *3.3.2.1 Pancreastatin (PST)*

PST was first isolated from the porcine pancreas in 1986, and it is usually derived from CHGA exon-VII cleavages [160, 161]. Composed of 49 amino acid ($CHGA_{273-301}$), PST requires an amidation at the carboxyl ends to be active [161]. PST is usually found in cohabitation with histamine and somatostatin in pancreatic islet cells, and under stress, its release is done by the sympathetic nervous system [162]. It is mostly modulating energy and lipid metabolism, as energy metabolism is necessary for immune response. PST decreases glucose uptake and enhances the spillover of free fatty acids [163]. This decrease happens via direct dysglycemic effect through alteration of glucose homeostasis and inhibition of insulin.

Two common clinical issues that coexistent in IBD and diabetes are characterized by an increase of proinflammatory cytokines and down-regulation of anti-inflammatory mediators, and alteration in the gut microbiota [164]. The hallmark of insulin resistance is an upregulation of inflammatory cascades; this effect is abolished in PST knock out mouse strain [163]. Overall, PST plays a proinflammatory role through the activation and suppression of pathways involved in IBD.

#### *3.3.2.2 Catestatin (CST)*

A highly conserved peptide, CST, is 21 amino acids peptide encoded by CHGA exon-VII (human $CHGA_{352-372}$ and rat $CHGA_{367-387}$) [162]. CST belongs to the nicotinic cholinergic receptor

family and can inhibit the secretion of catecholamines. CST has various functions; it can activate cardiovascular responses through histamines released by cells [165], but also can help in the proliferation and migration of endothelial cells and plays a role in the smooth muscle cells chemotaxis stimulation [149]. In IBD, a study has revealed that human CST functions can act as a regulator of the gut inflammation by inhibiting proinflammatory cytokine production via a signal transducer and activation of transcription STAR-3 dependent molecular mechanisms [140]. In a mouse model, the deleterious proinflammatory effect caused by DNBS and DSS is reduced by CST through the regulation of immune cells [154]. It also suppresses or inhibits the intestinal inflammation, and inflammatory cytokine by regulating the activity of classically activated MØs without affecting the alternatively activated MØs [160]. In sum, CST seems to be an anti-inflammatory peptide during intestinal inflammation.

#### *3.3.2.3 Vasostatin (VS)*

VS has two forms: VS-I ($CHGA_{1-76}$) and VS-II ($CHGA_{1-113}$), they are involved in a diverse range of functions, for example, inhibition of parathyroid hormone secretion, vasodilation, and antimicrobial effects [162]. Several studies have characterized VS-I to protect the integrity of the endothelial barrier, with inhibition of TNF-alpha induced vascular leakage [167], [168]. In other words, VS-I limit IFN-gamma and TNF-alpha increased permeability of colonic epithelial cells as it reduced IL-8 release in LPS-stimulated epithelial cells [169]. Some studies reported that VS promotes wound healing through increased migration of mechanically damaged Caco-2 cells [169]. The same review was able to show that oral administration of VS-I to DSS induced colitis mice can cause reduced weight loss and decrease colonic proinflammatory cytokine release.

# Chapter 3 | Rationale, hypothesis & aims

## 1 Rationale

CD and UC represent the two major form of the chronic idiopathic IBD with an estimated prevalence in Canada of around 15/100000 persons [3]. Biological agents are expensive, require indefinitely used to ensure a sustained response, and raise concerns about infections and other complications [5], thus limiting enthusiasm for introducing them earlier in the treatment for IBD. The essential cells in the innate immune system that coordinates the inflammatory process are the MØs, where they have been demonstrated to be increased in areas of inflammation in IBD [137, 152]. MØs are a heterogeneous population of two different phenotypes: group 1: "inflammatory, classically activated MØs: M1" and alternative activated MØs: M2. MØs polarization and plasticity depend on specific combinations of cytokines and peptides within the microenvironment [170, 171]. Mucosal changes in IBD are characterized by inflammation and are linked to hyperplasia in the chromogranin-A (CHGA)-producing enterochromaffin (EC) cells [151, 172]. CHGA cleavages generate several biologically active peptides, including CHR [151, 172]. Our lab demonstrated that CHR is an essential peptide in MØs regulation [112, 173]. Genome-wide associated studies in IBD have revealed that in IBD and within the epithelium, endoplasmic reticulum (ER) stress and intestinal epithelial apoptosis figure prominently [112, 123]. A p53-apoptotic pathway mediates intestinal epithelial apoptosis through the activation of the p53-upregulated modulator of apoptosis (PUMA), Bcl-2 associated-X protein (BAX), Bcl-2 associated death promoters (BAD) and Bcl-2 antagonist/killer-1 (BAK1) proteins [124, 125]. Polymorphisms of the X-box binding protein 1 (XBP1) gene, the main UPR signalling protein (GRP78), is associated

with an increased risk of IBD [112]. The deletion of CHGA leads to an increase in GRP78 expression in diabetes [153]. Currently, there is a gap of knowledge in understanding the influence of CHR on MØs, ER stress, and apoptosis markers during the development of experimental colitis.

# 2 Hypothesis

Chromofungin plays a critical role in colonic inflammation *via* modulating ER stress and apoptotic pathways in MØs.

# 3 Aims

1) To determine the actions of CHR on ER stress, UPR and apoptotic markers in MØs.
2) To determine the action of CHR on ER Stress, UPR pathway, and apoptotic markers using an experimental model of colitis.
3) To assess the expression of CHR, ER stress, UPR pathway, and apoptotic markers using biopsies collected from control patients and patients with active UC.

# Chapter 4 | Material & Methods

## 1 Human Samples

All studies were approved by the University of Manitoba Research Ethics Board [HS14878(E)]. Dr. Charles Bernstein collected the biopsies; mucosal biopsies samples were collected from patients with known IBD or without IBD and with their consent. A total of four biopsies were taken from the inflamed site of each patient (n=10). Healthy subjects (n=10) biopsies were taken from uninflamed sites, then biopsies were used for further RNA extraction and gene expression analysis. Patients were all older than 18 years, and they did not have any immune-suppressive therapy, and there was no treatment received by eight out of ten UC patients. Some of the patients who were taking thiopurines and anti-TNF medication were not included in the study. The information from healthy patients and UC patients are described in the following table.

| Status | Birth year | Endoscopic results | histology |
|---|---|---|---|
| Healthy | 1954 | Normal | Normal |
| Healthy | 1958 | Normal | Normal |
| Healthy | 1959 | Normal | Normal |
| Healthy | 1959 | Normal | Normal |
| Healthy | 1959 | Normal | Normal |
| Healthy | 1964 | Normal | Normal |
| Healthy | 1968 | Normal | Normal |
| Healthy | 1981 | Normal | Normal |
| Healthy | 1986 | Normal | Normal |
| Healthy | 1989 | Normal | Normal |

TABLE 1. DATA FROM HEALTHY INDIVIDUALS

| Status | Birth year | Endoscopic results | histology |
|---|---|---|---|
| UC | 1956 | Severely Active Colitis | Severely Active Colitis |
| UC | 1962 | Active Colitis | Active Colitis |
| UC | 1970 | Active Colitis | Active Colitis |
| UC | 1975 | Severely Active Colitis | Severely Active Colitis |
| UC | 1979 | Active Colitis | Active Colitis |
| UC | 1979 | Active colitis with ulcers | Severely Active Colitis |
| UC | 1981 | Severely Active Colitis | Severely Active Colitis |
| UC | 1990 | Severely Active Colitis | Severely Active Colitis |
| UC | 1993 | Severely Active Colitis | Severely Active Colitis |
| UC | 1996 | Active Colitis | Active Colitis |

TABLE 2. DATA FROM PATIENTS WITH ULCERATIVE COLITIS

# 4 Animals

All the experiments were conducted under two protocols #15-010 and #19-014, and the University of Manitoba ethics committee approved the study. C57BL/6 mice (males, 6 to 8 weeks old), with body-weight between 20 and 25 g, were purchased from Charles River, Sherbrooke, Canada. All animals were housed in the animal care facility at the University of Manitoba, where they are kept a pathogen-free barrier.

# 5 Peptides

Peptides of was obtained from Pepmic Co., Suzhou, China. Mass spectrometry and reverse-phase-high-performance liquid chromatography were used to process the peptide to give CHR ($ChgA_{47-66}$: RILSILRHQNLLKELQDLAL) [152]. The peptide purity was characterized by using mass spectrometry and reversed-phase high-performance liquid chromatography—lastly, a scrambled peptide ($ChgA_{47-66}$: RARDHQQENKILLLSILLL was used to confirm the peptide sequence specificity. According to previously published work from our laboratory, an appropriate dose of CHR to inject into mice is 2.5 mg/kg/day [152]. The CHR and 1% phosphate buffer saline (PBS) were given intra-rectally to the mice's different groups. Injections of both CHR and PBS were given one day before the DSS induced colitis and lasted for five days, before collection of whole colonic sections.

# 6 DSS-Induced Experimental Colitis

One percent PBS and CHR were injected intra-rectally to the colon one day before the induction of colitis and lasted for five days. DSS (molecular weight of 40 kDa, was purchased from MP biochemicals, Soho, OH, USA) was added to drinking water at a concentration of 5% (wt/vol) for five days till the mice are sacrificed [146]. For this study, only 6 to 8-week-old mice were used. DSS was freshly made every two days, and time matching of the controls was done with mice receiving normal drinking water only. The utilization of DSS was noted per cage each day.

# 7 Severity of DSS Colitis

The disease severity was recorded by measuring the weight loss by looking for rectal bleeding and checking the stool consistency. The record or scores were defined as follows: 0, no loss; 1, 5%-10%; 2, 10%-15%; 3, 15%-20%; and 4, 20% weight loss.

For Stoll consistency and bleeding scores were defined as follows: stool 0, standard; 2, loose stool; and 4, diarrhea; and the bleeding: 0, no blood; 2, presence of blood; and 4, gross blood. The hemoccult II test was used to assess the bleeding. The disease activity index was recorded every single day during the DSS treatment.

| Score | Rectal Bleeding | Rectal Prolapse | Stool Consistency | Blood |
|---|---|---|---|---|
| 0 | None | None | Normal | Normal |
| 1 | Red | Signs of prolapse | soft | Red |
| 2 | Dark red | Clear prolapse | Very soft | Dark Red |
| 3 | Gross bleeding | Extensive Prolapse | Diarrhea | Black |

TABLE 3. MACROSCOPIC SCORE

# 8 Peritoneal Macrophages Isolation & Culture

Peritoneal MØs were collected from C57BL/6 male mice following the protocol described by Mosser and Zhang [171]. Isolated MØs were cultured in 2 mL Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% deactivated fetal bovine serum (FBS). Cell cultures were stored in a humidified 5% CO2 incubator at 37 °C [152, 174]. The overall cell confluence of the adherent cell was more significant than

95%. Ex vivo, activated peritoneal MØs isolation; 5 days after the beginning of the DSS treatment, resident peritoneal MØs were collected from all groups and subjected to further analysis. For the in vitro experiment, peritoneal MØs were isolated from naive male C57BL/6 mice then collected non-complete in a DMEM medium. Macrophages were washed twice with 1% PBS solution and treated with CHR (200 ng/mL) for two h and then exposed for an additional 6 h LPS 100 ng/ml in medium to induce M1 MØs [171]. Cells and supernatant medium were harvested for analysis.

# 9 Protein Quantification

Western blot: Whole colonic sections were lysed using the NP-40 lysis buffer (50 mM Tris, pH7,4, 50 mM NaCl, five mM EDTA, 50 mM NaF, one mM $Na_3VO_4$, 1%Nonidet p40 and 0.002% $NaN_3$; Catalog number FNN0021; Invitrogen Corporation, Camarillo, CA, USA) containing a protease inhibitor cocktail (Roche Applied science, Madison, WI, USA). Proteins were isolated from the lysate using a centrifugation 14,000 x g for 20 min at 4 degrees. Protein samples (30 µg) were separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinyl difluoride membrane. The membrane is then blocked with 5% non-fat dried milk in tris-buffered saline with tween 20 for 1 hour at room temperature and then incubated overnight with a specific primary antibody (1:1000 dilution). Primary antibodies were anti-XBP1, anti-BAK, anti-BAX, anti-BAD, anti-CASP3, anti-CASP8, and anti-GAPH or B-actin were used as housekeeping genes. Goat-anti mouse, rabbit-anti mouse (1:2000 dilution) was incubated for an hour at room temperature. GE healthcare life sciences enhanced chemiluminescence-detecting reagent was used to visualize the membranes. The protein blots

were quantified by densitometry using one software (BioRad Laboratories, Inc., CA, USA), and the quantity was expressed relative to the housekeeping genes GAPDH or Beta-actin.

Enzyme-linked immunosorbent assays (ELISAs) were also used to quantify proteins from supernatants of the cell cultures. To homogenize the colonic samples, protease inhibitors were dissolved into Np-40 lysis buffer. The homogenized samples were then centrifuged for 30, and supernatants were collected and stored at -80 degrees until the assay is done. The total protein concentration was calculated using the Bradford protein assay (BioRad CA, USA). Caspase-3 colorimetric assay kit (ab39401, Abcam) was used to detect caspase-3 levels. Based on this kit, the enzymatic activity of caspase-3 using cell culture supernatants was quantified. This ready to use equipment is based on the formation of chromophore p-nitroaniline (p-NA) via cleavage from a labeled substrate. Lastly, Spectrophotometer reading absorbance at 405 nm to measure the P-NA formation is taken. Comparing the absorbance of p-NA from the apoptotic sample and an uninduced control allows us to determine the fold increase in caspase-3 activity.

# 10 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

## 10.1 RNA Extraction & Synthesis of cDNA

RNA was extracted from whole colonic sections and cell culture using the TRizol™ Plus RNA Purification kit (Life Technologies, NY, USA), according to the manufacturer's instructions. The quality and the quantity of the extracted RNA were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at the absorbance of 260 nm and 280 nm. The absorption ratio (A260/A290) was determined for all samples. One

microgram of each sample was treated with RQ1 RNase-free DNase (Promega Corporation, Madison, WI, USA) to remove any contamination from genomic DNA. The samples are then subjected to the Superscript VILO cDNA master mix (Introvigen, Grand Island, NY, USA) to perform the reverse transcription reaction. This reaction happens in an Eppendorf thermocycler, and the genes were amplified by the first step at 25 °C for 10 min, followed by 42 °C for 60 min and 85° for 5 min. all samples were cooled, and then the cDNA was stored at -20 °C before qPCR analysis.

## 10.2 Primers

Designing was done using the (http://blst.ncbi.nlm.nih.gov/Blast.cgi) NCBI BLAST. Nucleotide sequences and melting temperature ™ of 58-62°C, GC content of 45-55%, lengths 18-22 bp, and the amplicon sizes 75-175 bp were the criteria that were taken in consideration while designing the primers. All the primers were ordered from Life Technologies with their certificate of analysis. Here are two tables that show the primers used.

| Gene | Forward | Reverse |
|---|---|---|
| CHGA | TAAAGGGGATACCGAGGTGATG | TCGGAGTGTCTCAAAACATTCC |
| BAD | CCCAGAGTTTGAGCCGAGTG | CCCATCCCTTCGTCGTCCT |
| BAK | CATCAACCGACGCTATGACTC | GTCAGGCCATGCTGGTAGAC |
| BAX | CCCGAGAGGTCTTTTTCCGAG | CCAGCCCATGATGGTTCTGAT |
| PUMA | GAAGAGCAAATGAGCCAAACG | GGAGCAACCGGCAAACG |
| TRAIL | TGCGTGCTGATCGTGATCTTC | GCTCGTTGGTAAAGTACACGTA |
| XBP1 | CCTTCAGTGACATGTCTTCTCC | CCCAGTGTTATGTGGCTCTTTA |

| | | |
|---|---|---|
| CASP8 | CTCCCCAAACTTGCTTTATG | AAGACCCCAGAGCATTGTTA |

**TABLE 4. HUMAN PRIMERS SEQUENCES**

| Gene | Forward | Reverse |
|---|---|---|
| *Chga* | CACGGGAGACAGTGAGAGAG | TCCGACTGACCATCATCTTTCT |
| *Bad* | AAGTCCGATCCCGGAATCC | GCTCACTCGGCTCAAACTCT |
| *Bak* | CAACCCCGAGATGGACAACTT | CGTAGCGCCGGTTAATATCAT |
| *Bax* | TGAAGACAGGGGCCTTTTTG | AATTCGCCGGAGACACTCG |
| *Puma* | AGCAGCACTTAGAGTCGCC | CCTGGGTAAGGGGAGGAGT |
| *XBP1* | TGGACTCTGACACTGTTGCCTC | TAGACCTCTGGGAGTTCCTCCA |
| *Trail* | ATGATGGTGATTTGCATAGTGCT | AGCTGCTTCATCTCGTTGGTG |
| *Casp8* | CAACTTCCTAGACTGCAACCG | TCCAACTCGCTCACTTCTTCT |

**TABLE 5. MOUSE PRIMERS SEQUENCES**

## 10.3 Quantitative Real-time Polymerase Chain reaction (RT-qPCR)

All the reactions were performed in a final volume of 20 microliter. Manufacturer's instructions were followed to do the reaction; SYBR green master mix (Life Technologies) was added to the samples and primer in a Roche light Cycler 96 Real-time System. The conditions used for the reaction were 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 seconds and one period at 60 °C for 60 seconds. Duplicates of RT-qPCRs were run, and the average standard deviation within copies of all the samples was 0.25 cycles.

## 11 Fluorescent Imaging

Peritoneal MØs are collected from naïve C57BL/6 mice in RPMI media. The MØs are first counted using the hematocytometer, and then they are treated with ACK lysis buffer for a minute to remove all red blood cells. The MØs are then mixed to complete RPMI medium, and 0.5 microliter of the mix is aliquoted in a six-well cell culture plate containing coverslips for overnight. The next day the media is removed, and cells are fixed with 2% PFA for 15 min, and then they are washed with 1X PBS. After wash, cells are incubated with Chromofungin and Rhodamine at different times: 0 min, 5 min, 10 min, 20 min, 30 min, and 1 hour. After incubation, cells are washed the first time with 1X PBS and a second time with 1X PBS containing Dapi (Thermo Fisher Scientific, 1:10000 in PBS for 15 min) to counterstain the nuclei of the cells. Lastly, the coverslip containing the cells is placed microscope slides for imaging.

## 12 Statistical Analysis

Data are expressed as ± the mean error of the mean (SEM). Unpaired Mann-Whitney U test was applied to compare between two groups. One-way ANOVA, followed by a Tukey posthoc analysis, was used to compare more than two groups. The unpaired T-test was used to plot the western blot graph for protein quantification assay. Spearman's correlation test was used to study the association between different markers. The statistical two-tail significance level was determined at $p \leq 0.05$. GraphPad Prism software (version 8; GraphPad Software, Inc, La Jolla, CA, USA) was used for statistics analysis.

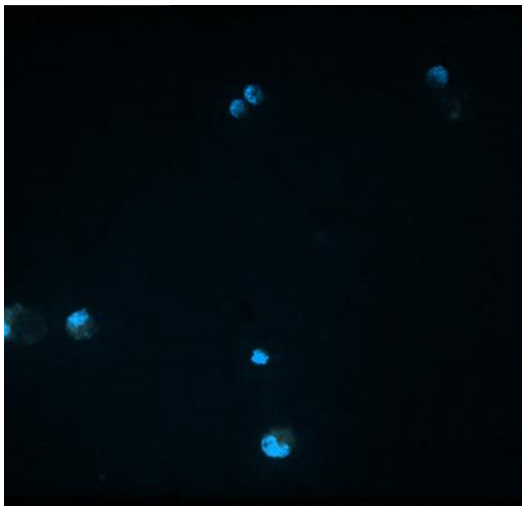
# Chapter 4 | Results

**CHR enters in peritoneal macrophages and resides in the intracellular compartment.**

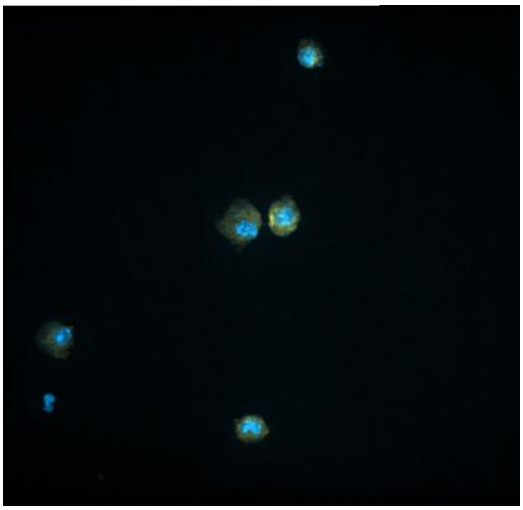
According to previous work performed by Dr. Nour Eissa, CHR protects against colitis through modulation of the functions of MØs and intestinal epithelial cells via the NF-kB dependent pathway [131]. To verify the capacity of entry of CHR inside the MØs, we did an imaging assay where the naïve MØs are incubated with CHR-linked to dye rhodamine.

Our results revealed that CHR enters the cells as early peritoneal MØs as early as 5 min of treatment **(Figure 9)**. The MØs show a bright yellow color surrounding the nuclei of the cell. The brightness of the yellow rhodamine increases inside the MØs with a higher time of incubation. It seems that the CHR enters the cell and resides in the intracellular compartment.

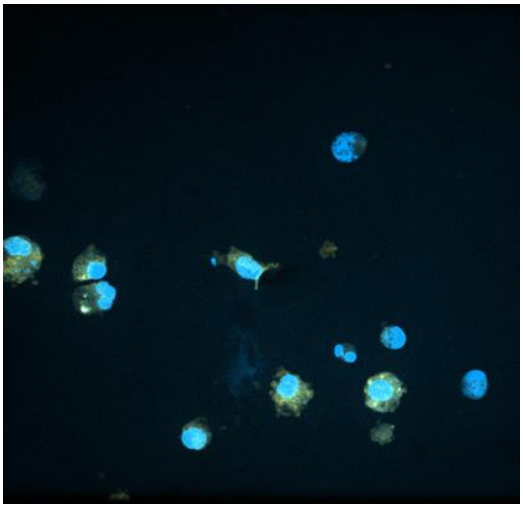
A Control



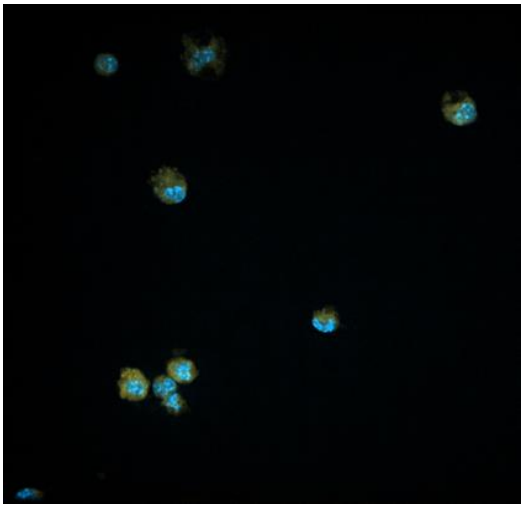
B 5 min in Rhodamine



C 10 min in Rhodamine



D 30 min in Rhodamine



DAPI (4.6-DIAMIDINO-2PHENYLINDOLE) BLUE FLUORESCENT DYE USED TO DETECT NUCLEI

RHODAMINE A TRACER DYE IS LINKED TO CHR, USED AS STAINING FLUORESCENT DYE TO LOCATE CHR

**FIGURE 9. CAPACITY OF RHODAMINED-CHROMOFUNGIN (ROD-CHR) PENETRATION IN MACROPHAGES. FLUORESCENT MICROSCOPY OF A) NAÏVE MACROPHAGES IN THE ABSENCE OF ROD-CHR TREATMENT. NAÏVE MACROPHAGES INCUBATED WITH ROD-CHR (IN YELLOW) AT B) 5 MIN, C) 10 MIN, AND D) 30 MIN AND THE DAPI NUCLEAR COUNTERSTAIN (IN BLUE). SCALE BAR: 40 X. N=6**

**CHR treatment decreased ER (XBP1) and apoptotic markers (Puma, BAK, Bad, Bax) but did not regulate gene expression of apoptotic markers Caspase-3, 8 and TRAIL in M1 polarized macrophages.**

First, we assessed the link between CHR and the genes expression of ER stress (Xbp1) and the genes expression of the intrinsic (Puma, Bad, Bax, Bak1) and extrinsic (Trail, Casp8) apoptotic markers. Xbp1 mRNA expression was significantly increased in M1 polarized after LPS treatment, but this effect was significantly decreased after treatment with CHR **(Figure 10)**. Similarly, we observed similar results with intrinsic apoptotic markers; the CHR treatment decreased mRNA expression of Puma, Bad, Bax, and Bak1 **(Figure 11)**. Additionally, using DSS as a stimulator we found that CHR treatment did not regulate gene expression of specific apoptotic markers (Casp-8, -3 and Trail), no significant up or downregulation were observed in the levels of expression of those genes **(Figure 12)**.

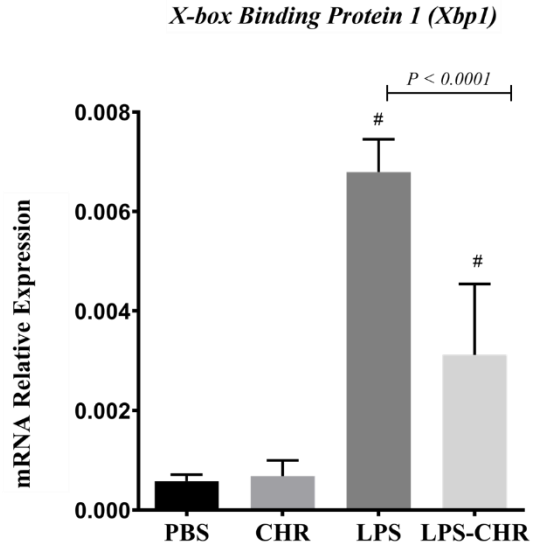
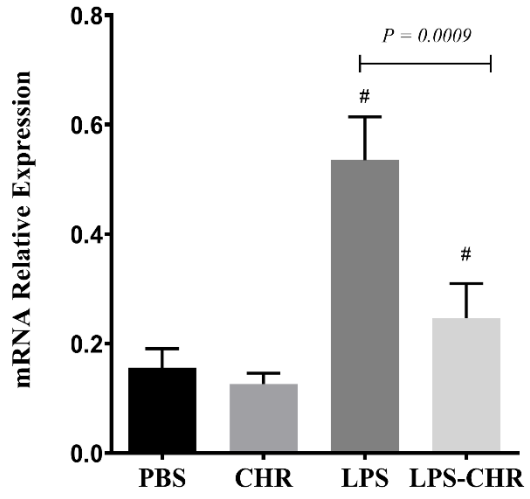


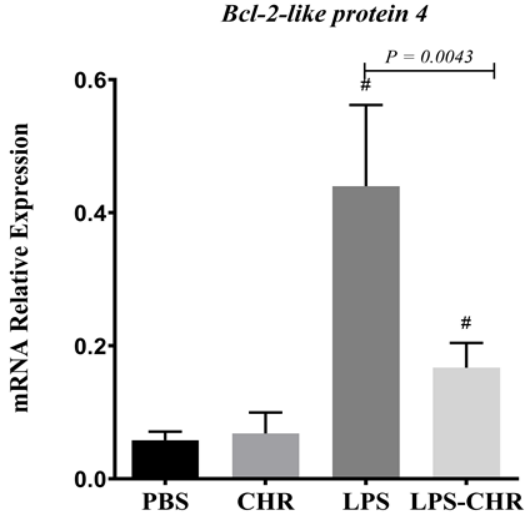

FIGURE 10. MACROPHAGES MRNA EXPRESSION OF ENDOPLASMIC RETICULUM STRESS MARKER, X-BINDING PROTEIN-1 (XBP1) IN NAÏVE POLARIZED M1 MACROPHAGES TREATED WITH LIPOPOLYSACCHARIDE (LPS) IN THE

PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR). ONE-WAY ANOVA FOLLOWED BY TUKEY'S MULTIPLE COMPARISON TESTS. EACH EXPERIMENT WAS DONE AT LEAST THREE TIMES., DATA REPRESENT MEAN ± SEM (N= 8).

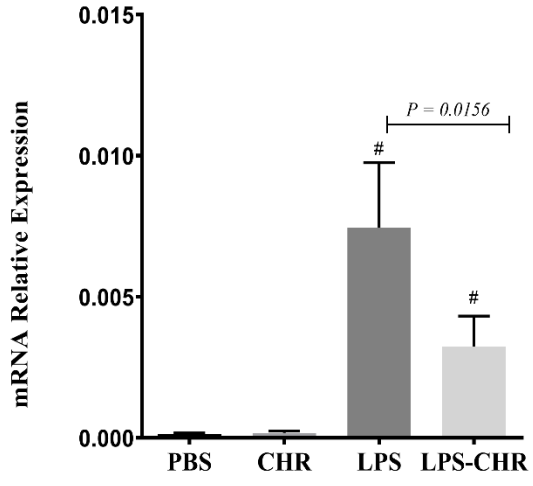
A




B




C




D

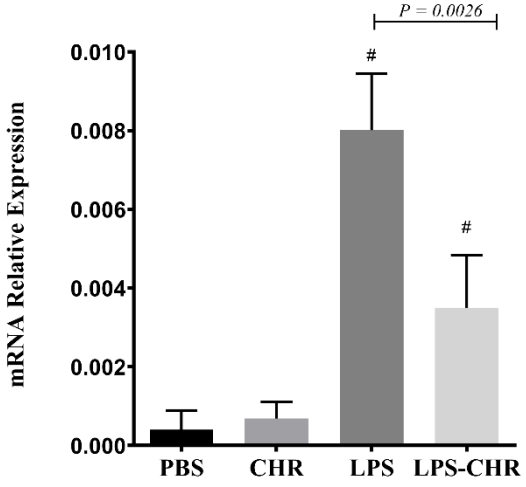
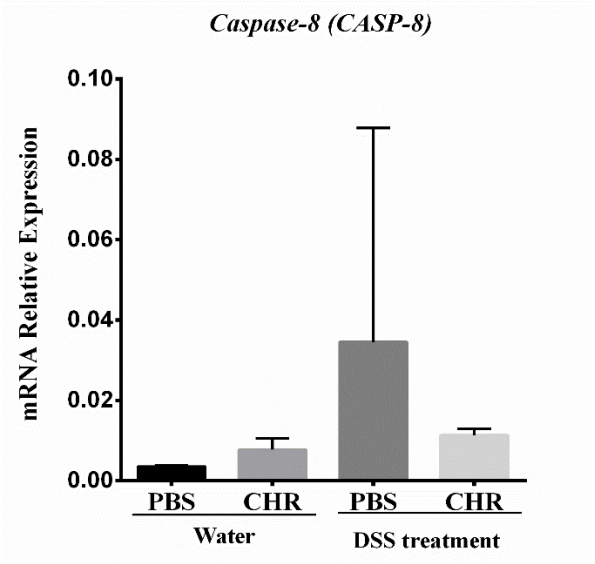
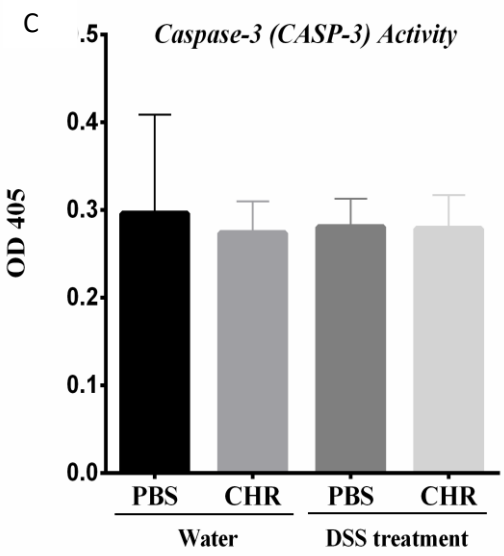


**Figure 11. Macrophages mRNA expression of apoptotic markers in naïve polarized M1 macrophages treated with lipopolysaccharide (LPS) in the presence or absence of chromofungin (CHR). A) Bcl-2 antagonist killer 1 (Bak1), B) Apoptosis regulator BCL-2 like protein 4 (Bax), C) p53 upregulated modulator of apoptosis (Puma), and D) Bcl associated against cell death (Bad). One-way ANOVA followed by Tukey's multiple comparison tests. Data represent mean ± SEM (n= 8). Each experiment was done at least three times.**

A




C




B

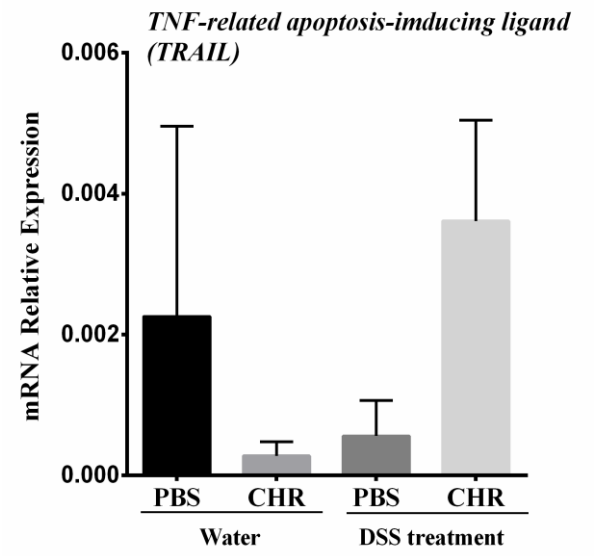


**FIGURE 12. MACROPHAGES MRNA EXPRESSION OF APOPTOTIC MARKERS IN NAÏVE POLARIZED M1 MACROPHAGES TREATED WITH DEXTRAN SULPHATE SODIUM (DSS) IN THE PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR) A) TUMOUR NECROSIS FACTOR (TNF)-A-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL), B) CASPASE (CASP) 8, C) CASP3. ONE-WAY ANOVA FOLLOWED BY TUKEY'S MULTIPLE COMPARISON TESTS. DATA REPRESENT MEAN ± SEM (N= 8). EACH EXPERIMENT WAS DONE AT LEAST THREE TIMES.**

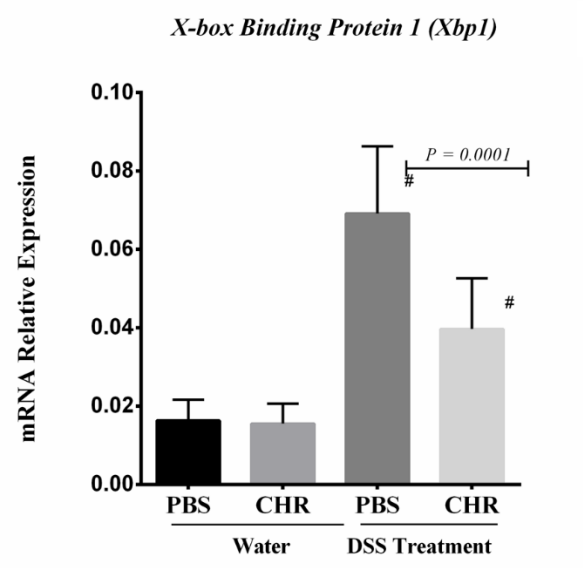
**CHR and XBP1 are decreased and increased during the development of colitis, and treatment with CHR upregulates the expression of Exon IV and downregulates XBP1 expression, respectively in whole colonic sections.**

Next, we investigated the transitional applicability to a mouse model of the result we obtained from our in vitro MØs data and other previous reports [131, 146]. We investigated the anti-inflammatory effect of the CHR. CHR (2.5 mg/kg/ day) peptide was given intra-rectally for five days to mice starting one day before initiation of the DSS treatment. Whole colonic sections were isolated, and CHR (Chga Exon-IV), ER stress, and apoptotic gene expression level were quantified using Q-RT-PCR.

We first observed a significant decrease in the mRNA expression of CHR (Chga Exon-IV) in the colitic group compared with the PBS control group. The CHR treatment was able to restore the CHR (Chga Exon-IV) expression **(Figure 13)**.

We found that colonic XBP1 expression was increased in the colitic group, and colitic mice treated with CHR, the treatment decreased the XBP1 marker within the colonic sections **(Figure 13)**.

A




B

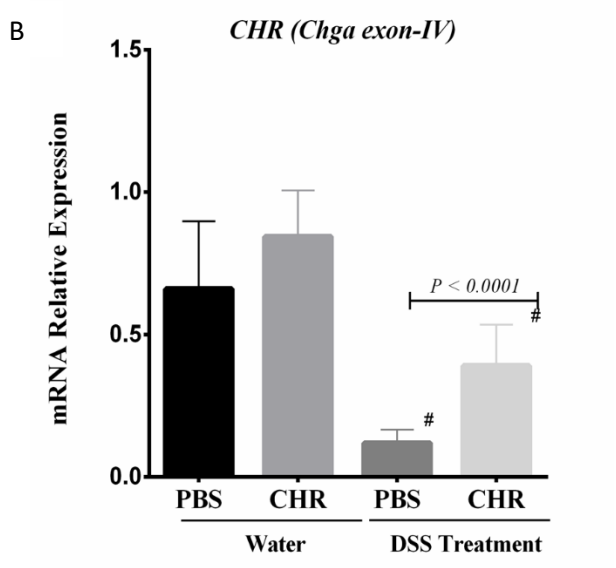


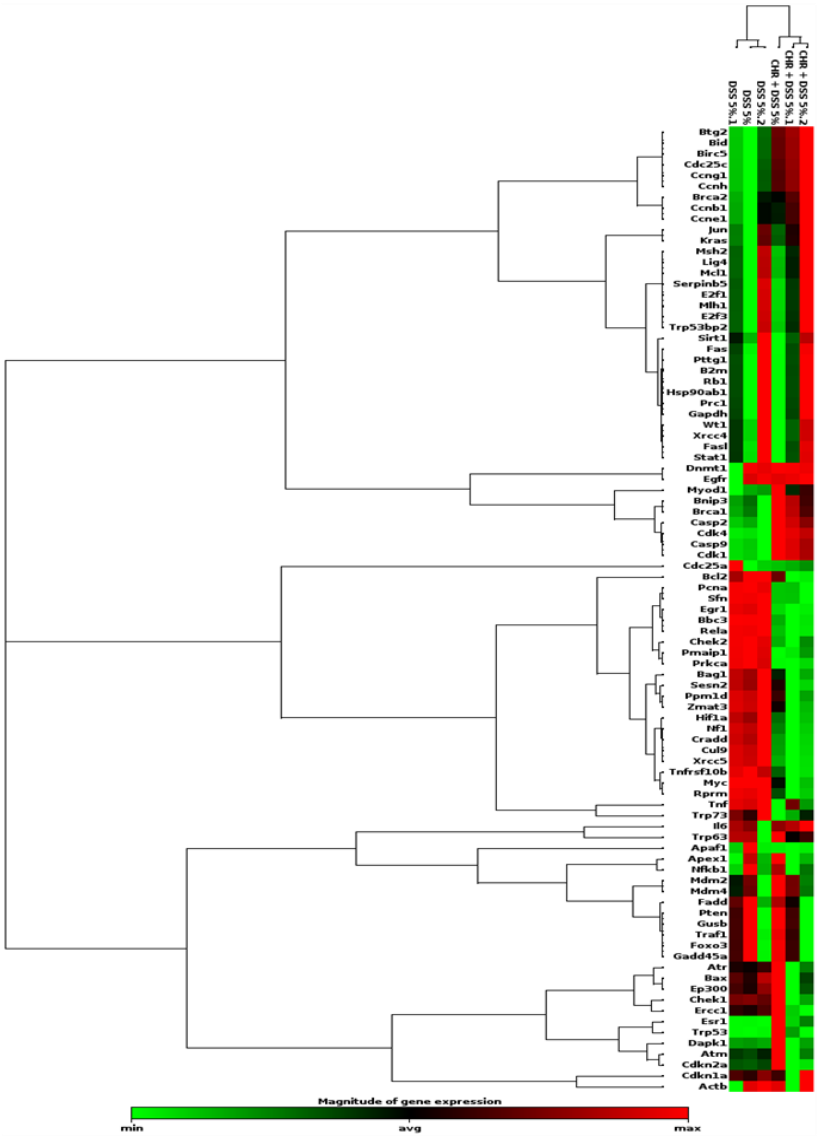

**FIGURE 13. WHOLE COLONIC MRNA EXPRESSION OF ENDOPLASMIC RETICULUM-STRESS AND CHROMOFUNGIN (CHR) (CHGA EXON-IV) IN COLITIC MICE TREATED WITH DEXTRAN SULPHATE SODIUM (DSS) IN THE PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR). A) X-BOX BINDING PROTEIN 1(XBP1), B) CHGA EXON-IV. ONE-WAY ANOVA FOLLOWED BY TUKEY'S MULTIPLE COMPARISON TESTS. DATA REPRESENT MEAN ± SEM (N= 6–8 PER GROUP).**

**In colitic conditions, CHR treatment downregulated 19 genes associated with the p53-downstream signalling pathway.**

In the next experience, we run an RT profile PCR array to analyze the gene expression of 84 genes related to the P53 apoptotic pathway. The PCR array shown all the 84 genes with different magnitude of expression. In colitic conditions, an up-regulation of 26 genes associated with the p53-dependent apoptosis pathway was detected, including Apaf1, Bax, Bbc3, Bcl2, Cradd, Fadd, Cul9, Pmaip1, Tnfrsf10b. In vivo, CHR treatment decreased colitis significantly and was associated with a significant downregulation of 19 genes, including the nine aforementioned when compared with biopsies from colitic groups. The Bcl-2 family genes were highly expressed

with in colitic conditions. Furthermore, the magnitude of expression of the Bcl-2 significantly decreased after CHR treatment **(Figure 14)**.

A



B

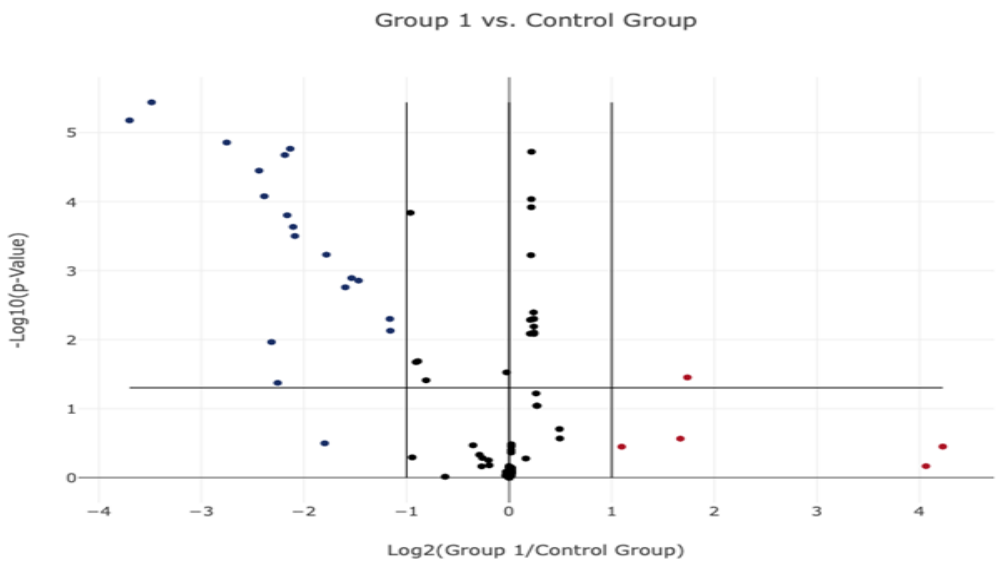
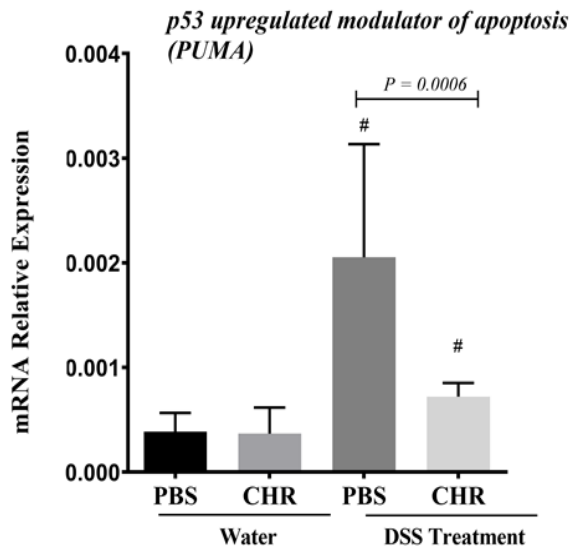



FIGURE 14. WHOLE COLONIC GENE EXPRESSION ANALYSIS OF P53 RELATED MARKERS PATHWAYS IN COLITIC MICE TREATED WITH DEXTRAN SULPHATE SODIUM (DSS) IN THE PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR). A) GENE EXPRESSION PROFILING OF P53 APOPTOTIC PATHWAYS AND B) ANALYSIS. 26 GENES WERE FOUND TO BE UP-REGULATED, 19 WERE DOWN REGULATED, AND THE REMAINING GENES DEPICTED NO CHANGE.

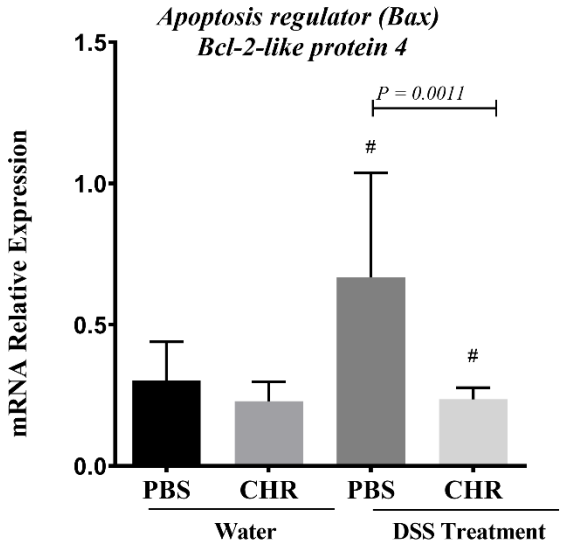
**CHR treatment decreased apoptotic markers (Puma, Bad, Bax, and Bak) but did not modify Caspase-8 and trail gene expression within the whole colonic sections.**

Using our experimental model of colitis, we confirmed our MØs data and demonstrated a significant upregulation of apoptotic expression in colitic wild-type mice. Intra-rectal administration of CHR in colitic mice significantly decreased gene expression of PUMA, BAK1, BAX, and BAD, but CHR administration did not modify the TRAIL gene and Caspase-8 expression within the colonic section **(Figure 15)**.

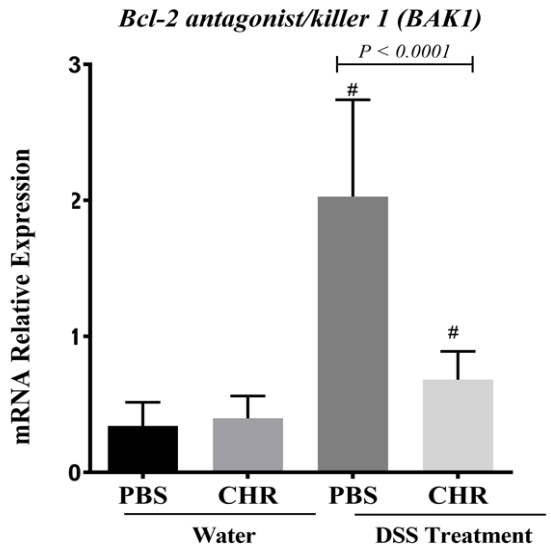
A


p53 upregulated modulator of apoptosis (PUMA)
mRNA Relative Expression
0.004
0.003
0.002
0.001
0.000
P = 0.0006
#
#
PBS
CHR
PBS
CHR
Water
DSS Treatment

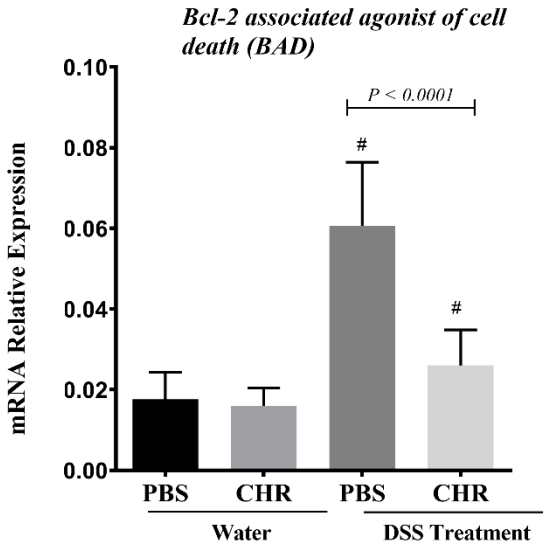
B


Apoptosis regulator (Bax)
Bcl-2-like protein 4
mRNA Relative Expression
1.5
1.0
0.5
0.0
P = 0.0011
#
#
PBS
CHR
PBS
CHR
Water
DSS Treatment

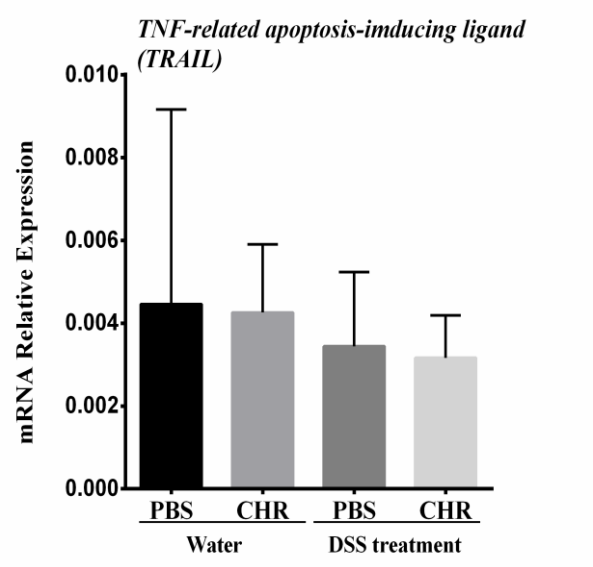
C


Bcl-2 antagonist/killer 1 (BAK1)
mRNA Relative Expression
3
2
1
0
P < 0.0001
#
#
PBS
CHR
PBS
CHR
Water
DSS Treatment

D


Bcl-2 associated agonist of cell death (BAD)
mRNA Relative Expression
0.10
0.08
0.06
0.04
0.02
0.00
P < 0.0001
#
#
PBS
CHR
PBS
CHR
Water
DSS Treatment

E




F

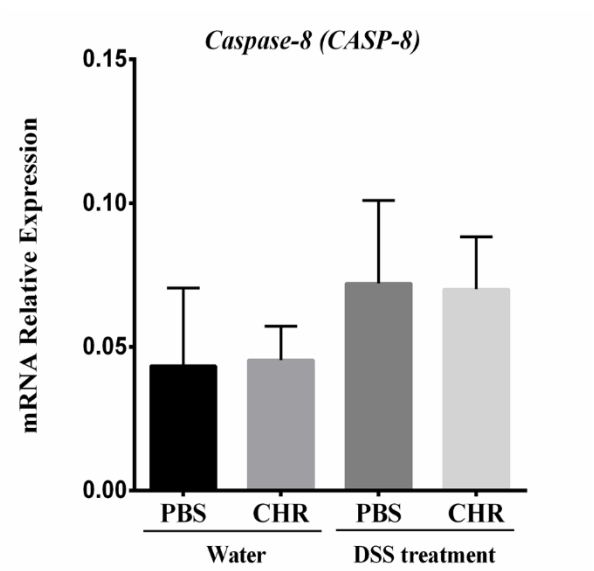


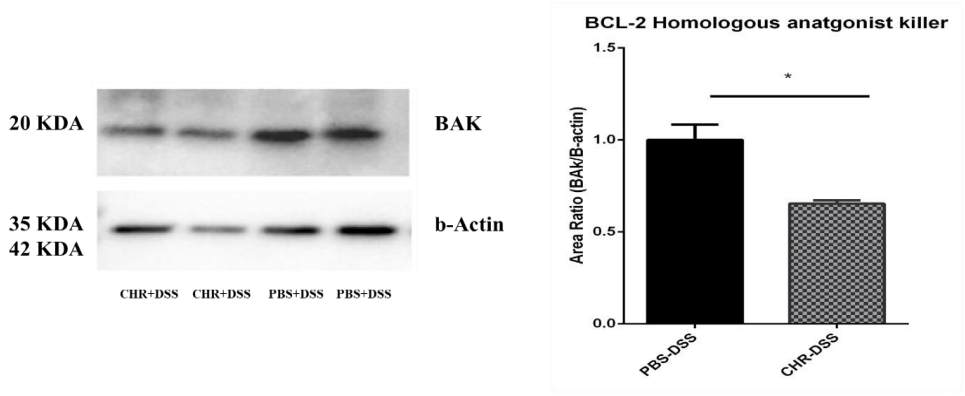

**FIGURE 15. WHOLE COLONIC MRNA EXPRESSION OF APOPTOTIC MARKERS IN COLITIC MICE TREATED WITH DEXTRAN SULPHATE SODIUM (DSS) IN THE PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR). A) P53 UPREGULATED MODULATOR OF APOPTOSIS, B) APOPTOSIS REGULATOR BCL-2 LIKE PROTEIN 4 (BAX), C) BCL-2 ANTAGONIST KILLER 1 (BAK), D) BCL ASSOCIATED AGAINST CELL DEATH, E) TUMOUR NECROSIS FACTOR (TNF)-A-RELATED APOPTOSIS-INDUCING LIGAND (TRAIL), AND F) CASPASE (CASP) 8. ONE-WAY ANOVA FOLLOWED BY TUKEY'S MULTIPLE COMPARISON TESTS. DATA REPRESENT MEAN ± SEM (N= 6–8 PER GROUP).**

**CHR treatment decreased BAK, BAX, CASP-3, CASP-8 & XBP1 protein level within the whole colonic sections.**

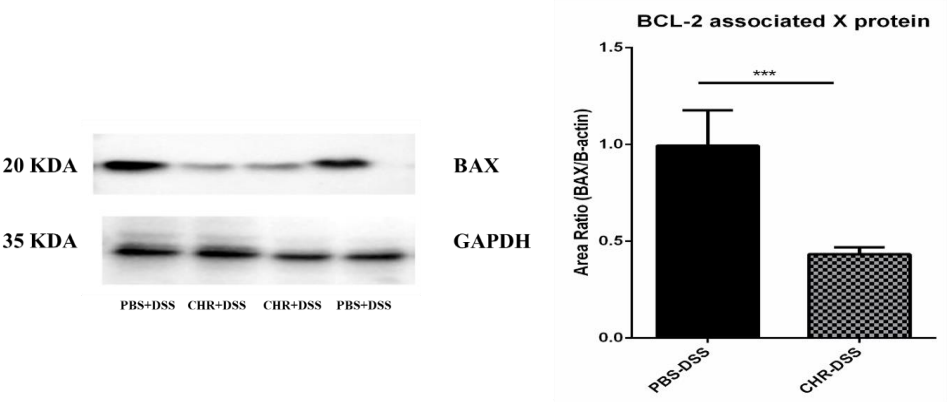
Next, we verified the effect of CHR on the protein level. To do so, we quantify the protein expression of ER stress and apoptotic markers using western blot technic from colonic tissue.

Compared to untreated groups, colitic mice treated with CHR demonstrated a significant decrease of BAX, BAK, and Caspase3, 8 protein, and the apoptotic ER stress inducer marker, X-Binding Protein 1 **(Figure 16)**.

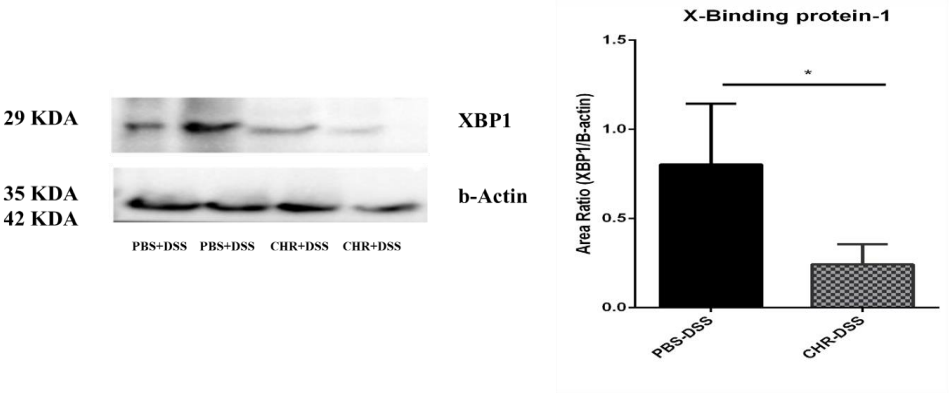
A




B




C



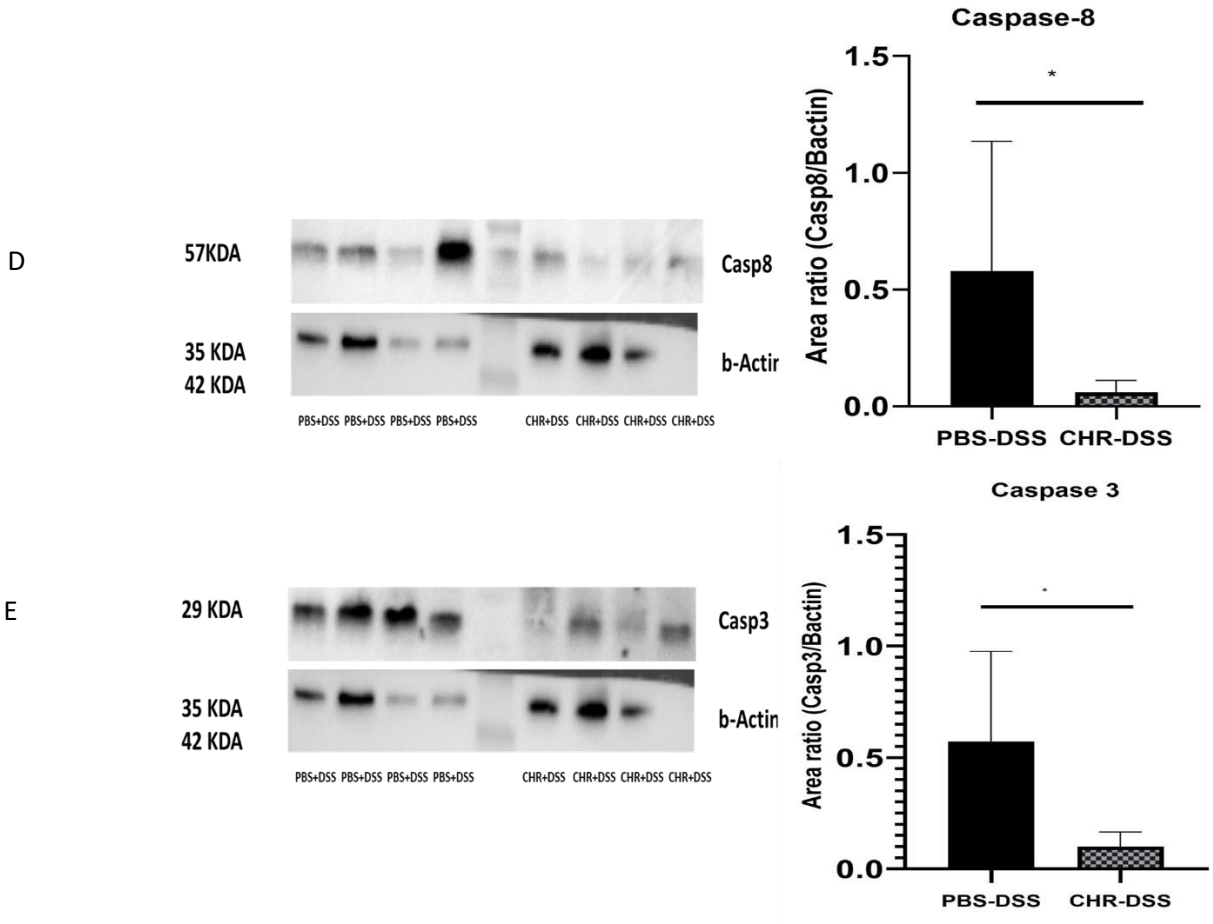
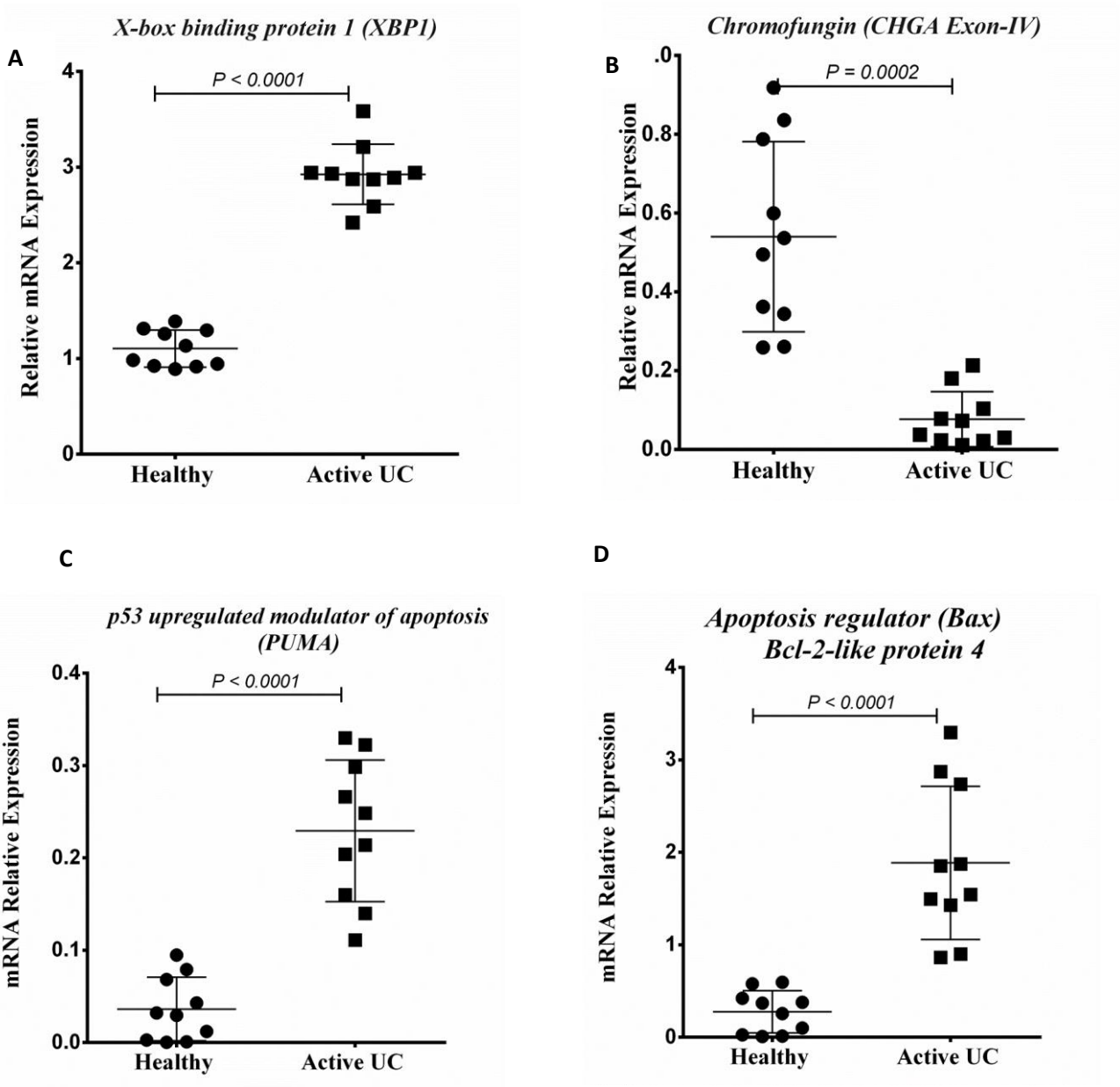



FIGURE 16. WHOLE COLONIC PROTEIN QUANTIFICATION OF ENDOPLASMIC RETICULUM-STRESS AND APOPTOTIC MARKERS IN COLITIC MICE TREATED WITH DEXTRAN SULPHATE SODIUM (DSS) IN THE PRESENCE OR ABSENCE OF CHROMOFUNGIN (CHR). IMMUNOBLOTS OF A) BCL-2 ANTAGONIST KILLER 1 (BAK), B) APOPTOSIS REGULATOR BCL-2 LIKE PROTEIN 4 (BAX), C) X-BINDING PROTEIN-1 (XBP1), D) CASPASE 3, AND E) CASPASE 8. MANN-WHITNEY TEST, N=10 PER GROUP P<0,05

**CHGA Exon-IV gene expression is decreased and associated with an increase in ER stress marker (XBP1), apoptotic markers within human colonic mucosa.**

We investigated the genes expression of CHR and XBP1 in biopsies from subjects with active UC when compared with healthy controls **(Figure 17a)**. mRNA level of CHR was significantly decreased in UC patients compared to healthy patients. mRNA level of XBP1 was singinifncalty increased within the colonic mucosa of active UC patients. Intrinsicapoptotic markers (PUMA, BAD, BAX, and BAK) are signifincalty increase within the colonic mucosa of active UC patients **(Figure 17 C, D, E, and F)**. Apoptotic marker Caspase 3 was also significnalty increased within the colonic mucosa of active UC patients **(Figure 17 G)**.



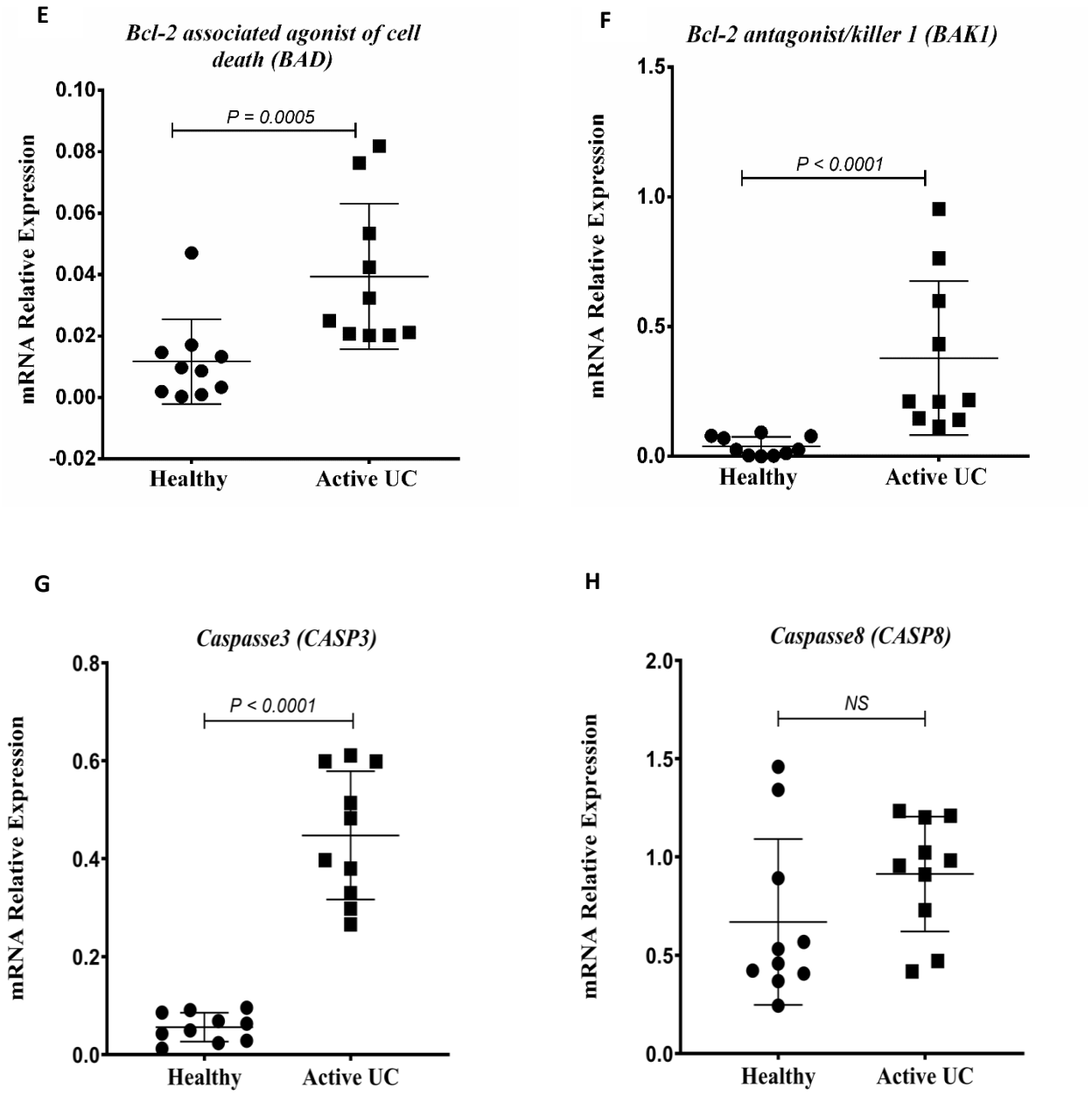
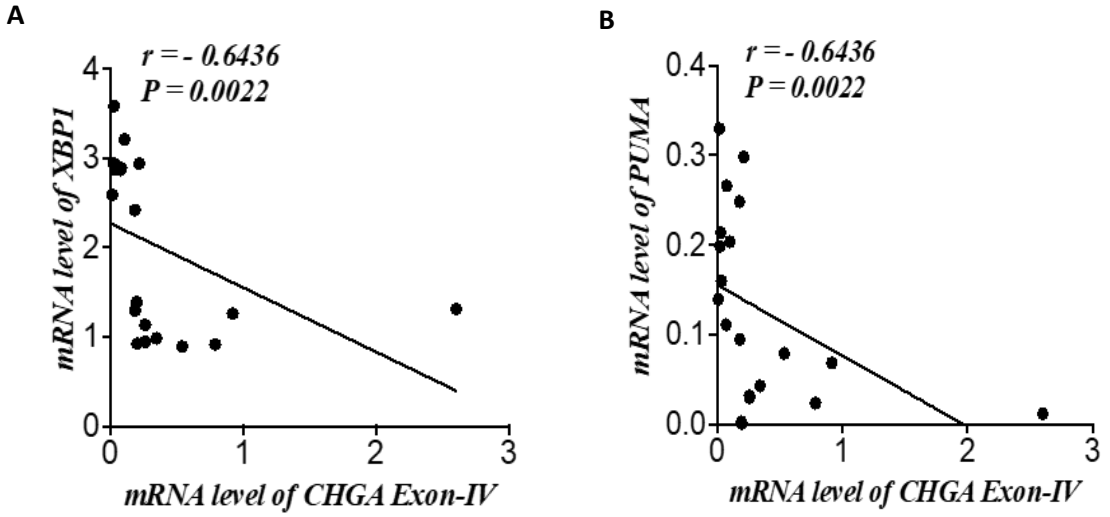



**FIGURE 17. MRNA LEVELS OF ER STRESS AND APOPTOTIC MARKERS IN PATIENTS WITH ACTIVE ULCERATIVE COLITIS (UC) AND HEALTHY CONTROL. A) X-BINDING PROTEIN-1 (XBP1), B) CHGA EXON IV, C) P53 UPREGULATED MODULATOR OF APOPTOSIS (PUMA), D) APOPTOSIS REGULATOR BCL-2 LIKE PROTEIN 4 (BAX), E) BCL ASSOCIATED AGAINST CELL DEATH (BAD), AND F) BCL-2 ANTAGONIST KILLER 1 (BAK), G) CASPASE (CASP) 3, AND H) CASP 8. MANN-WHITNEY TEST, N=10 PER GROUP P<0,05**

**CHR-Exon IV is correlated negatively with endoplasmic reticulum stress and apoptotic markers at the colonic mucosa but is not significantly negatively correlated with Caspase 3 and 8 markers.**

Finally, we assessed the relationship between CHR Exon-IV and the various surfaces and functional markers studied in UC and control patients, a correlation analysis was performed. Exon-IV showed a moderate to strong negative correlation with XBP1 (r=-0,6436, P= 0.0022), PUMA (r=-0,6436, P= 0.0022), BAD (r=-0,7185, P=0.0004), BAX (r=-0,6917, P= 0.0007), BAK (r=-0,6857, P= 0.0008) **(Figure 18 A, B, C, D and E)**. Further analysis of other apoptotic markers demonstrated a nonsignificant weak negative correlation Casp8 (r=-0,3618, P=0.1690), Casp3 (r=-0,3706, P=0.1577) (**Figure 18 F and G)**.



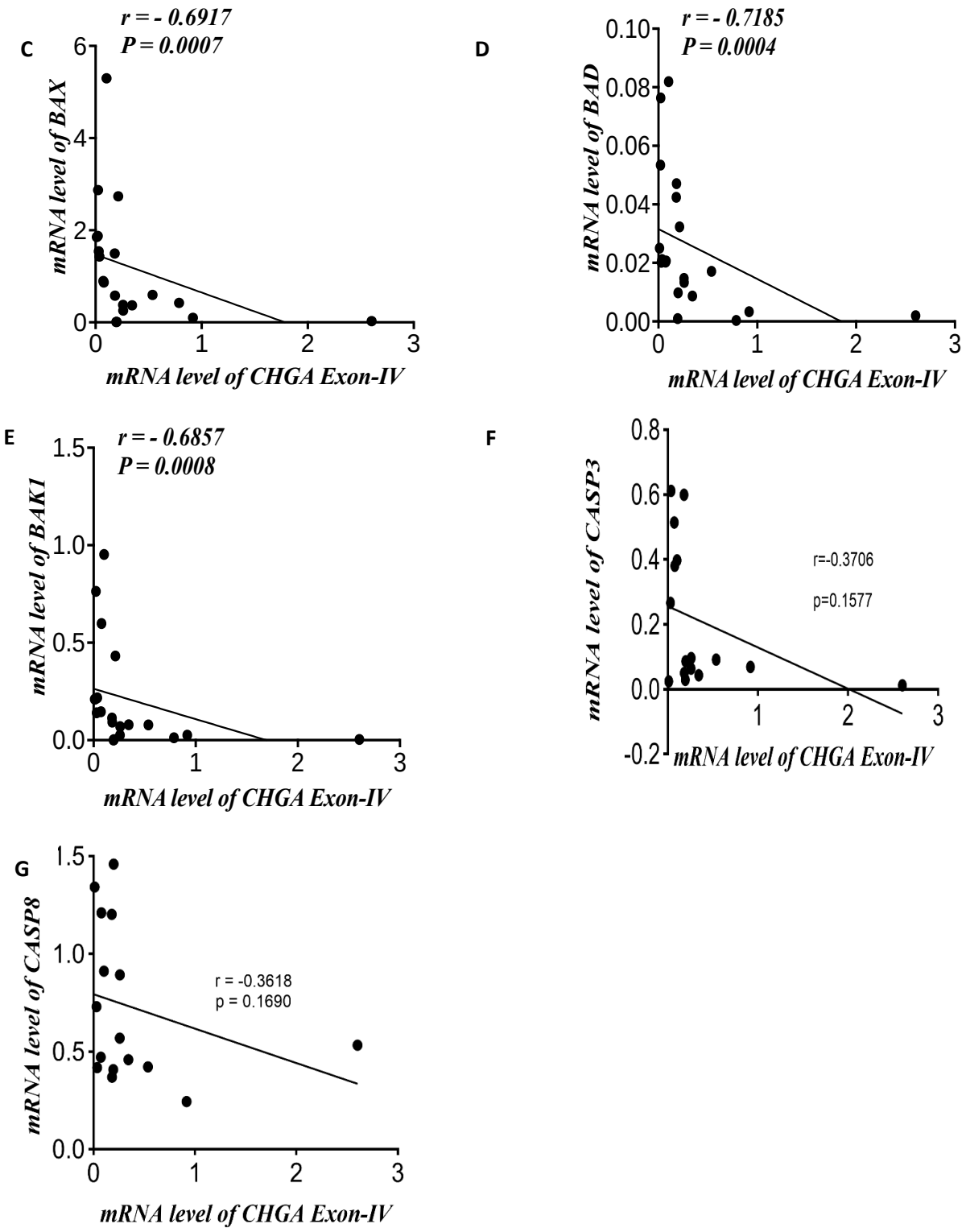



**FIGURE 18. CORRELATION ANALYSIS BETWEEN CHROMOGRANIN A (CHGA) EXON-IV GENE EXPRESSION AND ENDOPLASMIC RETICULUM (ER) STRESS AND APOPTOTIC MARKERS WITHIN THE COLONIC MUCOSA OF CONTROL**

PATIENTS AND PATIENTS WITH ACTIVE ULCERATIVE COLITIS. CHGA EXON-IV IS SIGNIFICANTLY NEGATIVELY CORRELATED WITH A) X-BINDING PROTEIN-1 (XBP1), B) P53 UPREGULATED MODULATOR OF APOPTOSIS (PUMA), C) APOPTOSIS REGULATOR BCL-2 LIKE PROTEIN 4 (BAX), D) BCL ASSOCIATED AGAINST CELL DEATH (BAD), AND E) BCL-2 ANTAGONIST KILLER 1 (BAK), F) CASPASE (CASP) 3, AND G) CASP 8. SPEARMAN CORRELATION FACTOR (R) & TWO TAILS SIGNIFICANCE (P) ADJUSTED AT 0.05., N=20.

# Chapter 5 | Discussion

IBD is an inflammatory condition in the gut divided into two different subclasses: UC and CD [169, 170]. IBD results from an unbalanced relationship within the environment, genes, and innate and adaptive immunity [175, 176]. The central management of IBD comprises pharmacological, nutritional, and surgical therapy [176]. The induction and maintenance of remission and prevention of complications are the primary goals of the treatment [177]. IBD generally results in a poor quality of life in affected patients and costs $2.6 billion/yr [5]. Currently, there is no cure for IBD, and the provided therapies are not entirely effective because of the long-term side effect of some of the treatments [3, 5]. Therefore, a lot of work is still needed to come up with a better treatment option.

Our laboratory recently demonstrated that CHR protects against colitis through modulation of the functions of MØs and intestinal epithelial cells via the NF-kB dependent pathway [137, p. 201]. Since MØs are implicated in the activation of multiple pathways, we decided to investigate the role of CHR in colonic inflammation and its potential modulating ER stress and apoptotic pathways in MØs. The study presented in this thesis shows possible mechanisms by which CHR ameliorates intestinal inflammation by regulating ER stress and apoptotic markers.

Confocal microscopy is a non-invasive tool that can be used to image cells isolated from the peritoneal cavity [178]. First, our results revealed that CHR enters the peritoneal cells MØs. This result is consistent with our prediction, and it confirms early data published by Lugardon et al. demonstrating that CHR can penetrate into lipid monolayers and fungal spores membranes at

specific concentrations [158]. The bright yellow colour surrounding the nuclei of the cell comes from the rhodamine, which is a fluorescent dye that usually works as a cell tracer [179]. Here the rhodamine was linked to CHR. We detected the yellow coloration of the rhodamine inside the MØs as early as five minutes. The brightness increases inside the MØs with a higher incubation time. Taken all together, it seems that the CHR enters the cell and resides in the intracellular compartment. As a control group, the mice which didn't receive any rhodamine-CHR treatment didn't depicted any yellow colour. One of the limitations of the study is the absence of one of the control groups where naïve MØs should have been treated with rhodamine alone. Due to the current COVID-19 situation it was not possible to perform them.

According to the literature, many events that happen at the cellular, molecular and tissue levels underlie the inflammation process seen during the development of IBD [152, 166]. It is suspected that CHR is one of the molecules produced during these events, and it has emerged to play a prominent role in the maintenance of the intestinal barrier function and the immune homeostasis [155, 156]. In our study using an in vitro culture system, we demonstrated that in M1 MØs treated with CHR induce a decrease expression of ER stress (XBP1) and intrinsic apoptotic markers such as PUMA, BAK, BAX, BAD in response to LPS stimulation were visible. These results confirm previous data demonstrating that when M1 MØs are activated many pro-inflammatory pathways are also activated, including ER stress and apoptotic pathways [13, 52]. Furthermore, we noticed a decrease in gene expression of Casp8, but we also showed that CHR does not seem to affect some of the extrinsic apoptotic markers such as Casp3, and Trail. As we found non-statistically significant results for each of them, p-value higher than 0.05, we concluded that CHR does not impact these markers.

To confirm the in vitro physiological effect of a CHR treatment, we decided to use an experimental animal model of colitis using DSS.

First, we detected the modification of expression of the CHR mRNA levels by quantifying the CHGA (Exon-IV) in our mice colon samples. To the best of our knowledge, there are no antibodies on the market that will quantify correctly CHR, therefore we used and indirect method. Herein, we show that CHGA (Exon-IV) is down-regulated when mice are treated with DSS. This demonstrated that the chemical DSS that mimics the disease or it subsequent effects on the host pathophysiology can modify the expression of the Exon-IV. Interestingly, the intra-rectal injection of CHR upregulated the expression of CHR. It is therefore possible that the level of CHR can autoregulate its own expression and more experiment are needed to study that mechanism of action.

Similarly, we observed high expression of XBP1 mRNA level in DSS treated mice. This result was expected as it has been reported in several publications that disruption of cellular homeostasis and evoke ER stress, leading to the development of IBD [104, 105]. CHR treatment decreased the ER stress (XBP1) marker within the colonic mucosa, it therefore suspected that CHR can regulate some of the pathways or protein implicated in the expression of XBP1.

Several studies have shown the implication of apoptosis with IBD, and most of these works have concluded that IBD patients have higher rates of apoptosis than healthy patients [124, 125]. The mechanism of apoptosis is complex and tightly regulated and involves an energy-dependent cascade of molecular events. We decided first to run an RT profile PCR Array to analyze the gene expression of 84 genes of the p53 apoptotic pathway. DSS treatment upregulated 26 genes

associated with the p53-dependent apoptosis for example Apaf1, Bax, Bbc3, Bcl2, Cradd, Fadd, Cul9, Pmaip1, Tnfrsf10b. The Bcl-2 gene family was highly activated in the colonic conditions. But administration of CHR cause the down regulation of 19 genes associated with the p53-downstream signalling pathways. This result is confirmed with the literature, the P53 gene is the caretaker of the genome, and it stimulates other proteins such as p53 upregulated modulatory of apoptosis (PUMA), Bcl-2 family proteins [122, 123]. This RT-PCR array data demonstrated the existence of modulation of the p53 apoptotic pathway after CHR treatment.

Next, using a q-RT-PCR we confirmed some of the results seen in our array. P53 apoptotic markers PUMA, Bcl-2 family (BAX, BAK, and BAK) had upregulated mRNA expression in colitic condition. CHR treatment decreased apoptotic markers within the colonic mucosa. We also demonstrated that CHR treatment did not modify TRAIL and Casp8 gene expression within the colonic mucosa on the gene level. These results are similar and confirm the conclusions from our in vitro study.

Besides that, we used a proteomics approach for protein quantification in colon collected from DSS treated mice. Under colitic condition we observed strong bands for each meaning strong protein expression of BAK, BAX, Casp3, XBP1 and Casp8. But more importantly our finding has revealed that mice CHR treatment decreased BAK, BAX, XBP1, Casp3, and Casp8 protein levels within the colonic mucosa. These results go along with the study that was done by Dr. Nour Eissa, where the deletion of Chromogranin-A reduce the disease severity and onset, pro-inflammatory mediators, M1, and p53/caspase-3 activation [152]. As we mentioned earlier Chromogranin-A is the precursor of multiple peptides including Chromofungin.

This result is yet surprising, as according to a molecular biology concept, which evokes that: DNA makes RNA makes proteins [180]. CHR had no effect Trail and Casp8 on the gene level, but on the protein level, CHR has a decreasing effect on these two. More study is needed to explain this phenomenon.

It is difficult to explain the exact mechanism of actions of CHR because of the lack of receptors for these peptides. But there are possibilities that the CHR is affecting the microbiota since it was reported in several papers that CHR can have antimicrobial function [152, 156, 158].

Lastly, we tried to translate the role of CHR to a human model. First, we confirmed the regulation of Exon-IV inactive UC patients [152] by demonstrating a significantly lower expression in IBD patients than healthy controls. This change was also associated with altered mRNA levels of ER stress and apoptotic markers. The literature supports our finding as active UC is characterized by increased intestinal epithelial cells apoptosis, which is associated with elevated pro-inflammatory (TNF-α, iNOS) and apoptotic mediators (CASPASE-3, P53, and PUMA) [181]. Moreover, immune activation is associated with iNOS stabilization of p53, followed by p53-mediated crypt cell apoptosis in IBD [181], and disruption of the epithelial barrier is linked to epithelial apoptosis that is mainly caused by immune cells [182]. We also found that CHGA Exon-IV gene expression is significantly negatively correlated with ER and apoptotic markers within the colonic mucosa of active UC and control patients. Correlation study of CHGA Exon-IV with Casp8 and Casp3 was not statistically significant. Some of these results can be explained by previous data that

demonstrated changes at the level of enterochromaffin cells during the inflammatory process [154, 174, 183]. Additionally, our data go in parallel with the remaining data in this study.

There are some limitations to our study. The patient selection included a strict inclusion and exclusion criteria, made human samples size too small, and additional biopsies needed to be added to have enough sample (n=20) to perform the correlation study. Moreover, one of our study criteria was limited to UC patients. It would have been necessary to demonstrate if we could have been reported the same result with CD patients and generalize the result for IBD. The same idea goes for our animal model; we used the DSS model because of its lower cost and ease of induction, which is focused on UC. It would have been interesting to see if we can extrapolate our same CD models. Moreover, a DSS model has some drawbacks. There is an inconsistent water uptake by mice and thus irregular exposure to DSS, causing a variation in the degree and distribution of mucosal injury and ulceration on the colon [184]. In our mice model, we used preventive and prophylactic treatment. Therefore, further experiments are needed to determine the efficacy of CHR in a healing model [185]. We cannot rule out the possibility that microbiota dysbiosis and permeability might lead to the changes seen post-treatment. Since there are shreds of evidence demonstrating the link between the gut microbiota and human IBD [186, 187] that CHR can have microbial function [156, 158]. They are chances that the intra-rectal injection of CHR could cause microbiota dysbiosis in favor of beneficial bacteria. But further studies are still required to investigate the role of CHR on the microbiota.

# Chapter 6 | Conclusions

We were able to confirm that the level of CHGA (*Exon-IV*) is significantly downregulated in the colonic samples from active UC patients compared to healthy patients showing a potential relationship between CHGA (Exon-IV) and UC. Our experiments have shown a negative correlation of CHR-Exon IV with endoplasmic reticulum stress and apoptotic markers at the colonic mucosa of control patients and patients with UC. Most importantly, we were able to report a CHR's protective effect during the development of colonic inflammation. Intra-rectal injection of CHR administration decreased the endoplasmic reticulum stress and some apoptotic markers in LPS-stimulated MØs. Within colonic mucosa of mice and patients with active UC as observed by RT-PCR and western blot. $RT^2$-PCR array showed the implication of the p-53 apoptotic pathway in the inflammation.

# Chapter 7 | Significance

My goal for this study was to have clinical relevance results, and a better idea of the pathophysiology of IBD and possibly develop a novel therapeutic target. This work is one of the first to show a relationship between CHGA Exon and apoptosis and ER stress marker using three approaches. CHR decreases the severity of colitis and the inflammatory process by suppressing mucosal and MØs-related ER stress/p53-dependent apoptosis. Our preliminary observations suggest that in the future, CHR be a potential new drug to reduce the inflammatory effect of M1 MØs and keep IBD on remission. Bunch of immunosuppressive medications is available on the market, but they still have a lot of have side effects. Therefore, better and safer treatment is needed to treat IBD. CHR might be a potential drug for obtaining more reliable therapeutic strategies in IBD treatment.

# Chapter 8 | Future Directions

In our project, we studied the role of CHR using an acute model of colitis; however, as discussed in the introduction, IBD is a chronic disease associated with active and remission phases. It is primordial to study the healing context of the CHR, for that the peptide will be given at the end of the acute period to measure the healing capacity of CHR at the mucosa by targeting factors like KLF5 which is DNA binding transcription factor that regulates several cellular processes such cell proliferation and differentiation [187]. Another way to look at the healing process will be to use a chronic model of DSS where 5% DSS is given to mice in their drinking water for seven days, followed by two to five weeks of consumption of normal water. We will introduce the treatment during remission and monitor for the speed of colitis setting up.

Furthermore, the antimicrobial functions described in multiple studies, but its microbial implication is still unknown. Dysbiosis is a significant factor that can lead to IBD. It will be interesting to check the relationship of CHR with microbiota and verify its effects on the good and bacteria in the gut. We can characterize microbial communities in the feces from the patient and mouse model by RT-PCR and determine the impact of CHR on the fecal microbiota.

So far, there is no data available for CHR kinetic; for example, half of this peptide is unknown. It will be useful to do pharmacokinetics to define the half-life, the optimal dose to have better efficacy and decrease toxicity or side effects if they exist.

Lastly, up to date, there are no data about the interaction between smooth muscle mechanic activity, permeability, and CHR. CHGA and its derived peptides have also been characterized to be interacting with calcium [135]. An altered colonic smooth muscle contraction due to a modification of calcium influx can

induce diarrhea. Therefore, taking these two observations together, it will be interesting to study the relation between CHR, calcium influx, and smooth muscle contraction. This study can be done by using the organ bath or the Ussing Chamber technic. The Ussing chamber allows measuring the transport of Ions, drugs, and other molecules across the various epithelial membrane, and the organ bath unit could detect the effect on different types of neurons present within the enteric nervous system. Over the last years, only a few data have reported the impact of CHR on colonic smooth muscle contraction [172, 188].

Our study focused only on one branch of endoplasmic reticulum stress (IRE1-alpha) by studying XBP1. As described early on in the literature review, ER stress can be divided into three categories IRE1, PERK, and ATF6-alpha. It will be interesting to explore the two other branches with more specific markers.

# Chapter 9 | References


[1] "Inflammatory bowel disease- ClinicalKey." https://www-clinicalkey-com.uml.idm.oclc.org/#!/content/playContent/1-s2.0-S016524781400073X?returnurl=null&referrer=null (accessed Apr. 15, 2020).

[2] B. R. R. de Mattos *et al.*, "Inflammatory Bowel Disease: An Overview of Immune Mechanisms and Biological Treatments," *Mediators Inflamm.*, vol. 2015, 2015, doi: 10.1155/2015/493012.

[3] "ccfc-ibd-impact-report-2012.pdf." Accessed: Apr. 15, 2020. [Online]. Available: http://www.crohnsandcolitis.ca/Crohns_and_Colitis/documents/reports/ccfc-ibd-impact-report-2012.pdf.

[4] G. G. Kaplan, "The global burden of IBD: from 2015 to 2025," *Nat. Rev. Gastroenterol. Hepatol.*, vol. 12, no. 12, pp. 720-, Dec. 2015.

[5] G. Kaplan *et al.*, "This Report was prepared by:," p. 232, 2018.

[6] D. Farrell, M. Artom, W. Czuber-Dochan, L. P. Jelsness-Jørgensen, C. Norton, and E. Savage, "Interventions for fatigue in inflammatory bowel disease," *Cochrane Database Syst. Rev.*, no. 4, 2020, doi: 10.1002/14651858.CD012005.pub2.

[7] "Inflammatory Bowel Disease." Accessed: Apr. 17, 2020. [Online]. Available: http://web.a.ebscohost.com.uml.idm.oclc.org/ehost/ebookviewer/ebook/ZTAwMHhuYV9fNDY3NjAzX19BTg2?sid=aa534f0f-bebe-4c3f-8fb5-67296438fa38@sdc-v-sessmgr02&vid=0&format=EB&lpid=lp_1&rid=0.

[8] “What are Crohn’s and Colitis? - What are Crohn’s and Colitis - Crohn’s and Colitis Canada.” https://crohnsandcolitis.ca/About-Crohn-s-Colitis/What-are-Crohns-and-Colitis (accessed Apr. 17, 2020).

[9] “A comprehensive review and update on ulcerative colitis , - ClinicalKey.” Accessed: Apr. 19, 2020. [Online]. Available: https://www-clinicalkey-com.uml.idm.oclc.org/#!/content/playContent/1-s2.0-S0011502919300318?returnurl=null&referrer=null.

[10] “A comprehensive review and update on ulcerative colitis , - ClinicalKey.” https://www-clinicalkey-com.uml.idm.oclc.org/#!/content/playContent/1-s2.0-S0011502919300318?returnurl=null&referrer=null (accessed Apr. 19, 2020).

[11] “Inflammatory Bowel Disease.” http://web.a.ebscohost.com.uml.idm.oclc.org/ehost/ebookviewer/ebook/ZTAwMHhuYV9fNDY3NjAzX19BTg2?sid=aa534f0f-bebe-4c3f-8fb5-67296438fa38@sdc-v-sessmgr02&vid=0&format=EB&lpid=lp_1&rid=0 (accessed Apr. 17, 2020).

[12] “33571_2901304_CMO_Chapter_8.pdf.” Accessed: Apr. 19, 2020. [Online]. Available: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/252658/33571_2901304_CMO_Chapter_8.pdf.

[13] Q. Guan, “A Comprehensive Review and Update on the Pathogenesis of Inflammatory Bowel Disease,” *Journal of Immunology Research*, 2019. https://www.hindawi.com/journals/jir/2019/7247238/ (accessed Apr. 19, 2020).

[14] J. M. Kim and J. H. Cheon, “Pathogenesis and clinical perspectives of extraintestinal manifestations in inflammatory bowel diseases,” *Intest. Res.*, Apr. 2020, doi: 10.5217/ir.2019.00128.

[15] “A comprehensive review and update on Crohn’s disease- ClinicalKey.” https://www-clinicalkey-com.uml.idm.oclc.org/#!/content/playContent/1-s2.0-S0011502917301530?returnurl=null&referrer=null (accessed Apr. 19, 2020).

[16] S. Alatab *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,” *Lancet Gastroenterol. Hepatol.*, vol. 5, no. 1, pp. 17–30, Jan. 2020, doi: 10.1016/S2468-1253(19)30333-4.

[17] I. Loddo and C. Romano, “Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis,” *Front. Immunol.*, vol. 6, Nov. 2015, doi: 10.3389/fimmu.2015.00551.

[18] “IBD risk loci are enriched in multigenic regulatory modules encompassing putative causative genes | Nature Communications.” https://www.nature.com/articles/s41467-018-04365-8 (accessed May 20, 2020).

[19] A. Kaser and B. Pasaniuc, “IBD Genetics: Focus on (Dys) Regulation in Immune Cells and the Epithelium,” *Gastroenterology*, vol. 146, no. 4, pp. 896–899, Apr. 2014, doi: 10.1053/j.gastro.2014.02.023.

[20] R. Cooney *et al.*, “NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation,” *Nat. Med.*, vol. 16, no. 1, pp. 90–97, Jan. 2010, doi: 10.1038/nm.2069.

[21] S. Lavoie *et al.*, “The Crohn’s disease polymorphism, ATG16L1 T300A, alters the gut microbiota and enhances the local Th1/Th17 response,” *eLife*, vol. 8, doi: 10.7554/eLife.39982.

[22] M. D. Jensen, R. F. Andersen, H. Christensen, T. Nathan, J. Kjeldsen, and J. S. Madsen, “Circulating microRNAs as biomarkers of adult Crohn’s disease,” *Eur. J. Gastroenterol. Hepatol.*, vol. 27, no. 9, pp. 1038–1044, Sep. 2015, doi: 10.1097/MEG.0000000000000430.

[23] B. Verstockt, K. G. Smith, and J. C. Lee, “Genome-wide association studies in Crohn’s disease: Past, present and future,” *Clin. Transl. Immunol.*, vol. 7, no. 1, Jan. 2018, doi: 10.1002/cti2.1001.

[24] A. T. Abegunde, B. H. Muhammad, O. Bhatti, and T. Ali, “Environmental risk factors for inflammatory bowel diseases: Evidence based literature review,” *World J. Gastroenterol.*, vol. 22, no. 27, pp. 6296–6317, Jul. 2016, doi: 10.3748/wjg.v22.i27.6296.

[25] D. P. Strachan, “Hay fever, hygiene, and household size,” *BMJ*, vol. 299, no. 6710, pp. 1259–1260, Nov. 1989, doi: 10.1136/bmj.299.6710.1259.

[26] J. K. Hou, B. Abraham, and H. El-Serag, “Dietary intake and risk of developing inflammatory bowel disease: a systematic review of the literature,” *Am. J. Gastroenterol.*, vol. 106, no. 4, pp. 563–573, Apr. 2011, doi: 10.1038/ajg.2011.44.

[27] D. S *et al.*, “Dietary-fat-induced Taurocholic Acid Promotes Pathobiont Expansion and Colitis in Il10-/- Mice,” *Nature*, Jul. 05, 2012. https://pubmed.ncbi.nlm.nih.gov/22722865/ (accessed May 19, 2020).

[28] J. Gj, C. J, and M. Jc, “Review Article: Smoking Cessation as Primary Therapy to Modify the Course of Crohn’s Disease,” *Alimentary pharmacology & therapeutics*, Apr. 15, 2005. https://pubmed.ncbi.nlm.nih.gov/15813828/ (accessed May 19, 2020).

[29] S. Y. Salim, G. G. Kaplan, and K. L. Madsen, “Air pollution effects on the gut microbiota,” *Gut Microbes*, vol. 5, no. 2, pp. 215–219, Mar. 2014, doi: 10.4161/gmic.27251.

[30] S. H. Lee, J. eun Kwon, and M.-L. Cho, “Immunological pathogenesis of inflammatory bowel disease,” *Intest. Res.*, vol. 16, no. 1, pp. 26–42, Jan. 2018, doi: 10.5217/ir.2018.16.1.26.

[31] M. G. Neuman, “Immune dysfunction in inflammatory bowel disease,” *Transl. Res.*, vol. 149, no. 4, pp. 173–186, Apr. 2007, doi: 10.1016/j.trsl.2006.11.009.

[32] M. E. McAlindon, C. J. Hawkey, and Y. R. Mahida, “Expression of interleukin 1 beta and interleukin 1 beta converting enzyme by intestinal macrophages in health and inflammatory bowel disease,” *Gut*, vol. 42, no. 2, pp. 214–219, Feb. 1998, doi: 10.1136/gut.42.2.214.

[33] C. A. Dinarello, “IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family,” *J. Allergy Clin. Immunol.*, vol. 103, no. 1 Pt 1, pp. 11–24, Jan. 1999, doi: 10.1016/s0091-6749(99)70518-x.

[34] S. H. Murch, C. P. Braegger, J. A. Walker-Smith, and T. T. MacDonald, “Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease,” *Gut*, vol. 34, no. 12, pp. 1705–1709, Dec. 1993, doi: 10.1136/gut.34.12.1705.

[35] S. Schreiber, T. Heinig, H. G. Thiele, and A. Raedler, “Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease,” *Gastroenterology*, vol. 108, no. 5, pp. 1434–1444, May 1995, doi: 10.1016/0016-5085(95)90692-4.

[36] O. H. Nielsen, T. Køppen, N. Rüdiger, T. Horn, J. Eriksen, and I. Kirman, "Involvement of interleukin-4 and -10 in inflammatory bowel disease," *Dig. Dis. Sci.*, vol. 41, no. 9, pp. 1786–1793, Sep. 1996, doi: 10.1007/BF02088746.

[37] W. O'Connor *et al.*, "A protective function for interleukin 17A in T cell-mediated intestinal inflammation," *Nat. Immunol.*, vol. 10, no. 6, pp. 603–609, Jun. 2009, doi: 10.1038/ni.1736.

[38] W. Strober, F. Zhang, A. Kitani, I. Fuss, and S. Fichtner-Feigl, "Pro-Inflammatory Cytokines Underlying the Inflammation of Crohn's Disease," *Curr. Opin. Gastroenterol.*, vol. 26, no. 4, pp. 310–317, Jul. 2010, doi: 10.1097/MOG.0b013e328339d099.

[39] M. C. Grimm and W. F. Doe, "Chemokines in Inflammatory Bowel Disease Mucosa: Expression of RANTES, Macrophage Inflammatory Protein (MIP)-1α, MIP-1β, and γ-Interferon-Inducible Protein-10 by Macrophages, Lymphocytes, Endothelial Cells, and Granulomas," *Inflamm. Bowel Dis.*, vol. 2, no. 2, pp. 88–96, 1996.

[40] M. Uguccioni *et al.*, "Increased expression of IP-10, IL-8, MCP-1, and MCP-3 in ulcerative colitis," *Am. J. Pathol.*, vol. 155, no. 2, pp. 331–336, Aug. 1999, doi: 10.1016/S0002-9440(10)65128-0.

[41] M. Rescigno and A. Di Sabatino, "Dendritic cells in intestinal homeostasis and disease," *J. Clin. Invest.*, vol. 119, no. 9, pp. 2441–2450, Sep. 2009, doi: 10.1172/JCI39134.

[42] A. Sakuraba, T. Sato, N. Kamada, M. Kitazume, A. Sugita, and T. Hibi, "Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease," *Gastroenterology*, vol. 137, no. 5, pp. 1736–1745, Nov. 2009, doi: 10.1053/j.gastro.2009.07.049.

[43] E. C. Steinbach and S. E. Plevy, “The role of macrophages and dendritic cells in the initiation of inflammation in IBD,” *Inflamm. Bowel Dis.*, vol. 20, no. 1, pp. 166–175, Jan. 2014, doi: 10.1097/MIB.0b013e3182a69dca.

[44] “Exploring the full spectrum of macrophage activation | Nature Reviews Immunology.” https://www.nature.com/articles/nri2448 (accessed Jun. 15, 2020).

[45] E. Bettelli *et al.*, “Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells,” *Nature*, vol. 441, no. 7090, pp. 235–238, May 2006, doi: 10.1038/nature04753.

[46] A. Sica and A. Mantovani, “Macrophage plasticity and polarization: in vivo veritas,” *J. Clin. Invest.*, vol. 122, no. 3, pp. 787–795, Mar. 2012, doi: 10.1172/JCI59643.

[47] J. L. Bishop, L. M. Sly, G. Krystal, and B. B. Finlay, “The inositol phosphatase SHIP controls Salmonella enterica serovar Typhimurium infection in vivo,” *Infect. Immun.*, vol. 76, no. 7, pp. 2913–2922, Jul. 2008, doi: 10.1128/IAI.01596-07.

[48] D. Fairweather and D. Cihakova, “Alternatively activated macrophages in infection and autoimmunity,” *J. Autoimmun.*, vol. 33, no. 3–4, pp. 222–230, Dec. 2009, doi: 10.1016/j.jaut.2009.09.012.

[49] S. Spence *et al.*, “Suppressors of cytokine signaling 2 and 3 diametrically control macrophage polarization,” *Immunity*, vol. 38, no. 1, pp. 66–78, Jan. 2013, doi: 10.1016/j.immuni.2012.09.013.

[50] S. Danese, “Immune and nonimmune components orchestrate the pathogenesis of inflammatory bowel disease,” *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 300, no. 5, pp. G716-722, May 2011, doi: 10.1152/ajpgi.00472.2010.

[51] N. Kamada *et al.*, “Unique CD14+ intestinal macrophages contribute to the pathogenesis of Crohn disease via IL-23/IFN-γ axis,” *J. Clin. Invest.*, vol. 118, no. 6, pp. 2269–2280, Jun. 2008, doi: 10.1172/JCI34610.

[52] E. Meroni, N. Stakenborg, M. F. Viola, and G. E. Boeckxstaens, “Intestinal macrophages and their interaction with the enteric nervous system in health and inflammatory bowel disease,” *Acta Physiol. Oxf. Engl.*, vol. 225, no. 3, p. e13163, 2019, doi: 10.1111/apha.13163.

[53] G. Monteleone, D. Fina, R. Caruso, and F. Pallone, “New mediators of immunity and inflammation in inflammatory bowel disease,” *Curr. Opin. Gastroenterol.*, vol. 22, no. 4, pp. 361–364, Jul. 2006, doi: 10.1097/01.mog.0000231808.10773.8e.

[54] “Crohn’s Disease: Th1, Th17 or Both? The Change of a Paradigm: New Immunological and Genetic Insights Implicate Th17 Cells in the Pathogenesis of Crohn’s Disease - PubMed.” https://pubmed.ncbi.nlm.nih.gov/19592695/ (accessed May 22, 2020).

[55] L. Zhou *et al.*, “IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways,” *Nat. Immunol.*, vol. 8, no. 9, pp. 967–974, Sep. 2007, doi: 10.1038/ni1488.

[56] J. E. Allen and T. E. Sutherland, “Host protective roles of type 2 immunity: Parasite killing and tissue repair, flip sides of the same coin,” *Semin. Immunol.*, vol. 26, no. 4, pp. 329–340, Aug. 2014, doi: 10.1016/j.smim.2014.06.003.

[57] F. Annunziato, C. Romagnani, and S. Romagnani, “The 3 major types of innate and adaptive cell-mediated effector immunity,” *J. Allergy Clin. Immunol.*, vol. 135, no. 3, pp. 626–635, Mar. 2015, doi: 10.1016/j.jaci.2014.11.001.

[58] W. C. Gause, T. A. Wynn, and J. E. Allen, “Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths,” *Nat. Rev. Immunol.*, vol. 13, no. 8, pp. 607–614, Aug. 2013, doi: 10.1038/nri3476.

[59] G. Bamias and F. Cominelli, “Role of Th2 immunity in intestinal inflammation,” *Curr. Opin. Gastroenterol.*, vol. 31, no. 6, pp. 471–476, Nov. 2015, doi: 10.1097/MOG.0000000000000212.

[60] L. R. Lopetuso, F. Scaldaferri, and T. T. Pizarro, “Emerging role of the interleukin (IL)-33/ST2 axis in gut mucosal wound healing and fibrosis,” *Fibrogenesis Tissue Repair*, vol. 5, no. 1, p. 18, Oct. 2012, doi: 10.1186/1755-1536-5-18.

[61] J. Bessa *et al.*, “Altered subcellular localization of IL-33 leads to non-resolving lethal inflammation,” *J. Autoimmun.*, vol. 55, pp. 33–41, Dec. 2014, doi: 10.1016/j.jaut.2014.02.012.

[62] N. Eastaff-Leung, N. Mabarrack, A. Barbour, A. Cummins, and S. Barry, “Foxp3+ regulatory T cells, Th17 effector cells, and cytokine environment in inflammatory bowel disease,” *J. Clin. Immunol.*, vol. 30, no. 1, pp. 80–89, Jan. 2010, doi: 10.1007/s10875-009-9345-1.

[63] G. Hardenberg, T. S. Steiner, and M. K. Levings, “Environmental influences on T regulatory cells in inflammatory bowel disease,” *Semin. Immunol.*, vol. 23, no. 2, pp. 130–138, Apr. 2011, doi: 10.1016/j.smim.2011.01.012.

[64] J. Maul *et al.*, “Peripheral and intestinal regulatory CD4+ CD25(high) T cells in inflammatory bowel disease,” *Gastroenterology*, vol. 128, no. 7, pp. 1868–1878, Jun. 2005, doi: 10.1053/j.gastro.2005.03.043.

[65] H. Tlaskalová-Hogenová *et al.*, "The role of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of inflammatory and autoimmune diseases and cancer: contribution of germ-free and gnotobiotic animal models of human diseases," *Cell. Mol. Immunol.*, vol. 8, no. 2, Art. no. 2, Mar. 2011, doi: 10.1038/cmi.2010.67.

[66] T. C. Fung, D. Artis, and G. F. Sonnenberg, "Anatomical localization of commensal bacteria in immune cell homeostasis and disease," *Immunol. Rev.*, vol. 260, no. 1, pp. 35–49, Jul. 2014, doi: 10.1111/imr.12186.

[67] A. K. DeGruttola, D. Low, A. Mizoguchi, and E. Mizoguchi, "Current understanding of dysbiosis in disease in human and animal models," *Inflamm. Bowel Dis.*, vol. 22, no. 5, pp. 1137–1150, May 2016, doi: 10.1097/MIB.0000000000000750.

[68] T. H. Mogensen, "Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses," *Clin. Microbiol. Rev.*, vol. 22, no. 2, pp. 240–273, Apr. 2009, doi: 10.1128/CMR.00046-08.

[69] N. Srinivasan, "Telling apart friend from foe: discriminating between commensals and pathogens at mucosal sites," *Innate Immun.*, vol. 16, no. 6, pp. 391–404, Dec. 2010, doi: 10.1177/1753425909357577.

[70] J. G. Ruseler-van Embden and H. C. Both-Patoir, "Anaerobic gram-negative faecal flora in patients with Crohn's disease and healthy subjects," *Antonie Van Leeuwenhoek*, vol. 49, no. 2, pp. 125–132, Jun. 1983, doi: 10.1007/BF00393670.

[71] H. Matsuda, Y. Fujiyama, A. Andoh, T. Ushijima, T. Kajinami, and T. Bamba, "Characterization of antibody responses against rectal mucosa-associated bacterial flora in

patients with ulcerative colitis," *J. Gastroenterol. Hepatol.*, vol. 15, no. 1, pp. 61–68, Jan. 2000, doi: 10.1046/j.1440-1746.2000.02045.x.

[72] C. P. Tamboli, C. Neut, P. Desreumaux, and J. F. Colombel, "Dysbiosis in inflammatory bowel disease," *Gut*, vol. 53, no. 1, pp. 1–4, Jan. 2004.

[73] C. Schultsz, F. M. Van Den Berg, F. W. Ten Kate, G. N. Tytgat, and J. Dankert, "The intestinal mucus layer from patients with inflammatory bowel disease harbors high numbers of bacteria compared with controls," *Gastroenterology*, vol. 117, no. 5, pp. 1089–1097, Nov. 1999, doi: 10.1016/s0016-5085(99)70393-8.

[74] A. Swidsinski *et al.*, "Mucosal flora in inflammatory bowel disease," *Gastroenterology*, vol. 122, no. 1, pp. 44–54, Jan. 2002, doi: 10.1053/gast.2002.30294.

[75] R. B. Sartor, "Pathogenesis and immune mechanisms of chronic inflammatory bowel diseases," *Am. J. Gastroenterol.*, vol. 92, no. 12 Suppl, pp. 5S-11S, Dec. 1997.

[76] A. N. Theofilopoulos, D. H. Kono, and R. Baccala, "The Multiple Pathways to Autoimmunity," *Nat. Immunol.*, vol. 18, no. 7, pp. 716–724, Jun. 2017, doi: 10.1038/ni.3731.

[77] C. D. Packey and R. B. Sartor, "Commensal Bacteria, Traditional and Opportunistic Pathogens, Dysbiosis and Bacterial Killing in Inflammatory Bowel Diseases," *Curr. Opin. Infect. Dis.*, vol. 22, no. 3, pp. 292–301, Jun. 2009, doi: 10.1097/QCO.0b013e32832a8a5d.

[78] "Inflammatory bowel disease: Causes, symptoms, and treatments." https://www.medicalnewstoday.com/articles/316395 (accessed May 28, 2020).

[79] E. I. Benchimol *et al.*, "Validation of international algorithms to identify adults with inflammatory bowel disease in health administrative data from Ontario, Canada," *J. Clin. Epidemiol.*, vol. 67, no. 8, pp. 887–896, Aug. 2014, doi: 10.1016/j.jclinepi.2014.02.019.

[80] A. Aujnarain, D. R. Mack, and E. I. Benchimol, “The role of the environment in the development of pediatric inflammatory bowel disease,” *Curr. Gastroenterol. Rep.*, vol. 15, no. 6, p. 326, Jun. 2013, doi: 10.1007/s11894-013-0326-4.

[81] L. Jean, M. Audrey, C. Beauchemin, and on behalf of the iGenoMed Consortium, “Economic Evaluations of Treatments for Inflammatory Bowel Diseases: A Literature Review,” *Can. J. Gastroenterol. Hepatol.*, vol. 2018, Jun. 2018, doi: 10.1155/2018/7439730.

[82] J. M. Baena-Díez *et al.*, “Association between chronic immune-mediated inflammatory diseases and cardiovascular risk,” *Heart Br. Card. Soc.*, vol. 104, no. 2, pp. 119–126, 2018, doi: 10.1136/heartjnl-2017-311279.

[83] J. Torres, M. Cravo, and J.-F. Colombel, “Anti-TNF Withdrawal in Inflammatory Bowel Disease,” *GE - Port. J. Gastroenterol.*, vol. 23, no. 3, pp. 153–161, May 2016, doi: 10.1016/j.jpge.2015.11.004.

[84] H. N. Iskandar, T. Dhere, and F. A. Farraye, “Ulcerative Colitis: Update on Medical Management,” *Curr. Gastroenterol. Rep.*, vol. 17, no. 11, p. 44, Nov. 2015, doi: 10.1007/s11894-015-0466-9.

[85] M. L. Scribano, “Vedolizumab for inflammatory bowel disease: From randomized controlled trials to real-life evidence,” *World J. Gastroenterol.*, vol. 24, no. 23, pp. 2457–2467, Jun. 2018, doi: 10.3748/wjg.v24.i23.2457.

[86] J. J. Hansen and R. B. Sartor, “Therapeutic Manipulation of the Microbiome in IBD: Current Results and Future Approaches,” *Curr. Treat. Options Gastroenterol.*, vol. 13, no. 1, pp. 105–120, Mar. 2015, doi: 10.1007/s11938-014-0042-7.

[87] J. D. Bennet and M. Brinkman, “Treatment of ulcerative colitis by implantation of normal colonic flora,” *Lancet Lond. Engl.*, vol. 1, no. 8630, p. 164, Jan. 1989, doi: 10.1016/s0140-6736(89)91183-5.

[88] E. van Nood *et al.*, “Duodenal infusion of donor feces for recurrent Clostridium difficile,” *N. Engl. J. Med.*, vol. 368, no. 5, pp. 407–415, Jan. 2013, doi: 10.1056/NEJMoa1205037.

[89] “Fecal Microbiota Transplantation Through Mid-Gut for Refractory Crohn’s Disease: Safety, Feasibility, and Efficacy Trial Results - PubMed.” https://pubmed.ncbi.nlm.nih.gov/25168749/ (accessed May 31, 2020).

[90] “Tu2033 Investigating the microbiome pre and post fecal microbiota therapy from active ulcerative colitis patients in a randomized placebo controlled trial - Gastroenterology.” https://www.gastrojournal.org/article/S0016-5085(14)63280-7/fulltext (accessed May 30, 2020).

[91] W. Selby *et al.*, “Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn’s disease,” *Gastroenterology*, vol. 132, no. 7, pp. 2313–2319, Jun. 2007, doi: 10.1053/j.gastro.2007.03.031.

[92] E. Miele, F. Pascarella, E. Giannetti, L. Quaglietta, R. N. Baldassano, and A. Staiano, “Effect of a probiotic preparation (VSL#3) on induction and maintenance of remission in children with ulcerative colitis,” *Am. J. Gastroenterol.*, vol. 104, no. 2, pp. 437–443, Feb. 2009, doi: 10.1038/ajg.2008.118.

[93] “The Probiotic Preparation, VSL#3 Induces Remission in Patients With Mild-To-Moderately Active Ulcerative Colitis - PubMed.” https://pubmed.ncbi.nlm.nih.gov/19631292/ (accessed May 31, 2020).

[94] J. A. Jiminez, T. C. Uwiera, G. Douglas Inglis, and R. R. E. Uwiera, “Animal models to study acute and chronic intestinal inflammation in mammals,” *Gut Pathog.*, vol. 7, no. 1, p. 29, Nov. 2015, doi: 10.1186/s13099-015-0076-y.

[95] M. E. Coors, J. J. Glover, E. T. Juengst, and J. M. Sikela, “The Ethics of Using Transgenic Non-Human Primates to Study What Makes Us Human,” *Nat. Rev. Genet.*, vol. 11, no. 9, pp. 658–662, Sep. 2010, doi: 10.1038/nrg2864.

[96] A. Kathrani *et al.*, “Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease,” *Vet. Immunol. Immunopathol.*, vol. 161, no. 1, pp. 32–41, Sep. 2014, doi: 10.1016/j.vetimm.2014.06.003.

[97] S. Wirtz, C. Neufert, B. Weigmann, and M. F. Neurath, “Chemically induced mouse models of intestinal inflammation,” *Nat. Protoc.*, vol. 2, no. 3, pp. 541–546, 2007, doi: 10.1038/nprot.2007.41.

[98] M. Kawada, A. Arihiro, and E. Mizoguchi, “Insights from advances in research of chemically induced experimental models of human inflammatory bowel disease,” *World J. Gastroenterol.*, vol. 13, no. 42, pp. 5581–5593, Nov. 2007, doi: 10.3748/wjg.v13.i42.5581.

[99] Y. Yan *et al.*, “Temporal and spatial analysis of clinical and molecular parameters in dextran sodium sulfate induced colitis,” *PloS One*, vol. 4, no. 6, p. e6073, Jun. 2009, doi: 10.1371/journal.pone.0006073.

[100] A. Mizoguchi, “Animal models of inflammatory bowel disease,” *Prog. Mol. Biol. Transl. Sci.*, vol. 105, pp. 263–320, 2012, doi: 10.1016/B978-0-12-394596-9.00009-3.

[101] L. R. Fitzpatrick, K. Meirelles, J. S. Small, F. J. Puleo, W. A. Koltun, and R. N. Cooney, “A new model of chronic hapten-induced colitis in young rats,” *J. Pediatr. Gastroenterol. Nutr.*, vol. 50, no. 3, pp. 240–250, Mar. 2010, doi: 10.1097/MPG.0b013e3181cb8f4a.

[102] C. O. Elson, R. B. Sartor, G. S. Tennyson, and R. H. Riddell, “Experimental models of inflammatory bowel disease,” *Gastroenterology*, vol. 109, no. 4, pp. 1344–1367, Oct. 1995, doi: 10.1016/0016-5085(95)90599-5.

[103] S. S. CAO *et al.*, “The Unfolded Protein Response and Chemical Chaperones Reduce Protein Misfolding and Colitis in Mice,” *Gastroenterology*, vol. 144, no. 5, pp. 989-1000.e6, May 2013, doi: 10.1053/j.gastro.2013.01.023.

[104] K. Luo and S. S. Cao, “frtiz,” *Gastroenterol. Res. Pract.*, vol. 2015, p. 328791, 2015, doi: 10.1155/2015/328791.

[105] P. Walter and D. Ron, “The unfolded protein response: from stress pathway to homeostatic regulation,” *Science*, vol. 334, no. 6059, pp. 1081–1086, Nov. 2011, doi: 10.1126/science.1209038.

[106] J. S. Cox, C. E. Shamu, and P. Walter, “Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase,” *Cell*, vol. 73, no. 6, pp. 1197–1206, Jun. 1993, doi: 10.1016/0092-8674(93)90648-a.

[107] A. Bertolotti, Y. Zhang, L. M. Hendershot, H. P. Harding, and D. Ron, “Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response,” *Nat. Cell Biol.*, vol. 2, no. 6, pp. 326–332, Jun. 2000, doi: 10.1038/35014014.

[108] X. Ma *et al.*, "Intestinal Epithelial Cell Endoplasmic Reticulum Stress and Inflammatory Bowel Disease Pathogenesis: An Update Review," *Front. Immunol.*, vol. 8, Oct. 2017, doi: 10.3389/fimmu.2017.01271.

[109] M. Calfon *et al.*, "IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA," *Nature*, vol. 415, no. 6867, pp. 92–96, Jan. 2002, doi: 10.1038/415092a.

[110] A. Bertolotti *et al.*, "Increased sensitivity to dextran sodium sulfate colitis in IRE1beta-deficient mice," *J. Clin. Invest.*, vol. 107, no. 5, pp. 585–593, Mar. 2001, doi: 10.1172/JCI11476.

[111] H.-S. Zhang *et al.*, "The Endoplasmic Reticulum Stress Sensor IRE1α in Intestinal Epithelial Cells Is Essential for Protecting against Colitis," *J. Biol. Chem.*, vol. 290, no. 24, pp. 15327–15336, Jun. 2015, doi: 10.1074/jbc.M114.633560.

[112] A. Kaser *et al.*, "XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease," *Cell*, vol. 134, no. 5, pp. 743–756, Sep. 2008, doi: 10.1016/j.cell.2008.07.021.

[113] X. Xue *et al.*, "Tumor necrosis factor alpha (TNFalpha) induces the unfolded protein response (UPR) in a reactive oxygen species (ROS)-dependent fashion, and the UPR counteracts ROS accumulation by TNFalpha," *J. Biol. Chem.*, vol. 280, no. 40, pp. 33917–33925, Oct. 2005, doi: 10.1074/jbc.M505818200.

[114] E. Tashiro *et al.*, "Trierixin, a novel Inhibitor of ER stress-induced XBP1 activation from Streptomyces sp. 1. Taxonomy, fermentation, isolation and biological activities," *J. Antibiot. (Tokyo)*, vol. 60, no. 9, pp. 547–553, Sep. 2007, doi: 10.1038/ja.2007.69.

[115] T. M. J. Fritz, L. Niederreiter, T. E. Adolph, R. S. Blumberg, and A. Kaser, “Crohn’s disease: NOD2, autophagy and ER stress converge,” *undefined*, 2011. /paper/Crohn's-disease%3A-NOD2%2C-autophagy-and-ER-stress-Fritz-Niederreiter/e4f79f5b18d0ed213c355490a600b8d06031d99e/figure/4 (accessed Jun. 06, 2020).

[116] S. Elmore, “Apoptosis: A Review of Programmed Cell Death,” *Toxicol. Pathol.*, vol. 35, no. 4, pp. 495–516, 2007, doi: 10.1080/01926230701320337.

[117] C. J. Norbury and I. D. Hickson, “Cellular responses to DNA damage,” *Annu. Rev. Pharmacol. Toxicol.*, vol. 41, pp. 367–401, 2001, doi: 10.1146/annurev.pharmtox.41.1.367.

[118] D. Martinvalet, P. Zhu, and J. Lieberman, “Granzyme A induces Epase-independent mitochondrial damage, a required first step for apoptosis,” *Immunity*, vol. 22, no. 3, pp. 355–370, Mar. 2005, doi: 10.1016/j.immuni.2005.02.004.

[119] G. Kroemer *et al.*, “Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009,” *Cell Death Differ.*, vol. 16, no. 1, pp. 3–11, Jan. 2009, doi: 10.1038/cdd.2008.150.

[120] A. Strasser, A. W. Harris, D. C. Huang, P. H. Krammer, and S. Cory, “Bcl-2 and Fas/APO-1 regulate distinct pathways to lymphocyte apoptosis,” *EMBO J.*, vol. 14, no. 24, pp. 6136–6147, Dec. 1995.

[121] K. H. Vousden and X. Lu, “Live or let die: the cell’s response to p53,” *Nat. Rev. Cancer*, vol. 2, no. 8, pp. 594–604, Aug. 2002, doi: 10.1038/nrc864.

[122] J. E. Chipuk, L. Bouchier-Hayes, T. Kuwana, D. D. Newmeyer, and D. R. Green, "PUMA couples the nuclear and cytoplasmic proapoptotic function of p53," *Science*, vol. 309, no. 5741, pp. 1732–1735, Sep. 2005, doi: 10.1126/science.1114297.

[123] T. Nunes, C. Bernardazzi, and H. S. de Souza, "Cell Death and Inflammatory Bowel Diseases: Apoptosis, Necrosis, and Autophagy in the Intestinal Epithelium," *BioMed Research International*, Jul. 14, 2014. https://www.hindawi.com/journals/bmri/2014/218493/ (accessed Jun. 03, 2020).

[124] H. S. P. Souza *et al.*, "Apoptosis in the intestinal mucosa of patients with inflammatory bowel disease: evidence of altered expression of FasL and perforin cytotoxic pathways," *Int. J. Colorectal Dis.*, vol. 20, no. 3, pp. 277–286, May 2005, doi: 10.1007/s00384-004-0639-8.

[125] M. Iwamoto, T. Koji, K. Makiyama, N. Kobayashi, and P. K. Nakane, "Apoptosis of crypt epithelial cells in ulcerative colitis," *J. Pathol.*, vol. 180, no. 2, pp. 152–159, Oct. 1996, doi: 10.1002/(SICI)1096-9896(199610)180:2<152::AID-PATH649>3.0.CO;2-Y.

[126] A. Shkoda, T. Werner, H. Daniel, M. Gunckel, G. Rogler, and D. Haller, "Differential protein expression profile in the intestinal epithelium from patients with inflammatory bowel disease," *J. Proteome Res.*, vol. 6, no. 3, pp. 1114–1125, Mar. 2007, doi: 10.1021/pr060433m.

[127] M. J. Rosen *et al.*, "STAT6 activation in ulcerative colitis: a new target for prevention of IL-13-induced colon epithelial cell dysfunction," *Inflamm. Bowel Dis.*, vol. 17, no. 11, pp. 2224–2234, Nov. 2011, doi: 10.1002/ibd.21628.

[128] K. A. Steinbrecher, E. Harmel-Laws, R. Sitcheran, and A. S. Baldwin, "Loss of epithelial RelA results in deregulated intestinal proliferative/apoptotic homeostasis and susceptibility

to inflammation," *J. Immunol. Baltim. Md 1950*, vol. 180, no. 4, pp. 2588–2599, Feb. 2008, doi: 10.4049/jimmunol.180.4.2588.

[129] A. Nenci *et al.*, "Epithelial NEMO links innate immunity to chronic intestinal inflammation," *Nature*, vol. 446, no. 7135, pp. 557–561, Mar. 2007, doi: 10.1038/nature05698.

[130] P. Gut *et al.*, "Chromogranin A – unspecific neuroendocrine marker. Clinical utility and potential diagnostic pitfalls," *Arch. Med. Sci. AMS*, vol. 12, no. 1, pp. 1–9, Feb. 2016, doi: 10.5114/aoms.2016.57577.

[131] L. Taupenot, K. L. Harper, and D. T. O'Connor, "The chromogranin-secretogranin family," *N. Engl. J. Med.*, vol. 348, no. 12, pp. 1134–1149, Mar. 2003, doi: 10.1056/NEJMra021405.

[132] S. Gkolfinopoulos, K. Tsapakidis, K. Papadimitriou, D. Papamichael, and P. Kountourakis, "Chromogranin A as a valid marker in oncology: Clinical application or false hopes?," *World J. Methodol.*, vol. 7, no. 1, pp. 9–15, Mar. 2017, doi: 10.5662/wjm.v7.i1.9.

[133] P. Banks and K. Helle, "The release of protein from the stimulated adrenal medulla," *Biochem. J.*, vol. 97, no. 3, pp. 40C-41C, Dec. 1965, doi: 10.1042/bj0970040c.

[134] H. Blaschko, R. S. Comline, F. H. Schneider, M. Silver, and A. D. Smith, "Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation," *Nature*, vol. 215, no. 5096, pp. 58–59, Jul. 1967, doi: 10.1038/215058a0.

[135] M. A. D'amico, B. Ghinassi, P. Izzicupo, L. Manzoli, and A. Di Baldassarre, "Biological function and clinical relevance of chromogranin A and derived peptides," *Endocr. Connect.*, vol. 3, no. 2, pp. R45–R54, Apr. 2014, doi: 10.1530/EC-14-0027.

[136] M. El-Salhy, A. Danielsson, R. Stenling, and L. Grimelius, “Colonic endocrine cells in inflammatory bowel disease,” *J. Intern. Med.*, vol. 242, no. 5, pp. 413–419, Nov. 1997, doi: 10.1046/j.1365-2796.1997.00237.x.

[137] N. Eissa *et al.*, “Chromogranin-A Regulates Macrophage Function and the Apoptotic Pathway in Murine DSS colitis,” *J. Mol. Med. Berl. Ger.*, vol. 96, no. 2, pp. 183–198, 2018, doi: 10.1007/s00109-017-1613-6.

[138] S. H. Yoo, Y. H. Huh, and Y. S. Hur, “Inositol 1,4,5-trisphosphate receptor in chromaffin secretory granules and its relation to chromogranins,” *Cell. Mol. Neurobiol.*, vol. 30, no. 8, pp. 1155–1161, Nov. 2010, doi: 10.1007/s10571-010-9564-2.

[139] H. I. Akbarali, C. Pothoulakis, and I. Castagliuolo, “Altered Ion Channel Activity in Murine Colonic Smooth Muscle Myocytes in an Experimental Colitis Model,” *Biochem. Biophys. Res. Commun.*, vol. 275, no. 2, pp. 637–642, Aug. 2000, doi: 10.1006/bbrc.2000.3346.

[140] N. Eissa, H. Hussein, G. N. Hendy, C. N. Bernstein, and J.-E. Ghia, “Chromogranin-A and its derived peptides and their pharmacological effects during intestinal inflammation,” *Biochem. Pharmacol.*, vol. 152, pp. 315–326, Jun. 2018, doi: 10.1016/j.bcp.2018.04.009.

[141] P. Arvan and D. Castle, “Sorting and storage during secretory granule biogenesis: looking backward and looking forward,” *Biochem. J.*, vol. 332 ( Pt 3), pp. 593–610, Jun. 1998, doi: 10.1042/bj3320593.

[142] G. N. Hendy *et al.*, “Targeted ablation of the chromogranin a (Chga) gene: normal neuroendocrine dense-core secretory granules and increased expression of other granins,” *Mol. Endocrinol. Baltim. Md*, vol. 20, no. 8, pp. 1935–1947, Aug. 2006, doi: 10.1210/me.2005-0398.

[143] H. Stettler, N. Beuret, C. Prescianotto-Baschong, B. Fayard, L. Taupenot, and M. Spiess, "Determinants for chromogranin A sorting into the regulated secretory pathway are also sufficient to generate granule-like structures in non-endocrine cells," *Biochem. J.*, vol. 418, no. 1, pp. 81–91, Feb. 2009, doi: 10.1042/BJ20071382.

[144] D. T. O'Connor and L. J. Deftos, "Secretion of chromogranin A by peptide-producing endocrine neoplasms," *N. Engl. J. Med.*, vol. 314, no. 18, pp. 1145–1151, May 1986, doi: 10.1056/NEJM198605013141803.

[145] J. Ciesielski-Treska *et al.*, "Mechanisms Underlying Neuronal Death Induced by Chromogranin A-activated Microglia," *J. Biol. Chem.*, vol. 276, no. 16, pp. 13113–13120, Apr. 2001, doi: 10.1074/jbc.M009711200.

[146] S. Danese *et al.*, "Angiogenesis as a novel component of inflammatory bowel disease pathogenesis," *Gastroenterology*, vol. 130, no. 7, pp. 2060–2073, Jun. 2006, doi: 10.1053/j.gastro.2006.03.054.

[147] D. Ribatti, "Endogenous inhibitors of angiogenesis: a historical review," *Leuk. Res.*, vol. 33, no. 5, pp. 638–644, May 2009, doi: 10.1016/j.leukres.2008.11.019.

[148] E. Ferrero *et al.*, "Chromogranin A protects vessels against tumor necrosis factor alpha-induced vascular leakage," *FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol.*, vol. 18, no. 3, pp. 554–556, Mar. 2004, doi: 10.1096/fj.03-0922fje.

[149] M. Theurl *et al.*, "The neuropeptide catestatin acts as a novel angiogenic cytokine via a basic fibroblast growth factor-dependent mechanism," *Circ. Res.*, vol. 107, no. 11, pp. 1326–1335, Nov. 2010, doi: 10.1161/CIRCRESAHA.110.219493.

[150] N. R. Mahapatra *et al.*, "Hypertension from targeted ablation of chromogranin A can be rescued by the human ortholog," *J. Clin. Invest.*, vol. 115, no. 7, pp. 1942–1952, Jul. 2005, doi: 10.1172/JCI24354.

[151] J.-E. Ghia, F. Crenner, M.-H. Metz-Boutigue, D. Aunis, and F. Angel, "The effect of a chromogranin A-derived peptide (CgA4-16) in the writhing nociceptive response induced by acetic acid in rats," *Life Sci.*, vol. 75, no. 15, pp. 1787–1799, Aug. 2004, doi: 10.1016/j.lfs.2004.02.035.

[152] N. Eissa *et al.*, "Chromofungin Ameliorates the Progression of Colitis by Regulating Alternatively Activated Macrophages," *Front. Immunol.*, vol. 8, p. 1131, 2017, doi: 10.3389/fimmu.2017.01131.

[153] N. Biswas, R. S. Friese, J. R. Gayen, G. Bandyopadhyay, S. K. Mahata, and D. T. O'Connor, "Discovery of a Novel Target for the Dysglycemic Chromogranin A Fragment Pancreastatin: Interaction with the Chaperone GRP78 to Influence Metabolism," *PLOS ONE*, vol. 9, no. 1, p. e84132, Jan. 2014, doi: 10.1371/journal.pone.0084132.

[154] M. F. Rabbi, B. Labis, M.-H. Metz-Boutigue, C. N. Bernstein, and J.-E. Ghia, "Catestatin decreases macrophage function in two mouse models of experimental colitis," *Biochem. Pharmacol.*, vol. 89, no. 3, pp. 386–398, Jun. 2014, doi: 10.1016/j.bcp.2014.03.003.

[155] M. J. Ostaff, E. F. Stange, and J. Wehkamp, "Antimicrobial peptides and gut microbiota in homeostasis and pathology," *EMBO Mol. Med.*, vol. 5, no. 10, pp. 1465–1483, Oct. 2013, doi: 10.1002/emmm.201201773.

[156] M. Metz-Boutigue, D. Zhang, T. Lavaux, F. Schneider, and D. Aunis, "Two chromogranin A-derived peptides, chromofungin and catestatin, induce neutrophil activation via a store-

operated channel-dependent mechanism," *Crit. Care*, vol. 14, no. Suppl 2, p. P32, 2010, doi: 10.1186/cc9135.

[157] S. H. Yoo, "Identification of the calcium-dependent calmodulin-binding region of chromogranin A," *Biochemistry*, vol. 31, no. 26, pp. 6134–6140, Jul. 1992, doi: 10.1021/bi00141a025.

[158] K. Lugardon *et al.*, "Structural and Biological Characterization of Chromofungin, the Antifungal Chromogranin A (47-66)-Derived Peptide," *Ann. N. Y. Acad. Sci.*, vol. 971, no. 1, pp. 359–361, 2002, doi: 10.1111/j.1749-6632.2002.tb04496.x.

[159] "Chromofungin, CgA47-66-derived Peptide, Produces Basal Cardiac Effects and Postconditioning Cardioprotective Action During Ischemia/Reperfusion Injury - PubMed." https://pubmed.ncbi.nlm.nih.gov/26151429/ (accessed Jun. 13, 2020).

[160] K. Tatemoto, S. Efendić, V. Mutt, G. Makk, G. J. Feistner, and J. D. Barchas, "Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion," *Nature*, vol. 324, no. 6096, Art. no. 6096, Dec. 1986, doi: 10.1038/324476a0.

[161] G. R. Valicherla, Z. Hossain, S. K. Mahata, and J. R. Gayen, "Pancreastatin is an endogenous peptide that regulates glucose homeostasis," *Physiol. Genomics*, vol. 45, no. 22, pp. 1060–1071, Nov. 2013, doi: 10.1152/physiolgenomics.00131.2013.

[162] J. Troger *et al.*, "Granin-derived peptides," *Prog. Neurobiol.*, vol. 154, pp. 37–61, Jul. 2017, doi: 10.1016/j.pneurobio.2017.04.003.

[163] D. T. O'Connor *et al.*, "Pancreastatin: multiple actions on human intermediary metabolism in vivo, variation in disease, and naturally occurring functional genetic

polymorphism," *J. Clin. Endocrinol. Metab.*, vol. 90, no. 9, pp. 5414–5425, Sep. 2005, doi: 10.1210/jc.2005-0408.

[164] A. Jurjus *et al.*, "Inflammatory bowel disease, colorectal cancer and type 2 diabetes mellitus: The links," *BBA Clin.*, vol. 5, pp. 16–24, Jun. 2016, doi: 10.1016/j.bbacli.2015.11.002.

[165] T. Angelone *et al.*, "The antihypertensive chromogranin a peptide catestatin acts as a novel endocrine/paracrine modulator of cardiac inotropism and lusitropism," *Endocrinology*, vol. 149, no. 10, pp. 4780–4793, Oct. 2008, doi: 10.1210/en.2008-0318.

[166] M. F. Rabbi, N. Eissa, P. M. Munyaka, A. Khafipour, E. Khafipour, and J.-E. Ghia, "Tu1893 Human Catestatin Represses Reactivation of Intestinal Inflammation in a Murine Model of Colitis Through the M1 Macrophages and Not the Gut Microbiota," *Gastroenterology*, vol. 150, no. 4, p. S969, Apr. 2016, doi: 10.1016/S0016-5085(16)33286-3.

[167] A. Corti, "Chromogranin A and the tumor microenvironment," *Cell. Mol. Neurobiol.*, vol. 30, no. 8, pp. 1163–1170, Nov. 2010, doi: 10.1007/s10571-010-9587-8.

[168] K. B. Helle, A. Corti, M. H. Metz-Boutigue, and B. Tota, "The endocrine role for chromogranin A: a prohormone for peptides with regulatory properties," *Cell. Mol. Life Sci. CMLS*, vol. 64, no. 22, pp. 2863–2886, Nov. 2007, doi: 10.1007/s00018-007-7254-0.

[169] C. Rumio *et al.*, "The N-terminal fragment of chromogranin A, vasostatin-1 protects mice from acute or chronic colitis upon oral administration," *Dig. Dis. Sci.*, vol. 57, no. 5, pp. 1227–1237, May 2012, doi: 10.1007/s10620-012-2031-9.

[170] T. Lawrence and G. Natoli, “Transcriptional regulation of macrophage polarization: enabling diversity with identity,” *Nat. Rev. Immunol.*, vol. 11, no. 11, pp. 750–761, Oct. 2011, doi: 10.1038/nri3088.

[171] D. M. Mosser and X. Zhang, “Activation of murine macrophages,” *Curr. Protoc. Immunol.*, vol. Chapter 14, p. Unit 14.2, Nov. 2008, doi: 10.1002/0471142735.im1402s83.

[172] J.-E. Ghia *et al.*, “A role for chromogranin A (4–16), a vasostatin-derived peptide, on human colonic motility. An in vitro study,” *Regul. Pept.*, vol. 121, no. 1, pp. 31–39, Sep. 2004, doi: 10.1016/j.regpep.2004.04.003.

[173] X. Tréton *et al.*, “Altered Endoplasmic Reticulum Stress Affects Translation in Inactive Colon Tissue From Patients With Ulcerative Colitis,” *Gastroenterology*, vol. 141, no. 3, pp. 1024–1035, Sep. 2011, doi: 10.1053/j.gastro.2011.05.033.

[174] M. F. Rabbi, B. Labis, M.-H. Metz-Boutigue, C. N. Bernstein, and J.-E. Ghia, “Catestatin decreases macrophage function in two mouse models of experimental colitis,” *Biochem. Pharmacol.*, vol. 89, no. 3, pp. 386–398, Jun. 2014, doi: 10.1016/j.bcp.2014.03.003.

[175] R. Marion-Letellier, G. Savoye, and S. Ghosh, “IBD: In Food We Trust,” *J. Crohns Colitis*, vol. 10, no. 11, pp. 1351–1361, Nov. 2016, doi: 10.1093/ecco-jcc/jjw106.

[176] A. Wędrychowicz, A. Zając, and P. Tomasik, “Advances in nutritional therapy in inflammatory bowel diseases: Review,” *World J. Gastroenterol.*, vol. 22, no. 3, pp. 1045–1066, Jan. 2016, doi: 10.3748/wjg.v22.i3.1045.

[177] S. B. Hanauer, “Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities,” *Inflamm. Bowel Dis.*, vol. 12 Suppl 1, pp. S3-9, Jan. 2006, doi: 10.1097/01.mib.0000195385.19268.68.

[178] S. Mathews *et al.*, "In vivo confocal microscopic analysis of normal human anterior limbal stroma," *Cornea*, vol. 34, no. 4, pp. 464–470, Apr. 2015, doi: 10.1097/ICO.0000000000000369.

[179] "Rhodamine B 83689," *Basic Violet 10*. https://www.sigmaaldrich.com/catalog/product/sigma/83689 (accessed Jul. 02, 2020).

[180] M. Gry *et al.*, "Correlations between RNA and protein expression profiles in 23 human cell lines," *BMC Genomics*, vol. 10, no. 1, p. 365, Aug. 2009, doi: 10.1186/1471-2164-10-365.

[181] T. Goretsky *et al.*, "p53 Mediates TNF-Induced Epithelial Cell Apoptosis in IBD," *Am. J. Pathol.*, vol. 181, no. 4, pp. 1306–1315, Oct. 2012, doi: 10.1016/j.ajpath.2012.06.016.

[182] K. O. Arseneau, H. Tamagawa, T. T. Pizarro, and F. Cominelli, "Innate and adaptive immune responses related to IBD pathogenesis," *Curr. Gastroenterol. Rep.*, vol. 9, no. 6, pp. 508–512, Dec. 2007, doi: 10.1007/s11894-007-0067-3.

[183] "Mucosal Macrophages in Intestinal Homeostasis and Inflammation." https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3224516/ (accessed Jul. 04, 2020).

[184] V. Morampudi *et al.*, "DNBS/TNBS Colitis Models: Providing Insights Into Inflammatory Bowel Disease and Effects of Dietary Fat," *J. Vis. Exp. JoVE*, no. 84, Feb. 2014, doi: 10.3791/51297.

[185] N. Eissa *et al.*, "Chromofungin Ameliorates the Progression of Colitis by Regulating Alternatively Activated Macrophages," *Front. Immunol.*, vol. 8, Sep. 2017, doi: 10.3389/fimmu.2017.01131.

[186] P. M. Munyaka, N. Eissa, C. N. Bernstein, E. Khafipour, and J.-E. Ghia, “Antepartum Antibiotic Treatment Increases Offspring Susceptibility to Experimental Colitis: A Role of the Gut Microbiota,” *PLoS ONE*, vol. 10, no. 11, Nov. 2015, doi: 10.1371/journal.pone.0142536.

[187] P. M. Munyaka, A. Khafipour, H. Wang, N. Eissa, E. Khafipour, and J.-E. Ghia, “Mo1774 Prenatal Antibiotic Treatment Increases Offspring’s Susceptibility to Experimental Colitis: A Role of the Gut Microbiota,” *Gastroenterology*, vol. 148, no. 4, p. S, Apr. 2015, doi: 10.1016/S0016-5085(15)32404-5.

[188] D. Ma *et al.*, “KLF5 promotes cervical cancer proliferation, migration and invasion in a manner partly dependent on TNFRSF11a expression,” *Sci. Rep.*, vol. 7, no. 1, Art. no. 1, Nov. 2017, doi: 10.1038/s41598-017-15979-1.