

Exploring Gene-Environment Interactions in Inflammatory Bowel Disease

by

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Abstract

Inflammatory bowel disease (IBD) is family of complex conditions of the gastrointestinal tract. Pathogenesis of IBD remains unknown and is postulated to be multifactorial, including contributions from host genetics, environmental factors, the gut microbiome, and the immune response. This work explored environmental influences and gene-environment interactions in IBD.

Vitamin D deficiency is an environmental factor involved in IBD pathogenesis. Although the mechanism remains unclear, previous studies suggest that a lack of vitamin D signaling causes a reduction in intestinal autophagy. A potential link between vitamin D deficiency and dysregulated autophagy is microRNA (miR)-142-3p, which suppresses autophagy. We found that vitamin D deficient mice had enhanced miR-142-3p expression in ileal tissues compared to control mice. Paneth cells of vitamin D deficient mice were morphologically abnormal and displayed evidence of an autophagy defect. These findings suggest that Paneth cells exhibit early markers of autophagy dysregulation within the intestinal epithelium in response to vitamin D deficiency and enhanced miR-142-3p expression. We demonstrated that treatment-naïve IBD patients with low vitamin D have increased miR-142-3p expression in colonic tissues procured

from 'involved' areas of disease. Taken together, our findings demonstrate that insufficient vitamin D levels alter expression of autophagy-regulating miR-142-3p in intestinal tissues of mice and IBD patients, providing insight into the mechanisms by which vitamin D deficiency modulates IBD pathogenesis.

We explored gene-environment interactions by evaluating the effects of vitamin D deficiency in mice that have an important IBD genetic risk variant, *Nod2fs*, which also disrupts autophagy. Interestingly, the combination of vitamin D deficiency and *Nod2fs* did not impact autophagy at baseline. However, upon microbial disturbances in the microbiome, the vitamin D deficient *Nod2fs* mice had pervasive autophagy defects. This is in line with the multi-hit model of IBD, which proposes that many insults converge to cause defects in the intestinal epithelium. In our gene-environment analyses, we also observed that the *Nod2fs* mutation led to reduced serum vitamin D, a novel interaction between an IBD genetic risk factor and environmental factor of interest. Through this work we were able to identify therapeutic targets of interest and contribute to the understanding of IBD pathogenesis.

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Statement of Contributions

This work was possible because of the contributions of my talented and generous colleagues. Dr. Dana Bronte-Tinkew assisted in performing the experiments associated with **Figure 2.2 A-D**, **Figure 2.3**, **Figure 2.4**, and **Figure 2.6 C**. Dr. Bronte-Tinkew designed and performed the experiments presented in **Figure 2.9**. Dr. Amanda Ricciuto performed the statistical analyses associated with **Figure 2.9 B** and **Tables 1-3**. Sunny Xia created the illustration associated with **Figure 2.10**. Dr. Iram Siddiqui and her team at the Department of Laboratory Medicine & Pathology processed all animal tissues. Dr. Siddiqui also served as the pathologist for blinded scoring of tissues presented in **Figure 2.2 F** and **Figure 2.9 C**. Members of Dr. Dana Philpott's lab kindly provided the serum samples that were analyzed in **Figure 4.2**.

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List of Abbreviations

5-ASA: 5-aminosalicylate

25(OH)D: 25-hydroxyvitamin D

AGO2: Argonaute 2

ANOVA: analysis of variance

ATG: autophagy-related

ATG16L1: autophagy-related 16-like 1

CARD: caspase-recruitment domain

CCL2: CC-chemokine ligand 2

CD: Crohn's disease

CRP: c-reactive protein

Ct: cycle threshold

DAPI: 4',6-diamidino-2-phenylindole (DAPI)

DGCR8: DiGeorge critical region 8

DNA: deoxyribonucleic acid

DSS: dextran sodium sulfate

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

ESR: erythrocyte sedimentation rate

FBS: fetal bovine serum

FFPE: formalin-fixed paraffin-embedded

GI: gastrointestinal

GWAS: genome-wide association studies

HCV: hepatitis C virus

HRP: horseradish peroxidase

IBD: inflammatory bowel disease

IFN γ : interferon γ

IL: interleukin

iNKT: invariant natural killer T

IRE1: inositol-requiring transmembrane kinase/endoribonuclease 1

JAK: janus kinase

KO: knockout

LC3II: microtubule-associated protein 1A/1B-light chain 3 II

LRR: leucine-rich repeat

MAPK: mitogen-activated protein kinase

MDP: muramyl dipeptide

miR, miRNA: microRNA

MNV: mouse norovirus

mRNA: messenger RNA

MUC2: mucin 2

NBD: nucleotide binding domain

NF- κ B: nuclear factor- κ B

NOD2: nucleotide oligomerization domain-containing protein 2

NSAID: non-steroidal anti-inflammatory drug

PBS: phosphate-buffered sodium

PPAR γ : peroxisome proliferator-activated receptor γ

pre-miRNA: precursor microRNA

pri-miRNA: primary microRNA

RegIII γ : regenerating islet-derived protein III γ

RIP2: receptor-interacting protein 2

RIPA: radioimmunoprecipitation assay

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RT-qPCR: real-time quantitative polymerase chain reaction

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

SNP: single nucleotide polymorphism

STAT: signal transducer and activator of transcription

T. muris: *Tritrichomonas muris*

TBS-T: Tris-buffered saline - Tween 20

Th: T helper

TNBS: trinitrobenzene sulfonic acid

TNF: tumour necrosis factor

TRBP: TAR RNA binding protein

Treg: T regulatory

UC: ulcerative colitis

UEA-I: Ulex europaeus agglutinin I

ULK1: UNC-51-like kinase 1

UTR: untranslated region

UVB: ultraviolet B

VDR: vitamin D receptor

VDRE: vitamin D response element

WBC: white blood cell

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Chapter 1

Introduction

1 Introduction

1.1 Background

1.1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a family of complex gastrointestinal (GI) disorders, including the two main forms, Crohn's disease (CD) and ulcerative colitis (UC). This group of idiopathic autoimmune conditions result in inflammation and damage to the GI tract. Peak onset of IBD is in early adulthood, although a diagnosis can occur at any age (Molodecky et al. 2012). Once diagnosed, IBD is a lifelong illness, with no preventive or curative measures available. These chronic conditions have a remitting and relapsing pattern, with periods of relative health interspersed with periods of moderate-to-severe illness resulting in GI symptoms such as abdominal pain, bloating, diarrhea, and rectal bleeding (Flynn and Eisenstein 2019). Extraintestinal manifestations are common, which may include inflammatory conditions of the skin, eyes, liver, and joints (Flynn and Eisenstein 2019). Symptoms vary between patients based on many factors, including the location and extent of disease.

The two main subtypes of IBD, CD and UC, share many similarities in terms of disease presentation but differ in several important ways (**Figure 1.1**). Crohn's disease can affect anywhere along the GI tract and most commonly manifests as inflammation of the ileum and colon in a discontinuous pattern (Flynn and Eisenstein 2019). In contrast, UC tends to involve the rectum with inflammation extending along the colon in a continuous pattern (Flynn and Eisenstein 2019). In addition, the inflammation in CD tends to be transmural, affecting all layers of the bowel, whereas the inflammation associated with UC is superficial and limited to the intestinal mucosa and submucosa (Flynn and Eisenstein 2019). A third type of IBD, IBD unclassified (IBDU), does not present with features aligning exactly with CD or UC and is more commonly observed in paediatric patients compared to adults (Thurgate et al. 2019). Whether IBDU is a distinct subtype of IBD or a reflection of the difficulties in diagnosing either CD or UC especially when disease presents at a young age remains a topic of debate (Thurgate et al. 2019). Irrespective of the subtype diagnosed, the goal of IBD management is to

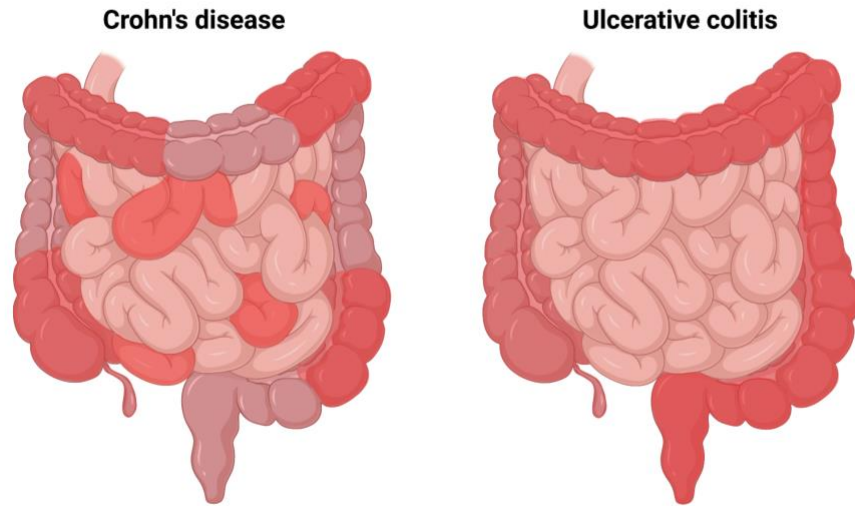


Figure 1.1: Crohn's disease and ulcerative colitis

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of inflammatory bowel disease. Although they have overlapping disease presentation, the pattern and extent of inflammation differ between the subtypes. Left: While CD can affect anywhere along the GI tract, it most commonly manifests as inflammation of the ileum and colon in a discontinuous pattern (Flynn and Eisenstein 2019). Right: In contrast, UC tends to involve the rectum with inflammation extending along the colon in a continuous pattern (Flynn and Eisenstein 2019).

promote and maintain disease remission in order to control disease and minimize symptoms.

Advances in IBD therapies in recent decades have revolutionized the care available to patients, although treatment options remain fairly limited. There are several immunomodulators and immunosuppressants available to manage symptoms and promote remission (Vedamurthy and Ananthakrishnan 2019). Unsuccessful disease management can result in irreversible intestinal damage that is no longer responsive to medical therapies and requires bowel resection surgery to remove the severely affected segments (Cosnes et al. 2011). Surgical intervention remains the fate for a substantial proportion of CD and UC patients, underscoring the need for enhanced understanding of IBD (Frolkis et al. 2013). The pathogenesis of IBD is extremely complex and remains unknown, and further research aimed at unraveling pathogenesis to identify new therapeutic targets and possibly prevention measures is required.

1.1.2 Personal impacts

A diagnosis of IBD has substantial implications on the lives of patients. Patients experience a lower health-related quality of life compared to the general population, including interferences with physical, mental, and emotional well-being (Jones et al. 2019). Disease onset often occurs in young adulthood and thus interferes with pursuits pertaining to education, employment, and family planning (Jones et al. 2019). Impairments in daily activities can affect interpersonal relationships, life activities, social participation, and mental well-being (Jones et al. 2019). Psychological distress is common in IBD patients and can stem from physical factors such as pain and other factors such as concern for the future and fear of stigmatization (Jones et al. 2019). Despite significant advancements in treatment options, there still remains a gap in life expectancy and health-adjusted life expectancy in IBD patients compared to those without IBD (Kuenzig et al. 2020). This contributes to a fear of dependency that is commonly observed among patients (Jones et al. 2019). Inflammatory bowel disease is a lifelong illness that can severely impact physical and psychological health of those affected, which is a contributing factor to its emergence as a research priority.

1.1.3 Economic impacts

The expenses associated with IBD care in Canada are substantial, and the trends in cost are increasing. It is estimated that Canada spent at least \$1 billion and possibly more than \$2 billion in direct health care costs due to IBD in 2018 (Kuenzig, Benchimol, et al. 2019). Direct costs include medically necessary health care services such as hospital-based care, outpatient consultations, diagnostic testing, prescription medications, and home care (Kuenzig, Benchimol, et al. 2019). There has been a rise in direct costs over the last 15 years that is predicted to continue to climb over the next decade, driven by the discovery and expanded use of expensive biologic therapies for the treatment of IBD (Kuenzig, Benchimol, et al. 2019). The indirect costs associated with IBD are also substantial, which include lost wages of patients and their caregivers. The indirect health-related costs of IBD in Canada were estimated to be in excess of \$1 billion in 2018, although this may be an underestimation (Kuenzig, Lee, et al. 2019). Creative solutions identified through research are required to alleviate the significant costs associated with IBD in Canada.

1.1.4 Epidemiology

The first sporadic cases of IBD reported around the time of the industrial revolution were confined to the western world, but IBD has since evolved into a global disease, with cases recorded on every populated continent in the world (Molodecky et al. 2012; Ng et al. 2017). Europe and North America have the highest rates of IBD worldwide, with prevalence exceeding 0.3% of the population in many countries (Ng et al. 2017). In Canada, the prevalence of IBD was estimated to be 0.7% in 2018, making it one of the most highly affected countries (Kaplan et al. 2019). With trends increasing year after year, prevalence is projected to hit 1.0% of the population by 2030 (Kaplan et al. 2019). This projection would amount to over 400 000 Canadians suffering with IBD (Kaplan and Windsor 2021). Although incidence is stabilizing and even declining in some regions of the country (Windsor and Kaplan 2019), incidence is accelerating in certain populations, such as in paediatric patients (Benchimol et al. 2014). On a global scale, IBD incidence is accelerating in newly industrialized countries in Africa, Asia, and South

America (Ng et al. 2017). It is clear that IBD is no longer a condition of the western world but has worldwide impacts.

Kaplan & Windsor (2021) outline 4 epidemiological states of IBD: Emergence, Acceleration in Incidence, Compounding Prevalence, and Prevalence Equilibrium. These stages act as a framework to understand the past and predict future global trends of IBD. Developing nations are in the Emergence stage, documenting their first cases of IBD. Newly industrialized countries are in the Acceleration in Incidence stage, with an exponential rise in incident cases, while prevalence remains relatively low. Canada and other western countries are currently in the Compounding Prevalence stage: incidence is still on the rise, but due to the low mortality rate of IBD and the aging population, prevalence is also high. It is thought that countries in this stage will soon reach the theoretical fourth stage, Prevalence Equilibrium, where the number of incident cases is approximately equal to the mortality rate of patients, leading to a constant number of total cases (Kaplan and Windsor 2021). The current epidemiological trends outline that IBD will continue to be a burden to health care systems all around the globe. This highlights the requirement for research into disease pathogenesis, so that preventive or curative measures can be developed.

1.2 Disease management & current therapies

1.2.1 Clinical management

Within the last decade, there has been a paradigm shift in the clinical management of IBD. Traditionally, IBD therapy had the goals of inducing and maintaining clinical remission, withdrawing the use of corticosteroids, and preventing post-operative disease recurrence (D'Haens et al. 2014). This was achieved through escalating treatment incrementally in response to progression of symptoms in what is called a 'step-up' approach (D'Haens et al. 2014; Bouguen et al. 2015). In recent years, however, the therapeutic approach has evolved as new therapies and technologies have become available (D'Haens et al. 2014). The more recent method of IBD management takes a 'top-down' approach centred on controlling disease and halting its progression using more aggressive therapies beginning early on in the disease course, before irreversible bowel damage occurs (D'Haens et al. 2008; Bouguen et al. 2015; Khanna et al. 2015; Singh and Loftus 2015). Whereas the step-up approach only takes clinical symptoms into consideration, the top-down approach integrates symptoms with laboratory values and imaging to more accurately calculate risk and develop an appropriate treatment strategy (Bouguen et al. 2015). Additional prognostic factors considered may include levels of inflammatory biomarkers and degree of mucosal healing (D'Haens et al. 2014). With multiple biologic agents that target specific proteins and pathways to control inflammation becoming available to complement the existing repertoire of anti-inflammatory and immunosuppressive therapies, there are now more treatment options than ever for IBD patients.

1.2.2 IBD Therapies

Classic IBD therapies include anti-inflammatory and immunosuppressive medications. Corticosteroids and 5-aminosalicylates (5-ASAs) are anti-inflammatory drugs that have been cornerstones in the treatment of IBD for decades (Williams et al. 2014). However, the efficacy of 5-ASAs is mostly limited to UC, and corticosteroids are not suitable for maintenance therapy due to their associated side effects (Vermeire, Van Assche, and

Rutgeerts 2007). Several immunomodulators have been used in the treatment of IBD, including 6-meraptopurine, azathioprine, methotrexate, and cyclosporine A (Zenlea and Peppercorn 2014). These therapies exert their effects on immune cells through various mechanisms to block their function, inhibit proliferation, or induce apoptosis (Zenlea and Peppercorn 2014). In addition to these conventional anti-inflammatory and immunomodulating agents, there are now biologic therapies emerging for the treatment of IBD.

Infliximab, which is a chimeric antibody against tumour necrosis factor (TNF), was the first biologic agent approved for the treatment of IBD (D'Haens et al. 2014). The cytokine TNF is key to imparting damage to the epithelium and perpetuating inflammation in IBD, and successfully targeting this cytokine to improve disease was a novel approach to IBD treatment that paved the way for many subsequent biologic therapies (Nielsen and Ainsworth 2013; Schreiner et al. 2019). Although the advent of anti-TNF therapies revolutionized IBD care, there are limitations to their use. Firstly, there are adverse events that have been documented with anti-TNF therapy, such as lymphoma (Lemaitre et al. 2017) and serious infections (Kirchgesner et al. 2018). In addition, there is a high level of non-response and loss of response to anti-TNF therapy. More than a third of patients are primary non-responders to anti-TNF therapy (Ford et al. 2011; D'Haens et al. 2014). Of the patients who initially respond to infliximab, it is estimated that approximately 40% will lose response over time (Gisbert and Panés 2009; D'Haens et al. 2014). This highlights the need to have multiple biologic therapies available that act on different pathways.

There have been many advances in biologic therapies for IBD in recent years. Ustekinumab, which has been approved to treat CD, antagonizes the p40 subunit of interleukin (IL)-12/IL-23 to suppress downstream immune activation and intestinal inflammation (D'Haens et al. 2014). Another pathway leveraged by biologics is cell trafficking and adhesion. Vedolizumab, which has been approved to treat both CD and UC, has a role in blocking the migration of gut-homing leukocytes (D'Haens et al. 2014; Neurath 2017). Vedolizumab antagonizes $\alpha 4\beta 7$ integrins, a class of adhesion molecules expressed on the surface of leukocytes, to ultimately inhibit their migration into tissues (D'Haens et al. 2014; Neurath 2017). While the aforementioned therapies are antibodies

that target specific proteins, there are also small molecule therapies available. For example, tofacitinib is a small molecule that has been approved for the treatment of UC, which inhibits janus kinase (JAK) signaling and production of downstream inflammatory cytokines (D'Haens et al. 2014; Neurath 2017). In addition to biologic agents, there are now biosimilars that are emerging on the market, which are highly similar versions of the original product (D'Haens et al. 2014). Because biologic agents are created through biological reactions, the copy version is not exact (D'Haens et al. 2014). Although relatively expensive compared to other pharmaceuticals, biosimilars are approximately 30% more affordable than the original product which can reduce the financial burden and increase access (Danese, Bonovas, and Peyrin-Biroulet 2017). There are many other biologic agents and biosimilars on the horizon, expanding the possibilities of personalized medicine in IBD.

Novel treatment strategies including biologic agents and biosimilars have expanded alongside our enhanced understanding of the pathophysiology of IBD. The expanding repertoire of treatment options will allow for personalized therapies in IBD. In the future, decision making in IBD care is likely to have a patient-tailored approach. With a wide range of biologic therapies available, clinicians will be able to utilize individual biomarkers and tissue signatures to select the appropriate treatment and monitor response (D'Haens et al. 2014). To facilitate this change toward precision medicine, the next step in IBD research involves bidirectional translational research. Clinical and epidemiological findings surrounding genetic defects and environmental exposures and their effects on intestinal inflammation should be connected to specific mechanisms through basic research in order to identify new therapeutic targets, so that new therapies may be developed and brought back into the clinic (Ananthakrishnan et al. 2018).

1.3 Mechanisms of disease

1.3.1 Introduction to the intestinal epithelium

The pathogenesis of IBD remains unknown due to its polygenic and multifactorial nature. It has long been postulated that IBD manifests due to the convergence of genetic, environmental, microbial, and immune factors (**Figure 1.2**) (Sartor 2006). The underlying mechanism is thought to involve an inappropriate immune response to intestinal microbes in genetically susceptible individuals exposed to environmental risk factors (Abraham and Cho 2009b; Khor, Gardet, and Xavier 2011). The initiating trigger in this cascade of events continues to be elusive and drives current IBD research efforts. In order to understand the complex chain of events that lead to the inflammation and damage characteristic of IBD, it is important to consider the unique composition of the intestine.

The intestinal epithelium has the tremendously important task of maintaining a barrier between the contents of the intestinal lumen and the underlying host tissues. The epithelium is a single cell layer in thickness, with adjacent cells sealed together by intercellular junctions (Abraham and Cho 2009b). The GI tract carries food and other exogenous factors, including microorganisms, and the primary role of the intestinal epithelium is to absorb dietary nutrients while preventing inappropriate immune activation by luminal antigens (Okumura and Takeda 2017). This role is complicated by the presence of the gut microbiome, a collection of microorganisms that normally maintain symbiosis with the host, which will be discussed in a later section. The epithelium has several strategies to protect its integrity, including physical and chemical barriers that spatially segregate the luminal microbes and other antigens from the host tissues (Okumura and Takeda 2017).

Specialized epithelial cell types are key in maintaining the integrity of the barrier. Two types of secretory cells, goblet cells and Paneth cells, contribute to the protective layer that overlies the epithelial cells (Abraham and Cho 2009b; Okumura and Takeda 2017). Goblet cells secrete mucins which form a viscous mucous layer that acts to physically separate the microbes from the host cells (Okumura and Takeda 2017).

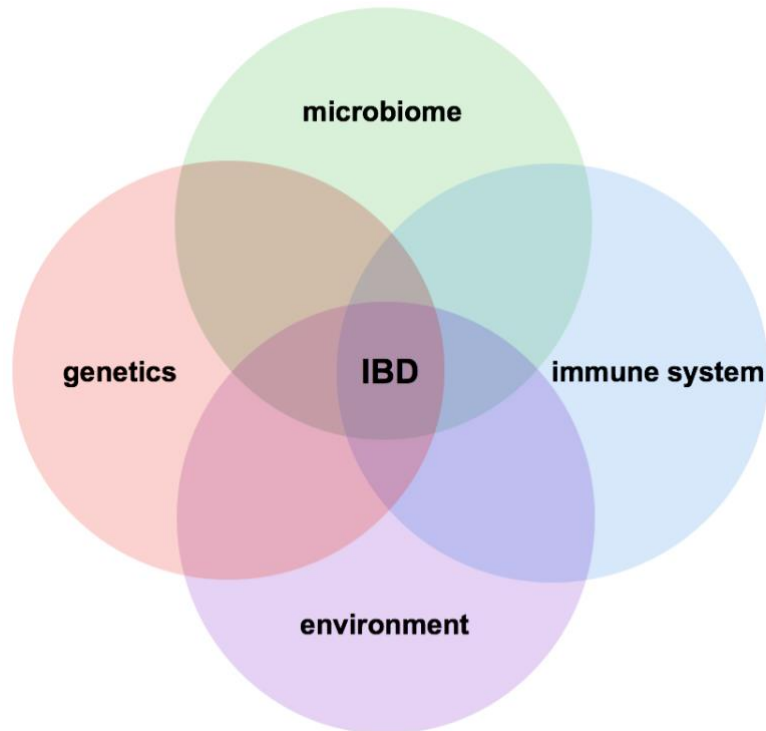


Figure 1.2: Multifactorial pathogenesis of IBD

The onset of inflammatory bowel disease (IBD) is extremely complex and involves contributions from host genetics, environmental factors, the gut microbiome, and the immune response. It is postulated that IBD occurs due to an inappropriate and hyperactive immune response to the gut microbiome in genetically susceptible individuals exposed to environmental triggers. Adapted from Sartor (2006) with permission.

Paneth cells secrete antimicrobial peptides to help maintain the physical separation (Okumura and Takeda 2017). Together, these cell types aim to maintain homeostasis by preventing the interaction of the gut microbiota and underlying lymphoid tissues.

The intestinal microenvironment must be tightly regulated in order to maintain homeostasis. Given the intricate structure of the mucosal barrier, it is not surprising that perturbances can cause barrier breakdown, invasion of enteric microbes, and an uncontrolled immune response that ultimately leads to tissue inflammation and damage, as is observed in IBD. The initial insult and exact mechanisms of barrier breakdown remain largely unknown. Research exploring the pathogenesis of IBD, taking into consideration host genetics, environmental exposures, contribution of the microbiome, and the immune response is critical so that understanding of IBD can be advanced and new therapeutic targets can be discovered.

1.3.2 The gut microbiome

The gut microbiome, which is the greatest reservoir of microorganisms in the human body, has gained attention and research interest for its role in many aspects of health and disease, including in the development of IBD. The gut microbiota is made up of a complex consortium of species from the Archaea, Eukarya, and Bacteria superkingdoms (Sender, Fuchs, and Milo 2016). The majority of research centres on bacterial populations because bacteria far outnumber the eukaryote and archaea populations (Sender, Fuchs, and Milo 2016). Furthermore, current technologies allow for proficient identification and functional analysis of bacterial communities within the microbiota (Sender, Fuchs, and Milo 2016; Turpin et al. 2018). The gastrointestinal tract hosts variable concentrations of bacteria along its length, with the colon harbouring the greatest concentration with approximately 10^{11} bacterial cells per gram of luminal contents (Sender, Fuchs, and Milo 2016). In a healthy human gut, the microbiome has a symbiotic relationship with the host. The microbial community carries out a range of functions, such as shaping and training the immune system, metabolizing food substrates, and controlling the growth of pathogenic microorganisms (Kostic, Xavier, and Gevers 2014). Many factors can influence the composition of the gut microbiota,

including genetics (Turpin et al. 2016), and environmental exposures such as antibiotics, smoking, and diet (Kostic, Xavier, and Gevers 2014). The role of the microbiome in IBD has been an area of research and debate.

The body of literature suggests that the microbiome is a key factor in the development of IBD. Early studies using mouse models of IBD concluded that the presence of the microbiome is essential for the development of intestinal inflammation, given that germ-free mice did not develop colitis (Dianda et al. 1997; Matsumoto et al. 1998; Sellon et al. 1998; Madsen et al. 1999). Subsequent studies in mice demonstrated that treatment with broad-spectrum antibiotics, reducing the total bacterial load, can both prevent and treat intestinal inflammation in several models of experimental colitis (Madsen et al. 2000; Bamias et al. 2002; Hoentjen et al. 2003; Kang et al. 2008). The results from studies involving antibiotics in the treatment of IBD patients are not as clear. Short-term treatment of CD patients with antibiotics can be beneficial in resolving intestinal inflammation and have been used in clinical settings to reduce disease activity and induce remission (Ursing et al. 1982; Sutherland et al. 1991; Sartor 2004). However, the use of antibiotics in the clinical treatment of UC is not supported, although there are studies that demonstrate minimal efficacy (Burke et al. 1990; Lobo et al. 1993; Sartor 2004). In addition, fecal diversions, which divert the fecal stream and associated microbes from inflamed intestinal segments, reduce inflammation and allow the inflamed area to heal in severe CD (Singh et al. 2015). Taken together, this data suggest that the gut microbiome is a critical driving factor in the development of inflammation in IBD.

The gut microbiota in IBD is dysbiotic, meaning that there is a loss of balance between protective and pathogenic microorganisms, with increased representation of pathogenic species. There is reduced alpha diversity, which is a measure of the unique taxa present, in the microbiota of IBD patients compared to healthy controls (Manichanh et al. 2006; Willing et al. 2010; Lepage et al. 2011). A relative reduction in Firmicutes and Bacteroidetes, and enrichment of Proteobacteria and Actinobacteria at the phylum level has been identified in the microbiome of IBD patients (Frank et al. 2007; Sokol et al. 2009; Turpin et al. 2018). At the genus level, there tends to be reduced abundance of *Bacteroides*, *Faecalibacterium*, *Rosburia*, *Blautia*, *Ruminococcus*, and *Coprococcus* in

IBD patients (Willing et al. 2010; Gevers et al. 2015). In CD, there is also reduced representation of the families *Ruminococcaceae* and *Lachnospiraceae*, and a relative increase in the family of *Enterobacteriaceae* (Gevers et al. 2015). In terms of specific bacterial species that have been associated with IBD, there is increased presence of *Escherichia coli* in intestinal lesions in both CD (Darfeuille-Michaud et al. 1998) and UC (Pilarczyk-Zurek et al. 2013) patients. In CD specifically, the adherent-invasive strain of *E. coli* has been associated with ileal lesions, indicating a potential role for this bacterial strain and disease development (Darfeuille-Michaud et al. 1998). There is also reduced representation of *Faecalibacterium prausnitzii* and *Roseburia hominis* in IBD, both of which are considered protective commensals with butyrate-producing and anti-inflammatory capacities (Sokol et al. 2008; Machiels et al. 2013; W. Wang et al. 2014). How these observed changes to the microbiota relate to disease development remains to be understood and is an active area of current research.

Recent studies have explored whether a dysbiotic microbiome can drive IBD development, or whether dysbiosis is secondary to the inflammation in IBD. Several studies have employed a technique in which the microbiome of IBD patients or healthy controls is transplanted into mice, and aspects of the immune and inflammatory responses are observed. A study that employed this strategy discovered that mice colonized with the microbiota of IBD patients had induction of a pro-inflammatory gene expression profile consistent with what is observed in CD patients. The CD microbiome in mice, compared with a healthy control, was responsible for an exaggerated inflammatory response when inoculated into an IL-10 deficient mouse model of colitis (Nagao-Kitamoto et al. 2016). Another study conducted by Britton et al. (2019) used a similar strategy and identified that mice colonized with the microbiome of IBD patients had increased numbers of intestinal T helper (Th)-17 and Th2 cells and a concurrent decrease in T regulatory (Treg) cells compared to mice colonized with the microbiome from healthy controls (Britton et al. 2019). The mice colonized with IBD microbiomes also had an exaggerated immune and inflammatory response in the context of a colitic trigger. Evidence from these studies suggest that that the microbiome itself is capable of driving disease and is not merely a by-product of inflammation.

Detecting early changes in the microbiome and linking them to changes in the host response in a genetically susceptible individual before and after disease onset would be instrumental to our understanding of the role of the microbiome in IBD development. This was the goal of a recent study in which germ-free mice were colonized with either the microbiome from healthy individuals at risk for developing IBD (pre-UC), the microbiome of the same individuals who later developed UC (post-UC), or the microbiome from healthy control individuals (Galipeau et al. 2021). The microbiome of post-UC samples was different from both pre-UC and healthy control samples, indicating a change in the microbiome with UC onset. However, there was increased proteolytic activity observed in fecal samples from mice inoculated with both pre- and post- UC microbiomes (Galipeau et al. 2021). This suggests that fecal proteolytic activity might be a key factor in driving IBD pathogenesis and could act as a biomarker in predicting who will develop IBD so that early interventions can be taken. Studies such as these are an exciting development in IBD research, and it is expected that many IBD triggers and mechanisms will be discovered through this modality.

Despite much of the current research being centered on bacteria, it remains possible that any member of the microbiota may contribute to IBD pathogenesis. Emerging research suggests that other populations, including viruses, fungi, helminths, and protozoa are important in the development of IBD. For example, it has been demonstrated that IBD patients have an abnormal enteric virome. Paediatric CD patients have an enrichment of *Caudovirales* bacteriophages in their ileal mucosa and gut washings compared to non-IBD controls (Wagner et al. 2013). Furthermore, *Caudovirales* enrichment has been identified in fecal samples from adult CD and UC patients compared to non-IBD household controls (Norman et al. 2015). In terms of fungal contributions, a study conducted by Chehoud et al. (2015) identified reduced fungal diversity in paediatric IBD patients and enrichment of specific *Candida* taxa (Chehoud et al. 2015). An additional study of fungal populations in IBD identified mucosa-associated fungal dysbiosis in inflamed biopsies from CD patients, further implicating a role for fungi in the development of IBD (Liguori et al. 2016). Recent work has demonstrated that helminths and protozoa play an important immunomodulatory role in the intestine and has begun to unravel the consequences of infection with

specific helminths or protozoan in the context of mouse models of IBD (Nadjsombati et al. 2018; Schneider et al. 2018; Escalante et al. 2016). Although a majority of the work to date has been focused on bacterial populations in IBD, it is clear that other microorganisms influence IBD onset. Further studies are required to identify which microbial species, bacterial or otherwise, contribute to the development of IBD, and to elucidate the specific mechanisms involved.

Research technologies enabling the exploration of the composition and function of the gut microbiome have advanced remarkably in recent decades and have allowed for enhanced understanding of the role of the microbiome in IBD. Based on current research, it appears that the microbiome is central to the development of IBD. However, there are still many questions pertaining to the microbiome that remain unanswered. The causes and consequences of shifts in microbial communities in IBD are an active area of research. Studies that track the changing gut microbiome of genetically susceptible individuals before and after IBD onset in order to identify novel biomarkers and potential therapeutic targets are emerging. There are still many unknowns regarding the role of the microbiome in the development and disease course of IBD and ongoing research in this area may allow for the discovery of creative solutions in the prevention or treatment of IBD.

1.3.3 The immune response

Damage to the intestinal epithelium that occurs in IBD is the result of a hyperactive and inappropriate immune response. The genetic polymorphisms that predispose to IBD tend to disrupt either innate or adaptive immunity. Although there may be no consequences at baseline, when additional insults occur, this can initiate a chain of events that lead to an uncontrolled immune response causing chronic inflammation and intestinal damage (Maloy and Powrie 2011; Philpott et al. 2014). Genetic variants affecting the innate immune response, such as *NOD2* or *ATG16L1* polymorphisms, generally diminish the capacity of the epithelium to properly recognize, handle, or protect against microbial threats, leaving the epithelium susceptible to breakdown with additional perturbances (Maloy and Powrie 2011; Philpott et al. 2014). Alternatively,

there are polymorphisms that directly affect the adaptive immune response, such as IBD variants within the IL-23 signaling pathway that either protect from or predispose to IBD by modulating the differentiation of CD4⁺ T cells (Abraham and Cho 2009a). It is clear that genetic differences in either innate or adaptive immunity can impair the resilience of the epithelium, thereby predisposing to IBD onset. The innate immune system is the first line of defense against enteric pathogens and perturbances, and in IBD, dysfunctional innate immune components can prime an aberrant adaptive immune response and the associated chronic inflammation and tissue damage.

The innate immune system encompasses the epithelial, endothelial, and mesenchymal cells that make up the physical barrier, as well as immune cells including neutrophils, monocytes, macrophages, and dendritic cells (de Souza and Fiocchi 2016). Infiltration of neutrophils into the mucosa is one of the first indications of inflammation in IBD, and the persistence of neutrophils is a hallmark of active disease (Brazil and Parkos 2013). Neutrophils contribute to tissue damage in IBD through releasing inflammatory mediators, disrupting barrier function, and initiating oxidative and proteolytic damage (Brazil and Parkos 2013). Macrophages, while anergic in the healthy gut mucosa, play a role in tissue inflammation in CD (Smythies et al. 2005). A subset of macrophages has been discovered in the CD-affected intestine which express both macrophage (CD14, CD33, CD68) and dendritic cell (CD205, CD209) markers and secrete large amounts of inflammatory cytokines including IL-6, IL-23 and TNF α (Kamada et al. 2008). Dendritic cells link the innate and adaptive immune responses by regularly sampling luminal constituents and presenting antigens to naïve CD4⁺ T cells (Rescigno and Di Sabatino 2009). Dendritic cells either induce tolerance to innocuous agents by stimulating Treg development or initiate a protective immune response to infectious agents by stimulating Th1, Th2, or Th17 cell development (Rescigno and Di Sabatino 2009). In IBD patients, dendritic cells have an activated phenotype and produce more inflammatory cytokines compared to those isolated from healthy controls (Hart et al. 2005). Dendritic cells are instrumental in IBD because of their ability to determine the fate of CD4⁺ T cells, thereby inducing an adaptive immune response that can impart damage to the intestinal epithelium.

The production of CD4+ T cells are central to the orchestration of protective responses within the gut and maintenance of homeostasis; however, these cells can also mediate chronic intestinal inflammation (Imam et al. 2018). Early studies demonstrated that therapies which either depleted or blocked CD4+ T cells led to disease remission in many IBD patients (Emmrich et al. 1991; Stronkhorst et al. 1997). This suggested that CD4+ T cells have an important role in propagating intestinal damage in IBD. A longstanding theory in the field claims that CD involves a Th1-type immune response, while UC involves a Th2-type immune response. Th1 cells have a crucial role in responding to infectious pathogens. They primarily produce interferon (IFN)- γ , which activate macrophages, and TNF, which directs cytotoxic CD8+ T cell responses (Manetti et al. 1993; Imam et al. 2018). These responses act to eliminate intracellular pathogens such as viruses and bacteria (Imam et al. 2018). The Th1/Th2 paradigm of CD and UC was found to be an over-simplified concept upon the discovery of Th17 cells, which are known for producing large amounts of inflammatory cytokine IL-17 (Weaver et al. 2013). A study by Sakuraba et al. (2009) revealed that in CD patients, the predominant lymphocytes present in mesenteric lymph nodes are Th1 and Th17 cells, and there is over production of IFN γ and IL-17 compared to UC patients and healthy controls (Sakuraba et al. 2009). The paradigm has since been revised to a Th1/Th17 response for CD. In UC, it is still widely accepted that a Th2 response is predominant (Li et al. 2016). The Th2 response has classically been associated with anti-parasite immunity, however; it is also recognized in chronic illnesses such as asthma (Walker and McKenzie 2018). Th2-like cells predominantly secrete IL-4, IL-5, IL-9, and IL-13 and drive a type 2 immune response (Walker 2018). The adaptive immune responses unique to CD and UC culminate in the chronic inflammation and intestinal pathologies that are characteristic of each disease.

The innate and adaptive immune system are key in maintaining intestinal homeostasis, and alterations in these compartments can predispose to immune-mediated diseases such as IBD. Current IBD therapies aim to blunt the aberrant immune response as a way to heal the epithelium and control symptoms. Identifying variants in immune-related genes and uncovering their mechanism of protection or predisposition to IBD will allow for the ongoing development of novel therapeutics. Leveraging components of the

immune system that have a role in IBD for targeted therapies will allow for a patient-tailored approach to IBD treatment in the future that takes into consideration each individual's dominant immunopathogenic phenotype.

1.3.4 Genetic predisposition

It has long been recognized that IBD has a strong genetic basis. Early work established the familial aspect of IBD (Orholm et al. 1991), and there have been many family and twin studies since that have aimed to quantify the heritability of IBD. A large Danish population-based cohort study conducted by Moller et al. (2015) calculated familial risk of IBD and found that up to 12% of all IBD patients report a family history of IBD. The same study determined that first-degree relatives (FDRs) of individuals with IBD are at increased risk compared to those without a family history, with an incidence ratio of 7.77 for CD and 4.08 for UC FDRs (Moller et al. 2015). This suggests that genetics play a larger role in CD than UC (Moller et al. 2015). In accordance with this, twin studies have demonstrated that among pairs of monozygotic twins, there is a concordance rate of 30%-35% for CD and 10%-15% for UC (Brant 2011). Concurrent studies have revealed many genetic loci that are responsible for driving the increased risk of IBD in individuals.

The development of technologies to identify genetic polymorphisms has remarkably advanced our understanding of many diseases, including IBD. Increased access to genome-wide association studies (GWAS), as well as new high-throughput technologies and global collaborations, have led to the discovery of over 230 independent genetic risk loci for IBD (Uniken Venema et al. 2016). Meta-analyses of IBD genetic studies have revealed substantial overlap between CD and UC risk loci, but have also revealed CD-specific and UC-specific loci (Jostins et al. 2012). This indicates both shared and distinct mechanisms of disease in the subtypes of IBD. A limitation with genetic studies is that association signals often span broad regions, and thus the causal gene and variants remain unknown for many of the identified loci (Uniken Venema et al. 2016; McGovern, Kugathasan, and Cho 2015). Multiple strategies, including targeted GWAS, imputation, resequencing of risk loci, and *in silico* fine-mapping of densely typed loci have allowed the identification of causal variants of IBD risk genes (Uniken Venema et

al. 2016). Linking variants with functional studies to understand altered gene function has provided insight into the mechanisms and pathways involved in IBD pathogenesis.

Discovery of the *ATG16L1* T300A mutation as a CD risk variant exemplifies the importance of identifying causal variants and linking them to functional studies to enhance our understanding of IBD pathogenesis (Rioux et al. 2007; Hampe et al. 2007). The autophagy-related 16-like 1 (*ATG16L1*) protein has a key role in autophagy, which is a conserved innate immune mechanism that promotes the degradation and recycling of cellular components and allows for elimination of intracellular pathogens to maintain homeostasis (**Figure 1.3**) (Levine, Mizushima, and Virgin 2011). Autophagy begins with a membranous crescent, or phagophore, that forms around cytosolic contents targeted for degradation (Levine, Mizushima, and Virgin 2011). The *ATG16L1* protein is part of a conjugation system that is essential for membrane formation as elongation occurs (Levine, Mizushima, and Virgin 2011). The membrane fully encloses the materials into a double-membrane bound autophagosome (Levine, Mizushima, and Virgin 2011). Autophagosome and lysosome fusion occurs, and hydrolytic enzymes originating from the lysosome degrade contents of the compartment (Levine, Mizushima, and Virgin 2011). The *ATG16L1* T300A polymorphism enhances degradation of the protein, leading to impaired function of the autophagy pathway (Murthy et al. 2014; Lassen et al. 2014). Importantly, the discovery of the *ATG16L1* T300A mutation as a genetic risk variant predisposing to IBD implicated dysfunctional autophagy in the pathogenesis of CD and has since accelerated research in this area. Further highlighting the importance of the autophagy pathway in IBD pathogenesis was the discovery of additional autophagy genes as risk loci, such as *NOD2* (Hugot et al. 2001; Ogura, Bonen, et al. 2001) and *IRGM* (Parkes et al. 2007). The ability to pinpoint causal variants and understand how they lead to dysfunction of major cellular pathways can provide new insights into disease pathogenesis.

Beyond the autophagy pathway, there have been several additional IBD risk loci associated with dysfunctional innate immunity. The intestinal epithelium is an important first line of defense in the gut, and there are several mutations that affect the proper

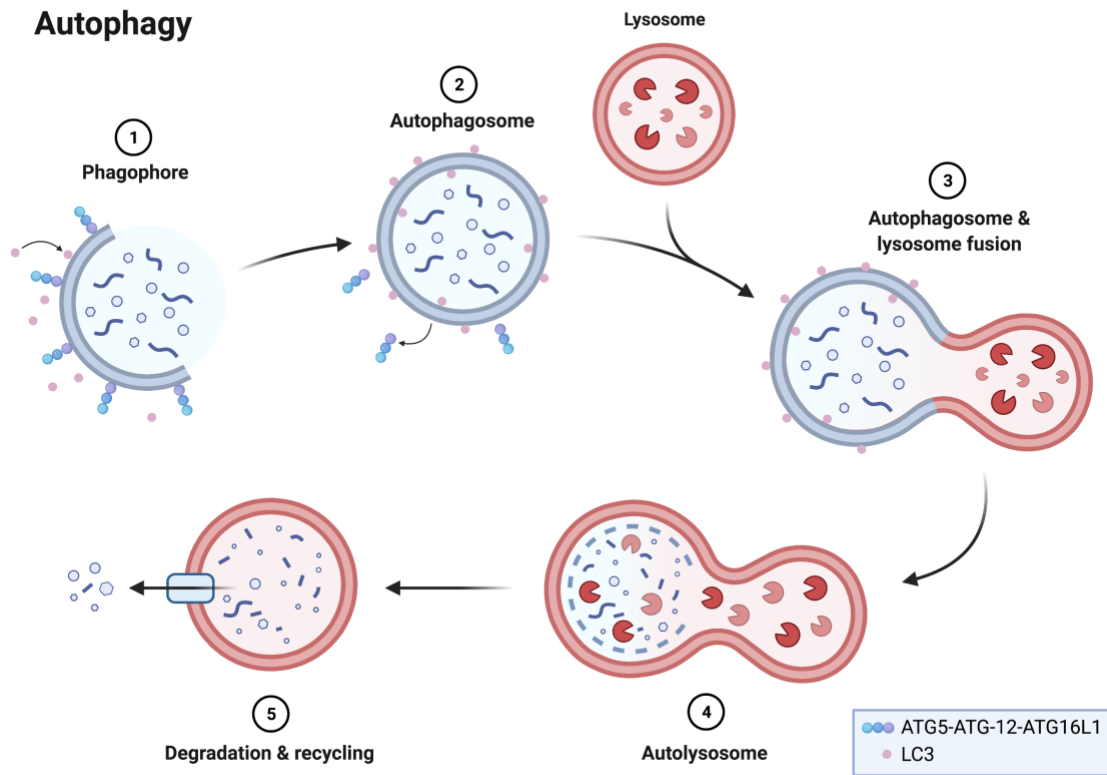


Figure 1.3: The autophagy pathway

Autophagy is key in maintaining cell and tissue homeostasis. Autophagosome formation is dependent on the autophagy-related (ATG)-5-ATG12-ATG16L1 and microtubule-associated light chain 3 (LC3) complexes. (1) The process begins with a membranous crescent, or phagophore, that begins to encapsulate the cytosolic components targeted for degradation. As the membrane elongates, cytosolic LC3 (termed LC3I) is conjugated to the forming autophagosome (once bound, it is termed LC3II). In the later stages of elongation, the ATG5-ATG12-ATG16L1 complex disassociates. (2) The membrane fully encloses the materials into a double-membrane bound autophagosome. (3) Autophagosome and lysosome fusion occurs, resulting in autolysosome formation. (4) Hydrolytic enzymes originating from the lysosome degrade the contents of the compartment (5) The contents are degraded or recycled, and the process of autophagy is complete.

functioning of the gut barrier and predispose to IBD (Turpin et al. 2018). For example, *FUT2* and *MUC19* are IBD risk genes that encode proteins involved in maintenance of the mucus layer (Rivas et al. 2012; McGovern et al. 2010). The mucus barrier serves to physically separate the gut microbes from host epithelial cells and these predisposing mutations likely enhance IBD risk by increasing microbial interaction with host tissues (Graham and Xavier 2013). Another risk gene associated with impaired barrier function is *XPB1* which regulates the cellular response to endoplasmic reticulum (ER) stress, and its proper function is required to maintain the health of epithelial cells (Kaser et al. 2008). While proper functioning of innate immunity is crucial to barrier maintenance and intestinal homeostasis, mutations in genes involved in adaptive immunity can also enhance risk of IBD.

There have been many IBD risk loci involved in adaptive immunity that are linked to IBD pathogenesis. For example, there have been multiple associations of components of the *IL23R* signaling pathway with IBD (Duerr et al. 2006). The *IL23R* gene encodes the IL-23 receptor complex which initiates a signaling cascade upon binding of its ligand, the pro-inflammatory cytokine IL-23 (Abraham and Cho 2009a). Once bound, the complex activates JAK2, and subsequently activates signal transducer and activator of transcription (STAT)-3 and STAT4 (Abraham and Cho 2009a). The result is the transcription of pro-inflammatory cytokines, which then promote the differentiation of CD4+ T cells into pro-inflammatory Th17 cells (Abraham and Cho 2009a). There are several protective single nucleotide polymorphisms (SNPs) located within *IL23R*, such as the R381Q variant which is a loss-of-function mutation (Parkes et al. 2007). The result of this SNP is attenuation of STAT3 and STAT4 activation to ultimately reduce the production of pro-inflammatory cytokines and Th17 cells (Abraham and Cho 2009a). Interestingly, *JAK2* and *STAT3* have been independently identified as IBD risk loci, highlighting the importance of this pathway in IBD pathogenesis (Barrett et al. 2008; Franke et al. 2008). A more recent IBD genetic study performed by de Lange et al. (2017) highlighted the involvement of multiple integrin genes in IBD, including *ITGA4*, *ITGAV*, *ITG8*, and *ITGAL*. Integrins are cell adhesion mediators that have a critical role in regulating leukocyte signaling, proliferation, and migration (Hynes 2002). The risk alleles were predicted to increase expression of their respective genes, identifying

enhanced integrin expression as a potential novel pathway linked to IBD pathogenesis, although functional analyses are required to confirm this association (de Lange et al. 2017). The identification of causal genes and variants and subsequent analyses to implicate the associated pathways have transformed the understanding of IBD pathogenesis.

Genetic studies have been instrumental in advancing our understanding of IBD pathogenesis and in developing new treatment options for patients. A genetic component of IBD was hypothesized for decades prior to the discovery of the first IBD risk gene, *NOD2*, which will be discussed in depth in the next section. In the 20 years that have passed since the identification of *NOD2* as a CD risk gene, the field has advanced substantially, with increased access to research technologies and high-throughput platforms that have allowed for the discovery of many more risk genes. However, there are still many research questions left to be answered. The priority of IBD genetic research presently is to continue elucidating the mechanisms of the predisposing SNPs so that more treatment options, and perhaps prevention measures, can be developed.

1.3.5 The role of *NOD2* in IBD

NOD2 was the first IBD susceptibility gene discovered and remains the most strongly correlated with disease risk. The *NOD2* gene, which encodes the nucleotide oligomerization domain-containing protein 2 (*NOD2*), was associated with CD risk in 2001 (Hugot et al. 2001; Ogura, Bonen, et al. 2001). There have since been several casual variants identified which reduce or eliminate function of the *NOD2* protein (Ogura, Bonen, et al. 2001; Ahmad et al. 2002). However, harbouring a *NOD2* mutation is not sufficient or necessary for the development of CD. It is estimated that almost 14% of the healthy Caucasian (non-Jewish) population carries a mutant *NOD2* allele, compared to 30-40% of CD patients (Ahmad et al. 2002; Economou et al. 2004). Individuals that have a copy of one of the three major common alleles have a 2- to 4-fold increased risk of developing CD (Economou et al. 2004). Individuals who are homozygous or compound heterozygous carriers have a 15- to 40-fold increase in risk

(Economou et al. 2004). Since the identification of *NOD2* as a CD risk gene, there has since been great interest in elucidating its function and exploring its role in maintaining gut epithelial homeostasis.

The NOD2 protein has an important role in regulating inflammation in the intestinal epithelium. It is expressed in haematopoietic cells of both myeloid and lymphoid lineage (Ogura, Inohara, et al. 2001; Gutierrez et al. 2002) and in intestinal epithelial cells (Gutierrez et al. 2002; Hisamatsu et al. 2003), including in Paneth cells (Ogura et al. 2003). Within these cell types, the cytosolic NOD2 protein acts as an intracellular sensor of bacteria. It recognizes muramyl dipeptide (MDP), a component of the bacterial cell wall molecule peptidoglycan, and triggers protective inflammatory and autophagic responses (Girardin et al. 2003). The NOD2 protein has a tripartite structure composed of tandem amino-terminal caspase-recruitment domains (CARDs), a central nucleotide-binding domain (NBD), and a series of carboxy-terminal leucine-rich repeats (LRRs) (Yasunori Ogura, Inohara, et al. 2001). Residues within the LRR are essential to the sensing of MDP by NOD2 (Tanabe et al. 2004). Recognition of MDP by the LRR domain leads to a conformational change and oligomerization of NOD2 proteins, which self-associate through the NBD (Inohara et al. 2000). Through an induced-proximity model, receptor-interacting protein 2 (RIP2) is recruited, forming a large signaling platform, and is activated to drive nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK)-dependent inflammatory gene transcription (Inohara et al. 2000; Ogura, Inohara, et al. 2001; Sorbara et al. 2013; Philpott et al. 2014). Upon sensing of MDP, the NOD2 protein also recruits autophagic machinery to the site of bacterial entry by targeting of the ATG16L1 protein (**Figure 1.4**) (Travassos et al. 2010). While this specific function of NOD2 is independent of RIP2 (Travassos et al. 2010), NOD2 also boosts basal autophagy by signaling through RIP2 to activate p38, extracellular signal-regulated kinase 1 (ERK1), ERK2, and UNC-51-like kinase 1 (ULK1) to promote autophagosome formation (Homer et al. 2012). These inflammatory and autophagic responses triggered by sensing of MDP by NOD2 allow for the cell to properly handle the potential pathogenic threat. The NOD2 protein is therefore important in maintaining homeostasis in the gut.

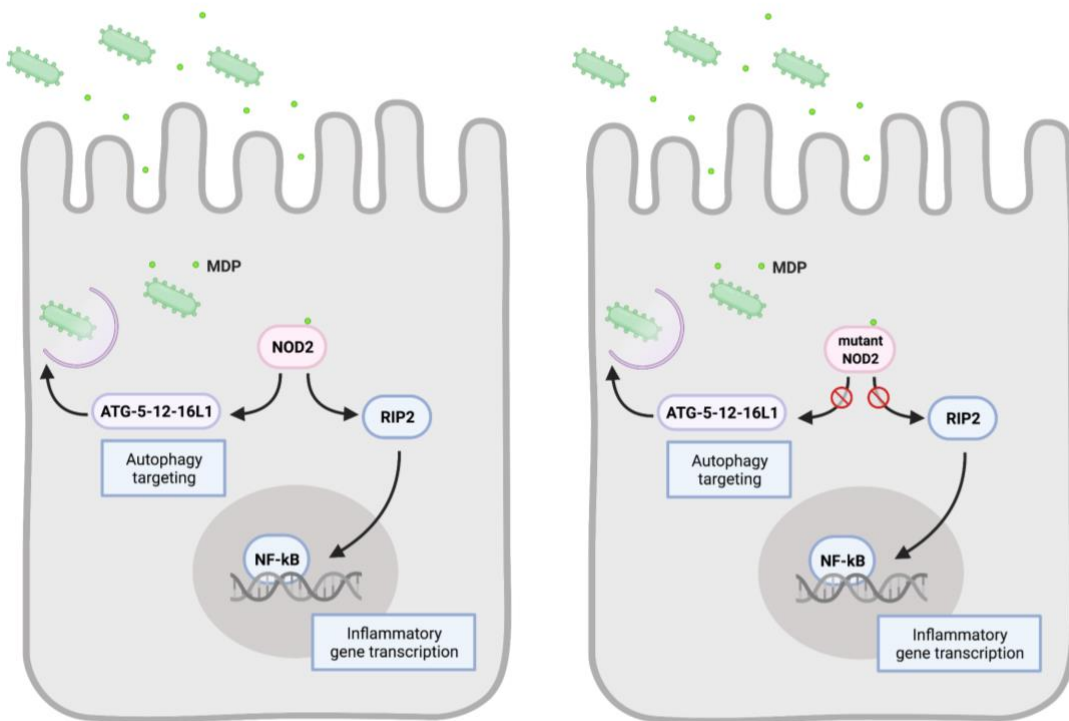


Figure 1.4: NOD2 signaling in the intestinal epithelium

Left: Upon detection of muramyl dipeptide (MDP), nucleotide oligomerization domain-containing protein 2 (NOD2) signals through receptor-interacting protein 2 (RIP2) to induce nuclear factor- κ B (NF- κ B)-dependent transcription of inflammatory mediators. Independent of RIP2, the NOD2 protein also directs autophagy machinery to the site of bacteria entry at the plasma membrane. Right: Mutant NOD2 no longer responds to MDP stimulation and the inflammatory gene transcription and autophagy targeting functions are ablated. Adapted from Philpott et. al (2014) with permission.

The use of mouse models deficient in Nod2 have enhanced understanding of the underlying mechanisms surrounding its protective role within the intestinal epithelium. Importantly, Nod2 deficiency alone does not lead to spontaneous colitis in mice, supporting the multi-hit hypothesis of CD development which highlights the importance of the convergence of genetic predisposition and environmental triggers in disease onset (Philpott et al. 2014). Early studies using *Nod2* knockout (KO) mice demonstrated that absence of the Nod2 protein increases susceptibility to chemical (Barreau et al. 2007), antigenic (Watanabe et al. 2006), and enteropathogenic (Biswas et al. 2010) models of colitis. In these studies, mice lacking Nod2 exhibited excessive intestinal inflammation in response to colitic agents, in comparison with wildtype mice exposed to the same stimulus (Rubino et al. 2012). To further explore the protective effect of Nod2 expression, Watanabe et al. (2008) performed an experiment in which wildtype mice were primed with peptidoglycan or MDP prior to exposure to trinitrobenzene sulfonic acid (TNBS)- and dextran sodium sulfate (DSS)- mediated colitis. Mice that received the Nod2 ligands as a pre-treatment exhibited protection from experimental colitis (Watanabe et al. 2008). The authors characterized intestinal changes amongst the mice exposed to the Nod2 ligands prior to TNBS colitis exposure and identified enhanced CD103+ dendritic cells and Foxp3+CD4+ Treg numbers in the mesenteric lymph nodes, along with increased expression of anti-inflammatory cytokine IL-10 in the colon (Watanabe et al. 2008). Together, these studies suggest that the basal stimulation of Nod2 induces a tolerogenic response that primes the intestinal epithelium to handle potential pathogenic threats.

The NOD2 protein, which is a component of the innate immune system, has a crucial role in the intestine of balancing tolerogenic responses to commensal microbes and protective responses to pathogenic microbes. At homeostasis, there is a baseline level of NOD2 activation which maintains tolerance. NOD2 is activated not only by invading bacteria, but also by free peptidoglycan fragments that have been acquired by other means, such as routine endocytosis (Philpott et al. 2014). The proximity of the microbiota to the NOD2-expressing epithelium causes baseline activation of the RIP2-NF- κ B axis, leading to the production of antimicrobial peptides and mucins that help to maintain a physical separation between the microbiota and the epithelium

(Philpott et al. 2014). Tonic stimulation also induces a Th17 response which protects the epithelial barrier by inducing secretion of IL-22 and regenerating islet-derived protein III γ (RegIII γ) (Geddes et al. 2011). There is also induction of CC-chemokine ligand 2 (CCL2) which recruits Ly6C^{hi} monocytes for barrier surveillance (Kim et al. 2011). Together, this leads to a functional epithelium that is primed to respond to bacterial threats. If the MDP sensing function is lost, as is the case with mutant NOD2, this protective program induced by the tonic stimulation of NOD2 is also lost (Philpott et al. 2014). This renders the epithelium susceptible to damage by a perturbation which in most cases remains unknown but can include antibiotics, dysbiosis, or an infection (Philpott et al. 2014). When this perturbation occurs, the result is a hyperactive compensatory immune response, which can lead to chronic inflammation as is seen in CD (**Figure 1.5**) (Philpott et al. 2014). The factor that is responsible for initiating the breakdown of the intestinal barrier and the NOD2-induced inflammatory cascade remains unknown.

Whether or not NOD2 influences the composition of microbiome has been a topic of debate within the field. There is high expression of NOD2 in Paneth cells, implicating a role for NOD2 in Paneth cell responses (Ogura et al. 2003). This presents a mechanism in which NOD2 could regulate the secretion of antimicrobial peptides and alter the microbiome. Some studies have identified alterations in the microbiome with a *Nod2* KO model, while other studies report no change (reviewed in Philpott et al. 2014). It appears as though studies which employ proper littermate and co-housing techniques find no association between *Nod2* expression and microbiome composition in mice (Philpott et al. 2014). This highlights a need for standardization of experimental practices in the field, so that replicable conclusions can be drawn within and between laboratories. Using littermates and co-housing is the gold standard for mouse experimental studies (Robertson et al. 2019), and so from this we can conclude that it is not likely that NOD2 has an effect on the composition of the microbiome. This conclusion fits with the literature surrounding NOD2 and the requirement of environmental perturbations to drive the chronic inflammatory cascade.

Although a genetic basis of IBD was postulated for decades, the identification of *NOD2* as the first IBD susceptibility gene was the beginning of an era of exponential learning about the pathogenesis of IBD. Mechanistic studies in murine models allowed for the

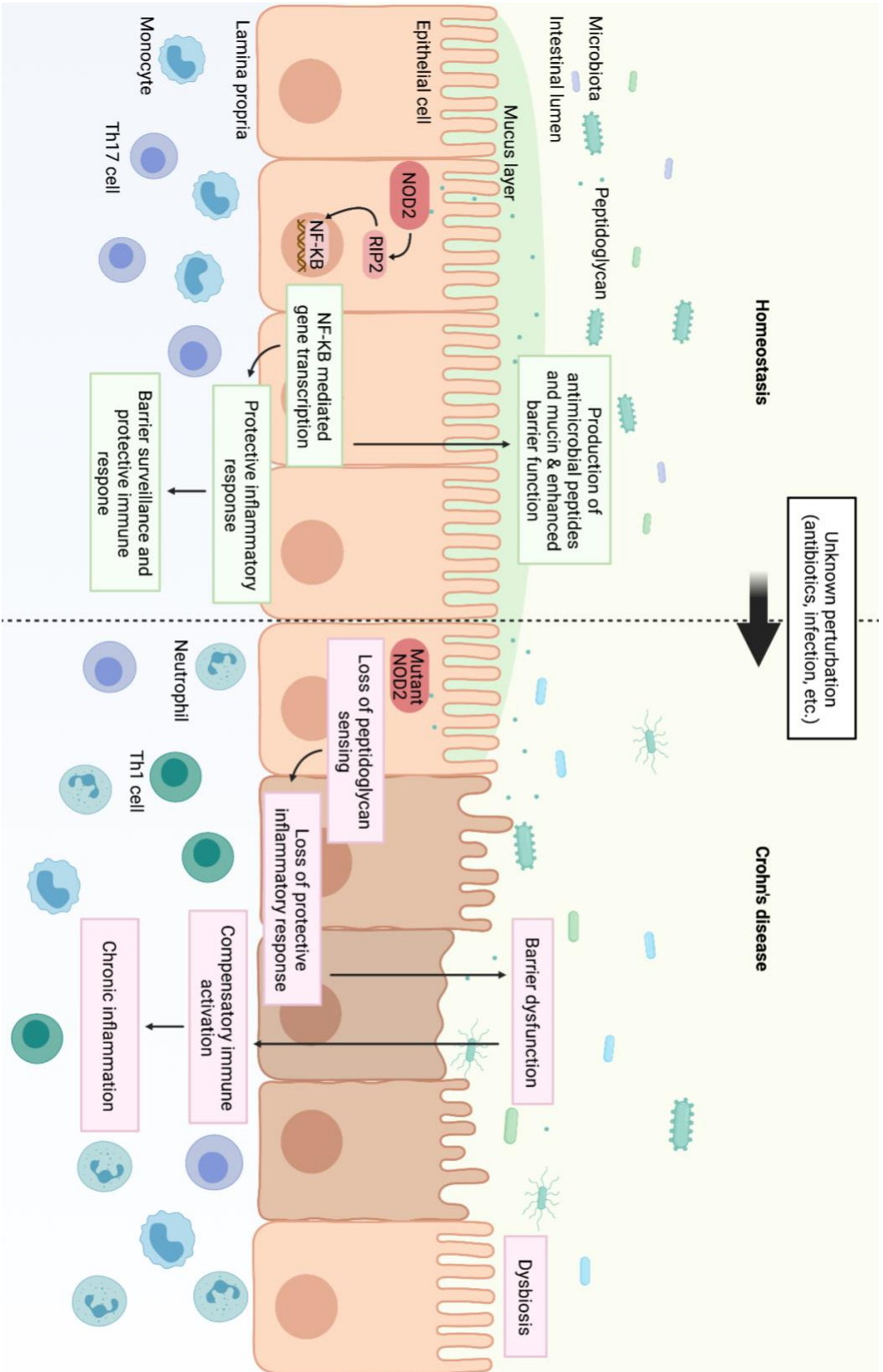


Figure 1.5: NOD2 in intestinal homeostasis and Crohn's disease

The peptidoglycan sensing capacity of nucleotide oligomerization domain containing protein 2 (NOD2) is important in maintaining intestinal homeostasis. Left: Sensing of microbiota-derived muramyl dipeptide (MDP) by NOD2 leads to a protective inflammatory response through receptor-interacting protein 2 (RIP2)-dependent nuclear factor- κ B (NF- κ B) gene transcription. Expression of antimicrobial peptides and mucins is enhanced which allow for maintenance of the mucus layer that physically separates the microbiome and host epithelial cells. Additionally, a protective immune response is established which involves an early Th17 response and chemokine-induced recruitment of LY6C^{hi} monocytes to promote barrier surveillance. Right: With loss-of-function mutations in NOD2, such as those that predispose to Crohn's disease (CD), mutant NOD2 loses its ability to sense MDP and trigger the protective inflammatory program. When an unknown perturbation occurs, which might include antibiotics or infection, mutant NOD2 contributes to barrier breakdown, dysbiosis, and a hyperactive compensatory immune response. The immune response includes recruitment of neutrophils and Th1 cells, which are hallmarks of the chronic inflammation observed in CD. Adapted from Philpott et al. (2014) with permission.

understanding of the role of Nod2 at homeostasis and explore how inactivating mutations may increase risk of CD. Concomitantly, further susceptibility genes were being discovered and studied. Genetic predisposition is necessary for the development of IBD in an individual, however research surrounding individual susceptibility genes highlights the requirement for other triggers for IBD onset to occur. Thus, studying compounding effects such as gene-environment interactions have been a recent focus of IBD research.

1.3.6 Environmental contributions

The sharp increase in incidence in the western world following the industrial revolution, together with the emergence of IBD in developing countries as they become 'westernized', suggest an especially important role for environmental factors in IBD onset (Kaplan 2015; Windsor and Kaplan 2019). Westernization encompasses the shift toward enhanced sanitation practices and establishment of urban centres, as well as diet, lifestyle, and behavioural changes (Kaplan and Ng 2016). Such practices affect the composition and function of the gut microbiome and have been implicated in the development of many immune-mediated diseases that are prevalent in western society (Ananthakrishnan et al. 2018). The gut microbiome has a central role in mediating inflammation in IBD, and many environmental factors associated with IBD act through the microbiome to modify disease risk. Understanding how environmental triggers accumulate throughout life and can lead to disease in genetically susceptible individuals is an important area of research. Knowledge of which factors influence IBD onset will allow individuals to reduce their risk, and the specific disease mechanisms behind environmental contributions will be key to developing new treatment options. Early life factors that shape the microbiome can modify risk of IBD later in life.

Early life factors have a substantial role in both shaping the microbiome and modulating risk for the development in IBD in childhood or later in life. The gut microbiome in early childhood is unstable and susceptible to perturbations that can affect its composition and function throughout life (Arrieta et al. 2014; Ananthakrishnan et al. 2018). Factors that affect the microbiome during this critical period can influence the development of

many diseases, including IBD (Arrieta et al. 2014; Ananthakrishnan et al. 2018). A meta-analysis conducted by Xu et al. (2017) concluded that a history of being breastfed is linked to reduced risk of both CD and UC. Additionally, there is a dose-dependent effect of breastfeeding with the strongest decrease in risk identified in those breastfed for a minimum of 12 months, as compared to 3 or 6 months (Xu et al. 2017). In CD, there was also an effect of ethnicity, with a stronger association amongst Asian compared to Caucasian participants (Xu et al. 2017). There are several mechanisms that have been proposed for the protection conferred by breastmilk, including modulation of the infant microbiome to promote oral tolerance, prevent infections, and maintain gut barrier integrity, in addition to direct immune effects (Xu et al. 2017). The protection of breastfed infants from infections may be two-fold, as antibiotic use increases risk of IBD.

Exposure to antibiotics can dramatically impact the composition of the gut microbiome and have long-lasting effects including increased risk for IBD, especially when administered early in life. A nested case-control study conducted by Shaw et al. (2010) demonstrated that individuals diagnosed with paediatric IBD are more likely to have received antibiotics in their first year of life compared to healthy control subjects. In this cohort, having received at least 1 course of antibiotics increased the odds of a paediatric IBD diagnosis by approximately 3-fold (Shaw, Blanchard, and Bernstein 2010). Similarly, a population-based cohort study by Kronman et al. (2012) concluded that exposure to antibiotics early in life was associated with a childhood IBD diagnosis. The association was strongest with the earliest introduction of antibiotics, with exposure before 1 year of age having the largest magnitude of effect (Kronman et al. 2012). Moreover, there was a dose-dependent effect, with receipt of more than 2 antibiotic dispensations being the most strongly associated with an IBD diagnosis (Kronman et al. 2012). A meta-analysis exploring the relationship between antibiotics and IBD by Ungaro et al. (2014) revealed that antibiotic exposure was associated with increased risk of CD but not UC. The association was stronger in paediatric CD compared to adult-onset CD (Ungaro et al. 2014). Taken together, these results suggest that antibiotic exposure increases risk for IBD in a manner that is dose-dependent and more severe with earlier exposure. Antibiotics reduce richness, diversity, and evenness of the

microbiome (Dethlefsen et al. 2008). The effects of antibiotics are extreme, with the shift occurring in 3 to 4 days after beginning treatment (Dethlefsen and Relman 2011). The microbiota begins to recover shortly after the course is finished, although return to the initial state is often incomplete (Dethlefsen and Relman 2011). The link between IBD and factors affecting the microbiome, such as antibiotic use, are of importance. The environment in which a child is raised can also modulate the microbiome and alter IBD risk.

The related factors of living in an urban area and exposure to pollution also enhance IBD risk. A systematic review and meta-analysis conducted by Soon et al. (2012) identified an association between living in an urban environment and risk of both CD and UC (Soon et al. 2012). This is a robust finding that has been demonstrated in several geographic areas and across ethnicities. For example, a study by Ng et al. (2013) which included 13 countries or regions in Asia-Pacific found that living in a densely populated area was associated with an increased incidence of IBD (Ng et al. 2019). There are many factors which could explain the correlation between IBD incidence and urbanization. One theory is that people living in urban centres have more exposure to pollution than people living in rural areas. Indeed, Elten et al. (2020) established that ambient air pollution is associated with paediatric IBD (Elten et al. 2020). In addition, the same group recently demonstrated that higher exposure to greenspace during childhood is associated with a reduced risk of paediatric IBD, which could be a confounding variable to urban dwelling and exposure to pollution (Elten et al. 2021). These studies suggest that factors impacting a child's physiology as they develop can put them at risk for developing IBD. In addition, many environmental risk factors for IBD have a dose-dependent effect, in which earlier and enhanced exposure to these factors can prime IBD onset at an earlier age. There are many other confounding lifestyle factors that could accompany the association between urbanization and modernization of societies and IBD risk, several of which have been independently associated with IBD.

Lifestyle factors such as cigarette smoking and dietary choices influence IBD risk. Cigarette smoking has long been associated with a more severe clinical course of CD (Cosnes et al. 1996; Cosnes et al. 1999). A meta-analysis performed by Higuchi et al.

(2012) explored the association between cigarette smoking and risk of developing IBD and revealed an interesting finding. There is an increased risk of CD in both current and former cigarette smokers. Former smokers are also at increased risk of developing UC; however, current smoking actually offers protection from UC (Higuchi et al. 2012). The differing effects of smoking on CD and UC highlights their distinct etiologies and the complexities in studying IBD pathogenesis. An additional lifestyle factor that can modulate risk of IBD is diet. A case-control study including 3 paediatric gastroenterology clinics across Canada identified an inverse association between risk of CD and consumption of healthy foods such as vegetables and fruits (Amre et al. 2007). Furthermore, a prospective cohort study tracked fibre intake of participating women and revealed that women with the highest quintile of dietary fibre intake were 40% less likely to develop CD compared to women in the lowest quintile of fibre intake, and the contribution of fibre from fruit had the strongest association (Ananthakrishnan et al. 2013). There was no association between fibre intake and risk of UC (Ananthakrishnan et al. 2013). Specific nutrients, such as zinc and vitamin D, also have a role in IBD pathogenesis. Low zinc has been shown to increase the risk of developing CD but not UC (Ananthakrishnan et al. 2015). Low zinc levels in IBD patients have been associated with an increased risk of hospitalizations, surgeries, and disease-related complications in both CD and UC (Siva et al. 2017). Zinc has an immunomodulatory role and aids in processes such as wound repair and tissue regeneration, and normalization of zinc levels corrects the adverse outcomes associated with deficiency (Siva et al. 2017). Similarly, vitamin D deficiency has been implicated in IBD, and will be discussed in detail in the next section. It is clear that diet has a role in the risk and outcomes of IBD, and dietary patterns associated with western culture function to enhance risk of IBD. There have been several other factors associated with IBD pathogenesis, including use of non-steroidal anti-inflammatory drugs (NSAIDs) and appendectomies.

Medical factors such as use of NSAIDs and history of appendectomy also influence IBD risk. A prospective cohort study which tracked NSAID use in women found that women who use NSAIDs at least 15 days per month had an increased risk of IBD compared to non-users (Ananthakrishnan et al. 2012). Furthermore, use of NSAIDs in individuals with established IBD leads to earlier relapse when compared to non-users with IBD

(Takeuchi et al. 2006; Long et al. 2016). The use of NSAIDs is common in both the general population and amongst IBD patients and their role in enteropathy is an area of research (Takeuchi et al. 2006). History of appendectomy has an interesting relationship with IBD risk. An early population-based study demonstrated that individuals who undergo appendectomy prior to 20 years of age have a low risk of developing UC (Andersson et al. 2001). A possible mechanism has been proposed, which implicates the appendix as a priming site for inflammation due an increased CD4/CD8 ratio and infiltration of CD4+CD69+ T cells (Matsushita et al. 2005). Conversely, a meta-analysis performed by Kaplan et al. (2008) identified an increase in risk of developing CD after appendectomy (Kaplan et al. 2008). The differential effect of appendectomy on risk of CD and UC serves a reminder of the nuances in pathogenesis between the IBDs. Exploring the mechanisms of IBD environmental risk factors to reveal how they contribute to inflammation and augment risk of either CD or UC will enhance understanding of the individual diseases.

Based on the body of literature, it is clear that genetics do not account for the full risk of IBD, and the contribution of environmental factors are extremely important to consider (**Figure 1.6**). Identifying factors contributing to IBD is imperative so that at-risk individuals, such as those with a family history of IBD, can control their exposure and possibly prevent IBD or modify disease course. Using cell and molecular biology to elucidate the mechanisms involved in the increased risk incurred by environmental factors in IBD pathogenesis and exploring gene-environment interactions are important strategies to uncover both mechanisms of pathogenesis and to identify new therapeutic targets of interest and treatment strategies.

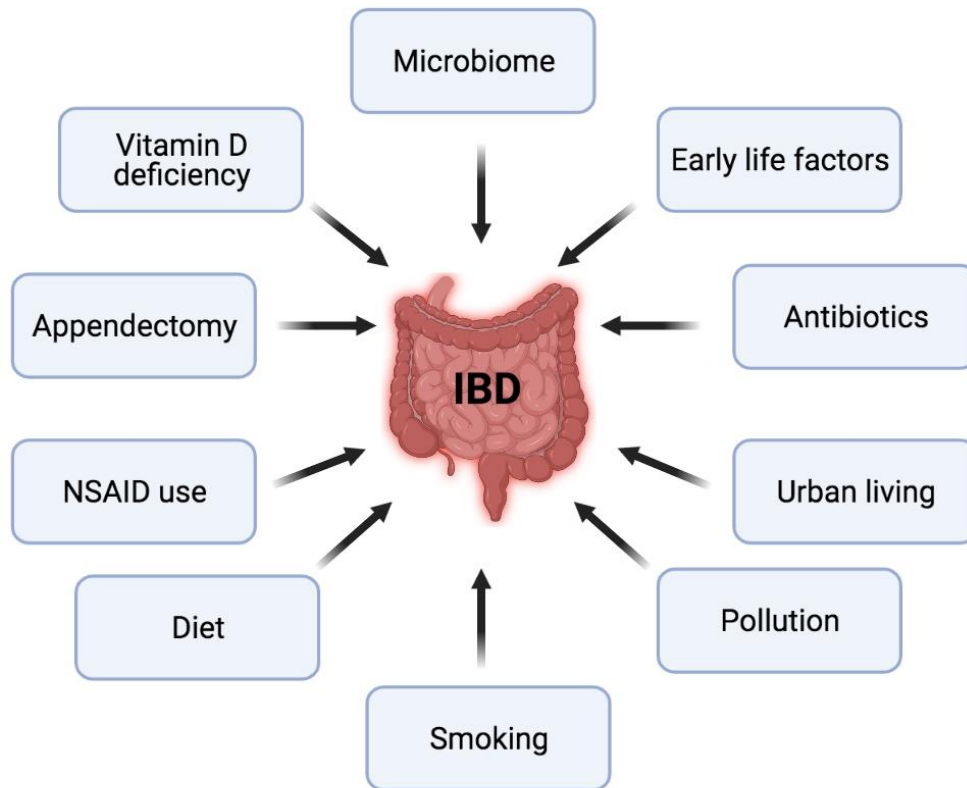


Figure 1.6: Environmental factors involved in IBD pathogenesis

Changes to the microbiome, early life factors (such as breastfeeding), use of antibiotics, exposure to urban environments and pollution, cigarette smoking, dietary choices, use of non-steroidal anti-inflammatory drugs (NSAIDs), appendectomies, and vitamin D deficiency are some of the environmental factors that can influence the onset of Crohn's disease and ulcerative colitis. Adapted from Ananthakrishnan et al. (2018) with permission.

1.3.7 The role of vitamin D deficiency in IBD

The geographical gradient of IBD prevalence in which rates of IBD are higher in regions farthest from the equator mirrors sunlight exposure and thus vitamin D levels, implicating vitamin D deficiency as a potential environmental factor involved in IBD pathogenesis (Armitage et al. 2004; Nerich et al. 2006; Khalili et al. 2012). Accordingly, it has been demonstrated that vitamin D deficiency is more common among IBD patients compared to the general population. Although there is variation in vitamin D levels according to season and skin tone, approximately one third of the healthy Canadian population has vitamin D deficiency, defined as <50 nmol/L (Janz and Pearson 2015). The proportion of IBD patients at a similar latitude that have vitamin D deficiency is closer to two thirds (Suibhne et al. 2012; Chatu et al. 2013). There are several possible explanations to account for the higher rates vitamin D deficiency in IBD patients, including impaired absorption due to intestinal pathology, restricted diet, less sunlight exposure, or genetic differences in the vitamin D pathway (Fletcher 2016). The increased rate of vitamin D deficiency in the IBD population suggests that vitamin D levels correlate with risk of IBD onset, however; the association has additional implications. Patients with vitamin D deficiency also tend to have worse disease outcomes compared to patients who are sufficient, indicating an association between vitamin D levels and IBD severity (Garg et al. 2013; Hassan et al. 2013; Jørgensen et al. 2013; Kabbani et al. 2016; Frigstad et al. 2017). A study by Kabbani et al. (2016) demonstrated that vitamin D deficient IBD patients experience increased emergency department visits and hospital admissions, require more pharmaceuticals to control their disease, and have increased rates of bowel resection surgery (Kabbani et al. 2016). In order to understand the potential basis for increased risk and severity of IBD with vitamin D deficiency, it is important to consider the vitamin D pathway.

The cellular actions of vitamin D are extremely important not only for skeletal health, but for extra-skeletal actions that influence human physiology. Exposure to solar ultraviolet B (UVB) radiation is the predominant source of vitamin D in humans (Holick 2007). The skin has a precursor, 7-dehydrocholesterol, which when exposed to UVB radiation is converted to previtamin D₃, which is then rapidly converted to vitamin D₃ (Holick 2007). Alternatively, vitamin D can be obtained through the diet, though its sources are limited.

Vitamin D is naturally present in mushrooms and fatty fish, and certain foods are fortified with vitamin D, such as dairy products (Holick 2007). Oral supplements are also a common source of vitamin D (Holick 2007). Once vitamin D enters the bloodstream, whether obtained through UVB exposure or the oral route, it is metabolized in the liver (Holick 2007). The liver enzyme vitamin D 25-hydroxylase converts vitamin D to 25-hydroxyvitamin D (Holick 2007). This is the stable circulating form, and the form that is measured when vitamin D levels are assessed (Holick 2007). However, this is not the final or active form of vitamin D. The enzyme 25-hydroxyvitamin D 1 α hydroxylase (*CYP27B1*) metabolizes 25-hydroxyvitamin D into its active form, 1,25-dihydroxyvitamin D, also called calcitriol (Holick 2007). This activating enzyme is expressed in the kidneys, and renal production of calcitriol is tightly controlled by parathyroid hormone and serum calcium and phosphorus levels (Holick 2007). However, it has been discovered that this enzyme is present in peripheral cell types, including intestinal epithelial and immune cells, allowing for local production of calcitriol (Abreu et al. 2004; Du et al. 2017). The vitamin D receptor (VDR) has a high level of expression in these cell types, which means that vitamin D can have local, non-skeletal actions in these tissues (Kongsbak et al. 2013). The VDR is a transcription factor that is activated upon binding of calcitriol (**Figure 1.7**) (Kongsbak et al. 2013). Calcitriol enters the cell and binds the cytosolic VDR. Once bound, vitamin D-VDR forms a complex with the retinoid X receptor and translocates into the nucleus (Kongsbak et al. 2013). The complex binds vitamin D response elements (VDREs) on DNA and can alter transcription of a wide range of genes involved in metabolism and immunity (Kongsbak et al. 2013). In the intestine, vitamin D and VDR are important in controlling many aspects of the immune response.

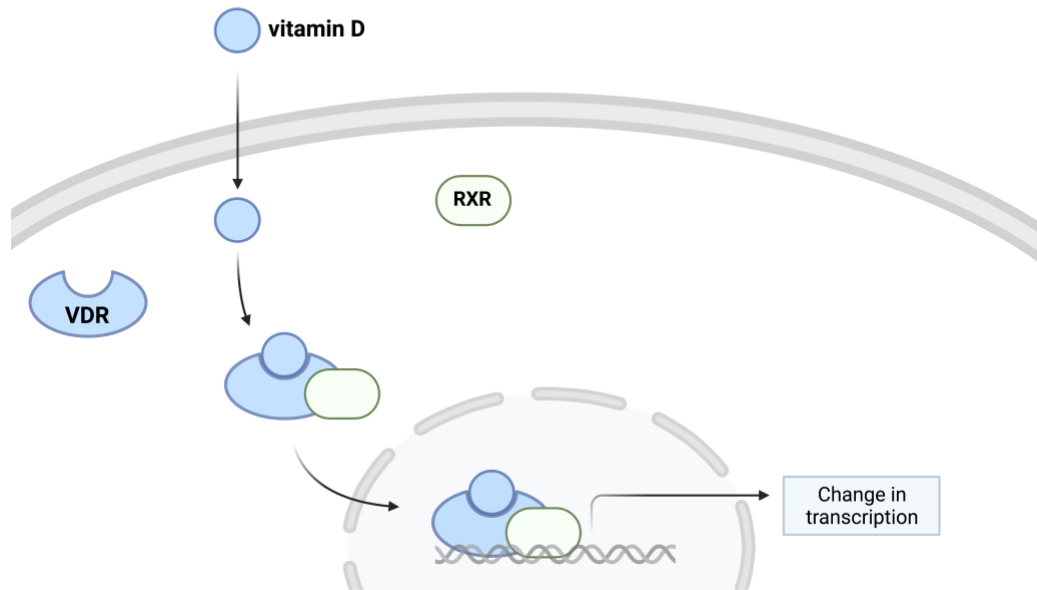


Figure 1.7: Vitamin D signaling pathway

The active form of vitamin D, 1,25-dihydroxyvitamin D (also called calcitriol), enters the cell and binds the cytosolic vitamin D receptor (VDR). The VDR heterodimerizes with the retinoid X receptor (RXR). This complex can translocate into the nucleus and bind vitamin D response elements (VDREs) on DNA to influence gene transcription.

Vitamin D and VDR have substantial control over both innate and adaptive immunity in the gut. Vitamin D regulates gut barrier function, an essential component of the innate immune system. A study conducted by Kong et al. (2007) sought to explore the effect of vitamin D on the intestinal epithelium. The human colon cancer cell line Caco-2 was treated with calcitriol and expression of crucial barrier proteins was measured. Treating the intestinal epithelial cells with calcitriol enhanced expression of tight junction protein zona occludin-1 and adherens junction protein E-cadherin (Kong et al. 2007). Knockdown of the VDR by small interfering RNA ablated this effect, suggesting that vitamin D signals through the VDR to maintain barrier integrity, and suggests that vitamin D deficiency may predispose to barrier dysfunction to enhance IBD risk (Kong et al. 2007). In terms of adaptive immunity, vitamin D and the VDR have several effects. Although *VdrKO* mice have normal numbers of CD4+, CD8+, and CD4+FoxP3+ regulatory T cells, they exhibit enhanced immune reactivity (Froicu et al. 2003; Yu et al. 2008). This was found to be mediated by the impaired development of two types of Tregs, invariant natural killer T cells (iNKT) (Yu and Cantorna 2008) and CD8 $\alpha\alpha$ T cells (Bruce and Cantorna 2011). iNKT cells have an important regulatory role in autoimmunity and infection (Godfrey et al. 2000), while CD8 $\alpha\alpha$ T cells aid in maintaining tolerogenic responses in the gut (Yu and Cantorna 2008). Furthermore, Bruce et al. (2011) discovered that the CD4+ T cells of *VdrKO* mice had an activated phenotype and readily developed into proinflammatory Th17 cells overproducing IL-17 (Bruce et al. 2011). These functions of vitamin D and VDR in regulating innate and adaptive immunity in the intestine highlight the importance of adequate vitamin D levels in maintaining homeostasis, however, it is also clear how a lack of vitamin D might predispose to chronic inflammation and immune-mediated damage that is present in IBD.

Mouse models are often used in IBD research to elucidate the mechanisms surrounding clinical observations, such as vitamin D deficiency predisposing to IBD. Studies have demonstrated that mice raised on a vitamin D deficient diet from weaning have increased susceptibility to experimental colitis (Lagishetty et al. 2010). Furthermore, mouse models in which the *Vdr* was deleted from the whole body (Froicu et al. 2003) or intestinal epithelial cells specifically (*Vdr^{IEC}*) (Wu et al. 2015) displayed increased

susceptibility to colitis alongside increases in intestinal permeability, an altered microbiome, and Paneth cell abnormalities. The specific mechanisms by which vitamin D deficient signaling may lead to increased susceptibility to colitis remain unclear. Interestingly, *Vdr*^{ΔIEC} mice were found to have reduced levels of Atg16l1 and other autophagy markers (Wu et al. 2015). This suggests that dysregulation of the autophagy pathway may contribute to colitis in this model, which is of interest given that dysfunctional autophagy predisposes to IBD. Although the *Vdr*^{ΔIEC} model has revealed important findings related to the absence of vitamin D signaling in the intestine, these findings require further investigation in the diet-induced vitamin D deficient model, which is more reflective of physiology. It is unclear if intestinal autophagy is impaired through diet-induced vitamin D deficiency, and what mechanisms may be involved. Together, these studies outline that impaired vitamin D signaling predisposes to colitis and offer a potential mechanism of vitamin D deficiency in IBD pathogenesis, though this association requires further exploration. Elucidating the mechanisms of vitamin D deficiency in predisposing to and enhancing colitis in animal models is important to uncovering new therapeutic targets, especially given that simply supplementing vitamin D in IBD patients may not correct the defects associated with deficiency.

The association between low vitamin D levels and increased IBD severity has fostered great interest surrounding the clinical utility of vitamin D supplementation in treating IBD symptoms. However, there are many complexities in evaluating the efficacy of vitamin D supplementation in IBD patients. The first challenge is that cut-off values for vitamin D deficiency and sufficiency are not well defined and vary across studies. Although there are no universally accepted cut-offs, the general consensus is that serum 25-hydroxyvitamin D levels above 75 nmol/L are sufficient (Hanley et al. 2010; Holick et al. 2011). Vitamin D deficiency is commonly regarded as levels less than 50 nmol/L, while levels between 50 and 75 nmol/L have been termed insufficiency (Holick et al. 2011). However, these values were established based on requirements of the skeletal system and do not take into consideration the optimal range for intestinal or immune function, thus the target serum value of IBD patients is unknown (Hanley et al. 2010). There have been several clinical studies evaluating the efficacy of vitamin D supplementation in IBD, which have been reviewed by Gubatan & Moss (2018) and Parizadeh et al. (2019).

Among the studies evaluated, some measured vitamin D levels pre- and post- vitamin D treatment, while others did not. This is an issue because most studies utilize oral supplements, and intestinal absorption of fat-soluble vitamins may vary between patients (Vítek 2015). Additionally, different doses and length of treatment were used. Outcome measures varied across studies, and while some studies only considered symptomatic outcomes, others compared endoscopic measures pre- and post- vitamin D treatment. Additionally, several studies included small sample sizes. With all of these limitations in mind, the outcomes of vitamin D supplementation in IBD patients are inconclusive. Overall, there tends to be a modest positive effect of vitamin D supplementation on IBD outcomes or no change (Gubatan and Moss 2018; Parizadeh et al. 2019). However, there is a need for standardization of experimental procedures and outcome measures across centres so that results can be properly compared. Because there appears to be no harm of vitamin D treatment, clinicians may recommend supplementation to their IBD patients (Nielsen et al. 2019). However, without clear guidelines regarding dosing regimens or the optimal target value, there can be no guarantee of clinical benefit.

The body of literature surrounding the role of vitamin D deficiency in IBD suggests it is important in both pathogenesis and disease outcomes. There are many important functions of vitamin D that contribute to the maintenance of intestinal epithelial homeostasis, although further research is required to determine specifically how vitamin D deficiency interacts with host factors to lead to IBD onset. The autophagy pathway is likely of importance in this association, given that autophagy is independently associated with both impaired vitamin D signaling and genetic polymorphisms predisposing to IBD (Wu et al. 2015). This indicates a potential for gene-environment interactions leading to synergistic dysregulation of innate immunity in the intestine, predisposing to mucosal damage and chronic inflammation. Vitamin D supplementation may be beneficial in IBD patients, although higher quality research is required to determine the proper dose, length of treatment, and target value. Overall, the role of vitamin D deficiency in IBD is of great interest and warrants further investigation.

1.3.8 microRNAs in IBD

An additional factor tied to IBD is the dysregulated expression of microRNAs (miRNAs). miRNAs are short, single-stranded RNA molecules that can bind and inhibit the translation of target messenger RNAs (mRNAs) (Winter et al. 2009). The biogenesis of miRNAs begins with the transcription of a primary miRNA (pri-miRNA) hairpin containing the functional miRNA sequence (Ha and Kim 2014). The pri-miRNA is cleaved by the endonuclease Drosha and its partner DiGeorge critical region 8 (DGCR8), releasing the precursor miRNA (pre-miRNA) (Ha and Kim 2014). The pre-miRNA is transported from the nucleus into the cytoplasm where it is recognized and cleaved by Dicer and TAR RNA binding protein (TRBP), releasing a mature length miRNA duplex (Ha and Kim 2014). The functional strand of the duplex is loaded into the RNA-induced silencing complex (RISC) with the core component Argonaute 2 (AGO2) (Winter et al. 2009). The miRNA guides the RISC to its target mRNAs where it binds to cause mRNA degradation or translational repression (**Figure 1.8**) (Winter et al. 2009). Through this mechanism, miRNAs have the ability to modulate gene expression and influence the onset of many conditions, including IBD (Schaefer 2016). The role of miRNAs in IBD has been a research area of interest given the possible applications for IBD diagnostics and therapeutics.

The aberrant miRNA signatures associated with IBD suggest that miRNAs have a role in IBD pathobiology. Schaefer et al. (2016) conducted a review focused on the differential miRNA expression in intestinal, blood, and saliva samples of IBD patients, revealing almost 50 distinct miRNAs with altered expression in CD and UC tissues (Schaefer 2016). Studies evaluating the altered expression of miRNAs in IBD patient tissues are important given the potential role for miRNAs as biomarkers and therapeutic targets in IBD. A study conducted by Schaefer et al. (2015) proposed a panel of miRNAs that have the potential to be used as biomarkers distinguishing CD from UC based on altered expression in whole blood and saliva samples of patients (Schaefer et al. 2015). Thus, assessing miRNA expression in accessible tissues such as the blood and saliva represent a potential future diagnostic tool in IBD. Additionally, miRNAs are emerging as therapeutic targets with the development of miRNA-blocking oligonucleotide-based therapies. Efficacy of miRNA-blocking therapies in reducing

intestinal inflammation has been demonstrated in several preclinical models of IBD (Soroosh et al. 2018). Although anti-miRNA therapies are not yet available for human use, a landmark study demonstrated the safety and efficacy of an anti-miRNA therapy in the treatment of hepatitis C virus (HCV) in humans (Janssen et al. 2013). A drug targeting miR-122, which is essential for HCV propagation, was tested in a phase 2a clinical trial and was shown to reduce viral RNA levels in a dose-dependent manner with no dose-limiting adverse events (Janssen et al. 2013). This study outlines the potential use of miRNA-targeting therapeutics as a strategy in the treatment of many conditions, including IBD. miRNAs are therefore an exciting area of research that may have future implications in the diagnostics and treatment of IBD.

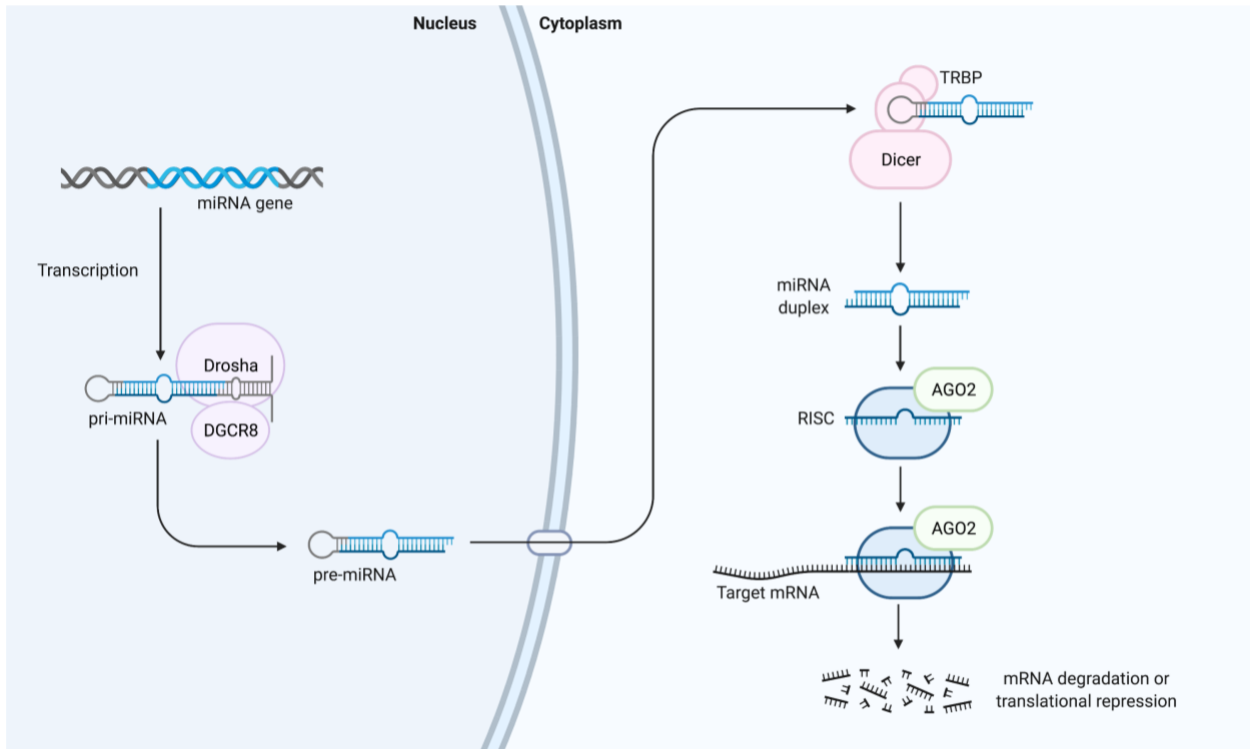


Figure 1.8: microRNA biogenesis and function

microRNA (miRNA) are transcribed as primary miRNA (pri-miRNA) which are long transcripts that form hairpin structures (Ha and Kim 2014). The pri-miRNA is cleaved by the endonuclease Drosha and its essential cofactor DiGeorge critical region 8 (DGCR8), releasing the precursor miRNA (pre-miRNA) (Ha and Kim 2014). The pre-miRNA is exported into the cytoplasm where it is recognized by Dicer and its partner TAR RNA binding protein (TRBP) (Ha and Kim 2014). Dicer cleaves the pre-miRNA into its mature length, leaving a miRNA duplex (Ha and Kim 2014). The functional strand of the duplex is loaded into the RNA-induced silencing complex (RISC) along with Argonaute 2 (AGO2) proteins (Winter et al. 2009). The miRNA will guide the RISC to its target mRNAs where it will bind and cause mRNA degradation or translational repression.

1.4 Gene-environment interactions in IBD

In IBD patients, many factors accumulate to lead to the eventual onset of IBD. It is therefore challenging to recapitulate this complexity in mouse models, which are inherently reductionist, making IBD pathogenesis a challenging topic of research. Despite this challenge, several studies have successfully identified complex gene-environment interactions in murine models. These studies have allowed for the understanding of the mechanisms involved in these regulating these complex interactions.

A study by Cadwell et al. (2010) demonstrated that a common IBD risk variant can be linked to a disease phenotype in mice only when an additional insult is present. Although approximately 50% of people of European ancestry carry the *ATG16L1* T300A CD risk variant, only a small portion will develop disease (Cadwell et al. 2010). This group established a mouse model with hypomorphic expression of *Atg16l1* (*Atg16l1^{HM}*) to mimic the human mutation. Although mice did not display an intestinal phenotype at baseline, when infected with mouse norovirus (MNV), the *Atg16l1^{HM}* mice developed abnormal Paneth cells compared to wildtype mice infected with MNV (Cadwell et al. 2010). *Atg16l1^{HM}* mice also displayed enhanced damage in response to DSS administration, but only in the presence of MNV infection (Cadwell et al. 2010). This study demonstrates that genetic susceptibility primes the intestine for disease onset, but additional factors must be present to cause dysregulation in the epithelium.

An example of how gene-environment interactions can be leveraged to identify therapeutic targets of interest was highlighted in a paper by Liu et al. (2018). In this study, mice homozygous for the *Atg16l1* T300A CD risk variant were exposed to cigarette smoke, an environmental factor that has been associated with CD onset (Liu et al. 2018). Mice with the predisposing *Atg16l1* mutation exposed to cigarette smoke developed Paneth cell abnormalities, while wildtype mice, wildtype mice exposed to cigarette smoke, and *Atg16l1* T300A mice did not exhibit Paneth cell defects. The authors performed transcriptional analysis of Paneth cells and found that the combination of the *Atg16l1* T300A mutation plus smoking triggered multiple changes in the Paneth cells, including selective downregulation of the peroxisome proliferator-

activated receptor γ (PPAR γ) pathway. Providing these mice with a PPAR γ agonist prevented the Paneth cell defects (Liu et al. 2018). This study utilized a murine model of a gene-environment interaction identified in a patient population to discover a relevant therapeutic target of interest.

Although there have been many genetic variants and environmental factors identified that increase risk of IBD, the individual alleles or environmental factors in isolation do not lead to disease onset. A combination of genetic and environmental factors must converge, and additional microbial or immune perturbations will lead to barrier breakdown and inflammation. Therefore, it is important to utilize murine models not just to study individual components predisposing to disease, but to understand the interactions between genetic, environmental, microbial, and immune factors that may lead to disease onset in IBD patients.

1.5 Rationale

IBD is a lifelong condition with no preventive or curative measures available, and treatment options remain limited. The incomplete understanding of IBD is due to its complex pathogenesis, with many predisposing factors accumulating in an individual over their lifetime to eventually lead to disease onset. Research focused on unraveling IBD pathogenesis will enhance understanding of disease mechanisms and lead to the discovery of novel therapeutic targets of interest. A challenge associated with studying IBD is successfully recapitulating the complexity of the condition in research models. Studies conducted in mice that harbour IBD-relevant genetic susceptibility alleles and involve compounding environmental, microbial, and immune insults better replicate the combinatorial nature of IBD and have started to unravel the complexity of IBD onset in the patient population, leading to clinically meaningful discoveries.

In the current study, we began by investigating mechanisms surrounding the intestinal effects of vitamin D deficiency, which is an important environmental factor involved in IBD pathogenesis. Upon recognizing that the intestinal effects of vitamin D deficiency, such as impaired autophagy, overlap with defects resulting from a significant CD risk variant *Nod2fs*, we next decided to study the combination of the two factors. Interestingly, we also had a serendipitous microbial insult through infection with *Trichomonas muris* which contributed to our understanding of the gene-environment interaction between *Nod2fs* and vitamin D deficiency. The overall goal of the study was to explore gene-environment interactions in IBD in order to enhance understanding of disease pathogenesis and identify therapeutic targets of interest.

1.6 Aims & hypotheses

The overarching goal of this study was to explore gene-environment interactions in IBD in order to gain insight into mechanisms of pathogenesis and uncover new therapeutic targets of interest. The specific aims and hypotheses were as follows:

1. To examine the effect of vitamin D deficiency on intestinal autophagy. We hypothesized that physiologic diet-induced vitamin D deficiency in mice leads to impairment of the autophagy pathway through epigenetic regulation by autophagy-targeting miR-142-3p.
2. To explore gene-environment interactions present upon the combination of vitamin D deficiency and the most significant genetic predisposing factor to IBD, *Nod2fs*, which increases risk for CD. We hypothesized that the convergence of vitamin D deficiency and mutant *Nod2*, each of which effect intestinal autophagy, would lead to synergistic dysregulation of autophagy in the intestine. We also noted serendipitous colonization with intestinal protozoan *Tritrichomonas muris* and sought to characterize our gene-environment interaction in the context of this additional microbial stimulus. We hypothesized that persistent infection would enhance defects present in the vitamin D deficient *Nod2fs* mice.
3. To explore the effect of IBD risk genes on the level of circulating vitamin D. Upon observing the novel effect of *Nod2* in regulating serum vitamin D levels, we hypothesized that the effect would be translatable to other IBD susceptibility genes, such as mutant *Atg16l1*, indicating shared mechanisms.

Chapter 2

Elucidating the effect of vitamin D deficiency on intestinal autophagy

This chapter was adapted from:

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2 Vitamin D deficiency, miR-142-3p, and intestinal autophagy

2.1 Introduction

Vitamin D deficiency is an important IBD environmental risk factor involved in both pathogenesis and outcomes. Although it has been established that vitamin D deficiency increases risk of IBD onset and contributes to a more severe disease course, the mechanisms responsible for these changes remain unknown (Garg et al. 2013; Hassan et al. 2013; Jørgensen et al. 2013; Kabbani et al. 2016; Frigstad et al. 2017; Fletcher et al. 2019). Unfortunately, simply correcting vitamin D deficiency in IBD patients is not consistent in reversing the increased disease severity (Gubatan and Moss 2018; Parizadeh et al. 2019). Therefore, it is important to elucidate the mechanisms of vitamin D deficiency using a cell and systems biology approach. This will enhance our understanding of its role in IBD and allow for the identification of potential therapeutic targets of interest.

Recent studies propose that vitamin D deficiency increases IBD risk through impairment of the autophagy pathway (Wu et al. 2015). Autophagy plays a key role in cell and tissue homeostasis and its dysregulation has been linked to inflammation (Deretic and Levine 2018). Genome-wide association studies have implicated the autophagy pathway in the pathogenesis of IBD through the *ATG16L1* T300A mutation (Hampe et al. 2007; Rioux et al. 2007). Normally, *ATG16L1* codes for a protein that has a key role in autophagosome formation (Glick, Barth, and Macleod 2010; B. Levine, Mizushima, and Virgin 2011). Several mechanisms have been proposed to explain the increase in risk incurred by the T300A variant, which enhances degradation of the protein, including reduced autophagy efficiency, altered Paneth cell function and enhanced inflammatory signaling (Cadwell et al. 2008). Interestingly, *Vdr^{ΔIEC}* mice were found to have reduced levels of Atg16l1 and autophagosome marker microtubule-associated protein 1A/1B-light chain 3 II (Lc3II) in the intestinal epithelium, indicating dysregulation of the autophagy pathway in the absence of Vdr signaling (Wu et al. 2015). Although the *Vdr^{ΔIEC}* model has revealed important findings related to the absence of vitamin D signaling in the intestine, these findings warrant further investigation in a model that is

more reflective of physiology, such as the diet-induced vitamin D deficient model. It is unclear as to whether autophagy is impaired through physiologic vitamin D deficiency, and what mechanisms may be involved in its regulation.

A potential link between vitamin D deficient signaling and reduced autophagy is enhanced expression of autophagy-targeting miRNAs. miRNAs are short, single-stranded RNA molecules that repress the translation of mRNAs by targeting the 3' untranslated region (UTR), thereby influencing protein expression and contributing to the onset of many diseases, including IBD (**Figure 1.8**) (Bartel 2009; Schaefer 2016). Previous studies have demonstrated that vitamin D regulates the transcription of miRNAs and that vitamin D deficiency is associated with altered miRNA expression in a variety of tissues and disease states (Dambal et al. 2017; Sheane et al. 2015; J. Xu et al. 2019). In addition, the upregulation of certain miRNAs can affect the autophagy pathway. For example, miR-142-3p has been shown to target the 3'UTR of *ATG16L1* and reduces its expression in human-derived intestinal and immune cell lines (Zhai et al. 2014; Y. Lu et al. 2018; Zhang et al. 2018). Furthermore, expression of miR-142-3p is increased in mouse models of IBD and in IBD patient tissues (Schaefer et al. 2011, 2015). We propose that the link between vitamin D deficient signaling and impaired intestinal autophagy involves altered expression of autophagy-regulating miRNAs, such as miR-142-3p, and that these associations are relevant in the context of IBD.

In the present study, we explored the dynamic between physiologic vitamin D deficiency, the autophagy pathway, and the expression of an autophagy- and IBD-related miRNA, miR-142-3p. We hypothesized that miR-142-3p serves as the link between vitamin D deficient signaling and impaired intestinal autophagy. We measured the expression of miR-142-3p and autophagy markers in intestinal tissues of vitamin D deficient mice. We also measured expression of miR-142-3p in intestinal biopsies procured from newly diagnosed, treatment-naïve paediatric IBD patients (**Figure 2.1**). We reported an upregulation of miR-142-3p in the ileum of vitamin D deficient mice. Interestingly, we found no impairments in autophagy in the ileal whole tissue of vitamin D deficient mice. However, upon further investigation, we observed an accumulation of the autophagy adaptor protein p62 in Paneth cells along with abnormal granule morphology, suggestive of an autophagy defect within this cell type. In our cohort of

paediatric IBD patients, we observed enhanced miR-142-3p expression in colonic tissues procured from 'involved' areas of disease in patients with low vitamin D. We propose miR-142-3p as a therapeutic target of interest that warrants further investigation and encourage the exploration of links between IBD environmental factors of interest and expression of miRNAs.

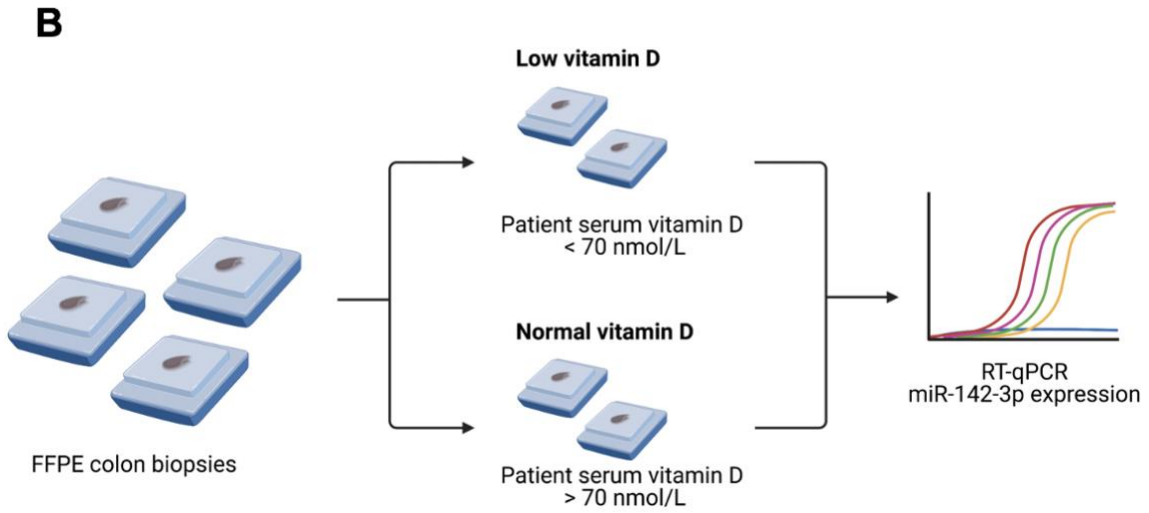
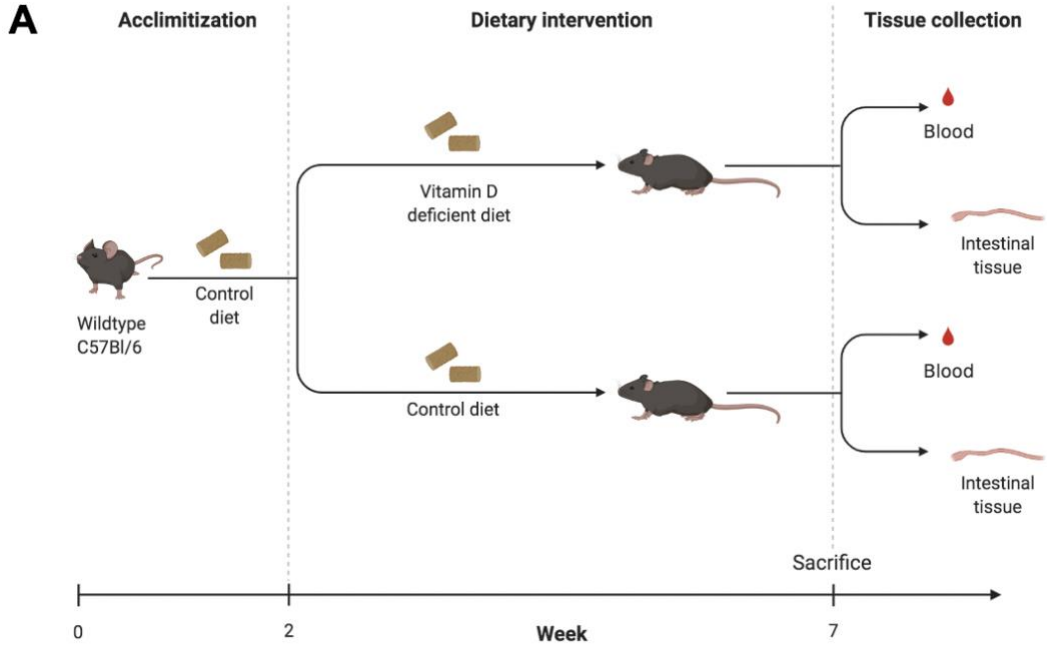


Figure 2.1: Chapter 2 experimental procedure

(A) Wildtype C57BL/6 mice were acclimatized on the control diet for 2 weeks before being randomized to receive either the vitamin D deficient diet or matched control diet for 5 weeks. After the feeding period, mice were sacrificed and blood and intestinal tissues were collected. (B) Formalin-fixed paraffin-embedded (FFPE) colon biopsy samples from newly diagnosed, treatment-naïve paediatric patients were obtained from the Hospital for Sick Children (SickKids; Toronto, ON). Biopsies were stratified into the 'normal vitamin D' or 'low vitamin D' group based on the serum vitamin D status of the patient. Serum 25-hydroxyvitamin D levels above 70 nmol/L were considered normal, and values below 70 nmol/L were considered low. miR-142-3p expression was measured in biopsies by real-time quantitative polymerase chain reaction (RT-qPCR).

2.2 Methods

2.2.1 Vitamin D deficient mouse model

All animal experiments were approved by the University of Toronto's Animal Care Committee and in accordance with the Canadian Council of Animal Care guidelines (protocol 20011441). Mice were housed under specific pathogen-free conditions on a 12-hour light/dark cycle with *ad libitum* access to food and water. Wildtype C57BL/6 female mice (3 weeks of age) were supplied by the Jackson Laboratory (Bar Harbor, ME, USA). The mice were acclimatized for 2 weeks before being randomized to receive either the vitamin D deficient diet (0 IU/g vitamin D₃) (57BQ; TestDiet, Richmond, IN, USA) or matched control diet (1.0 IU/g vitamin D₃) (58M1; TestDiet, Richmond, IN, USA) for 5 weeks.

2.2.2 Tissue preparation

Mice were euthanized by carbon dioxide exposure and cervical dislocation. Blood was collected post-euthanasia by cardiac puncture and serum was separated and collected. Ileum and colon samples were rinsed in phosphate-buffered sodium (PBS) and prepared for whole tissue lysates, isolation of epithelial cells, RNA extraction, and formalin-fixed paraffin-embedded (FFPE) samples. Whole tissue was frozen and stored at -80°C for subsequent lysis and RNA extraction. Tissue for epithelial cell isolation was placed directly into cell recovery solution (354253; Corning Life Sciences, Tewksbury, MA, USA) and processed as indicated below. Tissue for FFPE samples was placed into 10% formalin and later processed for histology.

2.2.3 Isolation of intestinal epithelial cells

Intestinal tissues were incubated in cell recovery solution overnight at 4°C. Samples were transferred to a conical tube containing PBS +1% fetal bovine serum (FBS) and shaken to release the epithelial cells. The epithelial cell-containing supernatant was collected and centrifuged at 1500 rpm for 10 mins at 4°C. Cell pellets were lysed for

western blotting or resuspended in RNAlater stabilization solution (Qiagen, Venlo, LI, NL) and stored at -20°C for later RNA extraction and analysis by real-time quantitative polymerase chain reaction (RT-qPCR).

2.2.4 Measurement of murine serum vitamin D levels

The circulating form of vitamin D, 25-hydroxyvitamin D [25(OH)D], was measured in murine serum using the 25-OH vitamin D enzyme-linked immunosorbent assay (ELISA) kit (VID31-K01; Eagle Biosciences, Amherst, NH, USA) as per the manufacturer's guidelines. Briefly, murine serum samples, calibrators, and controls were diluted with biotin-labeled 25(OH)D and added in duplicates to a 96-well microplate coated with monoclonal anti-25(OH)D antibodies detecting vitamin D₂ and D₃ with 100% specificity. In this assay, the known amount of biotin-labeled 25(OH)D competes with the unknown amount of 25(OH)D in the serum samples for the antibody binding sites on the coated wells during the incubation period. The unbound 25(OH)D was discarded and a second incubation using peroxidase-labeled streptavidin was performed to detect the bound biotin-labeled 25(OH)D. Finally, a third incubation was performed using the peroxidase substrate tetramethylbenzidine and the bound peroxidase caused a change in colour. The intensity of the colour is inversely proportional to the 25(OH)D concentration of the sample. The detection limit of the assay is 1.6 ng/mL.

As soon as possible and within 30 min, the plate was read at 450 and 650 nm wavelengths on a VersaMax 190 visible plate reader (Molecular Devices, San Jose, California, USA). The 450 nm wavelength detects the optical density of the sample, and the 650 nm reading detects background signal. The background signal was subtracted from the optical density for each well and duplicates for each sample were averaged. A standard curve was created using the values of the calibrators. An exponential trendline was plotted and the concentration of 25(OH)D was measured by inputting each value into the equation of the y-intercept of the standard curve.

2.2.5 Histological analysis of murine tissues

Murine intestinal FFPE samples were cut into 5 μm sections, deparaffinized, and stained with haematoxylin and eosin (H&E) by the Department of Laboratory Medicine and Pathology (SickKids, Toronto, ON). A blinded pathologist (I.S.) used a published histological inflammatory scoring system to score H&E-stained slides based on a scoring system developed for mouse intestinal tissues (Wirtz et al. 2017). Briefly, animal tissues were scored based on the following criteria: infrequent inflammatory infiltrate (0), increased chronic inflammatory infiltrate and some neutrophils (1), submucosal presence of inflammatory cell clusters (2), or transmural inflammatory cell infiltrates (3).

2.2.6 Immunofluorescent staining of murine tissues

Murine intestinal FFPE samples were cut into 5 μm sections and affixed onto a glass slide. Tissue sections were deparaffinized and rehydrated using the following steps:

1. Xylene (100%), 5 min x2
2. Xylene (100%) 1:1 with ethanol (100%), 5 min
3. Ethanol (100%), 3 min x2
4. Ethanol (95%), 3 min
5. Ethanol (70%), 3 min

Slides were then rinsed in running deionized water for 5 min or until ready to proceed to antigen retrieval. The slides were not allowed to dry at any point during the protocol in order to prevent non-specific antibody staining. Slides were then immersed in freshly prepared 10 mM sodium citrate buffer with 0.05% Tween-20, adjusted to pH 6.0. Heat-induced epitope retrieval was performed at 110°C for 7.5 min in a decloaking chamber (Biocare Medical, Pacheco, CA, USA). Following antigen retrieval, slides were washed in PBS and a barrier was created around the tissue using a hydrophobic pen. Blocking solution containing 10% goat serum in PBS was added to the tissues for 1 h. Tissues were incubated in primary antibodies in blocking solution overnight at 4°C in a humid chamber. The following day, slides were washed in PBS and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody was added to tissues for

1 h at room temperature. Slides were washed and nuclei were labeled using 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 1 µg/mL in PBS for 10 min. Slides were washed a final time before DAKO fluorescence mounting medium (Agilent Technologies, Santa Clara, CA, USA) was added onto the tissue and a glass coverslip was placed on top. Slides were left to dry overnight.

2.2.7 Confocal microscopy and quantitation

Images were acquired using a Quorum spinning disk confocal microscope using a 63x oil immersion objective with identical settings between conditions. Image acquisition and analysis were performed using Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA, USA). Unless indicated otherwise, images shown for immunofluorescent staining of murine tissues are merged z-planes.

2.2.8 Paneth cell analysis

For Paneth cell granule analysis, deparaffinized tissue sections were incubated with 1:2000 rhodamine-labeled Ulex europaeus agglutinin I (UEA-I) for 2 h at room temperature and co-stained with DAPI to label nuclei. Confocal images of well-oriented crypts were captured. UEA-I staining was used to count Paneth cell number per crypt and UEA-I fluorescence intensity was measured. For analysis of Paneth cell morphology, well-oriented crypts were blindly scored as normal (D0), disordered (D1), diminished (D2), diffuse (D3), excluded (D4) or enlarged (D5) based on a previously established scoring system (Cadwell et al. 2008, 2010; Liu et al. 2014; Vandussen et al. 2015). An average of 341 cells were scored per group and expressed as percentage of normal (D0) or abnormal (D1-D5) Paneth cells per crypt.

For p62 and Muc2 immunofluorescent staining, tissue sections were deparaffinized, blocked in 10% goat serum in PBS, and incubated with either a 1:200 dilution of anti-p62/SQSTM1 antibody (RRID:AB_10011069, NBP1-48320; Novus Biologicals, Littleton, CO, USA) or 1:100 anti-Muc2 antibody (RRID:AB_2146667, sc-15334; Santa Cruz

Biotechnology, Dallas, TX, USA) overnight at 4°C. The following day tissues were washed with PBS and incubated at room temperature with a 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Life Technologies, Carlsbad, CA, USA), washed with PBS, and co-stained with DAPI to label nuclei. Confocal images of well-oriented crypts were captured and p62 or Muc2 fluorescence intensity was measured blindly. For measurement of p62 fluorescence intensity in the full crypt, a region of interest was drawn around the crypt and mean intensity per crypt was compared between groups. An average of 96 crypts per group were included in the analysis. For measurement of p62 and Muc2 intensity in Paneth cells, tissues were co-stained with UEA-I to label Paneth cells as described in the previous section (RRID:AB_2336769, RL-1062; Vector Laboratories, Burlingame, CA, USA). A region of interest was drawn around each Paneth cell and the average p62 or Muc2 fluorescence intensity per crypt was determined and compared between groups.

2.2.9 Immunoblotting and quantitation

Murine intestinal tissues were homogenized in approximately 300 μ L radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl pH 8.0, 150mM sodium chloride, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with the following protease and phosphatase inhibitors: 1mM sodium orthovanadate, 50mM sodium fluoride, 1% protease inhibitor cocktail, and 0.5mM phenylmethylsulfonyl fluoride (all Sigma-Aldrich, St. Louis, MO) and lysed for 30 min on ice. Suspensions were centrifuged at 13 000 rpm for 15 min at 4°C. Supernatants were collected and a Bradford assay was performed to quantify protein concentration (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 60-80 μ g of whole intestinal tissue lysates were aliquoted. Laemmli sample buffer was added at 1x concentration and samples were heated at 95°C for 5 min. Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 135 volts for approximately 1 h 30 min. Proteins were transferred to nitrocellulose membrane at 70 volts for approximately 1 h 30 min at 4°C. Blots were blocked in 5% weight/volume non-fat milk powder prepared in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h

and subsequently incubated with primary antibodies overnight at 4°C in blocking solution. The following day, blots were washed in TBS-T and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature in blocking solution. Blots were washed in TBS-T before being visualized by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnologies, Dallas, TX, USA) using the LI-COR Odyssey FC imaging system (LI-COR Biosciences, Lincoln, NE, USA).

The following primary antibodies were used: 1:500 dilution anti-Lc3 (NB600-1384; Novus Biologicals, Littleton, CO, USA), 1:500 dilution of anti-Atg16l1 (RRID:AB_10950320, 80895; Cell Signaling Technology, Danvers, MA, USA) and 1:10000 dilution of anti- β -actin (A5441; Sigma-Aldrich, St. Louis, MO, USA). The appropriate HRP secondary was used (goat anti-mouse or goat anti-rabbit) at a dilution of 1:5000 (both Cedarlane, Burlington, ON).

Densitometric analysis was performed using ImageLab 6.0 Software (Bio-Rad Laboratories, Hercules, CA, USA). The densities of proteins of interest were quantified and normalized to the expression of the loading control protein and expressed as a fold change relative to the wildtype control value.

2.2.10 Paediatric IBD patient biopsy analysis

The SickKids Research Ethics Board approved the clinical study protocol (REB1000039604). The study was conducted in accordance with the Panel of Research Ethics and the regulations established in Canada for the Protection of Human Subjects (the Tri-Council Policy Statement: Ethical Conduct for Research Involving Human). We retrospectively identified a convenience cohort of newly diagnosed, treatment-naïve paediatric IBD patients from SickKids between the ages of 6-18 years. All patients with suspected new onset IBD systematically undergo upper endoscopy and ileocolonoscopy with mucosal biopsies collected for histologic examination, along with imaging of the small intestine by magnetic resonance enterography to assess type and macroscopic localization according to the Paris Classification (Levine et al. 2011).

Demographic and clinical parameters were extracted from patient charts and patients were categorized by disease diagnosis according to the Paris Classification (Levine et al. 2011), presence or absence of colitis, and grade of disease activity according to the pathology reports from the diagnostic colonoscopies. We included patients who had vitamin D levels obtained as part of routine clinical care within two weeks of the diagnostic endoscopy. Serum vitamin D was measured by liquid chromatography tandem mass spectrometry and a cut-off of >70nmol/L was considered sufficient.

'Involved' biopsies were defined as the presence of macroscopic disease and histological signs of chronic inflammation. Involved patient biopsies were scored for degree of chronic inflammation by a blinded pathologist (I.S.) according to previously established and accepted histopathological grading and reporting systems (Bessissow et al. 2012; Geboes et al. 2000). Briefly, this scoring system developed for IBD biopsies assesses the presence or absence and degree of structural abnormalities, chronic inflammatory infiltrate, lamina propria neutrophils and eosinophils, neutrophils in the epithelium, and crypt destruction and erosion or ulceration (Bessisow et al. 2012; Geboes et al. 2000).

For the isolation of RNA, 10 μ m FFPE intestinal samples were deparaffinized and total RNA was extracted using the miRNA extraction kit (Roche, Basel, CH). Synthesis of miR-142- 3p was performed using TaqMan microRNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA). qPCR was performed and miR-142-3p changes were normalized relative to endogenous control RNU48 (Applied Biosystems, Waltham, MA). Cycle threshold (Ct) values >34 were excluded from analysis.

2.2.11 RNA isolation, cDNA synthesis & RT-qPCR

Murine ileal and colonic tissues were homogenized and the MagMAX-96 total RNA isolation kit was used as per the manufacturer's instructions (Ambion, Life Technologies, Carlsbad, CA, USA). RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). The TaqMan microRNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA) was used for cDNA

synthesis of miR-142-3p and endogenous control SNO202RNA. miR-142-3p (assay ID 000464; Life Technologies, Carlsbad, CA, USA) and SNO202RNA (assay ID 001232; Life Technologies, Carlsbad, CA, USA) primers were used for reverse transcription and qPCR. Validated EvaGreen qPCR primer sets for ATG16L1 (qMmuCID0011303; Bio-Rad, Hercules, CA, USA) and β -actin (qMmuCED0027505; Bio-Rad, Hercules, CA, USA) were used. A validated primer set was used for detection of lysozyme 1 mRNA (fwd: GGAATGGATGGCTACCGTGG and rev: CATGCCACCCATGCTCGAAT).

2.2.12 Statistical analysis

The results are presented as the mean value \pm standard deviation (SD) or standard error of the mean (SEM) as indicated. The normality of distribution of all continuous variables was tested using the Shapiro-Wilk test and the proper parametric or non-parametric test was performed. Data from mouse studies were analyzed using unpaired student's t-test or Mann-Whitney U test as appropriate and the data are expressed as fold change \pm SEM relative to the control group. For these studies, each n represents data from one sample from one mouse unless indicated otherwise. For patient sample analyses, unpaired student's t-test or Mann-Whitney U test were performed as appropriate and the data are expressed as fold change \pm SEM relative to the control group. Categorical variables were summarized as frequencies with proportions and compared with the Chi-square test (or Fisher exact test if expected cell counts <5). The association between vitamin D status and miR-142-3p was examined using univariable and multivariable linear regression. Assumptions for linear regression were verified and satisfied. A p-value of <0.05 was considered statistically significant for all analyses. Statistical analyses were performed using GraphPad Prism v8.1.2 (GraphPad Software, San Diego, CA, USA) or SPSS v23 (IBM, Armonk, NY, USA).

2.3 Results

2.3.1 Vitamin D deficiency enhances expression of miR-142-3p in the ileum, but not the colon, of mice

It has been previously demonstrated that *Vdr*^{ΔIEC} mice have reduced Atg16l1 expression in the intestine, indicating that a lack of vitamin D signaling results in an autophagy defect (Wu et al. 2015). Furthermore, it has been established that vitamin D status can alter miRNA expression (Dambal et al. 2017; Sheane et al. 2015; Xu et al. 2019). Therefore, we measured expression of *ATG16L1*-targeting miR-142-3p in the intestinal tissues of our diet-induced vitamin D deficient mice. Because miR-142-3p targets *ATG16L1* and reduces its expression, we expected increased miR-142-3p expression in the intestinal tissues of vitamin D deficient mice.

We first sought to establish that mice fed the vitamin D deficient diet achieved deficiency. To do this, we assessed the serum vitamin D levels by performing a 25(OH)D ELISA (**Figure 2.2 A**). There was a 48% reduction in serum 25(OH)D in mice fed the vitamin D deficient diet compared to mice fed the control diet, confirming a relative deficiency.

We next measured expression of miR-142-3p in the intestinal tissues of our mice. Although there was no change in miR-142-3p expression in the colon (**Figure 2.2 B**), its expression was induced more than 3-fold relative to the endogenous control (SNO202RNA) in the ileum whole tissue of vitamin D deficient mice compared to control mice (**Figure 2.2 C**). The increase in miR-142-3p persisted in isolated epithelial cells from vitamin D deficient mice (**Figure 2.2 D**). A blinded pathologist (I.S.) assessed the inflammation status of the ileum by blindly scoring H&E-stained tissue sections and found that there was no intestinal inflammation in vitamin D deficient mice or control mice (**Figure 2.2 E,F**). This indicates that a change in immune cell infiltration did not account for alterations in miR-142-3p expression. Taken together, these results demonstrate that vitamin D deficiency leads to upregulation of autophagy-targeting miR-142-3p in the ileum of mice.

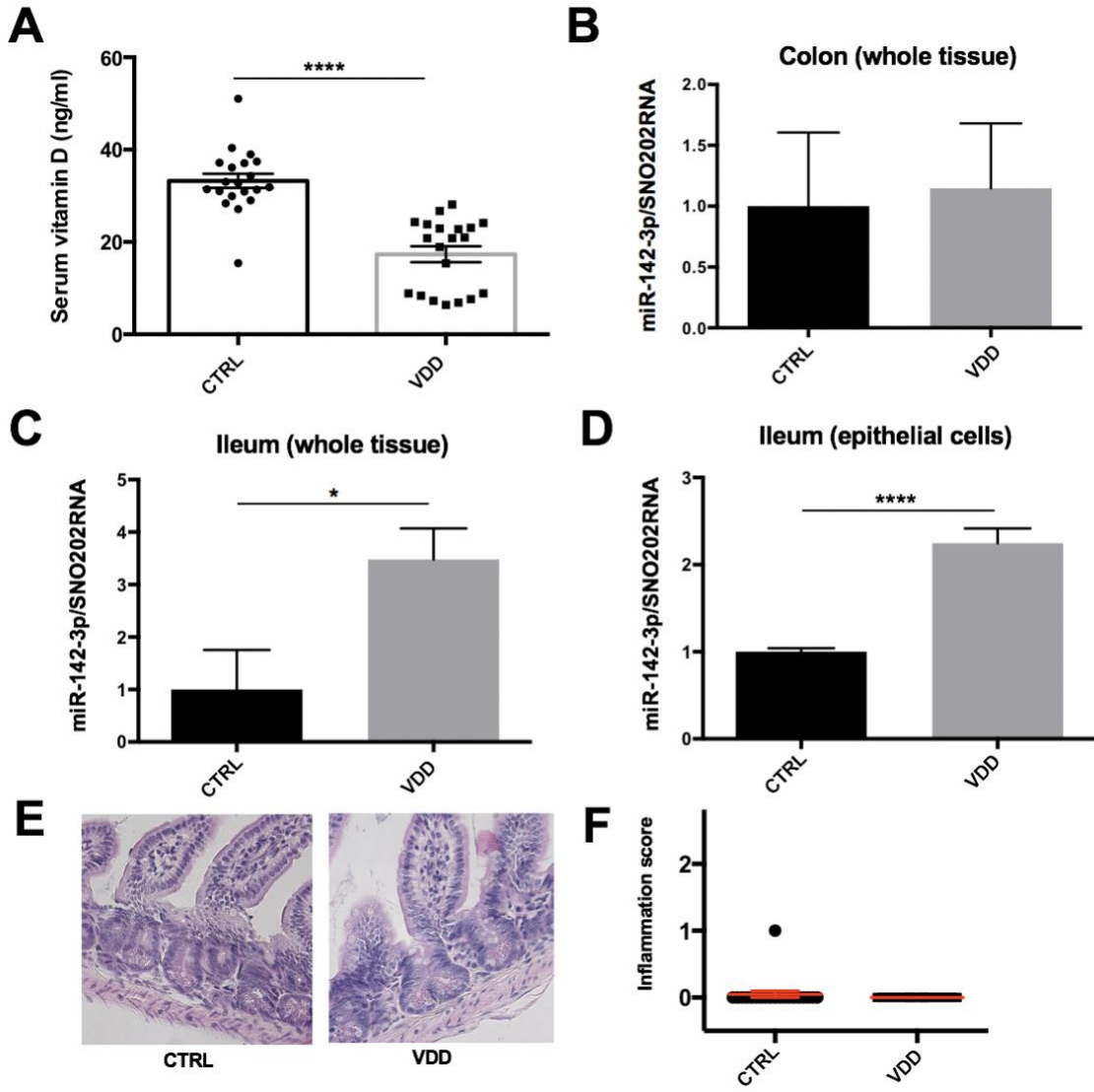


Figure 2.2: miR-142-3p expression is increased in the ileum of vitamin D deficient mice

(A) Serum 25-hydroxyvitamin D was measured in animals fed either the vitamin D deficient (VDD) or control (CTRL) diet after a 5-week feeding period. mRNA expression of miR-142-3p relative to endogenous control SNO202RNA was measured in (B) colon whole tissue, (C) ileum whole tissue, and (D) isolated epithelial cells of the ileum of vitamin D deficient mice compared to control mice. (E) Representative images of H&E-stained ileum tissues of vitamin D deficient and control mice. (F) Inflammation scores from (E) as assessed by a blinded pathologist using a previously described scoring system (Wirtz et al. 2017). Data were analyzed by student's t-test. Error bars represent \pm SEM. n=19-20/group. ****p<0.0001, *p<0.05

2.3.2 Autophagy is not impaired in intestinal whole tissue of vitamin D deficient mice

There is existing evidence to support an association between a lack of vitamin D signaling in the intestine and expression of autophagy protein Atg16L1 in the mouse intestinal epithelium. Wu et al. (2015) demonstrated that *Vdr^{ΔIEC}* mice have reduced Atg16L1 protein expression in intestine compared to *Vdr^{loxP}* control mice (Wu et al. 2015). However, a complete lack of vitamin D signaling in the intestine, as is present in the *Vdr^{ΔIEC}* mice, is not reflective of physiology. To determine if this association is replicable in our physiologic model of vitamin D deficiency, we measured expression of autophagy proteins in intestinal whole tissues of vitamin D deficient and control mice by western blotting (**Figure 2.3 A**).

In the ileum whole tissue, there were comparable levels of Atg16L1 between vitamin D deficient and control mice ($p=0.059$) (**Figure 2.3 A,B**). There was significant variability in expression of this protein within groups. Western blotting also revealed that there was no change in the expression of autophagosome marker Lc3II in ileum whole tissue between vitamin D deficient and control mice (**Figure 2.3 A,C**). Additionally, there was no difference in Atg16L1 mRNA in ileum whole tissue (**Figure 2.3 D**). Taken together, these results indicate that there is not a significant change in autophagy in the ileum whole tissue of vitamin D deficient mice compared to control mice.

There was also no difference in Atg16L1 (**Figure 2.4 A,B**) or cC3II (**Figure 2.4 A,C**) protein in the colon whole tissue as measured by western blotting. Additionally, Atg16L1 mRNA expression in colon whole tissue was unchanged between vitamin D deficient and control mice (**Figure 2.4 D**). These results in the colon were expected given that there was no change in autophagy-regulating miR-142-3p in this tissue.

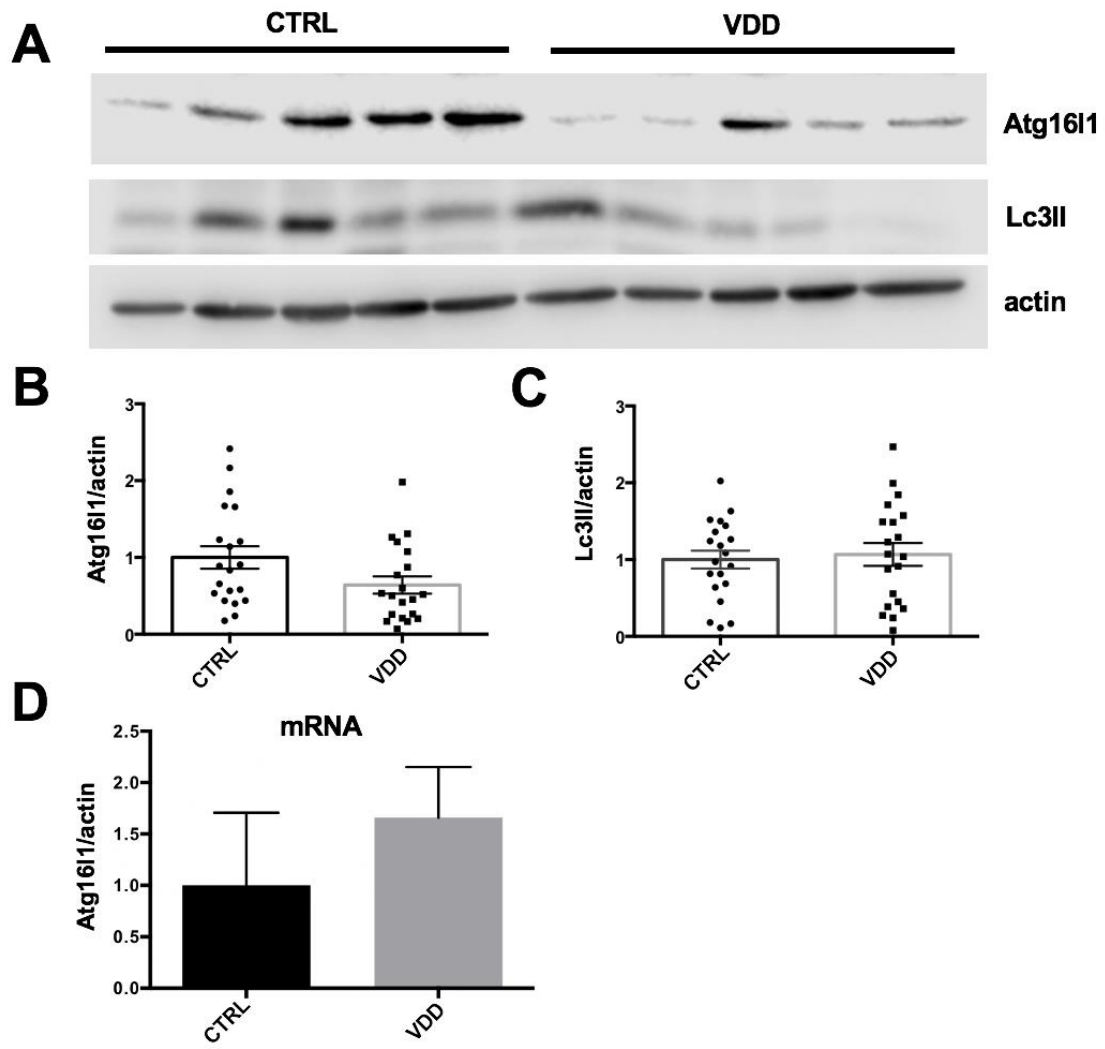


Figure 2.3: Autophagy is unchanged in ileum whole tissue of vitamin D deficient mice

(A) Ileal whole tissue from vitamin D deficient (VDD) and control (CTRL) mice was assessed for expression of Atg16l1 and Lc3II proteins by western blotting. A representative blot with 5 animals per group is shown. Densitometric analysis of (B) Atg16l1 ($p=0.059$) and (C) Lc3II expression from (A) was performed. Values were normalized to actin and expressed as a fold change relative to the control group. (D) mRNA expression of Atg16l1 relative to endogenous control SNO202RNA was measured and expressed as fold change relative to the control group. Data were analyzed by student's t-test. Error bars represent \pm SEM. $n=20$ /group

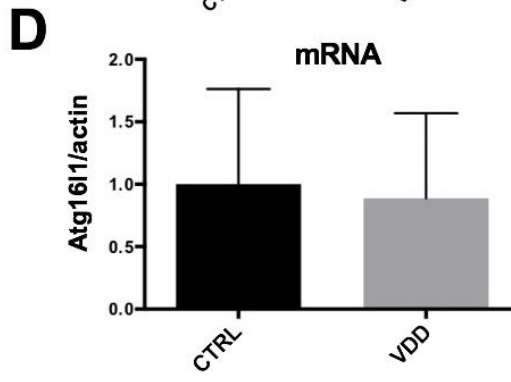
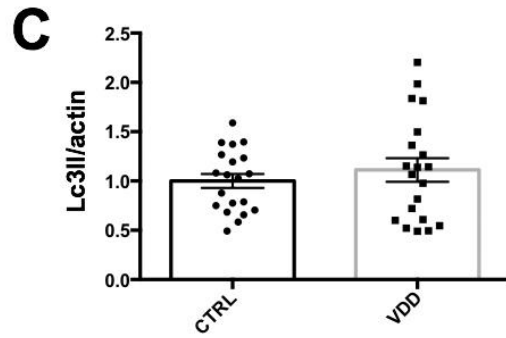
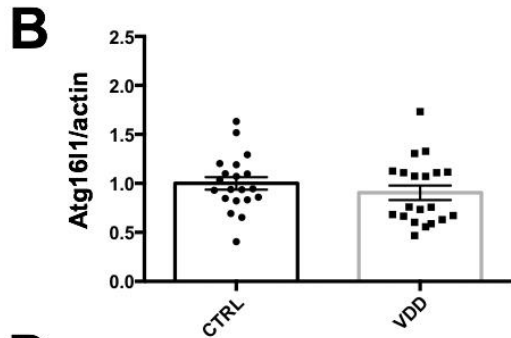
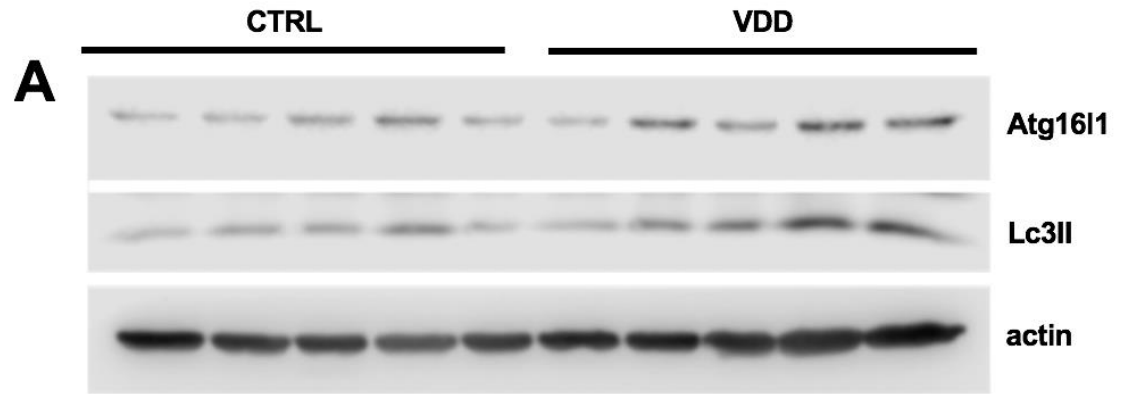


Figure 2.4: Autophagy is unchanged in colon whole tissue of vitamin D deficient mice

(A) Colon whole tissue from vitamin D deficient (VDD) and control (CTRL) mice were assessed for expression of Atg16l1 and Lc3II proteins by western blotting. A representative blot with 5 animals per group is shown. Densitometric analysis of (B) Atg16l1 and (C) Lc3II expression from (A) was performed. Values were normalized to actin and expressed as fold change relative to the control group. (D) mRNA expression of Atg16l1 in relative to endogenous control SNO202RNA was measured and expressed as a fold change relative to the control group. Data were analyzed by student's t-test. Error bars represent \pm SEM. n=20/group

2.3.3 Vitamin D deficient mice exhibit abnormal Paneth cell morphology

It has been previously demonstrated by Wu et al. (2015) that *Vdr^{ΔIEC}* mice display Paneth cell aberrations, including morphological abnormalities and a reduction in lysozyme expression compared to *Vdr^{loxP}* control mice. We explored whether our physiologic diet-induced vitamin D deficient model led to abnormalities in this cell type.

We utilized a previously defined scoring system which categorizes individual Paneth cells based on their morphology (**Figure 2.5 A**) (Cadwell et al. 2008; Cadwell et al. 2009; Liu et al. 2014; Vandussen et al. 2015). Briefly, a score of D0 is normal, and scores D1-D5 represent various morphological abnormalities that may be observed. After scoring over 300 cells per group, it was determined that vitamin D deficient mice have a reduced percentage of normal (D0) and increased percentage of abnormal (D1-D5) Paneth cells per crypt compared to control mice (**Figure 2.5 B,C**). We concluded that diet-induced vitamin D deficiency also leads to Paneth cell granule abnormalities.

Given this finding, we sought to measure additional parameters of Paneth cells. The average number of Paneth cells per crypt was unchanged between vitamin D deficient and control mice (**Figure 2.6 A**). Furthermore, the fluorescence intensity of UEA-I was not different between groups, indicating no change in expression (**Figure 2.6 B**). We also measured the expression of lysozyme, a key antimicrobial factor expressed in Paneth cells, in ileum whole tissue and found no change between vitamin D deficient and control mice (**Figure 2.6 C**). Although these additional characteristics of Paneth cells were unchanged, the morphological defect identified indicates that this cell type may be dysfunctional.

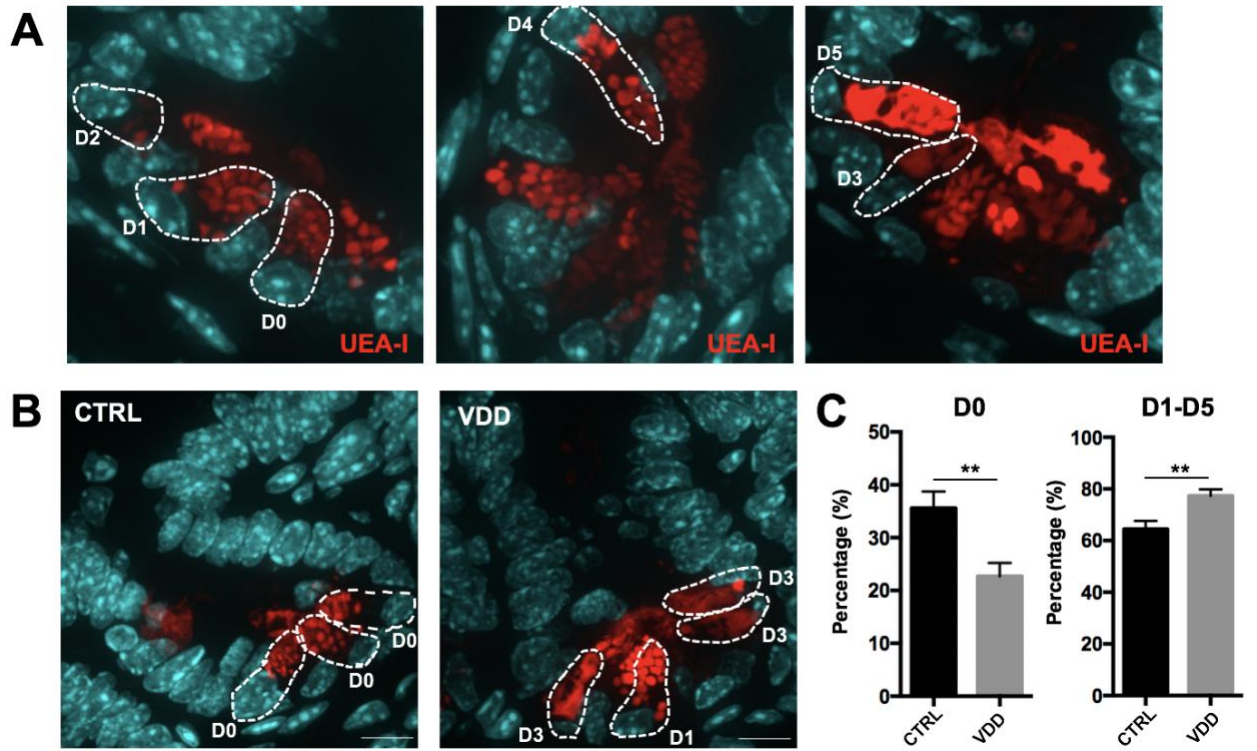


Figure 2.5: Morphological Paneth cell abnormalities are present in vitamin D deficient mice

(A) Confocal images of well-oriented crypts stained with UEA-I (Paneth cell granule marker) and DAPI (to label nuclei) are shown. Paneth cell granule morphology was scored in both vitamin D deficient (VDD) and control (CTRL) mice according to a previously established scoring system (Cadwell et al. 2008; Cadwell et al. 2009; Liu et al. 2014; Vandussen et al. 2015) for which an image highlighting a representative cell for each category is shown. D0=normal, D1=disordered, D2=diminished, D3=diffuse, D4=excluded (empty granules), D5=enlarged. (B) Paneth cells from each group were blindly given a score of D0-D5. Representative image from the vitamin D deficient and control groups are shown. Scale bar represents 10 μm . (C) Scores were represented as a percentage for each crypt assessed. Percentage of normal Paneth cells (D0) (left) and abnormal Paneth cells (D1-D5) (right) from vitamin D deficient and control mice are shown. Data were analyzed by Mann-Whitney U test. Error bars represent \pm SEM.

** $p < 0.01$

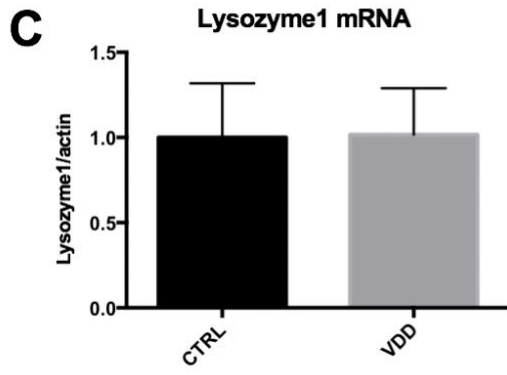
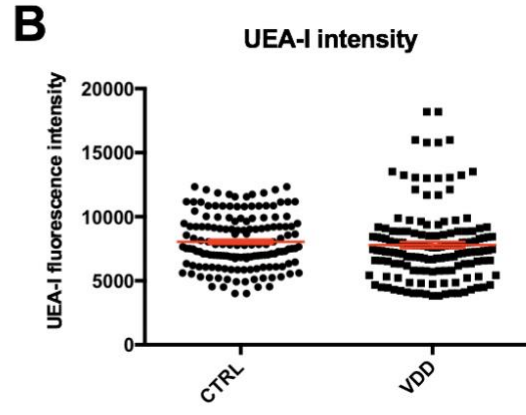
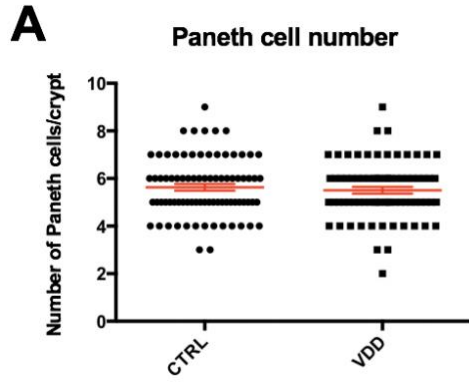


Figure 2.6: Paneth cell number, intensity of UEA-I staining, and lysozyme mRNA expression are unchanged between vitamin D deficient and control mice

(A) Number of Paneth cells in vitamin D deficient (VDD) and control (CTRL) mice were blindly counted. The average number of Paneth cells per crypt is shown. (B) UEA-I fluorescence intensity in Paneth cells was blindly measured. The average intensity of UEA-I in Paneth cells per crypt is shown. (C) mRNA expression of lysozyme, a key Paneth cell antimicrobial factor, was measured in vitamin D deficient and control mice and normalized to actin expression. Fold change relative to the control group is shown. Data were analyzed by student's t-test. Error bars represent \pm SEM.

2.3.4 Increased expression of Muc2 is present in Paneth cells of vitamin D deficient mice

Given the abnormal granule morphology present in Paneth cells of vitamin D deficient mice, we next sought to assess whether this observation translated to functional defects of the Paneth cells. A study by Stahl et al. (2018) identified that Muc2 coats murine Paneth cell granules to facilitate their release and dispersion (Stahl et al. 2018).

Therefore, differences in expression of Muc2 may indicate impaired function of the Paneth cells. We performed immunofluorescent staining of mucin 2 (Muc2) expression in ileal tissue sections from each group of mice

After comparing Muc2 expression between groups, we observed that vitamin D deficient mice have increased expression of Muc2 within Paneth cells (**Figure 2.7 A,B**) Increased Muc2 expression in the Paneth cells, along with abnormal granule morphology, suggest that there are morphological defects and possibly functional impairments within Paneth cells of vitamin D deficient mice.

In order to further characterize Paneth cell function, expression of key murine Paneth cell antimicrobials, such as lysozyme and cryptidins, should be measured in the luminal contents and connected with an analysis of the microbiome. Previous studies have indicated that gut microbiome dysbiosis is present in several models of impaired vitamin D signaling, including diet-induced vitamin D deficiency and a loss of the Vdr (Ooi et al. 2013; Wu et al. 2015; Cantorna et al. 2019). Complete characterization of a Paneth cell defect should evaluate these downstream effects which can have functional consequences within the epithelium and enhance susceptibility to IBD.

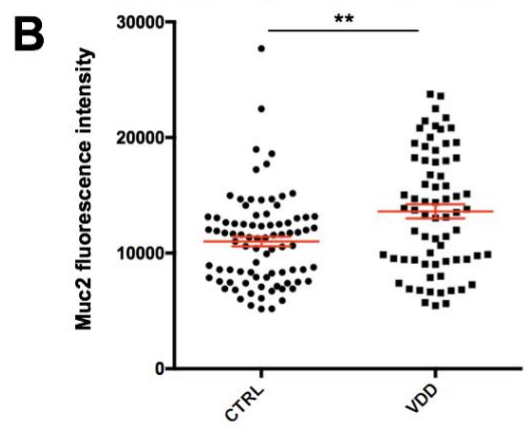
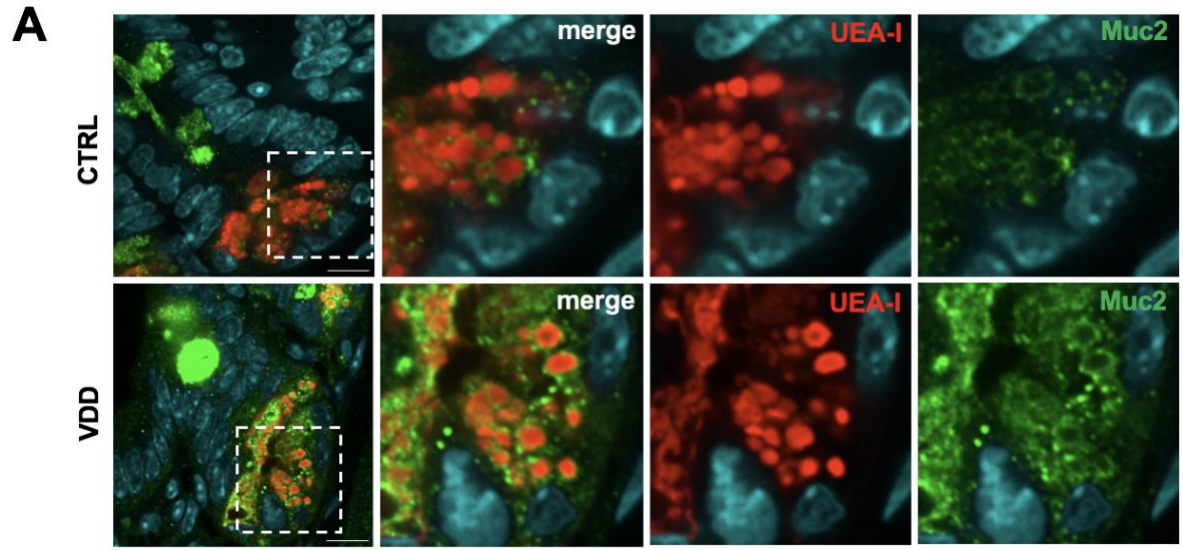


Figure 2.7: Paneth cells of vitamin D deficient mice have increased Muc2 expression

(A) Ileal tissue sections from vitamin D deficient (VDD) and control (CTRL) mice were co-stained for Muc2, Paneth cell marker UEA-I, and DAPI to label nuclei. Confocal images were captured of well-oriented crypts and Muc2 expression within the Paneth cells was blindly measured. Representative images of a single z-stack are shown. (B) Quantification of Muc2 expression within crypts from (A) is shown. Data were analyzed by student's t-test. Error bars represent \pm SEM. ** $p < 0.01$

2.3.5 Autophagy is impaired in Paneth cells of vitamin D deficient mice

We next aimed to further characterize a functional defect of Paneth cells given their abnormal granule morphology and accumulation of Muc2. Although there were no signs of autophagy dysregulation in the ileum whole tissue, current evidence indicates that Paneth cells are particularly sensitive to changes in autophagy (Cadwell et al. 2008, 2009). Importantly, Paneth cells are highly reliant on autophagy for the proper packaging and secretion of their cytoplasmic granules (Cadwell et al. 2008; Cadwell et al. 2009). Due to their function, Paneth cells require a high content of ER compared to other intestinal epithelial cell types and rely on proper functioning of autophagy for ER turnover and maintenance (Cadwell et al. 2009). In this case, assessing autophagy at the level of the whole tissue may cause an autophagy defect to be missed due to the small representation of Paneth cells within the ileal epithelium.

To account for the increased sensitivity of Paneth cells to changes in autophagy, we measured autophagic flux in Paneth cells. We performed immunofluorescent staining of p62 in combination with Paneth cell granule marker UEA-I on ileal tissue sections. The autophagy adaptor protein p62 targets cargo for autophagic degradation and is degraded with the process of autophagy and therefore serves as a marker of autophagic flux (Glick, Barth and MacLeod 2010; Levine, Mizushima and Virgin 2011). Compared to control mice, increased p62 accumulation was observed in the Paneth cells of vitamin D deficient mice (**Figure 2.8 A,B**), suggestive of an autophagy defect in these cells. In the same tissues, we measured p62 accumulation in the full crypt. Importantly, there were no differences in p62 accumulation in the full crypt epithelium between the two groups (**Figure 2.8 C**). Taken together, these results suggest that diet-induced vitamin D deficiency results in an intestinal autophagy defect that preferentially affects Paneth cells.

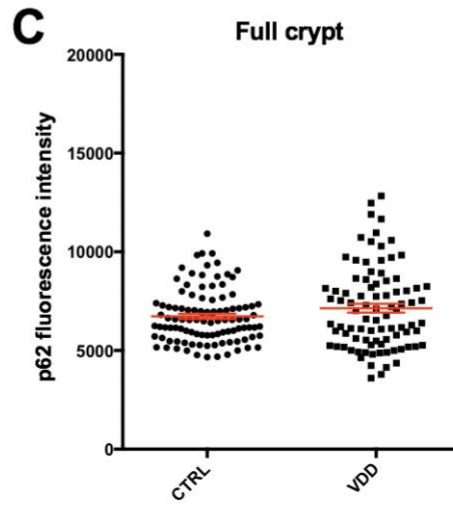
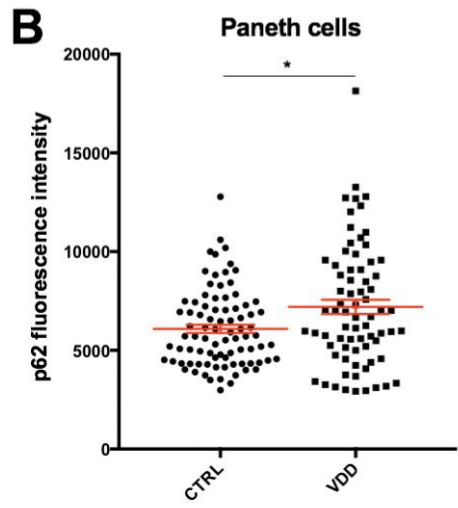
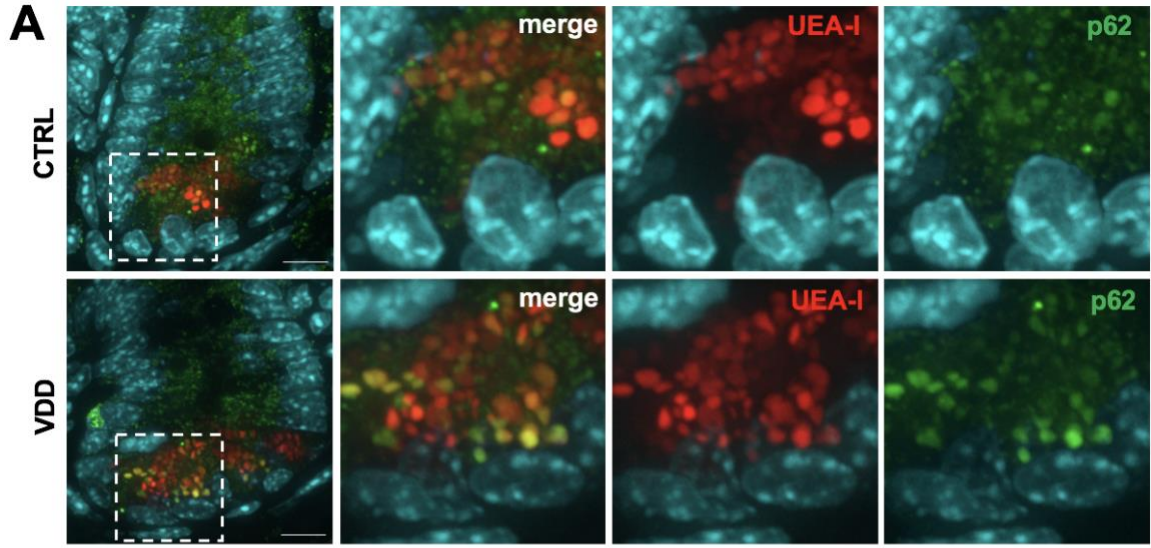


Figure 2.8: Accumulation of p62 in the Paneth cells of vitamin D deficient mice

(A) Ileal tissue sections from vitamin D deficient (VDD) and control (CTRL) mice were co-stained for autophagy adaptor protein p62, Paneth cell marker UEA-I, and DAPI to label nuclei. Confocal images of well-oriented crypts were captured. A representative image is shown. Fluorescence intensity of p62 was blindly measured in (B) Paneth cells and (C) the full crypt epithelium. Scale bar represents 10 μm . Average fluorescence intensity per crypt is shown. Data were analyzed by Mann-Whitney U test. Error bars represent \pm SEM. * $p < 0.05$

2.3.6 miR-142-3p expression is increased in ‘involved’ colonic biopsies from IBD patients with low vitamin D

Finally, to further explore the relationship between low vitamin D and expression of our miRNA of interest, we measured miR-142-3p in intestinal biopsies from a cohort of paediatric IBD patients. We used retrospectively collected colonic tissue biopsies obtained from newly diagnosed, treatment-naïve IBD patients who had serum 25(OH)D levels taken as part of routine clinical care within two weeks of their diagnostic endoscopy. Patient tissue samples were then stratified as originating from patients with normal (>70 nmol/L) or low (<70 nmol/L) vitamin D.

In total, 82 patients had colonic samples that could be assessed for expression of miR-142-3p (**Table 1**). There were no differences in important measures of disease characteristics such as location and extent of disease or laboratory measures of inflammatory status, including white blood cell (WBC) count, erythrocyte sedimentation rate (ESR), or c-reactive protein (CRP). Overall, there were no differences in miR-142-3p expression between colonic tissues from normal versus low vitamin D patients (**Figure 2.9 A**).

We then performed an analysis comparing biopsies procured from ‘uninvolved’ or ‘involved’ regions of disease. Involved regions were defined as macroscopically inflamed with histologic evidence of chronic inflammation (n=35). Interestingly, in this subgroup analysis, samples from involved areas of disease in patients with low vitamin D levels displayed increased miR-142-3p expression (**Figure 2.9 B**). Vitamin D status was the only difference in patient characteristics between groups (**Table 2**). There was also no difference in the severity of inflammation between the low and normal vitamin D biopsies from involved regions as assessed by a blinded pathologist (I.S.) (**Figure 2.9 C**). Thus, differences in inflammatory infiltrate did not explain the difference in miR-142-3p between the groups.

To further confirm an independent association between low vitamin D status and increased miR-142-3p in the subset of involved colonic samples, a multivariable linear regression analysis was performed. In this analysis, vitamin D status remained significantly associated with miR-142-3p after controlling for IBD type, sex, age at IBD

diagnosis and ethnicity (**Table 3**). As expected, no significant association was seen with any of the other factors. These results suggest that miR-142-3p may be tied to pathology in IBD patients with low vitamin D, and this association should be further explored.

Table 1: Comparison of patient characteristics between paediatric IBD patients with normal and low serum vitamin D

Patient Characteristics	Normal vitamin D (n=28)	Low vitamin D (n=54)	p-value
Age (years)	11.5 (9.0-14.0)	13.0 (10.0-15.2)	0.053
Females	16 (57%)	19 (35%)	0.057
Ethnicity			0.041
Caucasian	20 (83%)	31 (60%)	
Other	4 (17%)	21 (40%)	
Unknown	4	2	
IBD type and Paris classification			
Crohn's disease	19 (68%)	27 (50%)	0.122 (IBD type)
L1 (distal ileum ± cecum)	4 (21%)	4 (15%)	0.297 (CD classification)
L2 (colonic)	6 (31%)	8 (30%)	0.277 (UC classification)
L3 (ileocolonic)	7 (37%)	15 (55%)	
Macroscopically normal colonoscopy	2 (11%)	0	
Ulcerative colitis	9 (32%)	27 (50%)	
E1 (proctitis)	0	0	
E2 (distal to splenic flexure)	2 (22%)	1 (4%)	
E3 (distal to hepatic flexure)	1 (11%)	6 (22%)	
E4 (proximal to hepatic flexure)	6 (67%)	20 (74%)	
Serum vitamin D levels (nmol/L)	84.5 (73.5-97.0)	47.5 (40.8-58.2)	<0.001
Laboratory markers			
WBC ($\times 10^9/L$)	9.0 (6.2-11.5)	9.5 (7.6-11.8)	0.824
ESR (mm/h)	25 (12-36)	28 (17-52)	0.636
CRP (high sensitivity) ($\mu g/L$)	3.1 (1.0-9.6)	16.7 (1.6-34)	0.161

Results presented as median (interquartile range) or n (%) and compared using Mann-Whitney U test or Chi-square (or Fisher exact) test, respectively. Statistically significant differences are shown in bold ($p < 0.05$). WBC: white blood cell, ESR: erythrocyte sedimentation rate, CRP: c-reactive protein.

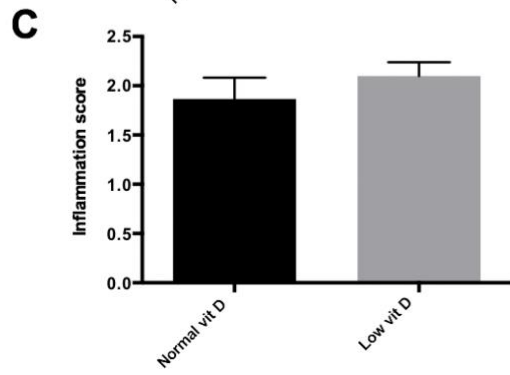
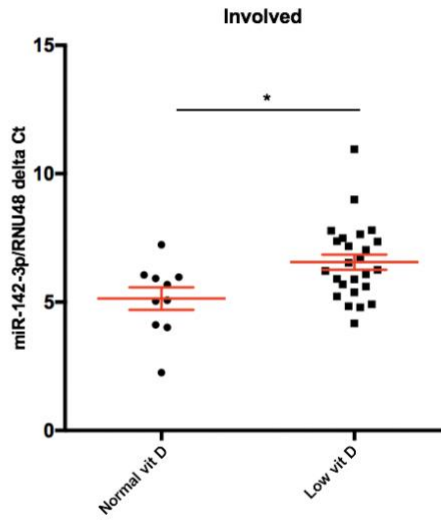
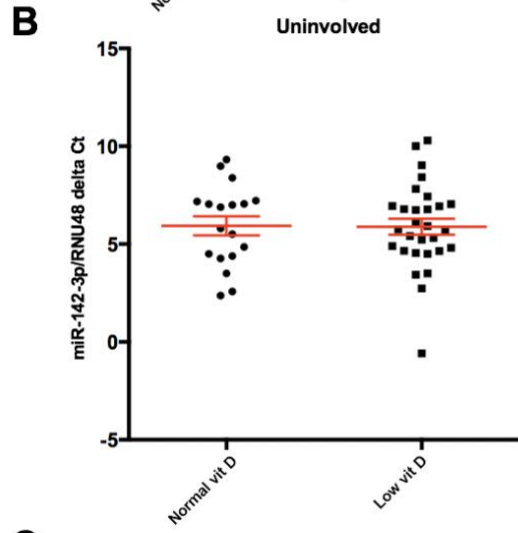
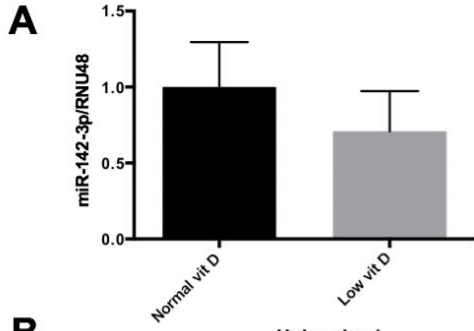


Figure 2.9: miR-142-3p expression is increased in ‘involved’ colonic biopsies of newly diagnosed, treatment-naïve paediatric IBD patients with low vitamin D

(A) mRNA expression of miR-142-3p normalized to housekeeping miRNA RNU48 was assessed and compared between normal and low vitamin D IBD patients. (B) miR-142-3p mRNA expression normalized to housekeeping miRNA RNU48 was assessed and compared between normal and low vitamin D patients in tissue collected from uninvolved (left) or involved (right) tissue regions. (C) Inflammatory status of tissues between low vitamin D (n=10) and normal vitamin D (n=25) patients was compared in the subgroup of biopsies collected from involved tissue regions in (B). Continuous variables were tested for normality of distribution and summarized with means \pm SD and compared with the (A,C) unpaired t-test or (B) \pm SEM with the Mann-Whitney U test.

*p<0.05

Table 2: Comparison of patient characteristics between paediatric IBD patients with low and normal serum vitamin D ('involved' biopsies only)

Patient Characteristics	Normal vitamin D (n=10)	Low vitamin D (n=25)	p-value
Age (years)	10.5 (6.0-12.5)	13.0 (10.0-15.0)	0.177
Females	5 (50%)	10 (40%)	0.712
Ethnicity			
Caucasian	7 (78%)	18 (75%)	1.0
Other	2 (22%)	6 (25%)	
Unknown	1	1	
IBD type and Paris classification			
Crohn's disease	4 (40%)	9 (36%)	1.00 (IBD type)
L1 (distal ileum ± cecum)	0	0	0.559 (CD classification)
L2 (colonic)	1 (25%)	5 (56%)	
L3 (ileocolonic)	3 (75%)	4 (44%)	⁻¹
Macroscopically normal colonoscopy	0	0	
Ulcerative colitis	6 (60%)	6 (64%)	
E1 (proctitis)	0	0	
E2 (distal to splenic flexure)	0	0	
E3 (distal to hepatic flexure)	0	0	
E4 (proximal to hepatic flexure)	6 (100%)	16 (100%)	
Serum vitamin D levels (nmol/L)	75.5 (71.8-85.5)	53.0 (42.5-59.5)	0.001
Laboratory markers			
WBC (x10 ⁹ /L)	8.6 (6.5-12.7)	10.1 (8.0-12.2)	0.789
ESR (mm/h)	41 (25-58)	28 (15-76)	0.915
CRP (high sensitivity) (µg/L)	9.2 (1.8-59.6)	14.5 (1.6-36.1)	0.703

Results presented as median (interquartile range) or n (%) and compared using Mann-Whitney U test or Chi-square (or Fisher exact) test, respectively. Statistically significant differences are shown in bold (p<0.05). ¹Not applicable as all patients in both groups had E4 distribution. WBC: white blood cell, ESR: erythrocyte sedimentation rate, CRP: c-reactive protein.

Table 3: Univariate and multivariable linear regression examining the association between miR-142-3p (delta Ct) and vitamin D status in ‘involved’ colonic biopsies (n=35).

Variable	Unadjusted Beta Coefficient (95% CI)	P-value	Adjusted Beta Coefficient (95% CI)	P-value
Low vitamin D (vs normal)	1.42 (0.306-2.53)	0.014	1.58 (0.190-2.97)	0.0274
Female (vs male)	-0.186 (-1.30-0.93)	0.736	0.017 (-1.18-1.22)	0.977
Age (years)	0.071 (-0.094-0.236)	0.390	-0.023 (-0.222-1.76)	0.814
UC (vs CD)	0.088 (-1.05-1.23)	0.876	- ¹	
Caucasian (vs other)	0.202 (-1.22-1.62)	0.774	-0.446 (-1.84-1.22)	0.518

$R^2 = 0.17$ indicates that low vitamin D status accounts for 17% of the variation in delta Ct. R^2 for multivariable model = 0.18 Statistically significant differences are shown in bold ($p < 0.05$).¹Excluded based on change in estimate approach. CI: confidence interval.

2.4 Discussion

Despite major advances in IBD research, pathogenesis remains unclear and treatment options are limited. Understanding how environmental triggers can lead to disease in susceptible individuals is crucial to uncovering disease pathogenesis and developing new treatment options. In this study, we demonstrated for the first time that vitamin D deficiency, an important environmental factor involved in IBD pathogenesis, can modulate the intestinal expression of an autophagy-regulating miRNA, miR-142-3p. We demonstrated this in both a mouse model of diet-induced vitamin D deficiency in association with abnormal Paneth cells exhibiting dysregulated autophagy (**Figure 2.10 A**), and most importantly, in 'involved' colonic tissues of newly diagnosed, treatment-naïve paediatric IBD patients with low vitamin D (**Figure 2.10 B**). Through this study we have identified a mechanism that links vitamin D deficiency to IBD pathogenesis and uncovered a potential therapeutic target.

Previous reports demonstrate that loss of the Vdr in the intestinal epithelium of mice results in reduced ileal autophagy and dysregulation of Paneth cells (Wu et al. 2015). Our findings similarly show that insufficient vitamin D signaling leads to Paneth cell abnormalities and we identified an autophagy defect in these cells. In ileal whole tissue, however, we observed no significant changes in autophagy. In contrast to these previous studies, our model involved a diet-induced reduction of vitamin D rather than a complete loss of its receptor, which likely more accurately reflects the physiologic effect of vitamin D deficiency on the autophagy pathway in the intestine. Based on these results, we propose that miR-142-3p upregulation, triggered by reduced vitamin D signaling, leads to susceptibility to IBD through intestinal autophagy disruption which preferentially affects the Paneth cells. We propose that the autophagy-sensitive Paneth cells were affected while other epithelial cell types were able to maintain homeostasis. Paneth cells rely on autophagic machinery for secretion of their cytoplasmic granules (Cadwell et al. 2008; Cadwell et al. 2009). Additionally, these cells require a higher content of ER compared to other intestinal epithelial cell types and rely on proper functioning of autophagy for ER turnover and maintenance (Cadwell et al. 2009). This provides an explanation for the observed autophagy defect which is present in Paneth cells and not in the total epithelium or whole tissue in our vitamin D deficient mice.

A recent study by Lu et al. (2021) reinforced the importance of Vdr signaling and maintenance of autophagy in Paneth cells by employing a mouse model in which the Vdr was specifically deleted from Paneth cells ($Vdr^{\Delta PC}$) (Lu et al. 2021). $Vdr^{\Delta PC}$ mice exhibited reduced intestinal Atg16l1 and lysozyme expression and had abnormal Paneth cell granule morphology. Furthermore, $Vdr^{\Delta PC}$ mice had increased susceptibility to *Salmonella* infection and small intestinal injury by indomethacin, an IBD-relevant NSAID. The $Vdr^{\Delta PC}$ mice also had increased susceptibility to DSS colitis, but this effect was lost upon co-housing with littermate control Vdr^{loxP} mice. Together, these findings highlight the importance of vitamin D/Vdr signaling in Paneth cells. Accordingly, assessing the impact of diet-induced vitamin D deficiency in models of IBD, such as the DSS colitis or SAMP1/YitFc ileitis, would be of particular interest. Previous reports have demonstrated that vitamin D deficient mice experience increased inflammation and damage in models of IBD (Lagishetty et al. 2010), and thus future studies should assess the role of miR-142-3p and Paneth cells in this context.

We consider the access to paediatric IBD patient biopsies a strength of this report. Since our cohort consists of newly diagnosed patients, it is likely that the characteristics of the tissue relate closely to disease pathogenesis and are not the result of prolonged exposure to chronic inflammation. Furthermore, these patients have not yet been exposed to therapeutics, which can also alter tissue characteristics. Therefore, it is likely that the increase in miR-142-3p observed in the involved colonic biopsies of patients with low vitamin D is tied to disease pathogenesis. Future studies can explore whether the increase in miR-142-3p is associated with a reduction in autophagy in these samples. A recent study revealed reduced intestinal Vdr, Atg16l1, and Paneth cell lysozyme expression, along with Paneth cell morphology defects, in patients with CD (Lu et al. 2021). Given this finding, and in the context of our findings in the ileum of mice, miR-142-3p should be measured in ileum tissues of CD patients.

In summary, we have demonstrated that miR-142-3p is upregulated with vitamin D deficiency in the ileum of mice and in active areas of inflammation in the colon of IBD patients with low levels of vitamin D, providing a potential explanation for the role of vitamin D in disease pathogenesis. Despite limitations in drawing conclusions between our mouse model and our patient cohort in the current study, we propose miR-142-3p

as a therapeutic target of interest. miRNAs represent potential therapeutic targets through the use of *in vivo* strategies to bind and inhibit specific miRNAs involved in disease. Such strategies have been employed in multiple preclinical models of colitis (Soroosh et al. 2018), suggesting relevance to IBD. Furthermore, anti-miRNA therapies are currently being tested in humans for the treatment of certain disorders such as hepatitis C virus infection (Janssen et al. 2013). The targeting of miRNAs involved in IBD, such as miR-142-3p, represents a potential future treatment option.

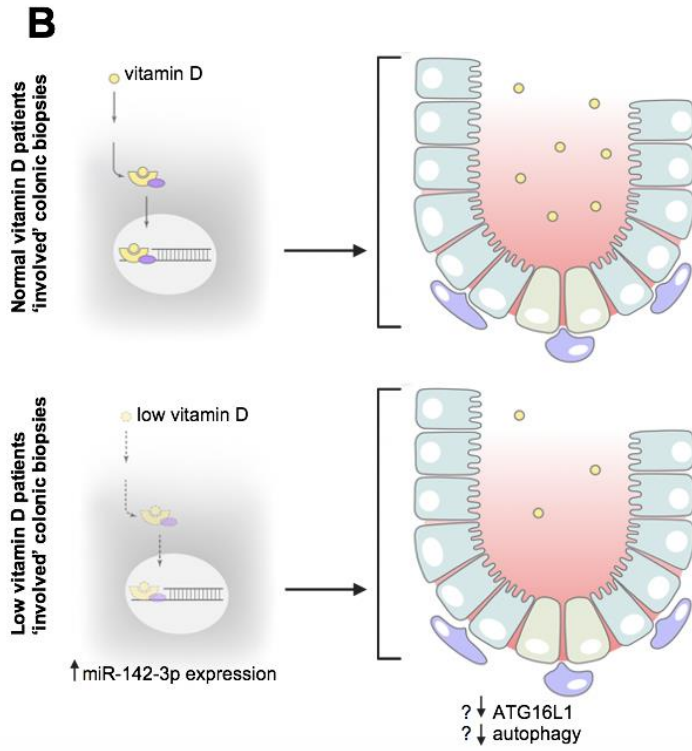
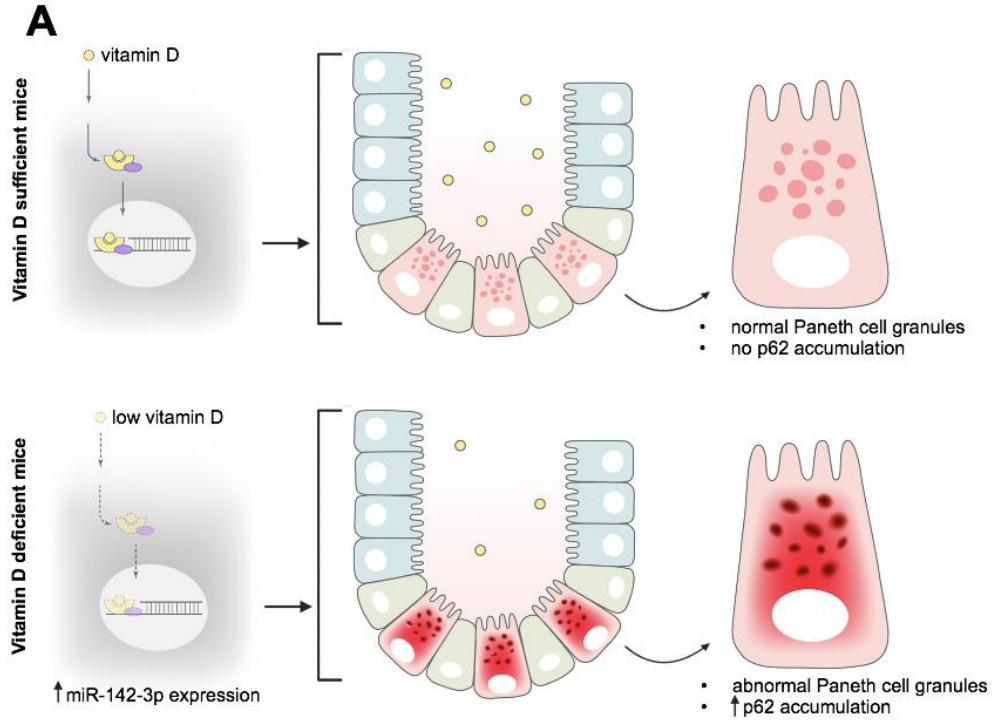


Figure 2.10: Chapter 2 model

(A) In mice, diet-induced vitamin D deficiency leads to increased expression of autophagy-modulating miR-142-3p in the ileal epithelium. Vitamin D deficiency and the corresponding increase in miR-142-3p is associated with p62 accumulation within Paneth cells and abnormal Paneth cell granule morphology, suggestive of dysregulation of the autophagy pathway within this cell type. miR-142-3p serves as a link between vitamin D deficiency and dysregulated autophagy, both of which contribute to the susceptibility of IBD. (B) In comparing colonic biopsies procured from 'involved' regions of disease from newly diagnosed paediatric IBD patients with and without low vitamin D status, patients with low vitamin D have an associated increase in expression of miR-142-3p. Future studies should address whether this increase in miR-142-3p leads to changes in autophagy in the epithelium.

Chapter 3
Linking gene-environment interactions in IBD: Vitamin D
deficiency and the *Nod2fs* mutation

3 Vitamin D deficiency and the *Nod2fs* mutation

3.1 Introduction

The multi-hit model of IBD pathogenesis suggests that IBD onset occurs due to an accumulation of genetic and environmental insults that eventually lead to the development of disease (Maloy and Powrie 2011; Philpott et al. 2014). The accumulation of many 'hits' to the epithelium makes it difficult to tease out the specific perturbation that ultimately causes disease onset. Similarly, early changes in the epithelium that are closely tied to pathogenesis are difficult to capture. Identifying research models that recapitulate the complex interactions that occur in the intestinal microenvironment of genetically susceptible hosts is challenging but will be instrumental to delineating pathogenesis and identifying therapeutic targets of interest.

A study conducted by Liu et al. (2018) demonstrated how combining genetic and environmental IBD risk factors in mice can lead to the discovery of therapeutic targets of interest. Gene-environment interactions present in *Atg16l1* T300A mice exposed to cigarette smoke led to metabolic dysregulation including Paneth cell defects and crypt base apoptosis, and targeting associated pathways therapeutically ameliorated intestinal defects in these mice (Liu et al. 2018). This work exemplifies how studying environmental triggers in genetically susceptible models can lead to the identification of clinically relevant targets. In the previous chapter, we identified an autophagy defect in the Paneth cells of vitamin D deficient mice (**Figure 2.10**). Several IBD risk genes, including *NOD2*, are also associated with autophagy defects in the intestinal epithelium. Given that predisposing mutations in *NOD2* have the strongest association with IBD risk and also lead to impairments in intestinal autophagy, we aimed to assess this pathway in mice homozygous for a mutant *Nod2* CD risk allele with concurrent vitamin D deficiency. We hypothesized that gene-environment interactions between mutant *Nod2* and vitamin D deficiency would lead to synergistic impairments in intestinal autophagy, thereby priming the intestine for barrier breakdown and onset of inflammation.

The current study aimed to combine a relevant environmental trigger in mice carrying mutant *Nod2* in order to gain a better understanding of gene-environment interactions in IBD. There are three common SNPs within the *NOD2* gene that predispose to CD,

including R702W, G908R, and L1007fs (Ahmad et al. 2002). Of the three most common SNPs, the L1007fs mutation, from here on referred to as the *NOD2* frameshift (*NOD2fs*), has the most dramatic effect on protein function and has the strongest correlation to CD (Ogura et al. 2001; Ahmad et al. 2002). The mutation involves a cytosine insertion in exon 11 at nucleotide 3020, resulting in a frameshift at the second nucleotide of codon 1007 (Ogura et al. 2001). At codon 1007 there is a leucine-to-proline substitution in the tenth LRR, followed by a premature stop codon (Ogura et al. 2001). The result is a truncated protein with only 1007 out of 1040 amino acids (**Figure 3.1**) (Ogura et al. 2001). This mutation within the LRR region results in loss of the ability for NOD2 to sense its ligand, and thus it was proposed that the NOD2fs protein is nonfunctional. Barnich *et al.* (2005) discovered that the Nod2fs mutant was unable to be recruited to the membrane during bacterial stimulation and the downstream NF- κ B signaling was lost (Barnich et al. 2005). Furthermore, Travassos et al. (2010) found that cells homozygous for the *Nod2fs* mutation have diminished induction of autophagy in response to invading bacteria (Travassos et al. 2010). These studies suggest that *Nod2fs* loses the ability to trigger both its inflammatory and autophagic defense mechanisms, rendering the epithelium susceptible to inflammation and barrier breakdown by invading pathogenic bacteria. Given the clinical importance of the mutation, we chose to use *Nod2fs* mice for the current study.

Throughout the course of this study, we identified persistent infection with *Tritrichomonas muris* (*T. muris*) in a subset of our mice. *T. muris* is a common mouse protozoa that has been traditionally regarded as nonpathogenic (Escalante et al. 2016). However, recent studies have found that the presence of *T. muris* can promote type 2 immune responses in the gut (Howitt et al. 2016). Furthermore, it has been demonstrated that mice infected with *T. muris* develop an exaggerated response to the T cell transfer model of colitis (Escalante et al. 2016). In this model, *T. muris*-infected mice exhibited an enhanced IFN γ + CD4 T cell response and epithelial damage in response to the colitic trigger in comparison with uninfected mice (Escalante et al. 2016). The same study also demonstrated that mice chronically colonized with *T. muris* have an altered baseline number of Th1 cells in the intestine (Escalante et al. 2016). Although *T. muris* may not cause overt disease in otherwise healthy mice, the current

evidence suggests that it can alter immune responses at baseline and in disease models. Given the association between perturbances in the microbiome and the development of disease in susceptible hosts, we conducted our analyses on both uninfected and infected subsets of mice.

In the current study, we measured intestinal autophagy in *Nod2fs* mice and wildtype littermate controls fed either a vitamin D deficient or control diet (**Figure 3.2 A**). We repeated the analyses for mice that were chronically infected with *T. muris* (**Figure 3.2 B**). There were no changes in our measures of intestinal autophagy in uninfected *Nod2fs* mice fed either a vitamin D deficient or control diet. However, in mice that were colonized with *T. muris*, we identified a reduction in whole tissue Atg16l1 expression in vitamin D deficient *Nod2fs* mice compared to *Nod2fs* littermates on the control diet. The dysregulation in autophagy had a strong association in the Paneth cells, but the effect persisted in the full crypt epithelium. This study highlights how multiple genetic and environmental hits can combine to disrupt intestinal epithelial homeostasis.

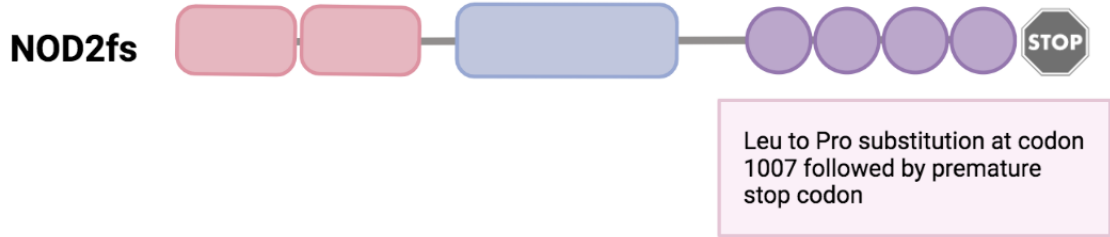
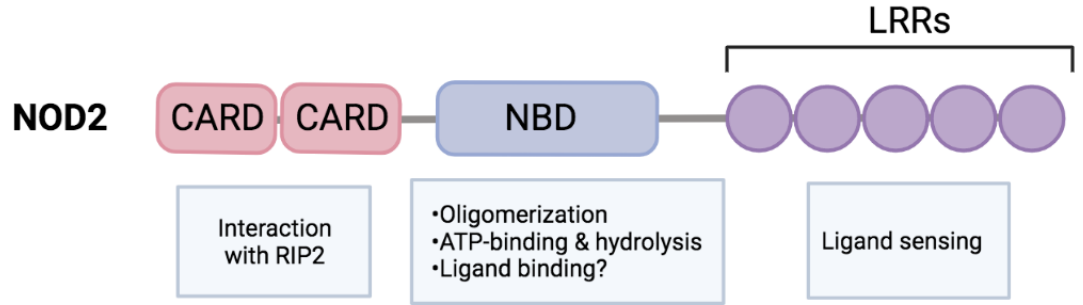


Figure 3.1: NOD2 and NOD2fs structure

Nucleotide oligomerization domain-containing protein 2 (NOD2) has a tripartite structure composed of tandem amino-terminal caspase-recruitment domains (CARDs), a central nucleotide-binding domain (NBD), and a series of carboxy-terminal leucine-rich repeats (LRRs). Residues within the LRR are essential to the sensing of muramyl dipeptide (MDP) by NOD2. The NBD facilitates oligomerization of NOD proteins and ATP binding. The CARDs are essential for interacting with receptor-interacting protein 2 (RIP2). The 1007fs/3020InsC mutation, also called the *NOD2* frameshift (*NOD2fs*) mutation, involves a cytosine insertion in exon 11 at nucleotide 3020, resulting in a frameshift at the second nucleotide of codon 1007. At codon 1007 there is a leucine-to-proline substitution in the tenth LRR, followed by a premature stop codon. The result is a truncated protein with only 1007 out of 1040 amino acids. This mutation within the LRR region results in loss of the ability for NOD2 to sense its ligand, and thus it was proposed that the *NOD2fs* protein is nonfunctional. Adapted from Philpott et al. (2014) with permission.

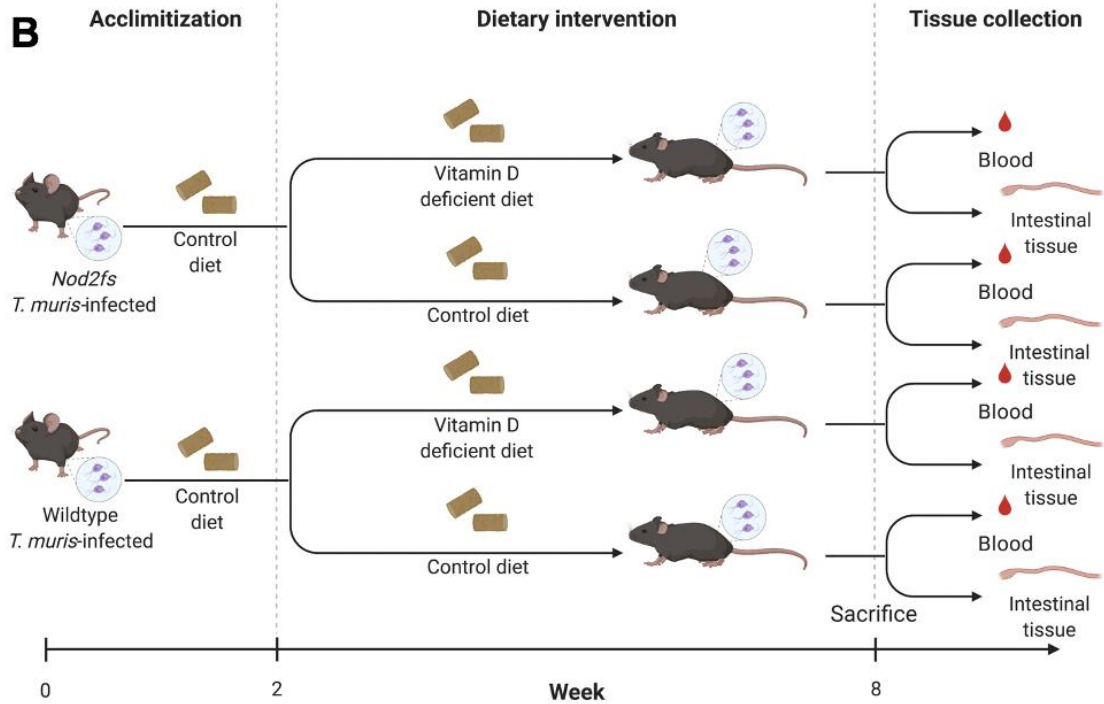
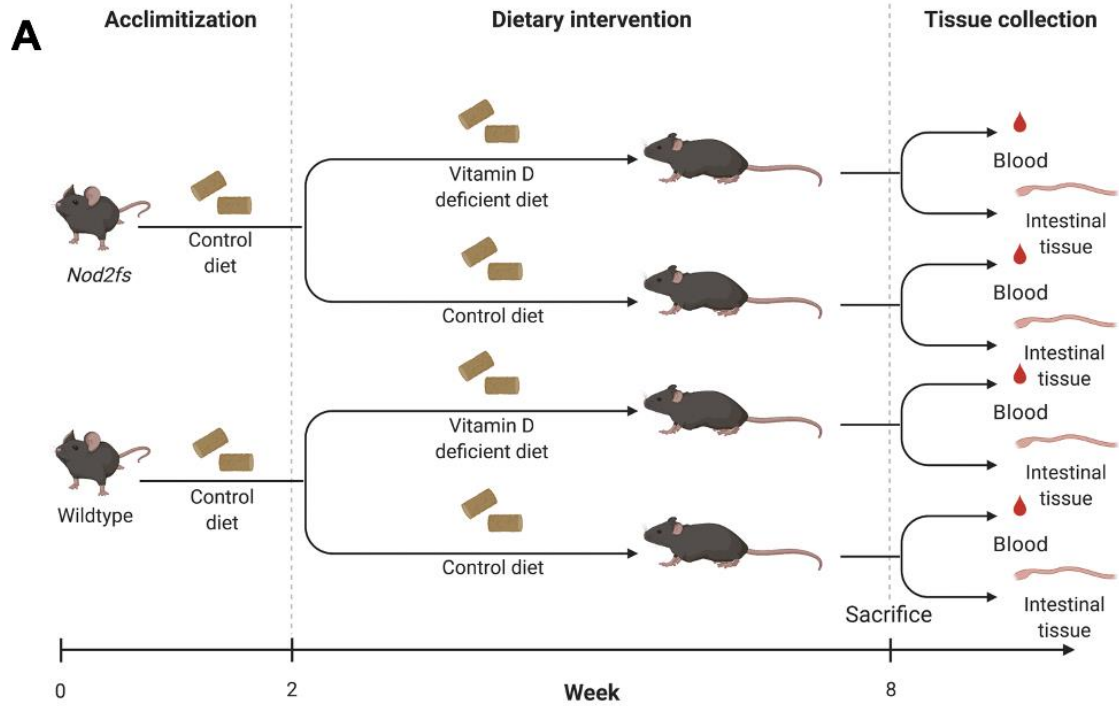


Figure 3.2: Chapter 3 experimental procedure

(A) *Nod2*^{fs} mice and wildtype littermate controls were acclimatized on the control diet for 2 weeks before being randomized to receive either the vitamin D deficient diet or matched control diet for 6-7 weeks. After the feeding period, mice were sacrificed and blood and intestinal tissues were collected. (B) The same experimental procedure from (A) was performed in mice infected with *Tritrichomonas muris*.

3.2 Methods

3.2.1 Vitamin D deficient animal model

All animal experiments were approved by the University of Toronto's Animal Care Committee and in accordance with the Canadian Council of Animal Care guidelines (protocol 20011441). Mice were housed under specific pathogen-free conditions on a 12-hour light/dark cycle with *ad libitum* access to food and water. Mice heterozygous for the *Nod2fs* mutation on C57BL/6 background were supplied by the lab of Dr. Dana Philpott at the University of Toronto. Heterozygotes were bred and homozygous *Nod2fs* mice and wildtype littermate controls, both male and female, were used in the experiments. At 3 weeks of age, mice were acclimatized to the control diet for 2 weeks before being randomized to receive either the vitamin D deficient diet (0 IU/g vitamin D₃) (57BQ; TestDiet, Richmond, IN, USA) or matched control diet (1.0 IU/g vitamin D₃) (58M1; TestDiet, Richmond, IN, USA) for 6-7 weeks.

3.2.2 Tissue preparation

Mice were euthanized by carbon dioxide exposure and cervical dislocation. Blood was collected post-euthanasia by cardiac puncture and serum was separated and collected. Ileum and colon samples were rinsed in PBS and prepared for whole tissue lysates, isolation of epithelial cells, and FFPE samples. Whole tissue was frozen and stored at -80°C for subsequent lysis for immunoblotting. Tissue for epithelial cell isolation was placed directly into cell recovery solution (354253; Corning Life Sciences, Tewksbury, MA, USA) and processed as indicated below. Tissue for FFPE samples was placed into 10% formalin and later processed for histology.

3.2.3 Isolation of intestinal epithelial cells

Intestinal tissues were incubated in cell recovery solution overnight at 4°C. Samples were transferred to a conical tube containing PBS +1% FBS and shaken to release the epithelial cells. The epithelial cell-containing supernatant was collected and centrifuged at 1500 rpm for 10 mins at 4°C. Cell pellets were lysed for western blotting.

3.2.4 Measurement of murine serum vitamin D levels

Serum 25(OH)D was measured in murine serum using the 25-OH vitamin D ELISA kit (VID31-K01; Eagle Biosciences, Amherst, NH, USA) as per the manufacturer's guidelines. Briefly, murine serum samples, calibrators, and controls were diluted with biotin-labeled 25(OH)D and added in duplicates to a 96-well microplate coated with monoclonal anti-25(OH)D antibodies detecting vitamin D₂ and D₃ with 100% specificity. In this assay, the known amount of biotin-labeled 25(OH)D competes with the unknown amount of 25(OH)D in the serum samples for the antibody binding sites on the coated wells during the incubation period. The unbound 25(OH)D was discarded and a second incubation using peroxidase-labeled streptavidin was performed to detect the bound biotin-labeled 25(OH)D. Finally, a third incubation was performed using the peroxidase substrate tetramethylbenzidine and the bound peroxidase caused a change in colour. The intensity of the colour is inversely proportional to the 25(OH)D concentration of the sample. The detection limit of the assay is 1.6 ng/mL.

As soon as possible and within 30 min, the plate was read at 450 and 650 nm wavelengths on a VersaMax 190 visible plate reader (Molecular Devices, San Jose, California, USA). The 450 nm wavelength detects the optical density of the sample, and the 650 nm reading detects background signal. The background signal was subtracted from the optical density for each well and duplicates for each sample were averaged. A standard curve was created using the values of the calibrators. An exponential trendline was plotted and the concentration of 25(OH)D was measured by inputting each value into the equation of the y-intercept of the standard curve.

3.2.5 Immunofluorescent staining of murine tissues

Murine intestinal FFPE samples were cut into 5 µm sections and affixed onto a glass slide. Tissue sections were deparaffinized and rehydrated using the following steps:

1. Xylene (100%), 5 min x2
2. Xylene (100%) 1:1 with ethanol (100%), 5 min
3. Ethanol (100%), 3 min x2

4. Ethanol (95%), 3 min
5. Ethanol (70%), 3 min

Slides were then rinsed in running deionized water for 5 min or until ready to proceed to antigen retrieval. The slides were not allowed to dry at any point during the protocol in order to prevent non-specific antibody staining. Slides were then immersed in freshly prepared 10 mM sodium citrate buffer with 0.05% Tween-20, adjusted to pH 6.0. Heat-induced epitope retrieval was performed at 110°C for 7.5 min in a decloaking chamber (Biocare Medical, Pacheco, CA, USA). Following antigen retrieval, slides were washed in PBS and a barrier was created around the tissue using a hydrophobic pen. Blocking solution containing 10% goat serum in PBS was added to the tissues for 1 h. Tissues were incubated in primary antibodies in blocking solution overnight at 4°C in a humid chamber. The following day, slides were washed in PBS and the appropriate HRP-conjugated secondary antibody was added to tissues for 1 h at room temperature. Slides were washed and nuclei were labeled using DAPI at a concentration of 1 µg/mL in PBS for 10 min. Slides were washed a final time before DAKO fluorescence mounting medium (Agilent Technologies, Santa Clara, CA, USA) was added onto the tissue and a glass coverslip was placed on top. Slides were left to dry overnight.

3.2.6 Confocal microscopy and quantitation

Images were acquired using a Quorum spinning disk confocal microscope using a 63x oil immersion objective with identical settings between conditions. Image acquisition and analysis was performed using Volocity 3D Image Analysis Software (PerkinElmer, Waltham, MA, USA). Unless indicated otherwise, images shown for immunofluorescent staining of murine tissues are merged z-planes.

3.2.7 Paneth cell analysis

For p62 immunofluorescent staining, tissue sections were deparaffinized, blocked in 10% goat serum in PBS, and incubated with 1:200 dilution of anti-p62/SQSTM1 antibody (RRID:AB_10011069, NBP1-48320; Novus Biologicals, Littleton, CO, USA) overnight at 4°C. The following day tissues were washed with PBS and incubated at room temperature with 1:1000 dilution of Alexa Fluor 488 goat anti-rabbit IgG secondary

antibody (Life Technologies, Carlsbad, CA, USA), washed with PBS, and co-stained with DAPI to label nuclei. Confocal images of well-oriented crypts were captured and p62 fluorescence intensity was measured blindly. For measurement of p62 fluorescence intensity in the full crypt, a region of interest was drawn around the crypt and mean intensity per crypt was compared between groups. For measurement of p62 intensity in Paneth cells, tissues were co-stained with rhodamine labeled UEA-I to label Paneth cells (RRID:AB_2336769, RL-1062; Vector Laboratories, Burlingame, CA, USA). Briefly, deparaffinized tissue sections were incubated with 1:2000 rhodamine-labeled UEA-I for 2 h at room temperature. A region of interest was drawn around each Paneth cell and the average p62 intensity was per crypt was determined and compared between groups.

3.2.8 Immunoblotting and quantitation

Murine intestinal tissues were homogenized in approximately 300 μ L RIPA buffer (50mM Tris-HCl pH 8.0, 150mM sodium chloride, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with the following protease and phosphatase inhibitors: 1mM sodium orthovanadate, 50mM sodium fluoride, 1% protease inhibitor cocktail, and 0.5mM phenylmethylsulfonyl fluoride (all Sigma-Aldrich, St. Louis, MO, USA) and lysed for 30 min on ice. Suspensions were centrifuged at 13 000 rpm for 15 min at 4°C. Supernatants were collected and a Bradford assay was performed to quantify protein concentration (Bio-Rad Laboratories, Hercules, CA, USA). Approximately 60-80 μ g of whole intestinal tissue lysates were aliquoted. Laemmli sample buffer was added at 1x concentration and samples were heated at 95°C for 5 min. Samples were separated by SDS-PAGE at 135 volts for approximately 1 h 30 min. Proteins were transferred to nitrocellulose membrane at 70 volts for approximately 1 h 30 min at 4°C. Blots were blocked in 5% weight/volume non-fat milk powder prepared in TBS-T for 1 h and subsequently incubated with primary antibodies overnight at 4°C in blocking solution. The following day, blots were washed in TBS-T and incubated with the appropriate HRP-conjugated secondary antibody for 1 h at room temperature in blocking solution. Blots were washed in TBS-T before being visualized by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnologies, Dallas, TX, USA) or high-sensitivity chemiluminescence (Clarity Max Enhanced

Chemiluminescence; Bio-Rad Laboratories, Hercules, CA, USA) as required using the LI-COR Odyssey FC imaging system (LI-COR Biosciences, Lincoln, NE, USA).

The following primary antibodies were used: 1:2000 dilution of anti-LC3 (NB600-1384; Novus Biologicals, Littleton, CO), 1:500 dilution of anti-ATG16L1 (RRID:AB_10950320, 80895; Cell Signaling Technology, Danvers, MA, USA) and 1:10000 dilution of anti- β -actin (A5441; Sigma-Aldrich, St. Louis, MO, USA). The appropriate HRP secondary was used (goat anti-mouse or goat anti-rabbit) at a dilution of 1:5000 (both Cedarlane, Burlington, ON).

Densitometric analysis was performed using ImageLab 6.0 Software (Bio-Rad Laboratories, Hercules, CA, USA). The densities of proteins of interest were quantified and normalized to the expression of the loading control protein and expressed as a fold change relative to the wildtype control value.

3.2.9 Statistical analysis

The results are presented as the mean value \pm SEM. The normality of distribution of all continuous variables was tested using the Shapiro-Wilk test and the proper parametric or non-parametric test was performed. Data were analyzed using unpaired Kruskal-Wallis test with multiple comparisons or ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test as appropriate and the data are expressed as the mean \pm SEM relative to the control group. For these studies, each n represents data from one sample from one mouse unless indicated otherwise. A p-value of <0.05 was considered statistically significant for all analyses. Statistical analyses were performed using GraphPad Prism v8.1.2 (GraphPad Software, San Diego, CA, USA) or SPSS v23 (IBM, Armonk, NY, USA).

3.3 Results

3.3.1 Vitamin D deficiency does not cause autophagy dysregulation in wildtype or *Nod2fs* mice

We first performed a vitamin D ELISA to confirm a relative deficiency was achieved in mice fed the vitamin D deficient diet compared to mice fed the control diet. We identified a reduction in vitamin D levels in both the wildtype and *Nod2fs* mice fed the vitamin D deficient diet compared to control (**Figure 3.3 A**). Unexpectedly, we also identified a reduction in serum vitamin D in the *Nod2fs* mice fed the control diet compared to wildtype mice on the same diet. We explored this association in the next chapter.

Although we previously identified no change in ileal whole tissue autophagy in wildtype mice with vitamin D deficiency (**Figure 2.3**), we hypothesized that the *Nod2fs* genotype in combination with vitamin D deficiency would act to synergistically dysregulate intestinal autophagy, given the involvement of both NOD2 and VDR in maintaining intestinal homeostasis through autophagy regulation. This is in line with the multi-hit model of IBD which suggests that genetic and environmental insults accumulate to lead to epithelial dysregulation and the onset of inflammation (Maloy and Powrie 2011).

We did not observe any changes in the expression of Atg16l1 or Lc3II in the ileum whole tissue, indicating that intestinal autophagy pathway was not defective in whole tissue, even with the combination of the *Nod2fs* genotype and vitamin D deficiency (**Figure 3.3 B-D**). There was also no change in Atg16l1 or Lc3II expression in isolated epithelial cells (**Figure 3.4**). Given that Paneth cells are particularly sensitive to changes in autophagy, we analyzed autophagic flux in these cells by immunostaining for autophagy adaptor protein p62 and Paneth cell marker UEA-I. Unexpectedly, there were no differences in p62 accumulation in Paneth cells (**Figure 3.5 A**) or in the full crypt epithelium (**Figure 3.5 B**) among mice.

The finding of no changes in p62 accumulation in Paneth cells in either wildtype or *Nod2fs* vitamin D deficient mice was unexpected given that our previous studies revealed an autophagy defect in Paneth cells of vitamin D deficient mice (**Figure 2.8**). This finding was not replicated in the current study, likely attributable to microbiome

differences in the mice. Mice used in the previous study were obtained through a vendor, while the mice in the current study were an in-house bred strain, and thus differences in the microbiome due to the source of the mice may have contributed to the divergent results. Taken together, these results suggest that vitamin D deficiency and the *Nod2fs* mutation either alone or in combination do not change levels of intestinal autophagy.

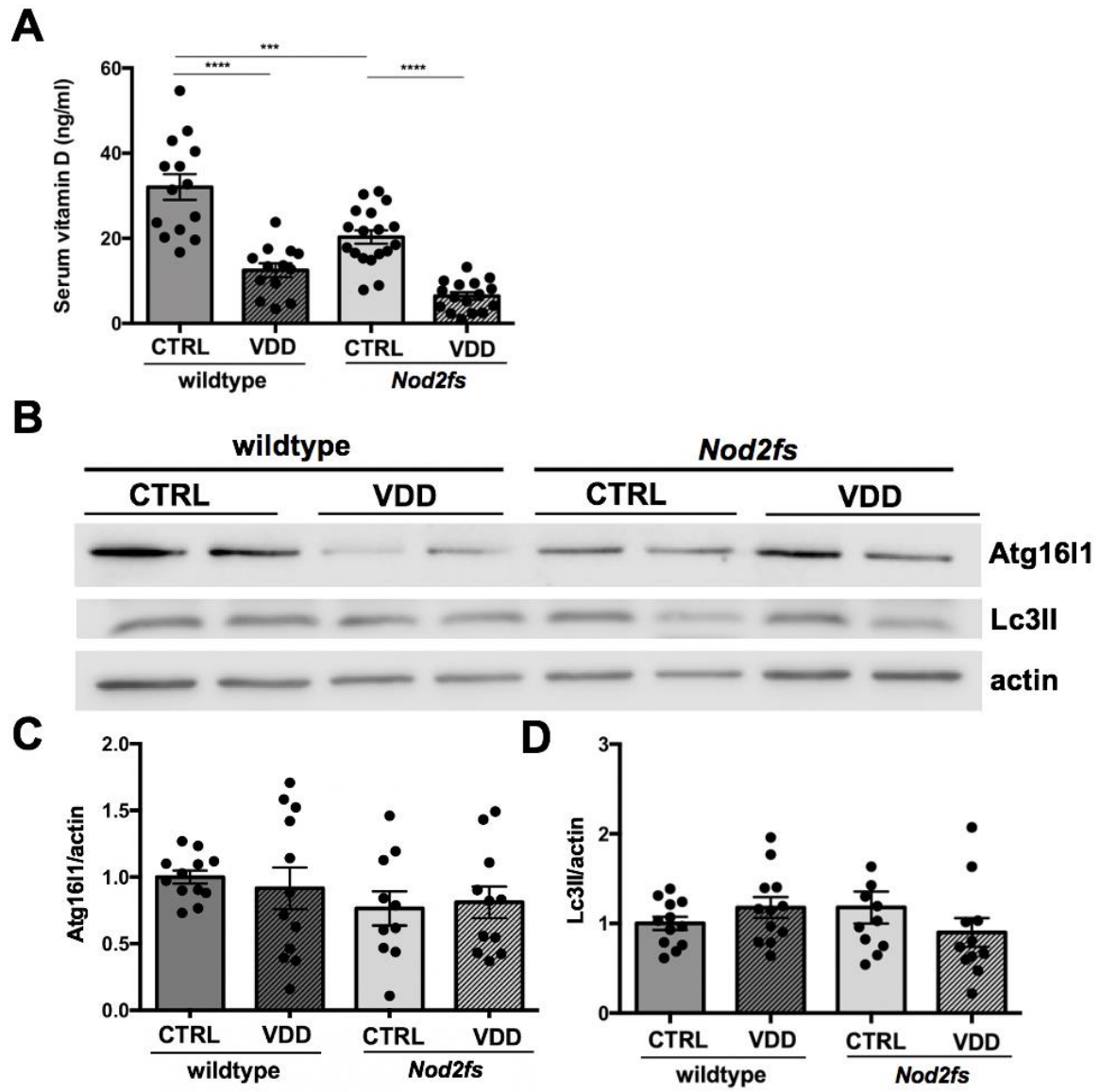


Figure 3.3: No change in autophagy proteins in whole tissue from vitamin D deficient wildtype or *Nod2fs* mice

(A) Serum 25-hydroxyvitamin D was measured in wildtype and *Nod2fs* mice fed either the vitamin D deficient (VDD) or control (CTRL) diet after a 6-7-week feeding period. (B) Ileal whole tissue from wildtype and *Nod2fs* mice fed either the vitamin D deficient or control diet was assessed for expression of Atg16l1 and Lc3II protein by western blotting. A representative blot with 2 animals per group is shown. Densitometric analysis of (C) Atg16l1 and (D) Lc3II expression from (B) was performed. Values were normalized to actin and expressed as a fold change relative to the control group. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Error bars represent \pm SEM. n=11-12/group. ***p<0.001, ****p<0.0001

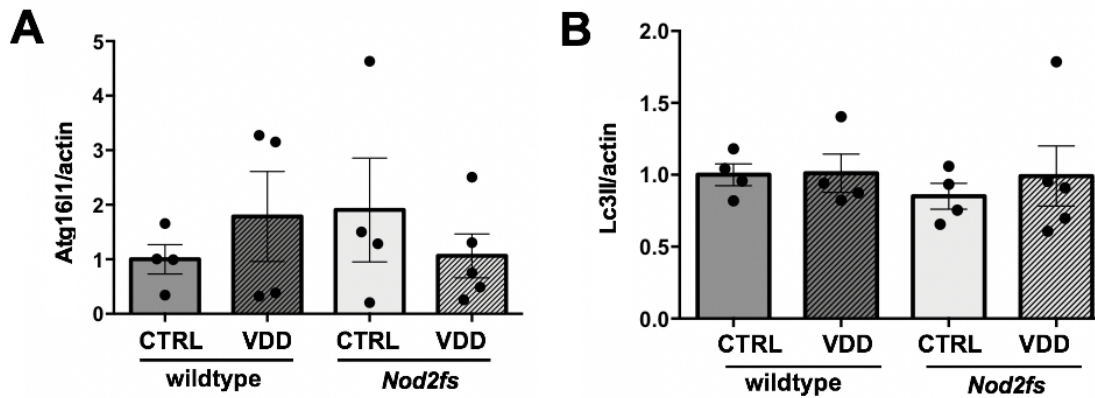


Figure 3.4: No change in autophagy proteins in isolated epithelial cells from vitamin D deficient wildtype or *Nod2fs* mice

Epithelial cells isolated from the ileum of wildtype and *Nod2fs* mice fed either the vitamin D deficient (VDD) or control (CTRL) diet were assessed for expression of Atg1611 and Lc3II protein by western blotting. Densitometric analysis of (A) Atg1611 and (B) Lc3II expression was performed. Values were normalized to actin and expressed as a fold change relative to the control group. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Error bars represent \pm SEM. n=4-5/group

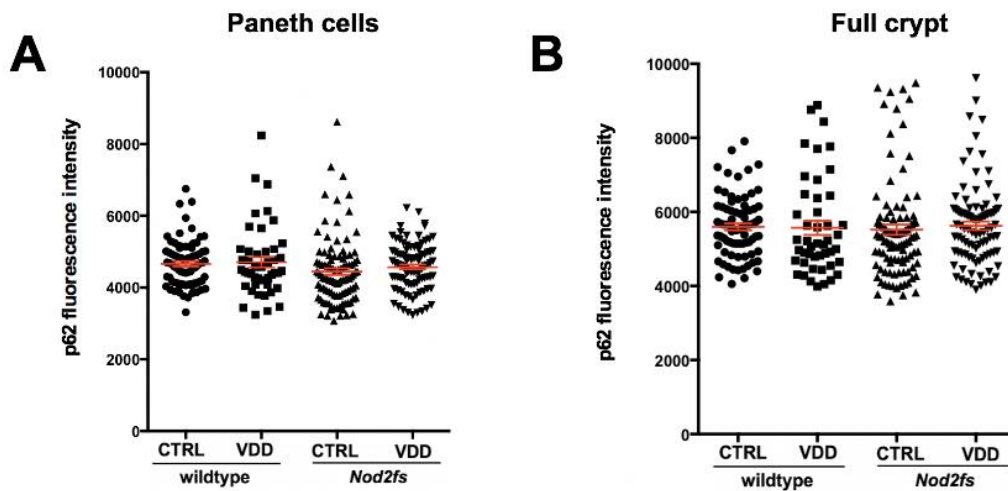


Figure 3.5: No change in p62 expression in Paneth cells or full crypt epithelium in ileal samples from vitamin D deficient wildtype or *Nod2fs* mice.

Ileal tissue sections from wildtype and *Nod2fs* mice fed either a vitamin D deficient or control diet were co-stained for autophagy adaptor protein p62, Paneth cell marker UEA-I, and DAPI to label nuclei. Confocal images of well-oriented crypts were captured. Fluorescence intensity of p62 was blindly measured in (A) Paneth cells and (B) the full crypt epithelium. Average fluorescence intensity per crypt is shown. Data were analyzed by Kruskal-Wallis test. Error bars represent \pm SEM. n=46-104/group

3.3.2 Colonization with *Tritrichomonas muris* causes autophagy dysregulation in vitamin D deficient *Nod2fs* mice

During the course of our studies, we became aware that our mice were colonized with *T. muris*, a protozoa common to North American animal facilities that has been recently shown to influence the immune response in the intestine. Upon discovering this, we re-bred our colony using *Nod2fs* mice that were housed in a separate room in the facility and were not infected with *T. muris*. The data presented thus far is from mice that were not colonized with *T. muris*, however, we conducted separate analyses on mice that tested positive for *T. muris*, which will be presented here.

We first performed a vitamin D ELISA to confirm that mice fed the vitamin D deficient diet achieved a relative deficiency. There was a reduction in serum vitamin D in wildtype and *Nod2fs* mice fed the vitamin D deficient diet, as expected (**Figure 3.6 A**).

Additionally, the *Nod2fs* mice on the control diet exhibited reduced serum vitamin D compared to wildtype mice on the same diet, as was observed in the *T.muris*-free cohort of mice. Together, these results indicate that the presence of *T. muris* did not affect the serum vitamin D status of mice.

We next measured autophagy in ileum whole tissue by western blotting for Atg16l1 and Lc3II (**Figure 3.6 B-D**). Interestingly, among these mice infected with *T. muris*, there was a reduction of Atg16l1 expression in the ileal whole tissue of vitamin D deficient *Nod2fs* mice compared to *Nod2fs* mice fed the control diet (**Figure 3.6 B,C**). However, there were no changes observed in the expression of autophagosome marker Lc3II (**Figure 3.6 B,D**). Taken together, these results demonstrate that there is dysregulation of Atg16l1 in vitamin D deficient *Nod2fs* mice colonized with *T. muris*.

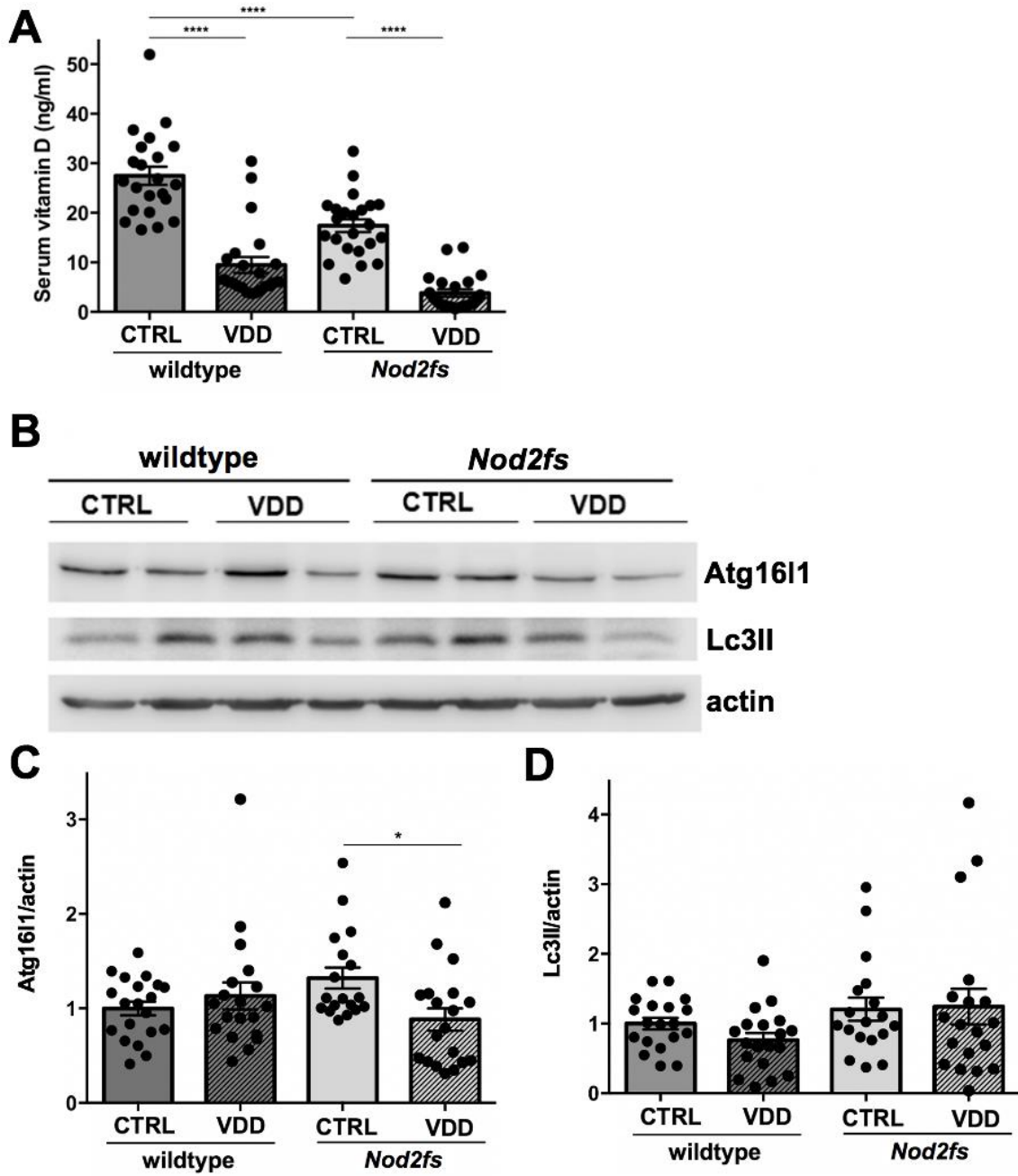


Figure 3.6: Atg16I1 expression is reduced in ileal whole tissue from *T. muris*-infected vitamin D deficient *Nod2fs* mice compared to infected *Nod2fs* mice fed a control diet

(A) Serum 25-hydroxyvitamin D was measured in *T. muris*-infected wildtype and *Nod2fs* mice fed either the vitamin D deficient (VDD) or control (CTRL) diet after a 6-7-week feeding period. n=22-23/group. (B) Ileal whole tissue from *T. muris*-infected vitamin D deficient and control mice was assessed for expression of Atg16I1 and Lc3II protein by western blotting. A representative blot with 2 animals per group is shown. Densitometric analysis of (C) Atg16I1 and (D) Lc3II expression from (B) was performed. Values were normalized to actin and expressed as a fold change relative to the control group. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Error bars represent \pm SEM. n=18-20/group. ****p<0.0001

To further characterize intestinal autophagy given the reduction in Atg16l1 expression in *T. muris*-infected vitamin D deficient *Nod2fs* mice, we performed immunostaining on ileal tissues to assess autophagic flux in Paneth cells and in the full crypt epithelium (**Figure 3.7 A**). We identified increased accumulation of p62 in Paneth cells of *T. muris*-infected vitamin D deficient *Nod2fs* mice compared to every other group of mice (**Figure 3.7 B**), suggesting that Paneth cells contribute to the autophagy dysregulation observed at the whole tissue level. In the full crypt epithelium, we similarly identified increased accumulation of p62 in *T. muris*-infected vitamin D deficient *Nod2fs* mice compared to every other group of mice (**Figure 3.7 C**). This suggests that in addition to Paneth cells, other epithelial cell types may contribute to the autophagy defect present.

Compared to *T. muris*-infected wildtype mice on the control diet, there was a reduction in p62 accumulation in Paneth cells of *T. muris*-infected wildtype mice on the vitamin D deficient diet and *T. muris*-infected *Nod2fs* mice on the control diet (**Figure 3.7 B**). In the full crypt epithelium, the reduction of p62 accumulation in *T. muris*-infected wildtype mice on the vitamin D deficient diet compared to *T. muris*-infected wildtype mice on a control diet persisted (**Figure 3.7 C**). Given that these effects were not present in uninfected mice, it is likely that *T. muris* is contributing to autophagy dysregulation in the epithelium.

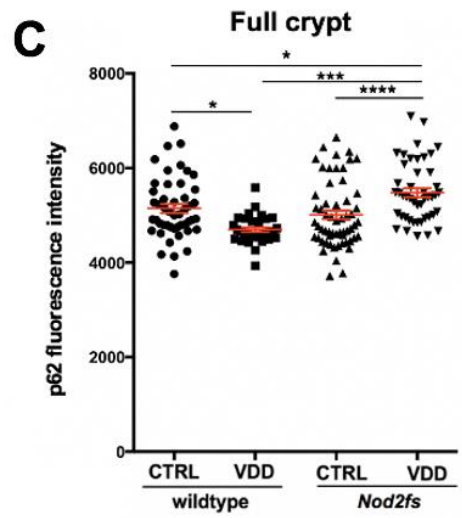
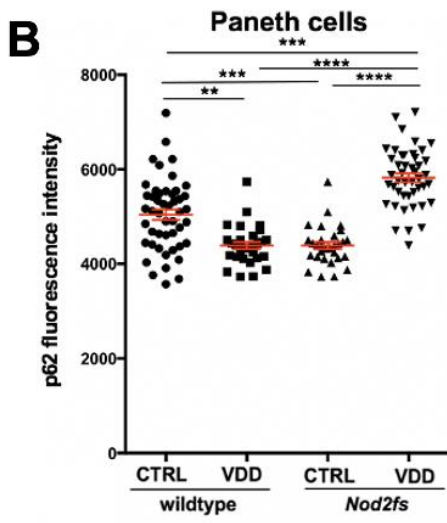
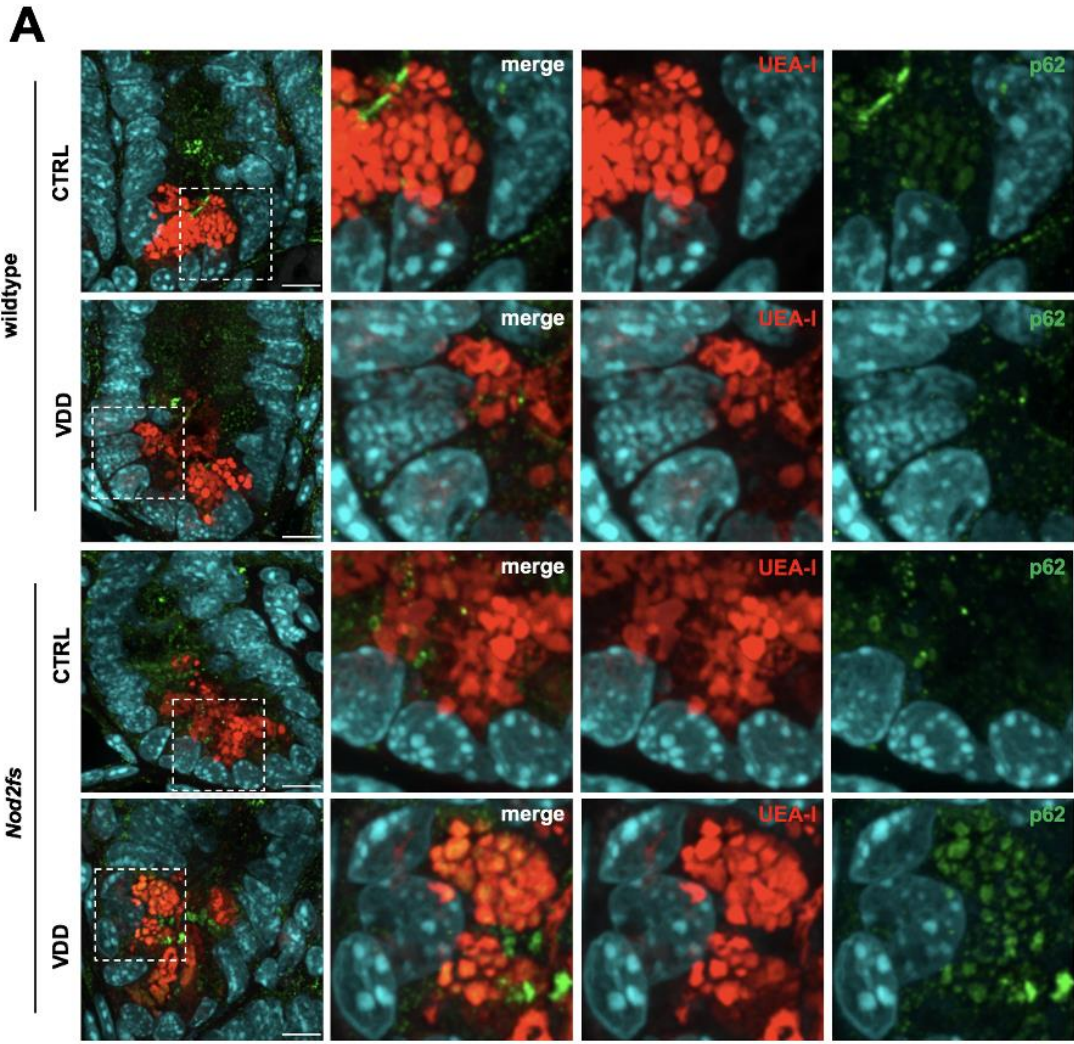


Figure 3.7: Accumulation of p62 in Paneth cells and the full crypt epithelium of *T. muris*-infected vitamin D deficient *Nod2fs* mice

Ileal tissue sections from *T. muris*-infected wildtype and *NOD2fs* mice fed either a vitamin D deficient (VDD) or control (CTRL) diet were co-stained for autophagy adaptor protein p62, Paneth cell marker UEA-I, and DAPI to label nuclei. (A) Confocal images of well-oriented crypts were captured. Scale bar represents 10 μm . Fluorescence intensity of p62 was blindly measured in (B) Paneth cells and (C) the full crypt epithelium.

Average fluorescence intensity per crypt is shown. Data were analyzed by Kruskal-Wallis test with multiple comparisons. Error bars represent \pm SEM. n=30-59/group.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

3.4 Discussion

Gene-environment interactions are an important component of IBD research and are beginning to provide insight on why some individuals with genetic susceptibility alleles develop disease while others remain healthy. In the current study, we explore the interaction between CD risk allele *Nod2fs* and vitamin D deficiency in mice. Both of these IBD risk factors have been independently linked with autophagy. Unexpectedly, the combination of these factors had no effect on intestinal autophagy at baseline in our model. However, persistent colonization with *T. muris* in addition to the *Nod2fs* genotype and vitamin D deficiency led to dysregulation of intestinal autophagy. *T. muris* infection caused a reduction in Atg16l1 expression in ileum whole tissue alongside accumulation of p62 in the full crypt epithelium and Paneth cells in vitamin D deficient *Nod2fs* mice. These findings are of interest given the central role of the microbiome in IBD pathogenesis.

The microbiome is an essential component of IBD development. Presence of the microbiome is required for the development of intestinal inflammation, given that germ-free mice do not develop colitis (Dianda et al. 1997; Matsumoto et al. 1998; Sellon et al. 1998; Madsen et al. 1999). Furthermore, IBD patients experience dysbiosis (Manichanh et al. 2006; Willing et al. 2010; Lepage et al. 2011) and changes in the microbiome have been demonstrated to precede the onset of disease (Galipeau et al. 2021). Accordingly, several studies have introduced microbial challenges into IBD-relevant mouse models. For example, a study conducted by Cadwell et al. (2010) demonstrated that mice with hypomorphic expression of Atg16l1 have no intestinal epithelial defects at baseline but develop Paneth cell abnormalities after MNV infection. Mice hypomorphic for Atg16l1 also displayed enhanced intestinal damage in response to DSS administration, but only in the presence of MNV infection (Cadwell et al. 2010). Similarly, a recent study by Lu et al. (2021) demonstrated that *Vdr^{ΔPC}* mice have an exaggerated response to *Salmonella* infection. Given these results, it is not surprising that in our susceptible model, *T. muris* infection leads to differences in the epithelium that are not observed otherwise. Future studies should focus on delineating the mechanisms through which *T. muris* leads to autophagy dysregulation in vitamin D deficient *Nod2fs* mice.

Interestingly, vitamin D deficiency, *Nod2*, and *T. muris* have each been independently associated with goblet cells, representing a shared feature that should be explored in future studies. *Nod2* signaling is associated with production of Muc2 and goblet cell hyperplasia in response to infection with an enteric pathogen in mice (Wang et al. 2016). Diet-induced vitamin D deficiency has been linked to a reduction of goblet cell expression in the epithelium (Su et al. 2016). Furthermore, *Vdr*KO mice have reduced expression of Muc2, suggesting that vitamin D signaling through *Vdr* directly influences Muc2 expression. Importantly, Muc2 expression and goblet cell hyperplasia are key features of the Th2 immune response against protist infection (Howitt et al. 2016). Mucin production is a protective host mechanism that is key to pathogen clearance. Therefore, it is reasonable to postulate that mice with the *Nod2fs* genotype and vitamin D deficiency would have an impaired response to protists, and the combination of the two factors may lead to a synergistic dysregulation of the epithelium in the presence of *T. muris*. Therefore, it is plausible that goblet cells, which are also highly dependent on autophagy and regulated by both vitamin D and *Nod2*, are contributing to the impairment of autophagy observed in the epithelium in vitamin D deficient *Nod2fs* mice infected with *T. muris*. In future studies, goblet cells should be assessed by Muc2 immunofluorescent staining. Co-staining of Muc2 and p62 can also be performed to determine if goblet cells have a significant contribution to the autophagy dysregulation observed in the epithelium.

There are several studies that we are interested in performing in order to build upon the results of the current study. It is important to note that in the current study, the colonization of *T. muris* was naturally occurring and not experimentally induced. Because infection was natural, the uninfected and infected mice are not littermates, and there are likely other aspects of the microbiome that differ between the two subsets of mice. Additionally, the pathogen burden is unknown within the *T. muris*-infected mice, and it could vary across groups, potentially influencing the results. We propose that experimental infection with *T. muris* should be performed in this model. We are also interested in delineating the cellular mechanism responsible for the intestinal autophagy defect observed in the current study in *T. muris*-infected vitamin D deficient *Nod2fs* mice. Given that there is p62 accumulation within the Paneth cells of these mice, we propose

that Paneth cells are further characterized. Paneth cell morphology, number of Paneth cells per crypt, and functional analyses of Paneth cells including measurement of key antimicrobial peptides and microbiome analysis should be performed. Additionally, transcriptional profiling of Paneth cells may reveal mechanistic pathways of interest that may explain the substantial p62 accumulation in this cell type. Given the high content of ER and autophagy defect in Paneth cells, it would be of interest to measure ER stress in this model through assessing levels of inositol-requiring transmembrane kinase/endoribonuclease 1 (IRE1) phosphorylation. Elucidating mechanisms of the autophagy defect incurred in our IBD-relevant model upon *T. muris* infection may allow for the identification of therapeutic targets of interest.

IBD is an extremely complex condition that occurs in patients due to the convergence of many genetic, environmental, and microbial insults that render the intestinal epithelium susceptible to breakdown and chronic inflammation. This study outlined how complex the interactions that lead to epithelial defects can be. Research models that capture the complexity of IBD and explore gene-environment interactions are required to identify targetable pathways of interest for the treatment of IBD.

Chapter 4
Exploring the association between *Nod2* and serum vitamin D

4 *Nod2* and serum vitamin D

4.1 Introduction

In Chapter 3, we came across an unanticipated finding related to a prominent IBD genetic risk variant that we sought to explore further. We identified that mice with the *Nod2fs* genotype had lower serum vitamin D compared to their wildtype counterparts (**Figure 3.3 A**). We replicated this finding many times in several groups of mice. The effect persisted in mice that were infected with *T. muris* (**Figure 3.6 A**). This is a novel finding that could have unidentified implications in IBD pathogenesis.

Vitamin D and *NOD2* have previously been linked to one another. It has been found that the active form of vitamin D, 1,25-dihydroxyvitamin D or calcitriol, induces *NOD2* expression (Wang et al. 2010). This is mediated by the VDR, which directly influences transcription of the *NOD2* gene (Wang et al. 2010). However, a relationship in the opposite direction has not been identified. The expression or function of *NOD2* has not been linked to changes in the vitamin D pathway. Given our unexpected finding of a possible role for *Nod2* in influencing the level of serum vitamin D, we aimed to explore aspects of this association to gain further insight into the potential mechanisms involved.

This finding of reduced serum vitamin D in mice homozygous for the *Nod2fs* variant is interesting given the implications of low serum vitamin D in individuals who are predisposed to IBD and in IBD patients. Low serum vitamin D has been associated with both increased risk for IBD onset and a more severe IBD course compared to vitamin D sufficient IBD patients (Garg et al. 2013; Hassan et al. 2013; Jørgensen et al. 2013; Kabbani et al. 2016; Frigstad et al. 2017). Therefore, if mutant *NOD2* has a role in controlling serum vitamin D status, this novel finding could have implications in IBD prevention and treatment strategies.

In the current study we sought to further characterize the association between mutant *Nod2* and serum vitamin D. We aimed to determine whether the *Nod2fs* genotype was specifically associated with reduced serum vitamin D, or if other factors in the serum were also changed, such as fat-soluble vitamin A and calcium. We also aimed to

determine if the reduction in serum vitamin D was specific to the *Nod2fs* genotype, or if the finding was replicated in *Nod2KO* mice. Finally, we aimed to determine if a mouse model of another prominent IBD genotype, *Atg16l1^{ΔIEC}* mice, had a similar association with vitamin D status. Exploring a potential effect in *Atg16l1^{ΔIEC}* mice also allowed us to determine if the autophagy pathway was involved in this association, given that dysregulated autophagy is a shared feature of mutant *Nod2* and *Atg16l1* mice. This unique association between *Nod2* and serum vitamin D is an interesting finding that warrants further investigation.

4.2 Methods

4.2.1 Vitamin D deficient animal model

All animal experiments were approved by the University of Toronto's Animal Care Committee and in accordance with the Canadian Council of Animal Care guidelines (protocol 20011441). Mice were housed under specific pathogen-free conditions on a 12-hour light/dark cycle with *ad libitum* access to food and water. Mice heterozygous for the *Nod2fs* mutation on C57BL/6 background were supplied by the lab of Dr. Dana Philpott at the University of Toronto. Heterozygotes were bred and homozygous *Nod2fs* and wildtype littermate controls, both male and female, were used in the experiments. At 3 weeks of age, mice were acclimatized to the control diet for 2 weeks before being randomized to receive either the vitamin D deficient diet (0 IU/g vitamin D₃) (57BQ; TestDiet, Richmond, IN, USA) or matched control diet (1.0 IU/g vitamin D₃) (58M1; TestDiet, Richmond, IN, USA) for 6-7 weeks.

4.2.2 Murine serum acquisition

In addition to the vitamin D deficient model, littermate mice raised from weaning on the standard animal facility chow (2918; Teklad Global Diets/Envigo, Indianapolis, IN, USA) were used. In these studies, homozygous *Nod2fs* mice and wildtype littermates, both male and female, that were originally acquired from the lab of Dr. Dana Philpott were used. Serum was also obtained from *Nod2KO*, *Atg16l1^{ΔIEC}*, and wildtype littermate mice, supplied generously by members of the Philpott lab.

Mice were euthanized by carbon dioxide exposure and cervical dislocation. Blood was collected post-euthanasia by cardiac puncture and transferred in 2 mL tubes. The samples were left standing upright at room temperature for 30 min and then centrifuged at 1000 rpm for 15 min at 4°C. After centrifugation, the serum accumulates on top of the sample and appears as a clear or light orange liquid. Serum was collected and aliquoted into new 2 mL tubes. Serum was used immediately or stored at -80°C. Repeated freeze-thaw cycles were avoided. Severely haemolytic samples were not used.

4.2.3 Measurement of murine serum vitamin A levels

Serum vitamin A was measured in murine serum using the mouse vitamin A colorimetric ELISA kit (NBP2-60192; Novus Biologicals, Littleton, CO, USA) according to the manufacturer's guidelines.

4.2.4 Measurement of murine serum calcium levels

Serum calcium was measured using a colorimetric serum calcium assay (ab102505; Abcam, Cambridge, UK) as per the manufacturer's guidelines. Briefly, samples and standards were loaded into a 96-well microplate and incubated with a chromogenic reagent that forms a complex between calcium ions and 0-cresolphthalein. The plate was read at 575 nm wavelength on a VersaMax 190 visible plate reader (Molecular Devices, San Jose, California, USA).

4.2.5 Measurement of murine serum vitamin D levels

Serum 25(OH)D was measured in mice using the 25-OH vitamin D ELISA kit (VID31-K01; Eagle Biosciences, Amherst, NH, USA) as per the manufacturer's guidelines. Briefly, murine serum samples, calibrators, and controls were diluted with biotin-labeled 25(OH)D and added in duplicates to a 96-well microplate coated with monoclonal anti-25(OH)D antibodies detecting vitamin D₂ and D₃ with 100% specificity. In this assay, the known amount of biotin-labeled 25(OH)D competes with the unknown amount of 25(OH)D in the serum samples for the antibody binding sites on the coated wells during the incubation period. The unbound 25(OH)D was discarded and a second incubation using peroxidase-labeled streptavidin was performed to detect the bound biotin-labeled 25(OH)D. Finally, a third incubation was performed using the peroxidase substrate tetramethylbenzidine and the bound peroxidase caused a change in colour. The intensity of the colour is inversely proportional to the 25(OH)D concentration of the sample. The detection limit of the assay is 1.6 ng/mL.

As soon as possible and within 30 min, the plate was read at 450 and 650 nm wavelengths on a VersaMax 190 visible plate reader (Molecular Devices, San Jose, California, USA). The 450 nm wavelength detects the optical density of the sample, and the 650 nm reading detects background signal. The background signal was subtracted

from the optical density for each well and duplicates for each sample were averaged. A standard curve was created using the values of the calibrators. An exponential trendline was plotted and the concentration of 25(OH)D was measured by inputting each value into the equation of the y-intercept of the standard curve. Data shown are littermate analyses. The average values of the wildtype control were set to 1.0 and the littermates were expressed as a relative fold change.

4.3 Results

4.3.1 *Nod2fs* mice have reduced serum vitamin D, but no changes in serum vitamin A or calcium levels

In our earlier studies, we came across the unexpected finding of reduced serum vitamin D in mice of the *Nod2fs* genotype compared to wildtype mice (**Figure 3.3 A**). This is an interesting finding given that *NOD2* has not been linked to vitamin D signaling, yet both vitamin D deficiency and *NOD2* mutations predispose to IBD. In fact, vitamin D directly regulates expression of *NOD2* (Wang et al. 2010), but the opposite has not been investigated. We were interested in probing this finding further.

We decided to first measure other factors in the serum of the *Nod2fs* mice to determine if the observed effect was specific to vitamin D. Given that vitamin D is a fat-soluble vitamin, we measured serum concentration of vitamin A, which is also fat-soluble. There were no changes in serum vitamin A in the same group of mice in which we observed the reduction in serum vitamin D (**Figure 4.1 A**). This suggests that there are no defects in the fat-soluble vitamin absorption pathway contributing to the observed reduction in serum vitamin D. We also measured serum calcium, given that vitamin D and calcium levels are inter-dependent factors of the parathyroid hormone signaling cascade (Holick 2007). There were no changes in serum calcium in vitamin D deficient or *Nod2fs* mice (**Figure 4.1 B**). Taken together, these results suggest that the effect of *Nod2fs* in reducing serum vitamin D is specific, although future studies should measure other nutrients and metabolites to confirm this.

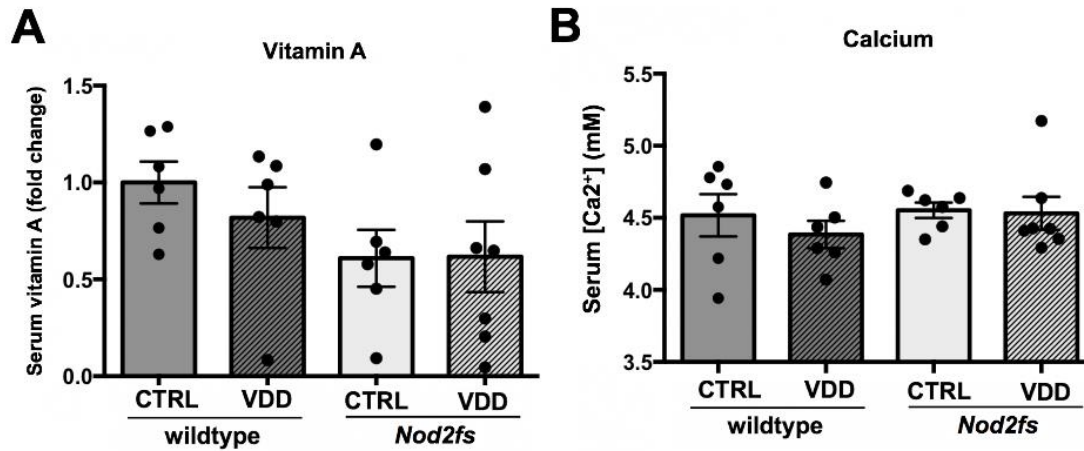


Figure 4.1: Serum levels of vitamin A and calcium are unchanged in *Nod2fs* mice

(A) Serum vitamin A and (B) serum calcium were measured in wildtype and *Nod2fs* mice fed either a vitamin D deficient (VDD) or control (CTRL) diet. Serum vitamin A is expressed as fold change relative to the wildtype control group. The millimolar concentration of serum calcium is shown. Data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. Error bars represent \pm SEM. n=6-7/group

4.3.2 Reduced serum vitamin D is a feature of mice lacking functional *Nod2*, and not *Atg16l1*

Given that we measured serum vitamin D only in mice of the *Nod2^{fs}* genotype, we sought to explore if the reduction in serum vitamin D was present in mice of other IBD-relevant genotypes. We next measured serum vitamin D in *Nod2KO* mice. Since *Nod2^{fs}* mice express a nonfunctional Nod2 protein, we hypothesized that the *Nod2KO* mice would similarly have reduced serum vitamin D. After performing a littermate analysis of mice fed the regular AIN-93 animal facility chow, both the *Nod2^{fs}* and *Nod2KO* mice displayed reduced serum vitamin D levels (**Figure 4.2 A,B**). This suggests that a lack of functional Nod2 is associated with reduced serum vitamin D.

In order to begin teasing apart the mechanism of reduced serum vitamin D in mice that lack Nod2, we measured vitamin D in mice lacking *Atg16l1* expression in the intestinal epithelium (*Atg16l1^{ΔIEC}*). This allowed us to begin to determine if the effect is specific to mice lacking functional Nod2, or if other IBD risk genes lead to a similar finding. We found no change in the serum vitamin D levels of homozygous *Atg16l1^{ΔIEC}* mice compared to wildtype littermate controls (**Figure 4.2 C**). This suggests that the association between serum vitamin D and *Nod2* is specific, and not a general effect of IBD risk genes. However, further risk genes should be explored to confirm this association. In addition, this finding suggests that the reduction in serum vitamin D caused by a lack of Nod2 signaling is independent of the autophagy pathway.

Figure 4.2: Serum vitamin D is reduced in *Nod2*^{fs} and *Nod2*^{KO} mice but is unchanged in *ATG16L1*^{ΔIEC} mice

Serum vitamin D was measured in (A) *Nod2*^{fs}, (B) *Nod2*^{KO}, and (C) *Atg16l1*^{ΔIEC} mice. Serum vitamin D is expressed as fold change relative to the littermate wildtype control. Data were analyzed by (A) unpaired student's t-test or (B,C) Mann-Whitney U test. Error bars represent \pm SEM. n=8-11/group. ****p<0.0001, *p<0.05

4.4 Discussion

The influence of *Nod2* on serum vitamin D levels was an unexpected and novel finding that could provide valuable insight into IBD pathogenesis. In the previous chapter, we identified that the *Nod2fs* genotype was associated with lower levels of serum vitamin D (**Figure 3.3 A**). In the current study, we determined that this effect was specific to serum vitamin D levels, given that serum vitamin A and serum calcium levels were unchanged (**Figure 4.1 A,B**). In our murine models, we identified that in addition to *Nod2fs* mice, *Nod2KO* mice had reduced serum vitamin D levels compared to wildtype littermates (**Figure 4.2 A,B**). Furthermore, the reduction in serum vitamin D was specific to a lack of *Nod2* signaling, as serum levels in *Atg16l1^{ΔIEC}* mice were unchanged (**Figure 4.2 C**). This finding suggests that there may be a link between *Nod2* mutations and vitamin D status and should be further explored.

The first research question we sought to address was whether the effect of *Nod2fs* in reducing serum vitamin D levels was specific. Given that vitamin D is a fat-soluble vitamin, we measured the serum levels of an additional fat-soluble vitamin, vitamin A. This would indicate whether or not the reduced serum vitamin D was due to a previously uncharacterized defect in the fat absorption pathway of *Nod2fs* mice. There were no changes in serum vitamin A (**Figure 4.1 A**), indicating that this was not characteristic of a general defect in the absorption of fat-soluble vitamins but an effect specific to vitamin D. Future studies should measure the serum levels of the other fat-soluble vitamins, vitamin E and vitamin K, to confirm this finding.

We next sought to discern whether the reduction in serum vitamin D observed in the *Nod2fs* mice was specific to this variant or applicable to other IBD genetic susceptibility mouse models. We first measured serum vitamin D in *Nod2KO* mice. Given that the *Nod2fs* mutation results in a nonfunctional *Nod2* protein, we hypothesized that *Nod2KO* mice, which completely lack the *Nod2* protein, would also have reduced serum vitamin D levels. Indeed, *Nod2KO* mice had reduced serum vitamin D compared to wildtype littermate controls (**Figure 4.2 B**), indicating that this is a broad effect of a lack of *Nod2* signaling. We next measured serum vitamin D in mice lacking expression of *Atg16l1* in intestinal epithelial cells. *ATG16L1* is another important CD susceptibility gene and the

AT16L1 and NOD2 proteins have overlapping functions, such as involvement in autophagy. There was no change in serum vitamin D between *Atg16l1^{ΔIEC}* mice and wildtype littermate controls (**Figure 4.2 C**). This further implies that this effect is specific to nonfunctional Nod2 and suggests that the mechanism does not involve the autophagic function of Nod2.

To explore this association in more depth, there are several future studies that could be performed. Several components of the vitamin D pathway that regulate the level of 25(OH)D in the serum could be measured. The liver enzyme responsible for producing 25(OH)D, 25-hydroxylase, should be measured by RT-qPCR. Similarly, the enzyme responsible for converting 25(OH)D into its active form, 1,25-hydroxyvitamin D 1 α hydroxylase, should be measured in intestinal tissues by RT-qPCR. These proposed experiments would allow for a better understanding of the source of reduced serum vitamin D, so that the mechanism can be further elucidated.

It would be interesting to determine the translatability of this association. To assess this, we could begin with measuring serum vitamin D in mice that are heterozygous for *Nod2^{fs}*, in order to determine if having one nonfunctional *Nod2* allele causes a reduction in serum vitamin D in mice, given that a large portion of patients have at least one mutant *NOD2* allele. In a patient population with sequencing data and vitamin D levels available, the association between mutant *NOD2* alleles and vitamin D levels could be assessed. If this association was present in an IBD patient population, this could partially explain why IBD patients, who have CD-associated *NOD2* risk alleles more frequently than the general population, have lower levels of vitamin D than the general population on average.

This association between Nod2 and serum vitamin D was a novel and unexpected finding. There is still much work that can be done in order to determine the mechanism and the translatability to IBD patients. This work exemplifies the complexity of gene-environment interactions in IBD and highlights the importance of studying gene-environment interactions in murine models.

Chapter 5 Conclusion

5 Conclusion

5.1 Discussion

There are several challenges inherent to studying the pathogenesis of complex conditions, such as IBD, in reductionist research models. In IBD, an intricate combination of genetic, environmental, microbial, and immune influences accumulate throughout the life of an individual and eventually lead to disease onset. These factors are extremely difficult to recapitulate in murine studies. The overarching aim of the current studies was to gain insight on the contribution of some of the factors involved in IBD pathogenesis, such as vitamin D deficiency, *Nod2* mutations, a microbial disturbance, and the effect of the combination of these factors on the intestinal innate immunity

5.1.1 Comparing murine models and IBD patients

There are limitations specific to murine models and patient studies that lead to many considerations when comparing the two models. In Chapter 2, some of the challenges in translating findings from mouse models to patient studies became apparent. The mice used in this study had diet-induced vitamin D deficiency but were healthy otherwise. In these mice, we observed increased expression of miR-142-3p in the ileum and not in the colon (**Figure 2.2**). Based on these findings, we explored miR-142-3p expression in a cohort of newly diagnosed, treatment-naïve paediatric IBD patients, a subset of which had low serum vitamin D levels. In this cohort, we identified miR-142-3p upregulation in the colon, only within active areas of inflammation in patients with low vitamin D (**Figure 2.9**). There are several discrepancies between the models that make it difficult to directly compare these results.

The first limitation we encountered when translating our findings from the mouse model to our patient cohort was the lack of access to patient tissues. Given the results of our mouse study, we attempted to measure miR-142-3p expression in the ileum of our patient cohort. Although preliminary results demonstrated a trend toward increased expression of miR-142-3p in the ileum of patients with low vitamin D (data not shown),

the integrity of the biopsies acquired during endoscopy was not sufficient to produce a reliable result. However, with the colon biopsies, which had better preservation of miRNA expression, we were able to reliably measure levels of miR-142-3p. This highlights the issue of access to appropriate biologic samples when conducting patient studies. Studies in mice are often used to overcome this issue, as the control over experimental conditions in murine models and a different set of ethical standards allows for the collection of tissues of higher quality.

An additional limitation in comparing the results from our mouse model and our IBD patient studies is the inherent differences in the tissues analyzed. In the colon of our vitamin D deficient mouse model, there was no change in expression of miR-142-3p (**Figure 2.2 B**). There was also no change in autophagy in this tissue (**Figure 2.4**), and no inflammation present (data not shown). In contrast, we observed that patients with low serum vitamin D had increased expression of miR-142-3p in the colon in biopsies that were procured from areas actively involved in disease (**Figure 2.9**). There are differences in the colon tissue of the otherwise-healthy vitamin D deficient mice and IBD patients that must be considered. In our patient cohort, low serum vitamin D is one of many factors that led to disease onset and active inflammation in the GI tract. In contrast, low vitamin D is the only dysregulated factor in the colon of our mice. Based on our regression analysis (**Table 3**), it is clear that the combination of active inflammation and low vitamin D led to the upregulation of miR-142-3p in our patient cohort. In order to make the mouse and patient models more comparable, it would be of interest to add an inflammatory stimulus to the vitamin D deficient mice and determine the impact on colonic miR-142-3p levels. The complexity and ambiguity of the insults that lead to IBD in patients must be considered when translating findings between mouse models and patients, and mouse models should attempt to recapitulate the level of complexity in patient tissues in order to better mimic the human pathology.

Although mouse models and patient studies are each extremely useful tools in studying the pathogenesis of IBD, each respective model comes with a set of limitations. In mouse models, the models are often reductionist and fail to recapitulate the full complexity of the IBD patient intestinal microenvironment. In contrast, patient tissues are extremely complex and involve the contribution of unknown factors and are

complicated by the presence of inflammation, making it difficult to delineate the contributions of specific elements involved in pathogenesis. However, both models can be used to gain insight on factors contributing to IBD and can advance our understanding of pathogenesis.

5.1.2 Replication of findings across murine studies

Murine studies are extremely useful in delineating pathogenesis of IBD and are viewed as simple models that can be used to tease apart the contributions of specific factors. However, there are limitations to drawing conclusions between studies conducted in mice, and mice may be a more complicated model than initially presumed. For example, in Chapter 2, we identified an accumulation of p62 in Paneth cells in wildtype vitamin D deficient mice, indicating an autophagy defect in these cells (**Figure 2.8**). In Chapter 3, the wildtype vitamin D deficient mice used in this study displayed no accumulation of p62 in Paneth cells (**Figure 3.7**). The reason for these discrepant findings is likely due to unmeasured factors, such as changes in the microbiome, that differ between the two studies. The mice used in Chapter 2 studies were obtained from a vendor, while mice used in Chapter 3 studies were bred in-house. Although we used the same strain of mice (C57BL/6), mice obtained from different sources have different microbiomes. Although we did not specifically analyze the gut microbiome in these studies, this is likely a factor that contributed to the discrepant results between the studies. The microbiome has a role in mediating changes in the intestinal microenvironment that can contribute to the onset of IBD and is an important factor to consider when employing murine models.

The gut microbiome is central to the pathogenesis of IBD and can drastically influence outcomes in murine IBD research. Because its role is still under investigation, it is difficult to know how changes in the microbiome may be influencing results of murine studies, unless it is explicitly being analyzed. In Chapter 3, we had a serendipitous microbial insult of *T. muris* infection present in a subset of our mice. Knowing that the presence of *T. muris* might confound the results of our study, we re-bred the mice to clear the parasite and re-performed our studies. This allowed us to compare results

between the mice that were *T. muris*-free compared to mice that were colonized with *T. muris*. We found that while the combination of vitamin D deficiency and *Nod2fs* was not associated with any changes in autophagy in the intestine at baseline (**Figure 3.3, 3.4, 3.5**), the presence of *T. muris* appeared to act as an additional insult which triggered autophagy defects in the ileal epithelium, including in Paneth cells (**Figure 3.6, 3.7**). This highlights the complexity of replicating and comparing results across murine studies, as the presence of unaccounted for factors such as differences in the microbiome may influence study results.

5.1.3 Identifying mechanistic insights

The ultimate goal of studying IBD pathogenesis is to gain insight on the mechanistic role of specific factors involved so that they may be targeted therapeutically to treat or prevent IBD. The findings identified in the current studies should be further delineated in order to reveal specific mechanisms that can be explored therapeutically. For example, in Chapter 3, the precise mechanism that allows for autophagy dysregulation in *T. muris*-infected mice remains unknown (**Figure 3.6, 3.7**). The mechanism likely involves the capacity of the epithelium to respond to the pathogen through innate and adaptive immune mechanisms. Further teasing out the details surrounding this association would allow for the identification of targetable factors. In Chapter 4, we explored the relationship of reduced serum vitamin D in *Nod2fs* mice, although the mechanisms involved remain unclear (**Figure 4.1, 4.2**). Further studies should be conducted in order to gain insight into the specific mechanisms involved in the identified associations, in order to identify therapeutic targets of interest.

5.2 Summary

The studies presented in this thesis have allowed for insight into the effects of several facets of IBD pathogenesis on the intestinal epithelium. In Chapter 2, we identified enhanced expression of an autophagy-targeting miRNA, miR-142-3p, in ileal tissues of vitamin D deficient mice. This increase in expression occurred in association with Paneth cell abnormalities and an autophagy defect in this cell type. Furthermore, we identified an increase in miR-142-3p in colonic biopsies procured from involved areas of disease from IBD patients with low vitamin D. In Chapter 3, we explored the effects of vitamin D deficiency in the context of genetic susceptibility to IBD, incurred by the *Nod2fs* mutation. The combination of these IBD genetic and environmental factors did not lead to detectable changes in autophagy in the intestinal epithelium. However, with the additional insult of *T. muris* infection, the combination of vitamin D deficiency and mutant *Nod2* led to dysregulation of autophagy in the intestinal epithelium, including in Paneth cells. In Chapter 4, we explored a novel association observed due to the *Nod2fs* mutation. We identified that a lack of *Nod2* function, but not a lack of *Atg16l1* function, led to reduced circulating vitamin D through an unknown mechanism. Taken together, these studies were able to reveal important aspects of IBD pathogenesis and have generated additional research questions to be explored.

Chapter 6 Future Directions

6 Future Directions

There are several studies that can be conducted to address limitations or unresolved research questions identified in the current studies. The focus of future work should be on utilizing models representative of IBD to uncover mechanistic actions so that results can be better translated to patients and clinically relevant targets can be identified.

6.1.1 Delineating the role of vitamin D deficiency in IBD

Future studies should follow up on the findings in Chapter 2 to further understand the role of vitamin D deficiency in IBD and to strengthen the results of the current study. Although we produced data to support an association between vitamin D deficiency and the upregulation of miR-142-3p, there are studies that can be conducted in order to bolster this finding. In future studies, re-feeding of vitamin D to a subset of vitamin D deficient mice should be conducted in order to observe if the expression of miR-142-3p returns to baseline. This would strengthen the association between vitamin D deficiency and expression of this miRNA and would indicate whether the effects of increased miR-142-3p expression and the associated autophagy defects in Paneth cells are reversible with correction of the deficiency. This proposed study is especially important given that the effects of correcting vitamin D deficiency in IBD patients are still unclear.

A longer vitamin D deficient diet feeding period and introduction of an additional inflammatory insult in the current murine model would be of interest. Our model included a 5-week feeding period. This was enough time to induce deficiency but is likely not optimal for recapitulating the human condition. It is likely that the vitamin D deficiency present in IBD patients is chronic rather than transient. Therefore, a longer vitamin D deficient feeding period is likely a better model and would allow for more time for changes in the intestinal epithelium to occur and accumulate. An additional stimulus should be introduced in the vitamin D deficient animal model in order to determine if the identified changes in the epithelium lead to enhanced susceptibility to intestinal inflammation and to elucidate the role of miR-142-3p in this association. Examples of stimuli that can be used include the DSS colitis or SAMP1/YitFc ileitis models of IBD. It has been previously shown that vitamin D deficient mice exhibit an enhanced response to DSS colitis (Lagishetty et al. 2010), and thus future studies should assess the role of

miR-142-3p, intestinal autophagy, and Paneth cells in this context. These studies would better model the intestinal microenvironment of IBD patients and would help to elucidate the role of inflammation in the association. This proposed study is of interest given our finding of enhanced miR-142-3p expression in 'involved' tissue regions in IBD patients with low vitamin D. If miR-142-3p is associated with low vitamin D and inflammation in our murine studies it can be targeted therapeutically. miRNAs represent potential therapeutic targets through the use anti-miRNA therapies which bind and inhibit specific miRNAs. The use of anti-miRNA therapies have been employed in multiple preclinical models of colitis (Soroosh et al. 2018) and are currently being tested for use in humans (Janssen et al. 2013). The proposed studies will delineate the role of miR-142-3p in the context of vitamin D deficiency and intestinal inflammation and will determine if it remains a therapeutic target of interest.

Additional experimental techniques can also be employed in the future in order to further uncover changes in the epithelium associated with vitamin D deficiency. In this study, we opted for a targeted approach in measuring our single microRNA of interest, miR-142-3p, and its effect on intestinal autophagy. However, it is likely that there are many other changes in microRNA and gene expression that have not been captured by our experimental techniques. To address this, methods such as next-generation sequencing (for example, RNA-Seq) should be employed so that a data-driven, rather than hypothesis-driven, approach can be taken. This would allow for enhanced understanding of the global role of vitamin D deficiency in the intestine and would lead to the discovery of more robust therapeutic targets of interest.

The proposed studies will help to further define the role of vitamin D deficiency in IBD pathogenesis by employing models that are more similar to the human condition and therefore more translatable in nature. Findings derived from these murine studies should be further explored in IBD patient tissues. Replicating findings of murine studies in patient tissues allows the research to be more impactful by demonstrating shared mechanisms between the murine models used and IBD patients.

6.1.2 Gene-environment interactions with additional insults

In Chapter 3, we sought to explore IBD-relevant gene-environment interactions in the intestine. In Chapter 2, we demonstrated that vitamin D deficiency alone causes some disturbances in intestinal homeostasis, including p62 accumulation in Paneth cells. Our goal for Chapter 3 was to assess the intestinal effects of vitamin D deficiency in combination with genetic susceptibility to IBD, in order to better mimic the complex interactions occurring in IBD patients. For these studies, we chose to combine vitamin D deficiency with *Nod2fs*, an important CD risk variant that also effects intestinal autophagy. Although neither vitamin D deficiency nor *Nod2fs* alone cause significant intestinal pathologies in mice, we were interested to assess the combination of the factors, especially given that previous studies have uncovered synergistic effects of IBD-relevant genetic and environmental risk factors in the intestine (Liu et al. 2018). In contrast to our hypothesis, there were no changes in autophagy at baseline in mice with vitamin D deficiency and the *Nod2fs* genotype. Interestingly, unintentional infection with *T. muris* in a group of our mice served as an additional insult that led to autophagy defects in the epithelium compared to the uninfected mice. Although inadvertent, these findings suggest that intestinal homeostasis may be maintained in the presence of both vitamin D deficiency and *Nod2fs* at baseline, however the inclusion of an additional insult may result in epithelial defects such as autophagy dysregulation.

Persistent colonization with *T. muris* served as an additional insult in our study that led to defects in intestinal autophagy that were not present in uninfected mice. However, *T. muris* infection was naturally occurring and not experimentally induced. Natural infection rather than experimental inoculation is not optimal due to the lack of control in natural infection. The uninfected and infected mice are not completely comparable because they are not littermates or co-housed and it is therefore likely that there are other differences in the microbiome in addition to the presence of *T. muris*. Additionally, because infection is natural rather than experimental, the parasitic load is not controlled. In experimental studies, a precise load of the parasite can be inoculated. However, with natural infection, it is likely that the infected mice have a variation in their parasitic load, which could lead to variability in the outcomes measured. To overcome these challenges associated with natural infection, future studies should introduce

experimental infection in these mice. This would ensure results are robust, comparable, and replicable, and would allow for enhanced understanding of the role of *T. muris* infection in this model.

Although *T. muris* infection elicits a response in our mouse model, there are other insults that could be introduced into our model to make it more translatable to IBD patients. An insult inducing inflammation would be of interest. In IBD patients, genetic and environmental factors contribute to barrier breakdown in the presence of an unknown insult to the epithelium. Future studies should model this by using the DSS colitis or SAMP1/YitFc ileitis models of IBD. The current study demonstrates that the combination of vitamin D deficiency and the *Nod2fs* genotype did not cause defects in autophagy at baseline, but the additional stimulus of *T. muris* infection led to dysregulation of intestinal autophagy. Given these results, we would expect that the vitamin D deficient *Nod2fs* mice would have an exaggerated response to an inflammatory insult. Pathways of particular interest to explore in this model would include autophagy and Paneth cells, given that these elements demonstrate signs of dysregulation in the *T. muris* model. Strategies such as transcriptional profiling of Paneth cells and RNA-Seq may allow for identification of therapeutic targets of interest. Differences in the inflammatory and immune response would also be of interest given the important role of both vitamin D and NOD2 in modulating intestinal inflammation and immunity. Introducing an inflammatory stimulus into our model would enable further exploration of IBD-relevant gene-environment interactions and would allow for identification of therapeutic targets of interest.

The studies described in this section will allow for enhanced translatability of the current research findings. Although reductionist models are important in elucidating specific aspects of IBD pathogenesis, more complex models that better mimic the combinatorial nature of IBD onset in patients will allow for the discovery of additive or synergistic effects of multiple factors involved in pathogenesis to provide a more complete understanding of the complex mechanisms leading to the onset of IBD. Future work should focus on identifying targetable pathways with cell and systems biology in order to lead to the discovery of novel IBD therapies and assist the transition toward personalized medicine in IBD.

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