Effect of Vitamin D Deficiency on Autophagy in the Intestine via MicroRNA Regulation

by

Adam Hsieh

A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Master of Science

Institute of Medical Science
University of Toronto

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Abstract

Rates of IBD have increased drastically over the past decade, greatest in children, asserting a role for the environment in disease pathogenesis. Emerging evidence has shown that vitamin D deficiency can affect the autophagy pathway and disrupt intestinal homeostasis, contributing to IBD. Epigenetic regulation through miRNAs may be a mechanism connecting vitamin D deficiency to dysregulated autophagy in IBD. Our study characterizes in the intestine the effect of vitamin D deficiency on autophagy via regulation by the miRNA, miR-142-3p. This research builds on data indicating miR-142-3p regulation of autophagy gene ATG16L1 and miR-142-3p overexpression in the intestine of IBD patients. Here, we demonstrate that vitamin D deficiency causes decreased ATG16L1 protein expression in the intestine. This poses a potential mechanism by which the environment interacts with genetics leading to IBD.
Acknowledgments

These past two years completing my Master’s has been an incredible experience, a journey full of highs and lows. Thus I would like to sincerely thank the many individuals who helped make my journey here possible.

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Contributions

The author performed all of the experiments, results, and analyses presented in this thesis, with assistance from Laura Greenfield and Mariana Capurro for animal sacrifice and tissue harvest.
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<tr>
<td>$1,25(OH)_2D$</td>
<td>1,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>1-OHase</td>
<td>25-hydroxyvitamin D-1α-hydroxylase</td>
</tr>
<tr>
<td>6-MP</td>
<td>6-mercaptopurine</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>$A_{260}$</td>
<td>Absorbance at wavelength 260 nm</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute</td>
</tr>
<tr>
<td>AI</td>
<td>Adequate intake</td>
</tr>
<tr>
<td>AIEC</td>
<td>Adherent-invasive <em>E. coli</em></td>
</tr>
<tr>
<td>Ambra1</td>
<td>Activating molecule in beclin-1-regulated autophagy 1</td>
</tr>
<tr>
<td>Ang4</td>
<td>Angiogenin-4</td>
</tr>
<tr>
<td>ASCA</td>
<td>Anti-<em>Saccharomyces cerevisae</em> antibodies</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related</td>
</tr>
<tr>
<td>ATG16L1</td>
<td>Autophagy-related 16-like 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CARD15</td>
<td>Caspase recruitment domain-containing protein 5</td>
</tr>
<tr>
<td>CASP3</td>
<td>Caspase-3</td>
</tr>
<tr>
<td>CCD</td>
<td>Coiled-coil domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
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<td>-------------</td>
<td>-----------</td>
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</table>
cDNA         | Complementary DNA |
<p>|CD           | Crohn’s disease |
|CEA          | Carcinoembryonic antigen |
|CT           | Computed tomography |
|CTL          | Control |
|DFCP1        | Double-FYVE containing protein-1 |
|DGCR8        | Di George syndrome critical region 8 |
|DKO          | Double knockout |
|DMEM         | Dulbecco’s modified Eagle’s medium |
|DMEM/F-12    | DMEM: nutrient mixture F-12 |
|DPBS         | Dulbecco’s PBS |
|DSS          | Dextran sulphate sodium |
|E. coli      | Escherichia coli |
|EDTA         | Ethylenediaminetetraacetic acid |
|eIF4E        | Eukaryotic translation initiation factor 4E |
|ELISA        | Enzyme-linked immunosorbent assay |
|ER           | Endoplasmic reticulum |
|F            | F statistic |
|FBS          | Fetal bovine serum |</p>
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF23</td>
<td>Fibroblast growth factor 23</td>
</tr>
<tr>
<td><em>F. prausnitzii</em></td>
<td><em>Faecalibacterium prausnitzii</em></td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen - D related</td>
</tr>
<tr>
<td>HOPS</td>
<td>Homotypic fusion and protein sorting</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IBS</td>
<td>Irritable bowel syndrome</td>
</tr>
<tr>
<td>IL23R</td>
<td>Interleukin-23 receptor</td>
</tr>
<tr>
<td>IRGM</td>
<td>Immunity-related guanosine triphosphatase family M</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LAMP-1</td>
<td>Lysosome-associated membrane protein-1</td>
</tr>
<tr>
<td>LC3</td>
<td>Microtubule-associated light chain-3</td>
</tr>
<tr>
<td>Lgr5</td>
<td>Leucine-rich repeat containing G protein-coupled receptor 5</td>
</tr>
<tr>
<td>LRRK2</td>
<td>Leucine-rich repeat kinase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MED</td>
<td>Minimal erythemal dose</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin complex 1</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor-κβ</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>NOD2fs</td>
<td>NOD2 frameshift mutation</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>P</td>
<td>P value</td>
</tr>
<tr>
<td>P0</td>
<td>Passage 0</td>
</tr>
<tr>
<td>P-ANCA</td>
<td>Perinuclear anti-neutrophilic cytoplasmic antibodies</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
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</table>
PI3P  Phosphatidylinositol-3-phosphate
PIK3C1  Phosphatidyinositol-3-kinase class 1
PMSF  Phenylmethylsulfonyl fluoride
PPAR  Peroxisome-activated receptor
PTGER4  Prostaglandin receptor EP4
PTH  Parathyroid hormone
PTPN2  Protein tyrosine phosphatase non-receptor type 2
qPCR  Quantitative polymerase chain reaction
Rab7  Ras-related GTP-binding protein 7
RISC  RNA-induced silencing complex
RIPA  Radioimmunoprecipitation assay
RNAi  RNA interference
RQ  Relative quantification
RT-qPCR  Reverse transcription qPCR
RXR  Retinoic X receptor
S. cerevisae  Saccharomyces cerevisae
SDS  Sodium dodecyl sulfate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
SEM  Standard error of the mean
<table>
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<tr>
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<th>Definition</th>
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<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>ULK1</td>
<td>Uncoordinated-51-like kinase 1</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultraviolet B</td>
</tr>
<tr>
<td>VDD</td>
<td>Vitamin D deficient</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D receptor element</td>
</tr>
<tr>
<td>WD</td>
<td>Tryptophan-aspartic acid</td>
</tr>
<tr>
<td>WIPI1</td>
<td>WD repeat domain phosphoinositide interacting 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
<tr>
<td>XPO5</td>
<td>Exportin-5</td>
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Chapter 1
Introduction

Inflammatory bowel disease (IBD) comprises a family of chronic, idiopathic inflammatory diseases of the gastrointestinal tract whose pathogenesis is complex and poorly understood. As with other complex diseases, interaction of environmental and genetic factors is suggested to be at the core of disease onset. Vitamin D deficiency has been increasingly observed in IBD patients. It is unclear how vitamin D deficiency promotes IBD susceptibility; however emerging evidence has implicated intestinal dysregulation of the autophagy pathway, a cellular recycling pathway crucial for maintaining intestinal homeostasis. This thesis will focus on exploring the effect of vitamin D deficiency on autophagy in the intestine. Previous work from our laboratory has identified the autophagy gene, ATG16L1, as a direct regulatory target of the microRNA, miR-142-3p. Therefore, we will determine if vitamin D deficiency alters levels of miR-142-3p targeting ATG16L1.

1.1 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is an umbrella term for a family of diseases causing chronic gastrointestinal inflammation. Crohn’s disease (CD) and ulcerative colitis (UC) are the predominant forms of IBD. Although their exact causations are unknown, inappropriate cell-mediated response to environmental triggers in genetically susceptible individuals is implicated (Knights et al., 2013). Onset can begin at any age, but the majority of cases occur in late childhood and adolescence (Ananthakrishnan, 2015). Few diseases match IBD’s devastating impact on patient quality of life and society. For patients, the lifelong debilitating symptoms, unpredictable relapses and exacerbations, and absence of a cure, pose an immense challenge for them and their families. At the societal level, there are wide implications on economics, healthcare, and social landscape (Rocchi et al., 2012).
Since IBD is increasing and most dramatically in children, investigating the disease is a priority (Molodecky et al., 2012). Thus much current work is focused on better understanding disease pathogenesis and pioneering novel therapeutics to combat IBD.

1.1.1 Pathology and Clinical Manifestations

CD and UC are the most common forms of IBD. While both diseases share many similarities, there are important differences in their pathologies and clinical manifestations.

CD was first described by Drs. Crohn, Ginzburg, and Oppenheimer in 1932. At Mount Sinai Hospital in New York City, the authors reported 14 cases of inflammation of the terminal ileum which they identified as a new pathologic and clinical entity, which they termed regional ileitis (Crohn et al., 1932). Since its original description, subsequent studies identified the potential of CD to affect any region of the gastrointestinal tract from the mouth to the anus. Not confined to the ileum, the disease name was correspondingly interchanged with CD. Of the gastrointestinal tract, CD most commonly targets the terminal ileum and colon (Hendrickson et al., 2002). Inflammation is segmental; diseased segments are separated by “skip areas” (Hendrickson et al., 2002). Depth of the inflammation is transmural; all layers of the intestine wall spanning the mucosa to the serosa are affected. In addition, intestinal wall thickening, lumen narrowing, deep ulcerations, perianal lesions, strictures, fistulas, discontinuous (skip) lesions, and granulomas are common (Hendrickson et al., 2002). Onset occurs across all ages but peaks in the third decade of life (Bernstein et al., 2006). CD is a lifelong, chronic, relapsing and remitting disease. Periods of remission can span many years, during which there is minimal distress, however symptoms eventually return. Location and extent of the disease will determine symptoms. Thus symptoms range from mild to severe and vary from patient to patient. Initial symptoms are usually subtle thus causing delay in
diagnosis; initial patient complaints are typically prolonged abdominal pain and diarrhea without blood (Hendrickson et al., 2002). If the small intestine is involved, persistent right lower quadrant pain or periumbilical pain can present (Hendrickson et al., 2002). The pain may be partially relieved by defecation. If present, perianal fissures can cause more pronounced pain and bleeding during bowel movements. However, the rectum is not commonly involved in CD. These intestinal manifestations may be accompanied by fever, nausea, fatigue, vomiting, dehydration, weight loss, anorexia, and anemia (Hendrickson et al., 2002). Extraintestinal effects can also manifest, either prior to or concurrently with intestinal symptoms. They are similar to those of UC, but more common in CD (Hendrickson et al., 2002). Manifestations affecting the eye (episcleritis, iritis, and uveitis), the skin (erythema nodosum, pyoderma gangrenosum, and aphthous ulcers), the joints (ranging from arthralgia to acute arthritis most common in the knees, ankles, and wrists), the liver (pericholangitis, sclerosing cholangitis, chronic active hepatitis progressing to cirrhosis), and bone (osteopenia and osteoporosis) have been associated with CD (Hendrickson et al., 2002). In children, growth failure and delayed sexual maturation are prominent concerns (Hendrickson et al., 2002). Chronic undernutrition due to abdominal pain is considered the main factor causing growth failure (Hendrickson et al., 2002). Finally, clinical features may extend beyond physical symptoms to affect mental health, manifesting as depression, anxiety, and distress (Nelson & Behrman, 1992).

In contrast, UC is confined to the colon. The rectum is involved in 95% of patients and the inflammation extends proximally from the rectum (Hendrickson et al., 2002). UC inflammation is continuous, where unlike CD, there are no skip areas. The inflammation is superficial and confined to the mucosa in UC (Hendrickson et al., 2002). Macroscopic features of affected tissue include distortion of colonic normal haustral pattern resulting in “lead-pipe” appearance, broad ulcers, and polypoid islands of inflamed mucosa, referred to as pseudopolyps because of their non-malignant potential (Hendrickson et al., 2002). Pathologically, features of UC include
irregular crypt architecture, neutrophil infiltration of crypts, crypt abscesses, goblet cells depletion, and disrupted surface epithelium (Hendrickson et al., 2002). Other aspects that distinguish UC from CD include no thickening of the intestinal wall, patchy and nonlinear ulcerations, and lack of perianal lesions, fistulas, and granulomas (Hendrickson et al., 2002). As with CD, disease onset can occur at all ages (Rocchi et al., 2012). In UC, there is a peak at 20-29 years of age, followed by a plateau, and a subsequent peak at 70-79 years of age (Bernstein et al., 2006). UC like CD, is a lifelong, chronic, relapsing and remitting disease. Onset of symptoms is gradual like CD. Initial symptoms are abdominal pain and diarrhea; however the most consistent feature is presentation of stool that is bloody and mixed with abundant mucus (Hendrickson et al., 2002). Fever, nausea, fatigue, vomiting, dehydration, weight loss, and anorexia commonly develop later. Extent of colon involved is correlated with severity of disease (CCFC, 2012). Extraintestinal manifestations are similar to CD but are less common. As in CD, the eyes, skin, joints, liver, bone, and mental health can be affected (Hendrickson et al., 2002). Children with UC have additional risk of stunted growth and maturation. The most serious exacerbation is toxic dilatation of the colon, also referred to as a megacolon, is rare but more common in UC than in CD (Hendrickson et al., 2002). Exact cause of aggravation is unclear, but severe colon inflammation, hypermotility agents for treatment of diarrhea, and cathartic administration and barium enema examination, are all possible contributing factors (Hendrickson et al., 2002). Toxic megacolon is a medical emergency because progression to perforation is associated with mortality of >30% (Isselbacher et al., 1994). Lastly, increased risk of cancer is associated with CD and UC. A meta-analysis study estimated that risk of colorectal cancer is 2.9% after 10 years of CD, 5.6% after 20 years, and 8.3% after 30 years (Canavan et al., 2006).
1.1.2 Diagnosis and Treatment

Due to symptom overlap with other conditions, multiple entities should be considered in the differential diagnosis. Gastrointestinal infectious agents can cause symptoms that mimic IBD. These include bacteria: *Campylobacter jejuni, Yersinia enterocolitica, Clostridium difficile, Escherichia coli, Salmonella, Shigella, Aeromonas hydrophilia, Plesiomonas, and Myobacterium tuberculosis*, and parasites: *Entamoeba histolytica and Giardia lamblia* (Hendrickson et al., 2002). Functional diarrhea can be difficult to distinguish from IBD. Identification of constitutional symptoms including fever, nausea, fatigue, vomiting, dehydration, weight loss, anorexia, and anemia, coupled with detection of leukocytes in stool samples, will implicate IBD (Hendrickson et al., 2002). Irritable bowel syndrome (IBS) produces abdominal pain and discomfort similar to IBD however inflammation is not involved. Thus sigmoidoscopy, rectal biopsy, and barium enema examination will be normal (Targan et al., 2005). Lactase intolerance should also be considered as part of differential diagnosis because of symptom overlap with irritable bowel syndrome (Evans & Meuser, 2006). Other conditions that should be included in differential diagnosis include acquired immune deficiency associated infections and diseases, Behçet’s disease, systemic vasculitis, diversion colitis, and radiation colitis (Targan et al., 2005).

Diagnosis of IBD should be entertained in all patients who present with non-bloody/bloody diarrhea or abdominal pain. Subsequent workup for diagnosis of IBD typically encompasses a combination of tests. Common laboratory tests include complete blood count to assess for anemia and leukocytosis, electrolytes test to assess for degree of diarrhea and small intestine malabsorption, inflammation marker panel checking for elevated erythrocyte sedimentation rate, liver function test to identify elevated liver enzyme levels, and stool cultures to rule out infection (Targan et al., 2005). Newer tests include perinuclear anti-neutrophilic cytoplasmic antibody (P-ANCA) and anti-*Saccharomyces cerevisae* antibodies (ASCA).
Once diagnosis of IBD is entertained, the best information about the location, extent, and severity of IBD is obtained by direct visualization of the intestines via endoscopy and radiologic examinations. Intestinal biopsies can be obtained to determine inflammation. Endoscopic procedures comprise of colonoscopies, sigmoidoscopies, and endoscopies. Radiologic procedures include computed tomography (CT) imaging, magnetic resonance imaging (MRI), and X-ray imaging. Diagnosis of CD is indicated by deep ulcerations, classic mucosal cobblestone appearance, skip areas, rectal sparing, and granulomatous inflammation (Hendrickson et al., 2002). Diagnosis of UC is indicated by superficial ulcerations, uniform mucosal appearance, mucosal friability, loss of mucosal vascularity, and bloody exudates (Hendrickson et al., 2002).

Since there is no cure, treatment is focused on achieving and maintaining remission. For treatment of mild to moderate disease, the principal drugs used are 5-aminosalicylic acids (5-ASA), namely mesalamine. Mesalamine can be administered orally, or topically if patients have limited distal colonic disease (Hendrickson et al., 2002). If this therapy is inadequate, corticosteroids such as prednisone and budesonide can be given. Although very effective, long-term steroid therapy is avoided to prevent side-effects and dependence. For patients who do not respond to corticosteroids, immunosuppressive drugs such as azathioprine, 6-mercaptopurine (6-MP), methotrexate, and cyclosporine can be employed (CCFC, 2012). In addition, biologicals such as infliximab and adalimumab are approved for treatment (CCFC, 2012). Both drugs block tumour necrosis factor-α (TNF-α) activation, leading to inflammation suppression (CCFC, 2012). While they are more costly, they are effective for moderate to severe disease, resulting in a shift to biologicals as the standard of care for severe disease (Rocchi et al., 2012).

Since there are discrepancies on the benefits of diet modification, a complete nutritious diet is recommended. However in certain patients, diet modification may help alleviate symptoms. Low-residue, low-fiber diet may benefit patients with
intestinal obstruction, small-intestine involvement, or extensive diarrhea and cramping (Isselbacher et al., 1994). Lactose-free diet should be instituted for patients with lactose intolerance (Hendrickson et al., 2002). For more severe disease, hospitalization is best. IBD can worsen rapidly, and course of disease can be unpredictable (Hendrickson et al., 2002). Since severely ill patients may not tolerate oral medications, and rapid therapy onset is desired, intravenous high-dose corticosteroid therapy is common (Hendrickson et al., 2002). Improvement after 7-10 days of such therapy is typical (Isselbacher et al., 1994). As well, intravenous fluid replacement therapy, electrolyte therapy, blood transfusions, and anti-diarrheal drugs (diphenoxylate, loperamide, codeine, anticholinergics) may be indicated (Rampton, 2000). If toxic megacolon presents, prompt care is vital. Nasogastric suction is typically instituted in combination with antibiotic and intravenous corticosteroid therapies (Hendrickson et al., 2002).

Surgical therapy is reserved as the last-resort for patients who do not respond to intensive management. Total colectomy and ileostomy are standard surgical procedure (R. T. Lewis & Maron, 2010). In contrast to UC, intestinal resection is not curative in CD (R. T. Lewis & Maron, 2010). Although resection permits improved symptoms for an interval of time, CD patients have high rates of recurrence following intestinal resection, approximately 50% over the subsequent 5 years (Rutgeerts, 2002). Thus, judicious use of surgery is advised. As with therapy of any chronic disease, physician empathy, compassion, and support are important to nurturing the therapeutic alliance with patients. For paediatric, geriatric, severely depressed, mentally ill, or chronically ill patients, additional psychiatric or counselling assistance may be needed to support them in their efforts to understand and cope with disease. Encouragement of living life as full as possible is recommended.
1.1.3 Burden of Disease

Canada has one of the highest IBD incidence and prevalence rates in the world. In 2012, an estimated 233,000 Canadians had IBD, 129,000 with CD and 104,000 with UC (Rocchi et al., 2012). This corresponded to a prevalence rate of 670 per 100,000 (Rocchi et al., 2012). The incidence rate was 16.3 per 100,000 for CD and 12.9 per 100,000 per UC, corresponding to an overall incidence rate of 29.2 per 100,000, equivalent to 10,200 new cases of IBD per year (Rocchi et al., 2012). Extrapolated to 2016 using the 2012 incidence rate, over 274,000 Canadians will have IBD, an 18% increase over 4 years. This number is expected to continue to grow going forward. Moreover, Canada also has one of the highest frequencies of paediatric onset IBD in the world. In 2012, 5,900 Canadian children <18 years of age were estimated to have IBD (Rocchi et al., 2012). IBD was diagnosed in children as early as 6 months of age (Benchimol et al., 2014). Most worrisome, data have shown that in Ontario, the most populous Canadian province, frequency of IBD increased most dramatically in children <10 years of age compared to other age groups over the past decade (Benchimol et al., 2014). Canadian findings are consistent with worldwide trends indicating increasing disease incidence and prevalence, particularly in historically low incidence countries (Molodecky et al., 2012). Thus the population burden of IBD is substantial.

The economic costs of IBD are enormous. In 2012, the total cost of IBD in Canada was estimated to be $2.8 billion, corresponding to $11,900 per patient (Rocchi et al., 2012). Since IBD is a lifelong disease with no cure, ongoing medical care is necessary and costly. As a result, direct medical costs accounted for $1.2 billion of total cost (Rocchi et al., 2012). The major contributors to the total direct medical costs of IBD were medications ($521 million), hospital inpatient care ($395 million), physician visits ($132 million), and hospital outpatient care ($61 million) (Rocchi et al., 2012). Indirect costs which comprise of non-medical costs to patients and society, accounted for $1.8 billion (Rocchi et al., 2012). The major contributors were
long-term work losses ($979 million), patient out-of-pocket expenses ($300 million), and short-term work losses ($181 million) (Rocchi et al., 2012). Using this data and the Consumer Price Index, the total IBD associated cost extrapolated to 2016 would be $3.4 billion. Compared to other countries, this cost profile is similar, although total expenditures differ largely due to number of patients. An American study estimated $6.3 billion in direct medical costs alone for 948,000 people with IBD in the United States in 2005 (Kappelman et al., 2008). Importantly, this study also noted that children with IBD incur significantly increased costs compared to adults (Kappelman et al., 2008). This is critical because IBD is increasing most in children. Given these are conservative estimates, actual costs may be higher, putting into perspective the tremendous financial burden of the disease.

Finally, IBD has broad implications for individuals with the disease, causing them to experience reduced quality of life compared to healthy people. Studies show quality of life is most affected by severity of disease; people with more severe disease reported poorest quality of life whereas people with more mild disease have less reduced quality of life (CCFC, 2012). Even patients in remission with no symptoms have reduced quality of life (CCFC, 2012). Furthermore, those with CD are associated with a 47% increased risk of premature mortality and an increased risk of colorectal cancer (CCFC, 2012). People with UC have no increased risk of death, although they do have an increased risk of colon cancer (CCFC, 2012). Beyond medical issues, patients must contend with numerous challenges from living with the disease. Awareness of IBD remains poor in Canada, resulting in persistence of social stigma, late diagnoses, and misdiagnoses (Rocchi et al., 2012). Diagnosis of IBD in childhood or early adulthood can interfere with education and preparation for a career (CCFC, 2012). Thus effective medical treatment is vital to minimize these challenges. As a result, there is great interest in elucidating the mechanism of IBD pathogenesis to achieve better prevention of the disease.
1.1.4 Etiology and Pathogenesis

Despite extensive effort during the past few decades, IBD etiology and pathogenesis remain poorly understood. Although many questions remain, research has implicated the convergence of four factors in the manifestation of IBD: genetics, microbiome, immune response, and environment (Figure 1). The exact causative mechanism for triggering of IBD has yet to be identified. Currently, it is postulated that certain environmental factors trigger a dysregulated immune response to gut microbiota in genetically susceptible individuals, leading to the chronic gastrointestinal inflammation that hallmarks IBD. Evidence for the potential roles of each of the four main contributing factors will be discussed here.

1.1.5 Genetics

Population based studies of IBD have shown support for the contribution of genetics to disease. IBD affects some ethnic groups more than others; IBD is most common in Caucasians while being rare among Hispanics and Asians (Matricon et al., 2010). In the United States, rates in African Americans were low historically, but have risen during recent years to rates similar for Caucasian Americans (Matricon et al., 2010). Frequency of IBD is significantly higher in Jews compared to non-Jews. For example, the Ashkenazi Jews, a subgroup of Central and European origins, have 5- to 8-fold higher frequency of disease compared to non-Jewish Caucasians (de Lange & Barrett, 2015). In addition to differences in the extent of ethnicities affected, studies have demonstrated the existence of familial clustering (Matricon et al., 2010). On average, between 5 to 10% of affected individuals have a relative with IBD, with some studies reporting frequencies as high as 20 to 30% (Matricon et al., 2010). For siblings of affected individuals, their risks of developing IBD are magnified to 30- to 40-fold higher for CD and 10- to 20-fold higher for UC (Matricon et al., 2010). Since these patterns reflect both genetic and environmental contributions, twin studies were conducted to assess the magnitudes of each
(Hendrickson et al., 2002). If concordance rates were higher in monozygotic twins compared to dizygotic twins, with the assumption that both types were brought up in the same environment, this variation would be attributed to genetics (Hendrickson et al., 2002). If 100% concordance was measured in monozygotic twins, this would implicate complete genetic contribution and no environmental contribution (Hendrickson et al., 2002). Subsequently, studies demonstrated significantly higher concordance rates of CD in monozygotic twins than dizygotic twins (50-58% and 0-12% respectively) and a similar trend for UC (6-14% and 0-5% respectively) (Matricon et al., 2010). Thus, there is a balance of genetic and environment contributions in IBD. Together, population based studies to date have provided strong support for the role of genetics in IBD.

Over the years, candidate gene studies have strived to identify single genes involved in IBD. A study in 1996 was the first to map a CD susceptibility region to chromosome 16, which was referred to as IBD1 (de Lange & Barrett, 2015). Subsequent studies on IBD1 determined that three variants, one frameshift (3020insC) and two missense polymorphisms (Arg702Trp, Gly908Arg), in the nucleotide-binding oligomerization domain-containing protein 2 (NOD2) gene were specifically responsible for susceptibility to disease (Hugot et al., 2001; Ogura et al., 2001). Also known as caspase recruitment domain-containing protein 5 (CARD15), NOD2 encodes for a protein with homology to disease resistance gene products in plants (de Lange & Barrett, 2015). In humans, the NOD2 protein is a cytosolic pattern recognition receptor that recognizes intracellular bacterial peptidoglycan, muramyl dipeptide, activating nuclear factor-κB (NF-κB) and mitogen-activated protein kinase signalling for subsequent inflammatory response (de Lange & Barrett, 2015). In the gastrointestinal tract, NOD2 is expressed in intestinal epithelial cells and Paneth cells (Philpott et al., 2014). The three aforementioned polymorphisms were observed to occur independently either within or nearby the NOD2 carboxy-terminal, leucine-rich repeat domain, distorting the architecture required for microbial pathogen sensing (Hugot et al., 2001; Ogura et al., 2001). In patients, mutated
NOD2 caused faulty NF-κB activation (Ogura et al., 2001; Hugot et al., 2001). In a meta-analysis of diverse populations, heterozygote carriers with one of the three risk alleles had a 2.4-fold increased risk of CD and homozygote carriers of one of the three risk alleles had a 17.4-fold increased risk (Economou et al., 2004). NOD2 variants were also found to be more common in Ashkenazi Jews compared to non-Jews (de Lange & Barrett, 2015). With this additional evidence, this lent credibility to the likely role of NOD2 variation in pathogenesis of CD. Although identifying NOD2 was a success story, progress in identifying IBD genetic risk loci was limited using single gene linkage analysis. This is because accumulating evidence suggests that IBD is not a monogenic disorder with genetic risk focused in one locus. Rather, IBD is a heterogeneous group of oligogenic diseases, each possessing a distinct association of multiple gene variants (Matricon et al., 2010). As a result, only highly penetrable variants such as NOD2 were capable of predicting similar phenotypic effect in different populations due to IBD’s genetic heterogeneity.

In light of this, key technological advances during recent years have led to the advent of genome-wide association studies (GWAS). Unlike single gene analysis, GWAS does not require prior hypotheses of the location or biology of predisposing genes, eliminating the limitation of needing to know which genes to test. Furthermore, because markers are assayed across the whole genome, GWAS have the advantage of extensive coverage. Thus, GWAS is a powerful technique, and has become a major tool for elucidating genomic loci associated with a complex disease, creating a revolution in IBD genetics. In GWAS, hundreds of thousands of genetic markers mapped throughout the whole genome are simultaneously genotyped in large population cohorts (Hendrickson et al., 2002). Since many disease associations may only confer moderate disease risks though, very large populations must be assayed. Affected cases and healthy controls are compared to identify markers with significant differences in frequency between the two groups (Hendrickson et al., 2002). These candidate markers suggest that their surrounding
chromosomal regions, ~150 kb long, contain genetic variants linked to increased disease susceptibility to IBD (Hardy & Singleton, 2009).

In this new era of GWAS technology, this major development has accelerated the identification of new IBD loci. Along with validation of the previously described association of NOD2 with CD in early studies using GWAS in 2006, new loci linked to IBD were rapidly identified (de Lange & Barrett, 2015). One study found an association between CD and the interleukin-23 receptor (IL23R) gene, which encodes for a subunit of a receptor for the IL-23 pro-inflammatory cytokine (Duerr et al., 2006). Arg381Gln mutation was found to confer protection against developing IBD in both non-Jewish and Jewish population cohorts (Duerr et al., 2006). Another study identified an association between CD and the autophagy-related 16-like 1 (ATG16L1) gene, which encodes for a protein involved in the autophagy pathway (Hampe et al., 2007). Since the autophagy pathway is involved in clearing of intracellular pathogens, this has fostered interest in the role of dysregulated autophagy in IBD patients, which will be discussed in greater detail in a later section. A separate early study also identified association of CD with a broad region on chromosome 5p13 not containing any genes, referred to as a “gene desert” (Libioulle et al., 2007). While prostaglandin receptor EP4 (PTGER4) is the closest gene to the associated region, unlike previous findings this study revealed the importance of regulatory and non-coding elements in IBD, whereby susceptibility loci could modulate expression of flanking genes (Libioulle et al., 2007). Although these are CD-specific associations, other studies have identified UC-specific associations, such as HNF4A, CDH1, and LAMB1, all three of which are involved in cell-to-cell interactions (de Lange & Barrett, 2015). With the help of GWAS, 163 risk loci were identified for IBD (de Lange & Barrett, 2015).

IBD-associated loci have implicated numerous pathways in the disease. These encompass the innate immune response (innate mucosal defence, autophagy, epithelial barrier function and repair, apoptosis/necroptosis, endoplasmic reticulum
stress regulation, reactive oxygen species and reactive nitrogen species production), activation of adaptive immune response (IL-23R, NF-κB), and regulation of adaptive immune response (Th17 cell differentiation, T-cell regulation, B-cell regulation) (de Lange & Barrett, 2015). Together, research has determined that 110 of the total 163 IBD risk loci currently identified are associated with CD and UC, indicating that both diseases share significant overlap of biological pathways (de Lange & Barrett, 2015). Furthermore, this suggests that the remaining loci that are specific for CD or UC drive the distinct clinical phenotypes of each disease (de Lange & Barrett, 2015). Due to the observation that many of the 163 loci were associated with pathways of the immune system, this led to the development of the Immunochip array in 2009 to identify links between IBD and other potentially related immune mediated diseases (de Lange & Barrett, 2015). The Immunochip is a custom genotyping array designed with dense coverage of genomic regions associated with immune-mediated diseases (Jostins et al., 2012). Employment of the Immunochip indicated linkage of 66 IBD loci with other immune-mediated diseases, strongest with ankylosing spondylitis and psoriasis (Jostins et al., 2012). This study also noted linkage to distantly related diseases, notably mycobacterial disease and leprosy, suggesting gastrointestinal infection by pathogenic organisms can contribute to IBD pathogenesis (Jostins et al., 2012). While polymerase chain reaction (PCR) studies have detected presence of mycobacterial sequences in patient intestinal biopsies, they do not show a specific association for CD (Hendrickson et al., 2002).

Going forward, because all of this data were obtained from analysis of populations of primarily European descent, a recent meta-analysis study assessed GWAS and Immunochip data for an expanded cohort of European, East Asian, Indian, and Middle Eastern descent (Liu et al., 2015). New susceptibility loci were identified, bringing IBD loci to a current total of 201 (Liu et al., 2015). While the direction and magnitude of effect of most loci were similar between European and non-European populations, a few loci such as NOD2 demonstrated discrepancies, thought to be a result of differences in allele frequencies between the populations (Liu et al., 2015).
With the discovery of over 200 IBD loci over the past decade, attention has shifted to narrowing down the associated loci to the causal variants, known as “fine-mapping”. Identification of the causal variants will help future elucidation of potential therapeutic targets for treatment of IBD.

1.1.6 Microbiome

Besides genetics, emerging data have indicated the importance of the host microbiome in the development of IBD. In animal studies, IBD mouse models displayed increased levels of *Bacteroides distasonis* and *Clostridium ramosum* in their intestinal tissue compared to healthy controls (Okayasu et al., 1990). IBD mouse models were also found to demonstrate reduced susceptibility to colitis when housed in a germ-free environment (Sellon et al., 1998). Furthermore, mouse models of IBD housed in a germ-free environment developed significantly fewer intestinal adenomas compared to controls housed in conventional, non-germ-free housing (Dove et al., 1997). Implication of the microbiome in IBD extends to humans, where comparisons of affected individuals and healthy controls have revealed differences in intestinal bacteria composition, such as increased *Escherichia coli* and *Clostridium perfringens* in patients (Matricon et al., 2010). In addition, studies exploring antibiotic use in CD have shown long-term alterations of microbiota while other studies have suggested potential therapeutic benefit of faecal microbial transplants in active CD, further implicating the role of the microbiome (Antonopoulos et al., 2009; Suskind et al., 2015).

Despite the alteration of numerous bacterial species in IBD, identities of the exact microbiota that cause IBD remain unclear pending further investigation. On the other hand, current evidence has suggested potential mechanisms of bacteria in the pathogenesis of IBD. One mechanism revolves around the mucosal-epithelial barrier in the intestine. Studies have shown abnormally elevated numbers of *E. coli* in the intestinal mucosa of patients with CD (Matricon et al., 2010). Identified as adherent-
invasive *E. coli* (AIEC), this bacterial strain reflected pathogenic characteristics that increased susceptibility to disease (Matricon et al., 2010). Specifically, AIEC demonstrated intimate association with intestinal epithelial cells, invaded intestinal epithelial cells by actin and microtubule dynamics, persisted within macrophages, and enhanced production of pro-inflammatory TNF-α and granulomas (Matricon et al., 2010). In multiple clinical studies on populations from North America and Europe, AIEC was reported to be more prevalent in CD cases compared to healthy controls (Matricon et al., 2010). Thus, leakiness of the intestinal mucosal-epithelial barrier seems to have implications for pronounced penetration of pathogenic bacteria towards the epithelium, leading to pronounced inflammatory response. The other potential mechanism of bacteria in development of IBD concerns the composition of the microbiome. It is hypothesized that when an imbalance of protective and pathogenic bacteria in the intestine occurs, known as dysbiosis, this increases susceptibility to IBD. This has been reconciled by studies that demonstrated in comparison to healthy controls, CD patients presented with less abundance of anti-inflammatory *Faecalibacterium prausnitzii* and increased numbers of pro-inflammatory *E. coli* (Matricon et al., 2010). Thus, dysbiosis of the normal microbiota may promote the growth of pathogenic bacteria causing a dysregulated inflammatory cascade. While microbiome studies in IBD are challenging because of influence by numerous factors such as antibiotics, diet, and other environmental factors, further investigation of the microbiota holds promise for better understanding the causes of IBD.

### 1.1.7 Immune Response

As reflected in previous discussion of genetics and the microbiome in IBD, inappropriate immune response plays a critical role in the disease. Immunopathogenesis of IBD can be classified into three main stages: (1) disruption and infiltration of the intestinal mucosal-epithelial barrier, (2) defective clearance of
foreign antigen influx, and (3) dysregulated, compensatory adaptive immune response (Matricon et al., 2010).

The intestine is host to millions of commensal and pathogenic microorganisms, emphasizing the intestinal epithelial barrier as a crucial barrier against the external environment. The barrier is composed of a single-cell layer of intestinal epithelium that is physically separated from the intestinal lumen by a mucus layer (Matricon et al., 2010). Cohesion and tightness of the epithelial cells are maintained by desmosomes, adherens junctions, and tight junctions (Matricon et al., 2010). Normally, the intestinal epithelial barrier is selectively permeable, permitting the transport of nutrients, electrolytes, and water through, but prohibiting entry of toxins and pathogenic organisms. However in IBD, studies have identified prevalent alterations of the barrier. Observations include reduced goblet cells that are responsible for secreting mucins that composes the mucus layer and disruption of intercellular junctions especially tight junctions, both of which compromise barrier permeability (Matricon et al., 2010). As a result, increased intestinal permeability to microorganisms is believed to be the first stage of IBD development (Matricon et al., 2010).

This is followed by the second stage of IBD immunopathogenesis, where there is dysregulation of the innate immune response causing impaired pathogen clearance. Once invading pathogens successfully penetrate into the epithelium, they are confronted by toll-like receptors (TLR) and NOD-like receptors (NLR) (Matricon et al., 2010). Following pathogen binding to TLR and NLR, these events signal the Paneth cells located in the intestinal crypts to secrete human α-defensins and the epithelial cells to secrete human β-defensins (Ramasundara et al., 2009). Research has indicated finding of modified defensin gene expression resulting in altered defensin production (Ramasundara et al., 2009). In contrast to enhanced production of defensins in UC, CD has been associated with defensin deficiency, particularly β-defensin attenuation in colonic CD and α-defensin reduction in ileal CD
While the exact mechanisms by which NOD2 mutation confers greater risk of CD are unclear, studies of NOD2 deficient mice indicated reduced defensin levels, increased commensal bacteria colonization, and defective pathogen clearance (Cho & Brant, 2011). In addition to defective defensin response, studies have identified impaired macrophage cytokine secretion in CD patients (Smith et al., 2009). Instead of release through the normal secretory pathway, cytokines were found to be rerouted to lysosomes for degradation, worsening clearance of injected bacteria (Smith et al., 2009). Additional evidence for dysregulation of innate immune response resides in the overactivation of dendritic cells in IBD, which are cells that link innate and adaptive immunity (Matricon et al., 2010).

Their overactivation causes progression to the final stage of IBD immunopathogenesis, where there is increased differentiation of native T cells into cytotoxic T cells (Tc cells) and helper T cells (Th cells), leading to differentiation of multiple types of effector T cells (Th1, Th2, Th17) (Hart et al., 2005). In turn, proliferation of these lymphocytes promotes production of pro-inflammatory cytokines. However, production of regulatory Th cells that mediate immunosuppression seems to be attenuated (Matricon et al., 2010). In addition, studies of IBD have found increased activation of chemokines and B cells contributing to amplified leukocyte infiltration of the mucosa and enhanced production of mucosal IgG antibodies directed against commensal bacteria respectively (Matricon et al., 2010). This combination of heightened inflammation to invaded pathogens as well as commensal bacteria normally tolerated by the host immune system perpetuates and gives rise to the chronic inflammation seen in IBD.

1.1.8 Environment

While genetic, microbial, and immunological factors are all important contributors to IBD pathogenesis, the environment has garnered much recent attention as a critical
factor. This is because in the past decade, rates of IBD have increased most dramatically in children (Molodecky et al., 2012). Historically, IBD was a Western civilization disease that was most prevalent in North America and Western Europe. In developing countries, such as in Eastern Europe, the Middle East, and Asia, rates of IBD were less compared to developed countries, indicating the influence of environmental and genetic factors in IBD. In addition, incidence was higher in urban populations compared to rural, suggesting that the Western lifestyle more prevalent in urban populations contribute to increased urban IBD incidence (Ananthakrishnan, 2015). Risk factors of the Western lifestyle provoking IBD have not been fully defined; however numerous factors have been explored. Some of the main environmental factors implicated in IBD pathogenesis will be discussed below.

Hygiene is one factor under investigation. According to the hygiene hypothesis, stringent hygiene limits early life exposure to antigens produced by infectious organisms, causing greater susceptibility of inappropriate immune response to these antigens later in life. Compared to developing countries, developed countries have improved hygiene, including increased access to clean water, reduced overcrowding, and increased access to hygiene products (Koloski et al., 2008). This has been proposed to account for the observation of increased immunological disorders such as IBD in developed countries (Matricon et al., 2010). However studies have disagreed on the strength of the role for hygiene in IBD. One study found that access to a hot water tap and separate bathroom at home increased risk of CD 5-fold, whereas another study found no significant association between access to hot water during childhood and IBD (Gent et al., 1994; Hampe et al., 2003). Owning a pet (animal exposure) during childhood was linked by a study to increased risk of CD but no such association was found by another (Amre et al., 2006; Gilat et al., 1987). It is plausible that hygiene may be involved in IBD pathogenesis, but further research is needed.

Smoking is another environmental factor that influences IBD. Since the original
observation that non-smoking was a feature of the majority of patients with UC (Harries et al., 1982), subsequent studies have identified a negative association of smoking with UC but a positive association with CD (Thomas et al., 2000). Research has also shown that smoking has little effect on clinical course of UC and conversely, worsens severity of CD (Hendrickson et al., 2002). In some studies, smoking even seemed to indicate potential benefit in UC, reflected in lower hospitalisation, colectomy, and relapse rates in active-smokers compared to non-smokers (Hendrickson et al., 2002). Despite these well-described associations, the mechanisms of smoking causing discordant effects in CD and UC are ill-defined.

Nicotine in tobacco is speculated to be the active agent, with potential regulation of nicotinic acetylcholine receptors expressed in mucosal epithelial cells and T-cells in the intestine (Molodecky & Kaplan, 2010). Ambient air pollution, a major by-product of Western industrialization, has also been investigated. It is well known that air pollution impairs development of lung function in children (Kaplan et al., 2010). Recent epidemiological research has indicated a novel association between certain air pollutants and IBD. IBD patients living in regions with high ambient NO\textsubscript{2} concentrations had increased risk of early onset CD while those living in regions with high SO\textsubscript{2} concentrations had increased risk of early onset UC (Kaplan et al., 2010). Thus smoking seems to negatively impact UC.

The effect of diet on IBD has been extensively studied however findings are inconsistent. Although some evidence indicates that high intake of total fats, polyunsaturated fatty acids, omega-6 fatty acids, and red meat as modifiers can increase risk of IBD, and high intake of fiber, fruit, and vegetables can decrease risk, other findings reported no such relationships (Hou et al., 2011). Numerous studies have addressed the influence of the microbiome by diet. One study showed enrichment of \textit{Bacteroides} in individuals on a high-protein, high-fat “Western” diet and enrichment of \textit{Prevotella} in individuals on a high-carbohydrate diet (G. D. Wu et al., 2011). This is consistent with data from other studies suggesting diet as a driving force in shaping different microbiota among different human populations across
geography (De Filippo et al., 2010; Yatsunenko et al., 2012). In the context of IBD, several studies have shown suppressed abundance of *F. prausnitzii* in the gut of IBD patients, likely a result of low-fiber dietary regimen (Cao et al., 2014; Miquel et al., 2013; Sokol et al., 2008). *F. prausnitzii* is one of the most abundant bacteria in the normal microbiome and is an important supplier of butyrate, an energy substrate for intestinal epithelial cells, without which barrier integrity is compromised enabling leaky bacterial translocation (Sokol et al., 2008). Therefore evidence suggests a role for diet in IBD.

The relationship between breastfeeding and risk of developing IBD has been under investigation. Breastfeeding enables transfer of breast milk antigen-immunoglobulin immune complexes from mothers to babies, helping them achieve tolerance induction which is important in intestinal immune development (Molodecky & Kaplan, 2010). Thus emerging studies have shown breastfeeding to be protective against developing IBD (Molodecky & Kaplan, 2010).

Several medications are thought to impact IBD development. Studies of oral contraceptives indicated positive association for IBD (Molodecky & Kaplan, 2010). Oral contraceptives contain estrogen, a hormone that can enhance the immune response in IBD by activating macrophages, the complement system, and B cells to increase antibody production (Molodecky & Kaplan, 2010). Use of antibiotics in childhood is a risk factor for developing IBD. Antibiotics are thought to perturb the normal microbiota, leading to disease (Koloski et al., 2008). Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) are another factor linked to increased risk of IBD. Several hypotheses have been proposed to explain the pathophysiology of intestinal damage by NSAIDs, including exacerbation of intestinal permeability, inhibition of cyclooxygenase, and reduction of prostaglandin production (Molodecky & Kaplan, 2010). Thus medications are a risk factor for IBD.

Appendectomy has been shown to have a protective effect against development of
Similar to smoking, there is no consensus on its effect on CD (Molodecky & Kaplan, 2010). Although there is significant risk of CD within the first year following an appendectomy, the risk is not significant after five years (Molodecky & Kaplan, 2010). Moreover, research has indicated benefit only in patients who undergo appendectomy before 20 years old (Andersson et al., 2001). Therefore, protective effect of appendectomy may be limited to early life.

Over the past few decades, there has been considerable globalization of IBD, paralleling the similar rise of Westernization. From the beginning of the 19th century, incidence rates of UC increased steadily in North America and Europe before plateauing in the 1960’s and 70’s (M’Koma, 2013). In contrast, over the same time period, UC emerged and steeply increased in historically low-incidence Eastern Europe, Asia, and Africa (M’Koma, 2013). Interestingly, as countries became more industrialized, rates of CD eventually predominate over UC with UC rates stabilizing (Ananthakrishnan, 2015). Reasons for this trend are unknown. Studies exploring risk of IBD in migrant populations have shown that immigrants who have migrated from low prevalence to Western countries adopt increased risk of IBD. For example in an epidemiological study on IBD rates in immigrants to Ontario, Canada between 1994 and 2010, the immigrants upon arrival had lower incidence of IBD compared to the indigenous, non-immigrant Canadian population (Benchimol et al., 2015). However it was found that younger age at immigration was associated with increased risk of IBD by 14% per younger decade of life at immigration (Benchimol et al., 2015). In contrast to their parents, children of immigrants from Africa, the Middle East, and South Asia had similar incidence of IBD compared to children of non-immigrants (Benchimol et al., 2015). Thus, these findings suggest a correlation between early life exposure to the Canadian environment in immigrants and increased risk of developing IBD.

In addition to the Westernization phenomenon, IBD epidemiology is influenced by geographical distance from the Earth equator. Countries further from the equator
receive less sunlight exposure compared to those nearer the equator. Studies have shown that occurrence of IBD has historically been greater in northern latitude countries compared to southern latitude countries (Molodecky et al., 2012). Even today with extensive migration of people between countries, IBD continues to be more prevalent in North American and northern Europe than in southern Europe, South America, Africa, and Asia (Molodecky et al., 2012). Since vitamin D is obtained through sunlight exposure, this finding suggests an important role for vitamin D in the pathogenesis of IBD, the focus of this project, to be expanded upon in a later section.

Altogether, IBD is a severe disease with widespread public health implications. To date, there is no cure for IBD. While considerable strides have been made in understanding the contribution of genetic, microbial, immune, and environmental factors to pathogenesis of IBD, much work remains to identify and describe their precise interactions.
**Figure 1. Multifactor pathogenesis of IBD.** The current model for pathogenesis of IBD is that convergence of genetics, microbiome, immune response, and environment triggers the chronic symptoms that hallmark the disease.
1.2 Autophagy: Genetic and Environmental Influences of the Pathway in IBD

Autophagy broadly refers to an intracellular degradation system that transports cytosolic material to lysosomes. Belgium scientist Christian de Duve was the first to describe autophagy (from the Greek “auto” meaning self and “phagein” mean to eat) in the 1960s (Deter & De Duve, 1967). Identification of autophagy-related (ATG) genes in the yeast *S. cerevisiae* in the 1990s, and subsequent discovery in various species led to enriched understanding (Salem et al., 2015). In recent years, growing evidence have recognized autophagy as a potent modulator of a wider range of cellular pathways than expected, such as cell survival, cell death, immunity, ageing, and cancer (Glick et al., 2010). In addition, autophagy occurs in all eukaryotes with many autophagy genes conserved across yeast, plants, worms, flies, and mammals (Glick et al., 2010; Wirawan et al., 2012). Given its importance, it is no surprise then that dysregulated autophagy has been implicated in many pathological diseases, including IBD. As a result, these findings have reinvigorated research of this field, with ongoing studies focused on better understanding how autophagy affects pathogenesis of IBD.

1.2.1 The Autophagy Pathway

In autophagy, cytosolic cargo is sequestered by a double-membraned vesicle, called an autophagosome, and targeted to a lysosome for fusion, causing degradation of the autophagosomal cargo by lysosomal hydrolases (Figure 2). While nutrient starvation is the most potent stimulus of autophagy in yeast, induction in mammalian cells is more complex (Mizushima, 2007). Induction can be triggered in response to diverse signals such as nutrient starvation, hypoxia, damaged protein or organelle accumulation, and pathogen invasion (Wirawan et al., 2012). These signalling pathways converge on the mammalian target of rapamycin complex 1 (mTORC1).
mTORC1 is involved in regulation of broad cellular processes that encompass cell growth, proliferation, protein synthesis, and autophagy (Wirawan et al., 2012). In autophagy, mTORC1 is the key regulator of autophagy induction. When nutrients, adenosine triphosphate (ATP), and growth factors are available, it is thought that mTORC1 remains activated by phosphatidylinositol-3-kinase class 1 (PIK3C1), enabling constitutive phosphorylation of ATG13, uncoordinated-51-like kinase 1 (ULK1), and ULK2 (Wirawan et al., 2012). This keeps them in a conformational state that renders them in an inactive form, causing inhibition of autophagy (Jung et al., 2010). On the other hand, during starvation conditions, autophagy is induced. Here, PIK3C1 is inactivated and mTORC1 is inhibited, leading to dephosphorylation and activation of ATG13, ULK1, and ULK2 (Jung et al., 2010). This leads to phosphorylation of Ambra1, release of activating molecule in beclin-1-regulated autophagy 1 (Ambra1) and PIK3C3 from microtubules, and their translocation from the cytosol to the endoplasmic reticulum (ER), regarded as the major site of autophagosome formation or nucleation (Wirawan et al., 2012). While the ER is the primary site, studies have shown that the trans-Golgi and late endosomes can serve as additional sites of autophagosome formation (Glick et al., 2010).

Upon association with the ER, PIK3C3 generates phosphatidylinositol-3-phosphate (PI3P), which recruits additional autophagy machinery to the autophagosome formation site (Wirawan et al., 2012). Research has identified some of these proteins to include WD repeat domain phosphoinositide interacting 1 (WIPI1), WIPI2, ATG2, and double-FYVE containing protein-1 (DFCP1), although their precise functions are unknown (Wirawan et al., 2012). In the first step of autophagosome formation, a portion of the cytoplasm is sequestered by a sequestration crescent (Mizushima, 2007). During this time, cytosolic contents, such as misfolded proteins, aggregated proteins, damaged organelles, and pathogenic microorganisms, are captured. While autophagic selection of cargo was widely believed to be non-selective because a portion of the cytoplasm is engulfed by the autophagosome, recent evidence has shown that autophagosomes are capable of
selective cargo sequestration (Glick et al., 2010; Mizushima, 2007). The terms mitophagy, peroxphagy, reticulophagy, nucleophagy, lipophagy, and xenophagy have been recently coined to describe the selective autophagic degradation of mitochondria, peroxisomes, ER, nuclei, lipids, and pathogens, respectively (Wirawan et al., 2012).

The next step is autophagosome elongation, where two ubiquitin-like conjugation systems are involved. The first is the ATG conjugation cascade pathway. ATG7, an E1-like ubiquitin activating enzyme, activates ATG12 causing its covalent linkage to ATG5 via ATG10, an E2-like ubiquitin conjugating protein (Wirawan et al., 2012). ATG16L1 is subsequently linked, creating an ATG5-ATG12-ATG16L1 complex (Wirawan et al., 2012). This complex is transiently associated with the autophagosomal membrane during its elongation phase, during which it is suggested to manipulate the membrane’s curvature (Wirawan et al., 2012). Formation of the multimeric ATG complex acts results in progression to the second ubiquitin-like conjugation reaction. Microtubule-associated light chain-3 (LC3) is cleaved by ATG4 to generate LC3-I with an exposed glycine at the carboxy terminal (Wirawan et al., 2012). This glycine is activated by E1-like ATG7, causing LC3-I transfer to E2-like ATG3, leading to conjugation with phosphatidylethanolamine (PE) via the ATG16L1 complex serving as an E3-like ubiquitin ligase, to become LC3-II (Wirawan et al., 2012). LC3-II remains associated with the autophagosomal membrane and only becomes degraded after autophagosome fusion with the lysosome (Wirawan et al., 2012). Since LC3-II is localized to the autophagosomal membrane and LC3-II synthesis is increased during autophagy, LC3-II is a widely used autophagy marker (Glick et al., 2010). It is thought that selective capture of cargo is mediated through recognition of target adaptor molecules by the LC3-interacting region (LIR) of autophagosome-associated LC3-II, acting as receptors (Glick et al., 2010).
Once the autophagosome is fully enclosed, the autophagosome undergoes maturation. Here, microtubules facilitate the fusion of autophagosomes with lysosomes to form degradative autolysosomes (Köchli et al., 2006). At the lysosome, multiple proteins are recruited for facilitating tethering and fusion. These include lysosome-associated membrane protein-1 (LAMP-1), LAMP-2, Ras-related GTP-binding protein 7 (Rab7), homotypic fusion and protein sorting (HOPS), and soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) (Wirawan et al., 2012). After autophagosomal membrane fusion with the lysosome, the contents carried by the autophagosome are exposed to the acidic lumen of the lysosome, and degraded by the lysosomal hydrolases (Wirawan et al., 2012). Amino acids, fatty acids, and nucleosides left over from the autophagic degradation are exported back into the cytosol by lysosomal efflux transporters for use by the cell (Z. Yang et al., 2006). Thus in addition to promoting clearance of excess, damaged, and harmful contents, autophagy enables cells to adapt to starvation conditions by recycling, making the pathway a key player in maintaining cellular homeostasis.
Figure 2. The autophagy pathway. In response to starvation or stress, cytosolic cargo is sequestered by a double-membraned vesicle called the autophagosome. ATG5-ATG12-ATG16L1 complex and LC3-II conjugation are necessary for autophagosome formation. Autophagosomal fusion with lysosomes facilitates degradation of sequestered cargo and recycling, completing the autophagy pathway. Reproduced and modified with permission from Greenfield and Jones; Trends in Microbiology, 2013 (Greenfield & Jones, 2013).
1.2.2 Implication in Disease

Given that autophagy is an integral mechanism of defending cells against starvation and stress, dysregulated autophagy has been linked to pathogenesis of diverse diseases. Pathologies include gastrointestinal diseases, neurological diseases, heart diseases, liver diseases, muscle diseases, cancer, aging, infections, inflammatory diseases, and more (Levine & Kroemer, 2008). Interestingly, despite ample literature indicating the role of autophagy to be primarily protective; emerging evidence has suggested that it is equally plausible that autophagy activation has maladaptive implications leading to disease (Levine & Kroemer, 2008). Since the precise involvement of autophagy in the context of specific diseases remains ambiguous, this pathway remains under intense investigation.

1.2.3 Dysregulation of ATG16L1 in Crohn’s Disease

In CD, the most commonly involved sites are the terminal ileum and colon. These regions are associated with high bacterial loads, implicating a prominent role for dysregulated antibacterial autophagy in the development of CD. This is reconciled by numerous genetic studies linking single-nucleotide polymorphisms (SNPs) in autophagy genes to pathogenesis of CD. Autophagy genes linked to CD include: immunity-related guanosine triphosphatase family M (IRGM), protein tyrosine phosphatase non-receptor type 2 (PTPN2), X-box binding protein 1 (XBP1), leucine-rich repeat kinase 2 (LRRK2), ULK1, and ATG16L1 (Salem et al., 2015).

Dysregulation of ATG16L1 in particular has been implicated in disease by multiple studies. ATG16L1 is essential for autophagy; it regulates localization of the ATG5-ATG12 complex to the sequestration crescent in autophagosome formation. Using GWAS, there have been nine SNPs in ATG16L1 associated with CD identified so far: rs13412102, rs12471449, rs6431660, rs1441090, rs2289472, rs2241880, rs2241879, rs3792106, and rs4663396 (Salem et al., 2015). Rs2241880, also
known as T300A, was the first variant to be discovered (Hampe et al., 2007). Subsequent studies have consistently reported T300A to be strongly associated with developing CD, with an increased odds ratio of between 1.4 and 1.9, and not associated with developing UC (Hampe et al., 2007; H.-F. Zhang et al., 2009). T300A is also the most prevalent variant among patients, accounting for 55% in Europeans and 20-40% in other populations (Hampe et al., 2007).

ATG16L1 protein structure is comprised of an amino-terminal ATG5-binding region, coiled-coil domain (CCD), and seven carboxy-terminal tryptophan-aspartic acid (WD) repeat regions (Salem et al., 2015). In the presence of the T300A variant, there is an adenine to guanine missense SNP, encoding for an alanine instead of a threonine at position 300 in the carboxy-terminal WD region (Salem et al., 2015). A recent study has shown that this mutation is located in a caspase cleavage site (Murthy et al., 2014). Compared to the controls, mice harbouring the variant demonstrated significantly increased susceptibility of ATG16L1 to caspase-3 degradation, resulting in reduced autophagy (Murthy et al., 2014). Deletion of the caspase-3 gene (CASP3) rescued starvation-induced autophagy, confirming that T300A is sensitive to accelerated degradation mediated by caspase-3 (Murthy et al., 2014). While other molecular consequences are under investigation, multiple studies have explored the physiological consequences of dysregulated ATG16L1. They encompass impaired pathogen clearance, abnormal Paneth cell function, and aberrant inflammatory signalling (Murthy et al., 2014).

Since autophagy mediates sequestration and degradation of pathogens, research has reported an association of dysregulated autophagy and increased bacterial colonization (Salem et al., 2015). In the previous study discussed, ATG16L1 mutation in mice resulted in increased susceptibility to infection with Yersinia enterocolitica, indicating impaired autophagic pathogen clearance (Murthy et al., 2014). This was consistent with studies in human epithelial cells which have linked the CD ATG16L1 variant to increased bacterial load of Salmonella, Escherichia coli,
and *Helicobacter pylori* (Deuring et al., 2014; Kuballa et al., 2008; Raju et al., 2012). Subsequently, accumulation of bacteria in CD patients may be associated with increased inflammatory response such as increased pro-inflammatory cytokine and antimicrobial antibody release, higher disease activity, and necessity for more aggressive biological therapy to suppress inflammation (Gutiérrez et al., 2014; Lapaquette et al., 2012; Murdoch et al., 2012). Thus this evidence indicates the importance of intact ATG16L1 in the maintenance of pathogen clearance.

Dysregulated ATG16L1 also affects Paneth cells. Paneth cells are specialized epithelial cells that mediate innate immune protection in the small intestine from enteric pathogens, through secretion of granule contents containing defensins and lysozymes. The importance of ATG16L1 was demonstrated by a study showing that ATG16L1-deficient mouse Paneth cells had abnormal cell morphology (aberrant and disorganized granules) and granule exocytosis (reduced granule numbers and diminished lysozyme exocytosis to the ileal mucus layer) (Cadwell et al., 2008). Furthermore, the ATG16L1-deficient mouse Paneth cells showed increased peroxisome-activated receptor (PPPAR), leptin, and adiponectin gene expression, which are linked to pro-inflammatory signalling (Cadwell et al., 2008). Similar observations of granule exocytosis abnormalities and increased leptin expression were reported in human Paneth cells from patients homozygous for CD ATG16L1 mutation, furthering support for the role of ATG16L1 in Paneth cell function (Cadwell et al., 2008).

Lastly, ATG16L1 dysregulation influences development of the inflammatory response. ATG16L1-deficient macrophages demonstrated increased pro-inflammatory IL-1β and IL-18 production in response to lipopolysaccharide challenge (Saitoh et al., 2008). Mice with ATG16L1 knockout (KO) in their haematopoietic cells were more susceptible to dextran sulphate sodium (DSS)-induced colitis (Saitoh et al., 2008). Injection of anti-IL-1β and anti-IL-18 antibodies ameliorated the effect (Saitoh et al., 2008). Another study showed that dendritic cells cultured from
ATG16L1 T300A patients had diminished antigen uptake and processing compared to dendritic cells from healthy patients (Strisciuglio, Miele, et al., 2013). In a confocal microscopy study, reduced autophagy in dendritic cells was associated with decreased trans-epithelial protrusions by dendritic cells, further implicating diminished luminal antigen-dendritic cell sampling in the altered abnormal immune response (Strisciuglio, Duijvestein, et al., 2013). Following bacterial exposure, T300A dendritic cells showed reduced expression of human leukocyte antigen – D related (HLA-DR) and co-stimulator CD86, both involved in pro-inflammatory signalling (Strisciuglio, Miele, et al., 2013). Evidence has also implicated crosstalk between ATG16L1 and NOD2 in the autophagic response to invasive bacteria, linking this to bacterial clearance. This study showed that in cells homozygous for CD NOD2 mutation, dysfunctional NOD2 abolished the ability of ATG16L1 to localize to the plasma membrane at the site of bacteria entry, leading to failed autophagosome formation and diminished autophagic response (Travassos et al., 2010). Together, abundant evidence indicate the importance of ATG16L1 in host immunity.

Thus, autophagy is a strong risk factor for CD, having been genetically and physiologically associated with disease pathogenesis. T300A disrupts stability of ATG16L1, making it more susceptible to caspase degradation. Subsequently, ATG16L1 dysregulation contributes to impaired pathogen clearance, abnormal Paneth cell function, and aberrant inflammatory signalling (Figure 3). These consequences all increase susceptibility to CD, emphasizing autophagy as an important pathway that warrants further study on how dysregulation causes disease.
Figure 3. Consequences of ATG16L1 dysregulation. Autophagy is involved in maintaining intestinal homeostasis, thus ATG16L1 dysregulation can give rise to multiple consequences that increase susceptibility to CD.
1.3 Vitamin D Deficiency

Previously perceived as a problem of solely northern latitude countries with limited exposure to sunlight, new insight into this health condition have led to resurgence of vitamin D deficiency as a global public health concern in the 21st century. Prevalence of low vitamin D has been observed even in areas of the world with abundant sun exposure. Vitamin D deficiency has wide implications for disease, with numerous studies indicating linkage to cancers, cardiovascular diseases, endocrine diseases, autoimmune diseases, and many other common and serious pathologies (R. Zhang & Naughton, 2010). With growing data implicating low vitamin D status in development of IBD, the potential mechanistic role of vitamin D deficiency merits consideration.

1.3.1 Vitamin D

Vitamin D, long recognized as the “sunshine vitamin”, is an essential nutrient that has two major forms: D₂ (ergocalciferol) and D₃ (cholecalciferol). While sunlight is the dominant source of vitamin D, vitamin D can also be obtained from diet. There are only a few natural foods high in vitamin D. These include oily fish (salmon, trout, mackerel, etc.), cod liver oil, shiitake mushrooms, and to a lesser extent, egg yolk (R. Zhang & Naughton, 2010). Some foods are fortified with vitamin D, such as butter, milk, orange juice, yogurt, margarine, cheese, bread, and breakfast cereals (R. Zhang & Naughton, 2010). Vitamin D acquired from either sunlight exposure or diet is biologically inert, and so it must undergo metabolism in the body in order to become bioactive.

Metabolic activation of vitamin D is a multi-step process (Figure 4). During exposure to sunlight, solar ultraviolet B (UVB) radiation in the 290 to 315 nm wavelength range penetrates the skin, converting cutaneous 7-dehydrocholesterol to previtamin D₃, followed by isomerization to vitamin D₃ (Holick, 2007). Excess sunlight exposure
degrades previtamin D₃, preventing vitamin D intoxication (Holick, 2007). Alternatively, vitamins D₂ and D₃ ingested from dietary sources are taken up by chylomicrons which are lipoprotein transporters, and transported by the lymphatic system into the venous circulation (Holick, 2007). Given it is fat-soluble, vitamin D can also be stored in adipose cells (Holick, 2007). Circulating vitamin D is bound to vitamin D-binding protein in the plasma before being hydroxylated in the liver by vitamin D 25-hydroxylase to 25-hydroxyvitamin D [25(OH)D] (Holick, 2007). As the major circulating form of vitamin D, 25(OH)D is used to determine a person’s vitamin D status, and has a half-life of 3-4 weeks (Mølgaard & Michaelsen, 2003). Once formed, 25(OH)D is released back into the plasma where it is bound again by vitamin D-binding protein. This vitamin D metabolite is further hydroxylated in the kidneys to 1,25-dihydroxyvitamin D [1,25(OH)₂D] (Holick, 2007). This hydroxylation reaction is catalyzed by 25-hydroxyvitamin D-1α-hydroxylase (1-OHase). The final product - 1,25(OH)₂D, is the active form of vitamin D and exerts its physiological effects through binding of the vitamin D receptor (VDR). VDR is a nuclear receptor and serves as a DNA-binding transcription factor that dimerizes with a retinoic X receptor (RXR) to form a VDR-RXR complex (Holick, 2007). The VDR-RXR complex recognizes vitamin D responsive elements (VDREs) in the DNA sequence of genes regulated by vitamin D (Haussler et al., 2011). Activated VDR can subsequently modulate transcription of these genes by recruiting coactivators or corepressors (Haussler et al., 2011). Vitamin D metabolism is a tightly regulated pathway, with stimulation of 1-OHase by parathyroid hormone (PTH) and suppression by calcium, phosphate, 1,25(OH)₂D, and fibroblast growth factor 23 (FGF23) (Holick, 2007). 1,25(OH)₂D is catabolized to calcitroic acid by the enzyme 25-hydroxyvitamin D-24-hydroxylase, which is excreted in the bile (Holick, 2007).

Vitamin D plays a pivotal role in regulation of circulating calcium and phosphate levels. Without vitamin D, absorbance of dietary calcium and phosphate is limited to 15% and 60%, respectively (Holick, 2007). Thus when levels of calcium and phosphate are low, PTH stimulates metabolism of vitamin D. In the intestine, vitamin
Vitamin D promotes calcium and phosphate absorption (Holick, 2007). In bone, vitamin D activates osteoclasts to promote bone resorption, replenishing serum calcium levels (Holick, 2007). In the kidney, vitamin D increases tubular resorption of calcium and phosphate (Holick, 2007). Besides maintenance of calcium and phosphate, vitamin D mediates effects in other organs of the body. Including the intestine, bone, and kidney, VDR is expressed in 38 tissues throughout the body, such as the brain, prostate, breast, and immune system (Holick, 2007). Furthermore, vitamin D is estimated to regulate more than 200 genes, including cell proliferation and differentiation, cell death, and angiogenesis (Holick, 2007).
Figure 4. Vitamin D synthesis and metabolism. Cutaneous conversion of 7-dehydrocholesterol to previtamin D₃ is triggered by sunlight exposure. After isomerization to vitamin D₃, vitamin D₃ obtained from photochemical synthesis or dietary absorption is converted to 25(OH)D in the liver. The kidneys metabolize 25(OH)D to 1,25(OH)₂D, the active form of vitamin D. Reproduced and modified with permission from Deeb et al.; Nature Reviews Cancer, 2007 (Deeb et al., 2007).
1.3.2 Relationship between Vitamin D and IBD

Vitamin D deficiency occurs when sunlight exposure and dietary intake are insufficient. As mentioned previously, serum 25(OH)D is used as a biomarker of a person’s vitamin D status. While there is no consensus on standard reference values for 25(OH)D, the predominant classifications are: optimal (> 75 nmol/L), sufficient (> 50 nmol/L), insufficient (27.5-50 nmol/L), and deficient (<27.5 nmol/L) (Bischoff-Ferrari et al., 2006; Institute of Medicine (US), 2011). The American National Academy of Medicine currently recommends the Adequate Intake (AI) for vitamin D, AI defined as the average daily nutrient intake level recommended based on nutrient intake by healthy people, to be 200 international units (IU)/day for children and adults ≤ 50 years old, 400 IU/day for adults 51-70 years old, and 600 IU/day for adults ≥ 71 years old (Holick, 2007). However without adequate sun exposure, especially for population in northern countries such as Canada, the scientific community proposes increased AI to at least 800-1000 IU/day (Holick, 2007). It is challenging to achieve dietary intake of this amount daily, thus sensible sunlight exposure and assistance from multivitamin or single vitamin supplements are recommended. Research shows that exposure of the body in a bathing suit to one minimal erythemal dose (MED), defined as the minimum amount of UVB radiation that causes redness 24 hours after exposure, is equivalent to ingesting 10,000 IU of vitamin D (Holick, 2007). Thus, exposure of 6-10% of the body to 1 MED is equivalent to 600-1000 IU, fulfilling the AI recommendations (Holick, 2007). An advantage of sunlight exposure-mediated synthesis of vitamin D is that excess production of previtamin D_3 triggers degradation, avoiding intoxication (Holick, 2007).

More than 1 billion people worldwide are estimated to be either vitamin D deficient or insufficient (Holick, 2007). There are many reasons for this prevalence of low vitamin D. First, people in northern countries receive less sunlight exposure compared to those living closer to the equator due to northern latitude and extended winter seasons (Webb et al., 1988). Second, people with darker skin have increased
melanin that competes with 7-dehydrocholesterol for UVB absorption, causing reduced vitamin D synthesis (Clemens et al., 1982). Third, use of sunscreen blocks UVB radiation interaction with 7-dehydrocholesterol (Matsuoka et al., 1987). Fourth, lifestyles have shifted to significantly increased time indoors, drastically reducing outdoors sunlight exposure (Owen et al., 2010). Fifth, individuals who have undergone surgical procedure to remove part of their stomach or intestines develop malabsorption of fat-soluble vitamins including vitamin D (Lo et al., 1985). Sixth, obesity has been associated with vitamin D deficiency, speculated to be an issue of reduced UVB exposure and increased fat storage along with restricted release into the circulation compared to non-obese cohorts (Wortsman et al., 2000). Seventh, VDR polymorphisms causing VDR dysfunction have been identified, however influence of IBD risk remains poorly defined (Ardesia et al., 2015). Lastly, some people obtain insufficient vitamin D from their nutrition. Thus many reasons exist for vitamin D deficiency.

Growing evidence from animal and human studies dictates that vitamin D deficiency is an important environmental factor contributing to IBD pathogenesis. IL-10 is an anti-inflammatory cytokine of which knockout leads to spontaneous development of CD-like colitis, making IL-10 KO mice a common IBD animal model (Madsen, 2001). One study showed that 3 week old vitamin D deficient IL-10 KO mice were growth retarded and had significantly more intestinal inflammation compared to vitamin D sufficient wild type (WT) and IL-10 KO mice (Cantorna et al., 2000). Furthermore, the vitamin D deficient IL-10 mice showed considerable mortality by 9 weeks of age, whereas the WT and IL-10 KO vitamin D sufficient mice were healthy even at 13 weeks (Cantorna et al., 2000). In 7 week old vitamin D deficient IL-10 KO mice, intestinal inflammation was ameliorated after 2 weeks of vitamin D supplementation (Cantorna et al., 2000). In another study, DSS-treated C57BL/6 mice fed vitamin D sufficient diet for 6 weeks had reduced severity of DSS-induced colitis compared to mice fed vitamin D insufficient diet for the same time period (Lagishetty et al., 2010). In addition, the vitamin D deficient DSS-treated mice showed elevated levels of
bacteria infiltration compared to controls (Lagishetty et al., 2010). 27 genes including angiogenin-4 (Ang4) were altered more than 2-fold in vitamin D deficient mice colon tissue compared to vitamin D sufficient controls (Lagishetty et al., 2010). Ang4 encodes for an antimicrobial peptide that is involved in minimizing enteric bacterial invasion. Ang4 was reduced more than 5-fold in the vitamin D deficient mice compared to controls, suggesting that vitamin D deficiency increases mouse susceptibility to colitis by dysregulation of antimicrobial activity (Lagishetty et al., 2010). In another study also using the DSS-induced colitis model, mice were administered daily intra-rectal injections of BXL-62 (anti-inflammatory VDR agonist) or 1,25(OH)₂D (Laverny et al., 2010). The BXL-62-treated mice demonstrated reduced weight loss, diarrhea, and colitis compared to the 1,25(OH)₂D-treated mice, suggesting that VDR agonists may be an effective intervention for IBD (Laverny et al., 2010). Lastly, a study conducting research on double IL-10 KO/VDR KO (DKO) mice showed greater acceleration and severity of experimental colitis compared to IL-10 KO mice with functional VDR (Froicu et al., 2006). This observation is evidence that VDR signalling is implicated in development of IBD.

In addition to animal studies, human studies support the role of vitamin D deficiency in IBD pathogenesis. Literature has shown that prevalence of IBD is higher in northern latitude countries than in southern latitude countries (Molodecky et al., 2012). This is consistent with reduced sunlight exposure as distance is increased from the equator. In another study that focused only on the United States, increased latitude of residence was associated with increased incidence of CD and UC, with residence at age 30 years and later most strongly associated with risk (Khalili et al., 2012). Various studies have reported vitamin D deficiency in patients with IBD. In a 2012-2013 survey, 25% of the Canadian population was considered to be vitamin D insufficient and 10% to be vitamin D insufficient (Statistics Canada, 2014). Studies of other IBD patient cohorts around the world during the past 5 years have reported vitamin D deficiency prevalence ranging from 19 to 95% (Mouli & Ananthakrishnan, 2014).
supplementation in patients with IBD, 94 CD patients were randomized to receive oral treatment of either 1200 IU vitamin D or placebo once daily for one calendar year (Jørgensen et al., 2010). Vitamin D treatment raised mean serum vitamin D levels by 39% after just 3 months (Jørgensen et al., 2010). Although not significant because of sample size statistical power, disease relapse rate was reduced in the vitamin D treated patients compared to placebo (Jørgensen et al., 2010). Thus, vitamin D treatment in this study showed promise in managing CD. However other vitamin D intervention studies have shown variable improvement in disease outcome (Grunbaum et al., 2013). This may be a consequence of underlying VDR polymorphisms or an inverse correlation between inflammation and VDR expression (Mangin et al., 2014). More recent studies have shown that low vitamin D is associated with increased relapses, hospitalizations, surgeries, risk of developing cancer, and loss of response to TNF-α therapy in CD patients (Ananthakrishnan et al., 2013, 2014; Zator et al., 2014). Overall, evidence from animal and human studies has implicated vitamin D deficiency in IBD, where further research is necessary to illuminate this pathway.

### 1.3.3 Relationship between Vitamin D and Autophagy

Over recent years, increasing evidence has shown interaction of vitamin D signalling with autophagy. Studies have shown that vitamin D signalling is able to regulate autophagy at (1) induction, (2) autophagosome nucleation, and (3) maturation (S. Wu & Sun, 2011). mTORC1 inhibition is needed for autophagy induction. Vitamin D has been recognized to exert mTOR inhibition by increase of free cytosolic calcium (Maria Høyer-Hansen et al., 2007). Vitamin D has been shown in human leukemia cells to regulate nucleation through upregulation of beclin-1 and PI3KC3, which are both involved in localization of ATG proteins to the nucleation site (J. Wang et al., 2008). One VDR-regulated gene is cathelicidin, which encodes for an antimicrobial protein (S. Wu & Sun, 2011). Autophagy in human monocytes has been shown to be induced by vitamin D via cathelicidin (Yuk et al., 2009). Moreover, autophagosome
maturation was observed in a cathelicidin-dependent manner (Yuk et al., 2009). Besides cathelicidin, vitamin D is also recognized to be a potent activator of the NOD2 gene (T.-T. Wang et al., 2010). Since NOD2 recruits ATG16L1 to the site of bacteria entry, vitamin D affects autophagosome formation through the NOD2 pathway (Travassos et al., 2010). The ATG16L1 gene is also transcriptionally regulated by vitamin D, where VDR deletion in the intestine has been associated with abnormal Paneth cell morphology, ATG16L1 reduction, autophagy deficiency, and bacterial dysbiosis (Sun, 2015; S. Wu et al., 2015). Another study investigating vitamin D analog EB1089 found that EB1089 induced autophagy in human breast cancer cells and triggered dramatic lysosomal changes, where lysosome volume and protease activity were both greatly increased (M Høyer-Hansen et al., 2005).

Overall, vitamin D is essential for health. Development of IBD has been widely associated with vitamin D deficiency. Findings from animal and human studies suggest that dysregulation of autophagy in IBD may be influenced by vitamin D deficiency at different levels of the autophagy pathway. Many questions remain about the mechanistic overlap of vitamin D, autophagy, and IBD pathogenesis, thus further study is warranted.
1.4 Epigenetic Mechanisms of Environment-Gene Regulation

Until the 1940s, the environment and heredity were thought to be independent entities (Dupont et al., 2009). However this changed with the discovery of epigenetics as a bridge of these two spheres. Epigenetics is defined as heritable changes in gene function that do not involve change in DNA sequence. In light of this finding, multiple types of epigenetic modifications have been identified, such as DNA methylation, histone methylation and acetylation, X chromosome inactivation, genomic imprinting, and gene silencing (Dupont et al., 2009). Over the years, epigenetics has become one of the most rapidly growing fields in medical sciences research. Epigenetics has been proposed as an explanation for the missing heritability in complex genetic diseases not identified in GWAS (Slatkin, 2009). Thus environmentally-triggered heritable epigenetic changes may be common and can influence risk of disease. Epigenetic regulation through microRNAs poses a potential mechanism by which vitamin D deficiency alters autophagy in IBD.

1.4.1 MicroRNA

MicroRNAs (miRNAs) constitute one epigenetic mechanism that functions in gene silencing, also known as RNA interference (RNAi). miRNAs are small, non-coding, single stranded ribonucleic acids (RNAs) generally 19-25 nucleotides long. miRNAs are highly conserved, they have been identified in over 140 of vertebrate and invertebrate species (Kozomara & Griffiths-Jones, 2011). In humans, more than 1400 miRNAs have been either functionally confirmed or predicted (Jansson & Lund, 2012). It has been estimated that a single miRNA is on average capable of regulating 400 messenger RNA (mRNA) targets, each of which can be regulated by multiple miRNAs (B. P. Lewis et al., 2003). Inferred from this, 60% or more of the human genome is predicted to be under miRNA regulation (Jansson & Lund, 2012). Due to the breadth of genes regulated by miRNAs, miRNAs are accordingly involved in numerous cellular processes such as growth, proliferation, differentiation,
apoptosis, migration, and metabolism (Esquela-Kerscher & Slack, 2006). Thus, miRNAs comprise a profound gene expression regulation mechanism.

1.4.2 MicroRNA Biogenesis and Gene Silencing

miRNA biogenesis requires multiple processing steps in order to yield mature miRNAs that mediate gene silencing (Figure 5). Most miRNA gene loci are located in the introns and exons of both protein-coding and non-coding transcripts (Jansson & Lund, 2012). While less common, recent studies have identified some miRNA loci located in transposable elements and pseudogenes (Devor, 2006; Smalheiser & Torvik, 2005). The miRNA biogenesis pathway begins with transcription of miRNA genes into miRNA primary transcripts (pri-miRNAs) by RNA polymerase II in the nucleus (Rodriguez et al., 2004). Pri-miRNAs are cleaved in the nucleus by the RNase III enzyme, Drosha, and co-factor Di George syndrome critical region 8 (DGCR8) (Y. Lee et al., 2003). The products are shorter stem-loop intermediates termed pre-miRNAs, approximately 70 nucleotides long (Y. Lee et al., 2003). The two nucleotide overhang left by Drosha at the 3’ end of the pre-miRNA stem-loop must be recognized by exportin-5 (XPO5) for pre-miRNA export (Muqbil et al., 2013). Upon recognition of this motif, the pre-miRNAs are exported from the nucleus to the cytoplasm in a guanosine triphosphate (GTP)-dependent manner (Y. Lee et al., 2003). In the cytoplasm, the pre-miRNAs are further processed by Dicer, another RNase III enzyme (Y. Lee et al., 2003). During this step, the pre-miRNAs are cleaved at the base of their stem-loops by Dicer and arms ligated together into 19-25 nucleotides long, double stranded miRNAs (Barrero et al., 2011). These duplex structures are unstable, activation of their gene silencing activity requires maturation to their active forms (X. Yang et al., 2013). While evidence has shown that either strand of the miRNA can be processed into mature miRNA, typically one strand undergoes maturation while the passenger strand undergoes degradation (X. Yang et al., 2013). In some cases, both strands are viable and become unique functional miRNAs that target distinctive mRNA populations (Okamura et al., 2008). The
mature, single stranded miRNAs are known as guide strands and are loaded onto a RNA-induced silencing complex (RISC), containing key proteins such as argonaute (AGO) and scaffold protein GW182 (Jansson & Lund, 2012).

Construction of RISC enables induction of posttranscriptional gene silencing. Although core components are shared between plants and animals, difference lies in complementarity of target recognition. In plants, miRNAs must have near-perfect or perfect pairing with their mRNA targets for tethering (Chen, 2005). However in animals, miRNA hybridization to their mRNA targets can be less stringent (Axtell et al., 2011; Bartel, 2009). Target recognition is conducted through pairing of a 6-8 nucleotides long sequence in the miRNA, termed the seed sequence, to complementary sequence motifs within the 3’ untranslated region (3’ UTR) of a mRNA (Jansson & Lund, 2012). More recently, studies have shown that a new class of miRNAs can also bind the mRNA 5’ UTR (I. Lee et al., 2009). There are two consequences of target recognition: (1) mRNA cleavage and (2) translational repression (Pratt & MacRae, 2009).

Near perfect complementarity between the miRNA and mRNA target promotes mRNA cleavage (Ryan et al., 2015). AGO is central to mRNA cleavage. AGO protein conformation is bilobed, where each lobe binds opposite ends of the guide (Pratt & MacRae, 2009). The carboxy-terminal lobe contains a Mid domain that binds the 5’ end of the guide RNA and the amino-terminal lobe contains a PAZ domain that binds the 3’ end of the guide RNA (Pratt & MacRae, 2009). The carboxy-terminal lobe also contains the PIWI domain (Pratt & MacRae, 2009). The PIWI domain structurally resembles ribonuclease H with a conserved aspartate-aspartate-glutamate active site, implicating AGO as the “slicer” (Song et al., 2004). Of AGO1-4 in humans, only AGO2 possesses cleavage activity (Pratt & MacRae, 2009). The target mRNA is always cleaved between bases 10 and 11 via hydrolysis reaction (Pratt & MacRae, 2009). Studies show that multiple rounds of slicing can occur (Pratt & MacRae, 2009).
Imperfect complementarity between the miRNA and mRNA target promotes translational repression (Ryan et al., 2015). Although the exact mechanisms and circumstances have yet to be determined, preliminary studies have identified two different mechanisms in flies. One mechanism involved competitive blockage of eukaryotic translation initiation factors 4E and 4G (eIF4E, eIF4G) interaction, resulting in impaired ribosome formation and inhibition of translation initiation (Iwasaki et al., 2009). The other mechanism involved GW182 disruption of eIF4G association with poly-A binding protein, resulting in impaired mRNA circularization and translational repression (Zekri et al., 2009).
Figure 5. MicroRNA biogenesis and gene silencing. DNA is transcribed into pri-miRNA by RNA polymerase II and then cleaved by Drosha. The resultant pre-miRNA is exported into the cytoplasm and processed into a miRNA duplex by Dicer. The mature miRNA strand is loaded onto a RNA-induced silencing complex which mediates gene silencing by mRNA degradation or translational repression. Reproduced and modified with permission from O’Kelly et al.; Nature Reviews Urology, 2012 (O’Kelly et al., 2012).
1.4.4 MicroRNAs Implicated in IBD

Given that miRNAs regulate numerous cellular processes, the emerging picture is that miRNAs serve as key players in driving many diseases, including IBD. This is supported by increasing evidence of aberrant miRNA expression in IBD. One study demonstrated differential expression of 5 miRNAs in intestinal biopsies from patients with colon CD and differential expression of 4 miRNAs in those with ileal CD (F. Wu et al., 2010). While none of the 5 colonic CD-associated miRNAs were altered in UC tissues, 2 of the 4 miRNAs differentially expressed in ileal CD were found altered in UC (F. Wu et al., 2008, 2010). In addition to alteration of tissue miRNA profiles in IBD, studies have also shown changes in peripheral blood miRNA profiles. Supporting evidence comes from a study that demonstrated significant increase of 5 miRNAs and decrease of 2 miRNAs in CD patient blood compared to healthy controls (F. Wu et al., 2011). Blood from UC patients revealed significant increase of 12 miRNAs and decrease of 1 miRNA compared to healthy controls (F. Wu et al., 2011). Thus aberrant miRNA expression exists in IBD, and specific miRNAs in intestinal tissue and circulating blood may serve as biomarkers for the diagnosis and prognosis of disease. However it remains unknown how alteration of miRNA levels occurs.

Since dysregulated autophagy is proposed to be a central mechanism of IBD pathogenesis, miRNAs that regulate autophagy genes are of great interest. miR-130a has been shown to reduce autophagy through suppression of ATG2B and DICER1 in leukemia cells (Kovaleva et al., 2012). miR-196 was shown in another study to be elevated in intestinal epithelia of patients with CD (Brest et al., 2011). Experiments demonstrated downregulation of the autophagy associated IRGM gene by miR-196, resulting in compromised autophagy and increased bacterial invasion by AIEC (Brest et al., 2011). In a different study, miR-106b and miR-93 were observed to be capable of targeting ATG16L1, another autophagy gene, causing reduced autophagy and impaired clearance of AIEC in intestinal epithelial cells (Lu
et al., 2014; Zhai et al., 2014). Altogether, more than a dozen miRNAs have been so far identified to regulate autophagic signalling (Zhai et al., 2014). Using miRecords (http://c1.accurascience.com/miRecords/index.php), a database that integrates predicted miRNAs from 11 established bioinformatics target prediction programs, our laboratory has identified 36 miRNAs predicted to target ATG16L1 (Table 1). Of these, miR-142-3p was chosen for further study because of elevated expression in animal colitis models and in IBD patients (Schaefer et al., 2011, 2015).
### Predicted miRNAs targeting ATG16L1

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<td>hsa-miR-96</td>
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<td>hsa-miR-130a</td>
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<td>hsa-miR-106b</td>
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<td>hsa-miR-17</td>
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<td>hsa-miR-519d</td>
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<td>hsa-miR-106a</td>
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<td>hsa-miR-93</td>
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<td>hsa-miR-609</td>
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<td>hsa-miR-502-5p</td>
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**Table 1. miRNAs predicted *in silico* to target ATG16L1.** miRNAs predicted to target the ATG16L1 3’UTR were identified using miRecords (Xiao et al., 2009), an integrated miRNA-target interactions database. Study conducted by Michael Sibony.
1.4.5 miR-142-3p Directly Regulates ATG16L1 and the Autophagy Pathway

Preliminary work from our laboratory investigated how miR-142-3p, predicted in silico to target ATG16L1, affects the autophagy pathway.

To determine whether miR-142-3p targets the 3’ UTR of ATG16L1, a dual luciferase reporter vector was generated containing the 3’ UTR of ATG16L1, and co-transfected into human cervical carcinoma epithelial (HeLa) cells with either a miR-142-3p mimic, miR-142-3p inhibitor, or non-specific miRNA negative control (Figure 6). Analysis using a dual-luciferase reporter assay system on a luminometer demonstrated significantly decreased luciferase activity in cells co-transfected with the miR-142-3p mimic compared to cells with the miRNA negative control, indicating miR-142-3p targets the 3’ UTR of ATG16L1 in vitro, confirming the in silico prediction.

Next, the effects of miR-142-3p on the autophagy pathway were characterized in HeLa cells. HeLa cells were co-transfected with miR-142-3p mimic, miR-142-3p inhibitor, or miRNA negative control. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis showed significantly reduced ATG16L1 mRNA expression in the miR-142-3p mimic cells compared to control (Figure 7A). Furthermore, immunoblot analysis showed significantly reduced ATG16L1 protein expression in the miR-142-3p mimic cells (Figure 7B). Together, these findings indicate that miR-142-3p suppresses ATG16L1 mRNA and protein expression, repressing autophagy.

Expression of the miR-142-3p inhibitor in these experiments resulted in significantly reduced ATG16L1 3’UTR targeting (Figure 6), increased ATG16L1 mRNA expression (Figure 7A), and increased ATG16L1 protein expression (Figure 7B).
These findings show that miR-142-3p function is effectively blocked by specific inhibition.

To determine the functional effect of miR-142-3p in a physiological model of the intestine, intestinal organoids were employed. Intestinal organoids are 3-dimensional miniature cellular constructs of the intestinal epithelium. Upon generation of intestinal organoids from mice, they were transduced with a green fluorescent protein (GFP)-tagged lentivirus expressing miR-142-3p or miRNA scramble control. Successful transduction was confirmed by positive detection of the GFP reporter 2 days post-transduction by confocal microscopy (Figure 8).

RT-qPCR analysis showed significantly increased miR-142-3p expression in the miR-142-3p organoids compared to the control organoids, confirming effective miR-142-3p overexpression (Figure 9). ATG16L1 mRNA expression was significantly decreased in the miR-142-3p organoids compared to control, demonstrating robust cleavage of ATG16L1 mRNA by the miR-142-3p enrichment (Figure 9). Under basal conditions, LC3-II levels in miR-142-3p enriched organoids were reduced compared to scramble control, while ATG16L1 levels were similar. After 17 hours starvation-induced autophagy, the miR-142-3p enriched organoids demonstrated reduced ATG16L1 and LC3-II protein levels compared to scramble control (Figure 10). These results indicate overall reduction of autophagy by miR-142-3p in transduced organoids, whether at baseline or after challenge.

miRNAs has been increasingly recognized as an important mechanism of connecting environmental and genetic risk factors of disease. Due to evidence of altered miRNA expression in IBD and implication in regulation of numerous autophagy genes, miRNAs represent a pathway for new understanding of IBD pathogenesis. Once the precise functions and consequences of miRNAs are established, they may become the focus of novel diagnostics and therapeutics. Preliminary work from our laboratory has shown that miR-142-3p can directly impact
the autophagy pathway through ATG16L1, making this an intriguing area of study in the context of vitamin D deficiency.
Figure 6. miR-142-3p directly targets the ATG16L1 3’ UTR. HeLa cells co-transfected with ATG16L1 3’ UTR and one of miR-142-3p mimic, inhibitor, or miRNA negative control. Firefly luciferase activity was measured and normalized to renilla luciferase activity to assess 3’ UTR targeting. Reduced luciferase activity (increased 3’ UTR targeting) was demonstrated in miR-142-3p cells. Work courtesy of Frances Dang.
Figure 7. miR-142-3p reduces ATG16L1 mRNA and protein expression. HeLa cells co-transfected with ATG16L1 3’ UTR and one of miR-142-3p mimic, inhibitor, or miRNA negative control. (A) miR-142-3p mimic cells showed reduced ATG16L1 mRNA expression by RT-qPCR. (B) miR-142-3p mimic cells showed reduced ATG16L1 protein expression by immunoblotting. Work courtesy of Frances Dang.
Figure 8. Lentiviral transduction of miR-142-3p into intestinal organoids.
Intestinal organoids were lentiviral transduced with a GFP-miR-142-3p or –scramble control expressing construct. Positive GFP detection by confocal microscopy indicated successful transduction. Work courtesy of Inez Verpalen and Ryan Murchie.
Figure 9. miR-142-3p transduced organoids have increased miR-142-3p and decreased ATG16L1 mRNA. miR-142-3p and ATG16L1 mRNA expression in intestinal organoids were assessed by RT-qPCR. miR-142-3p transduced organoids showed miR-142-3p overexpression and decreased ATG16L1 mRNA. * indicates P < 0.05.
Figure 10. miR-142-3p reduces autophagy in intestinal organoids. ATG16L1 and LC3-II protein levels were assessed by immunoblotting under basal and starvation conditions. miR-142-3p transduced organoids showed reduced ATG16L1 and LC3-II protein expression. * indicates P < 0.05. Work courtesy of Inez Verpalen.
1.5 Summary, Hypothesis, and Research Aims

1.5.1 Summary

Inflammatory bowel disease (IBD) is a family of severe autoimmune diseases whose hallmark is chronic, relapsing inflammation of the gastrointestinal tract. Given its lifelong symptoms and lack of a cure, IBD is a devastating disease. Pathogenesis of IBD remains complex and poorly understood. While genetic, microbial, and immunological factors may all be important contributors, dramatically increasing rates of IBD in children indicate the environment as a critical factor in disease pathogenesis. Vitamin D has been implicated as a major environmental risk factor. It is unclear how vitamin D deficiency promotes IBD susceptibility; however evidence implicates dysregulation of autophagy, a highly conserved cellular recycling pathway. Epigenetic regulation through microRNAs (miRNAs) may be a mechanism by which vitamin D deficiency disrupts autophagy. Work from our lab has demonstrated that the miRNA, miR-142-3p, directly targets autophagy. To date, there has been no evidence detailing vitamin D deficiency effect on autophagy in the intestine via miRNA regulation.

1.5.2 Hypothesis

We hypothesize that vitamin D deficiency alters miRNAs that target the autophagy gene ATG16L1, thereby leading to increased susceptibility to IBD.

1.5.3 Research Aims

Aim: Determine the effect of vitamin D deficiency on autophagy in the intestine via miRNA regulation.
Chapter 2
Materials and Methods

2.1 Animals

3 week old female C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All animals were maintained at the University of Toronto Donnelly Centre for Cellular and Biomolecular Research (CCBR) animal facility (Toronto, ON, Canada). Upon arrival, the mice were fed *ad libitum* either vitamin D deficient (VDD) diet (TestDiet AIN-93M/No Vitamin D; Ren’s Pet Depot, Canada) or control (CTL) diet (TestDiet AIN-93M Maintenance; Envigo, Canada) for 5 weeks (Table 2). For the second set of experiments, different VDD diet (Teklad Diet 89123; Envigo, Alconbury, CAM, UK) and CTL diet (Teklad Diet 89124; Envigo, Alconbury, CAM, UK) were used, and feeding duration was 6 weeks (Table 3). Mouse holding rooms at the animal facility were illuminated by overhead fluorescent light fixtures, thus UVB light exposure for epidermal vitamin D synthesis was negligible. At termination of feeding regimen, mice were euthanized, blood collected, and intestinal biopsies taken. Intestinal biopsies consisted of 1.5 inch long samples of terminal colon, 1 inch distal from rectum, and terminal ileum, 1 inch distal from cecum. Tissue samples were rinsed in PBS and sectioned longitudinally for whole tissue collection, epithelial cell isolation, organoid culture generation, and paraffin embedding. Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care and experimental procedures were approved by the University of Toronto Animal Care Committee.

2.2 Enzyme-Linked Immunosorbent Assay

Whole blood was collected from mice in terminal anesthesia via cardiac puncture. Samples were rocked gently for 1 hour at 4 °C then centrifuged at 26.6 x g for 10
minutes at 4 °C. Serum was isolated and stored at -80 °C until analyzed. Enzyme-linked immunosorbent assay (ELISA) (25-OH Vitamin D ELISA Assay Kit; Eagle Biosciences, Nashua, NH, USA) was used to measure serum vitamin D levels. In summary, all reagents of the ELISA kit were first brought to room temperature 30 minutes before use. Next, standards, controls, and animal samples were diluted with 1% biotin-labelled 25-OH vitamin D and added to microplate wells pre-coated with monoclonal antibody 100% specific for both vitamin D2 and vitamin D3. The wells were incubated for 2 hours at room temperature, and then followed by 3 rigorous washes. For detection of bound biotin-labelled vitamin D, peroxidase-labelled streptavidin was added for 30 minutes incubation at room temperature. After 3 rigorous washes, the wells were incubated with peroxidase substrate tetramethylbenzidine (TMB) for 15 minutes at room temperature, stopped with sulphuric acid stop solution. Prior to photometric measurement of optical intensity (OD), the microplate was shaken for 15 seconds for homogeneous distribution of well solutions. Finally, OD was measured at wavelength 450 nm (OD 450) and reference wavelength 650 nm (OD 650) using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.3 Determination of Vitamin D Levels

For determination of vitamin D concentrations in animal samples, OD 450 – 650 was plotted against known vitamin D concentration for the 6 calibration standards using Excel software (version 14.0.7166.5000; Microsoft, Redmond, WA, USA). A curvilinear regression line was plotted through the standard points. The equation given by the trend line was used to interpolate the vitamin D concentrations for the animal samples.
2.4 Isolation of Intestinal Epithelial Cells

Colon and ileum samples isolated from mice were promptly immersed in cell recovery solution (Corning Life Sciences, Tewksbury, MA, USA) overnight at 4 °C. Epithelial cells were released by mechanical shaking in phosphate buffered saline (PBS) and collected, followed by centrifugation at 500 x g for 10 minutes.

After supernatant was aspirated, cell pellets for immunoblotting were resuspended in 300 µL radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM sodium chloride, 1% IGEPAL CA-630 [NP-40], 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing phosphatase inhibitors 50 mM sodium fluoride, 1 mM sodium orthovanadate (Na₃VO₄), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1% protease inhibitor cocktail for 1 hour on ice (all Sigma-Aldrich, St. Louis, MO, USA). Lysates were centrifuged at maximum speed for 5 minutes at 4 °C and the supernatants were collected for storage at -20 °C.

For reverse transcription quantitative polymerase chain reaction (RT-qPCR), cell pellets were resuspended in 300 µL RNA/later stabilization solution (Qiagen, Hilden, NW, Germany) instead of RIPA buffer.

2.5 Culture of Intestinal Organoids

Colon samples isolated from mice were promptly rocked in cold AdvanceSTEM ES qualified Dulbecco’s phosphate buffered saline (DPBS) (HyClone, GE Healthcare, Little Chalfont, BKM, UK) with 5 mM ethylenediaminetetraacetic acid (EDTA) for 1 hour at 4 °C. Following incubation, whole and fragmented intestinal crypts containing leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) stem cells were released by mechanical shaking in cold 1% fetal bovine serum (FBS) (Wisent, Saint-Jean-Baptiste, QC, Canada) in PBS. Light microscopy was used to assess crypt density. Crypts were isolated by centrifugation at 500 x g for 10 minutes at 4 °C and embedded in Matrigel basement membrane matrix (BD Biosciences, Franklin Lakes,
NJ, USA) on 24-well tissue culture plates (25 μL/well). After Matrigel polymerization at 37 °C for 30 minutes, 500 μL of complete intestinal organoid medium was added to each well and incubated at 37 °C and 5% CO₂. Culture medium comprised of advanced Dulbecco’s modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) (Life Technologies, Carlsbad, CA, USA) containing 65% condition Wnt3a-media, 25% conditioned R-spondin-1 (Rspo1)-media, and 10% condition Noggin-media, supplemented with 1% GlutaMAX media (L-alanyl-L-glutamine dipeptide), 10 mM HEPES, 1% N2, 2% B27 (all Life Technologies, Carlsbad, CA, USA), 50 ng/mL EGF (R&D Systems, Minneapolis, MN, USA), 100 ng/mL FGF10 (PeproTech, Rocky Hill, NJ, USA), 1 μM N-acetylcysteine (NAC), 10 μM Y-27632, 10 mM nicotinamide (all Sigma-Aldrich, St. Louis, MO, USA), 1 μM TGFβ-RI inhibitor (A 83-01; Tocris Bioscience, Bristol, BST, UK) and 1% penicillin/streptomycin (Figure 11).

Within 24 hours in medium, the Lgr5⁺ stem cells begin proliferation into 3-dimensional intestinal organoids. Colon organoids had their medium changed every 2-3 days with Wnt3a and Rspo1 supplementation to maintain stemness. Organoids were passaged every 5-6 days according to growth. For passaging, culture medium was aspirated and Matrigel was dissolved in cold DMEM/F-12 media with 10mM HEPES buffer. Released organoids were collected and mechanically dissociated by vigorous pipetting with a flamed glass Pasteur pipette. The sheared organoids were centrifuged at 500 x g for 10 minutes at 4 °C and resuspended in Matrigel on 24-well tissue culture plates, followed by incubation at 37 °C and 5% CO₂. For organoid differentiation, culture medium was exchanged after 4 days culture to complete medium lacking Wnt3a (0% Wnt3a) to induce crypt budding and proliferation. After 24 hours of additional growth in Wnt3a-free medium to achieve sufficient size and complexity, the organoids were harvested for experimentation.

For RT-qPCR, medium was aspirated and replaced with 250 μL Dispase (354235, Corning Life Sciences, Tewksbury, MA, USA) per well. After 1 hour incubation at 37 °C, Matrigel was solubilized and Dispase was inactivated by addition of 5 mM EDTA.
Organoids were centrifuged at 500 x g for 2 minutes at 4°C, washed 3 times with PBS, and lysed in 100 μL RNA later solution with phosphatase and protease inhibitors for 15 minutes on ice. Lysates were centrifuged at maximum speed for 5 minutes at 4 °C and the supernatants were collected for storage at -20 °C.
Figure 11. Culture of intestinal organoids. Intestinal crypts were isolated from mice intestine tissue by EDTA incubation, agitation, and serial dilutions. Next, crypts were centrifuged, embedded in Matrigel on tissue culture plates, incubated in growth media, and grown over 5-6 days. Reproduced and modified with permission from Clevers; Nature, 2013 (Clevers, 2013).
2.6 Determination of Protein Concentration

Bradford assay was used to determine lysate protein concentration for preparation of samples for immunoblotting. In summary, dye reagent (Protein Assay Dye Reagent Concentrate; Bio-Rad Laboratories, Hercules, CA, USA) was added to standards or lysates in polystyrene cuvettes. Cuvettes were incubated for 5 minutes at room temperature. Absorbance was measured at wavelength 595 nm using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.7 Immunoblotting

Lysates were heated at 95°C with 4X Laemmli sample buffer for 3 minutes. Equal amounts of protein from lysates were loaded and separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 135 V for 75 minutes. Proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) at 70 V for 70 minutes. Membranes were blocked with 5% w/v non-fat powdered milk (Bio Basic Canada, Markham, ON, Canada) in tris-buffered saline, pH 7.4, with 0.05% v/v Tween 20 (TBST) for one hour at room temperature with shaking. For protein detection, membranes were incubated overnight with appropriate primary antibodies in 5% w/v milk in TBST overnight at 4°C with shaking. The primary antibodies used were as follows: ATG16L1 rabbit monoclonal antibody (8089, 1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), GAPDH mouse monoclonal antibody (MA5-15738, 1:5000 dilution; Pierce Biotechnology, Rockford, IL, USA), and β-actin mouse monoclonal antibody (A4700, 1:5000 dilution; Sigma-Aldrich, St. Louis, MO, USA). Incubation of anti-ATG16L1 antibody and corresponding secondary antibody was performed in 2.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) in TBST instead of 5% milk in TBST. Membranes were washed 10 minutes with TBST 3 times, and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies.
(1:3000 dilution) in 5% w/v milk in TBST for one hour at room temperature with shaking. Membranes were washed 10 minutes with TBST 3 times. Target proteins were detected by chemiluminescence (Western Blotting Luminol Reagent; Santa Cruz Biotechnologies, Dallas, TX, USA) using an Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE, USA).

2.8 Densitometry Analysis

Immunoblot images were captured and quantitated using Image Studio (version 5.2; LI-COR Biosciences, Lincoln, NE, USA). Densities of target protein bands were measured and expressed as a ratio of protein of interest over loading control (e.g. ATG16L1/GAPDH). Immunoblotting with LC3 antibody generated two bands, representing LC3-I and LC3-II. Only signal density of LC3-II was measured as it correlates with autophagosome levels. For graphic representation, ratio for VDD treatment was expressed relative to the measured ratio for CTL and depicted as fold change.

2.9 RNA Extraction

Total RNA was extracted from the intestinal epithelial cells and organoids using the MagMAX-96 for Microarrays Total RNA Isolation Kit protocol (Life Technologies, Carlsbad, CA, USA). In summary, samples stored in RNALater solution were lysed in TRI Reagent (guanidinium thiocyanate-phenol-based solution) and mixed with isopropanol. After homogenization, the lysates were transferred to a 96-well processing plate and mixed with RNA binding magnetic beads. After incubation on an orbital shaker for 3 minutes, the processing plate was coupled to a magnetic stand to capture the beads. After 3 minute capture time, the supernatant was discarded and the beads were gently washed with shaking 2 times. Finally, the beads were dried and hot elution buffer was used to elute the total RNA. Absorbance was measured at wavelength 260 nm ($A_{260}$) and 280 nm ($A_{280}$) using a
BioPhotometer Plus (Eppendorf, Hamburg, HH, Germany). The RNA concentration was determined using the formula $A_{260} \times 40 = \mu g \ RNA / mL$ while the RNA purity was determined using the $A_{260}/A_{280}$ ratio. RNA samples were converted immediately to complementary DNA (cDNA).

### 2.10 cDNA Synthesis

To prepare template cDNA for TaqMan-based quantitative polymerase chain reaction (qPCR) detection of miR-142-3p, cDNA was synthesized from total RNA using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). In summary, RNA template was combined with master mix containing 100 mM dNTPs with oligo(dT), 50 U reverse transcriptase, 10X buffer with MgCl$_2$, 3.8 U RNase inhibitor, and nuclease-free water, and either reverse transcription miR-142-3p (assay ID 000464; Life Technologies, Carlsbad, CA, USA) or snoRNA202 (assay ID 001232; Life Technologies, Carlsbad, CA, USA) (control gene) primer in 0.2 mL polypropylene reaction tubes on ice. After each reaction was centrifuged, the reverse transcription was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the following thermal profile: 30 minutes priming at 16°C, 30 minutes reverse transcription at 42°C, and 5 minutes reverse transcription inactivation at 85°C, followed by storage at 4°C.

To prepare template cDNA for SYBR Green-based qPCR detection of ATG16L1, cDNA was synthesized from total RNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA, USA) containing dNTPs, oligo(dT), reverse transcriptase, buffer, MgCl$_2$, RNase inhibitor, and random primers. In summary, the RNA template was combined with 5X Supermix and nuclease-free water and centrifuged. The reactions were incubated in the thermal cycler using the following thermal profile: 5 minutes priming at 25°C, 30 minutes reverse transcription at 42°C, and 5 minutes reverse transcription inactivation at 85°C, followed by storage at 4°C.
2.11 Quantitative Polymerase Chain Reaction

qPCR amplification of miR-142-3p was performed by TaqMan probe assay. The cDNA template product from the reverse transcription reaction was combined with 2X SensiFAST Probe Hi-ROX mix (Bioline, London, LND, UK), nuclease-free water, and either 20X miR-142-3p (assay ID 000464; Life Technologies, Carlsbad, CA, USA) or 20X snoRNA202 (assay ID 001232; Life Technologies, Carlsbad, CA, USA) primer on ice. Each qPCR reaction mix was transferred to triplicate wells on a 96-well plate. The plate was sealed with optically clear film, centrifuged at 500 x g for 1 minute, and loaded into a StepOnePlus System (Applied Biosystems, Foster City, CA, USA). The qPCR was performed using the following thermal profile: 1 cycle of polymerase activation for 2 minutes at 95°C, followed by 40 cycles of denaturation for 10 seconds at 95°C and annealing for 20 seconds at 60°C.

qPCR amplification of ATG16L1 was performed by SYBR Green dye assay. The cDNA template product from the reverse transcription reaction was combined with 2X SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA), nuclease-free water, and either 20X ATG16L1 (assay ID qMmuCID0011303; Bio-Rad Laboratories, Hercules, CA, USA) or 20X β-actin (assay ID qMmuCED0027505; Bio-Rad Laboratories, Hercules, CA, USA) (control gene) primer on ice. Each qPCR reaction mix was transferred to triplicate wells on a 96-well plate. The plate was sealed with optically clear film, centrifuged at 500 x g for 1 minute, and loaded into a StepOnePlus System (Applied Biosystems, Foster City, CA, USA). The qPCR was performed using the following thermal profile: 1 cycle of polymerase activation for 2 minutes at 95°C, 40 cycles of denaturation for 5 seconds at 95°C and annealing for 30 seconds at 60°C, followed by a step and hold melt curve stage.
2.12 qPCR Amplification Data Analysis

qPCR amplification data were collected and analyzed using StepOne software (version 2.3; Applied Biosystems, Foster City, CA, USA). For miR-142-3p gene expression analysis, snoRNA202 was used as the control gene. For ATG16L1 gene expression analysis, β-actin was used as the control gene. Gene expression analysis was performed by comparative Ct (ΔΔCt) method since the qPCR efficiencies between the target and control genes were relatively equivalent. For graphic representation, relative quantification (RQ) for each gene of interest was determined using the formula: RQ = 2^{-ΔΔCt}, where ΔΔCt = (Ct_target - Ct_control) VDD - (Ct_target - Ct_control) CTL, and depicted as fold change.

2.13 Statistical Analysis

Statistical analyses were performed using GraphPad Prism (version 6.07; GraphPad Software, San Diego, CA, USA). Results were expressed as mean ± standard error of the mean (SEM). Statistical comparisons were carried out using unpaired two-sample Student’s t-test or two-way ANOVA. A P value (P) of < 0.05 was considered as statistically significant.
Table 2. Formulation of TestDiet® VDD and CTL mouse diets.

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<td>Vitamin Mix, with D (1.0U/g)‡ ‡</td>
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†: Additional ingredients in VDD diet (AIN-93M/No Vitamin D).

‡ ‡: Additional ingredient in CTL diet (AIN-93M Maintenance).
Table 3. Formulation of Teklad® VDD and CTL mouse diets.

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†: Additional ingredients in VDD diet (TD.89123).

‡: Additional ingredient in CTL diet (TD.89124).
Chapter 3

Results

3.1 Vitamin D deficient diet reduces mice serum vitamin D levels

To study the effect of vitamin D deficiency on autophagy via miRNA regulation, C57BL/6 mice were randomized into two groups and either fed TestDiet® vitamin D deficient (VDD) diet or control diet supplemented with vitamin D (CTL) for 5 weeks. After 5 weeks, serum vitamin D levels were measured using an enzyme-linked immunosorbent assay (ELISA). Serum vitamin D levels were significantly lower in the VDD diet fed group compared to the CTL diet fed group (Figure 12A).

This study was replicated with an additional group of C57BL/6 mice fed Teklad® VDD or CTL diets for 6 weeks. However in this group, 2 animals died unnaturally during the 6 weeks feeding duration, and another 3 animals were close to death at time of sacrifice. The sick mice presented signs of severe weight loss, ruffled fur, hunched posture, lethargy, abdominal distension, and diarrhea, suggesting pathogenic infection. These 5 animals were all on VDD diet, suggesting that vitamin D deficiency predisposed the animals to the unknown disease. Of the animals assessed (all except the two animals that died), serum vitamin D levels were significantly lower in the VDD mice compared to CTL mice (Figure 12B), recapitulating previous findings.

Taken together, these results indicate that feeding for at least 5 weeks with either TestDiet® or Teklad® VDD diets was sufficient to generate a vitamin D deficient mouse model. This model was subsequently used to investigate the effects of vitamin D deficiency on autophagy via miRNA regulation.
Figure 12. Vitamin D deficient diet reduces mice serum vitamin D levels. Mice serum vitamin D levels were measured by ELISA. (A) Mice fed TestDiet® VDD diet for 5 weeks had significantly reduced serum vitamin D levels compared to CTL diet mice. (B) Mice fed Teklad® VDD diet for 6 weeks had significantly reduced serum vitamin D levels compared to CTL diet mice. Unpaired t-test was used to determine statistical significance. **** indicates P < 0.0001.
3.2 Vitamin D deficiency does not affect miR-142-3p expression in the colon

Having generated a diet-induced vitamin D deficient mouse model, the effect of vitamin D deficiency on miR-142-3p expression in the intestine was assessed to determine whether vitamin D deficiency alters expression of miRNAs that regulate autophagy. Colonic and ileal whole tissue samples were obtained and total RNA isolated from the animals fed the TestDiet® diet for 5 weeks. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis demonstrated no significant effect of vitamin D deficiency on miR-142-3p expression in colon whole tissue, although there was a trend towards increased miR-142-3p in VDD (Figure 4A). Ileum whole tissue miR-142-3p expression was unchanged by vitamin D status (Figure 13A).

From the 6 weeks Teklad® diet animals, RT-qPCR was performed on total RNA isolated from colon and ileum epithelial cells isolated from whole intestinal tissue. There was no significant effect of vitamin D deficiency on miR-142-3p expression in either colon or ileum epithelial cells, although there was a trend towards increased miR-142-3p in colon (Figure 13B). However, as noted above, two of the vitamin D deficient animals had died and three were very sick at the time of sacrifice.

Although vitamin D deficiency does not affect miR-142-3p expression in the intestine, data indicated a trend towards increased miR-142-3p expression in VDD mice whole colon tissue, and even greater miR-142-3p expression in VDD mice colon epithelial cells.
Figure 13. Vitamin D deficiency does not affect miR-142-3p expression in the colon. Mice intestine miR-142-3p expression was assessed by RT-qPCR. (A) VDD diet did not significantly affect miR-142-3p expression in intestinal whole tissue; however there was a trend towards increased expression in colon. (B) VDD diet did not significantly affect miR-142-3p expression in intestinal epithelial cells; however there was a trend towards increased expression in colon. Unpaired t-test was used to determine statistical significance.
3.3 Vitamin D deficiency does not affect ATG16L1 mRNA expression in the colon

To determine if vitamin D deficiency affects autophagy in the intestine via miR-142-3p regulation, intestine ATG16L1 mRNA expression was first assessed. Colon and ileum epithelial cells from the 5 weeks TestDiet® diet animals were analyzed by RT-qPCR. After normalization to β-actin levels, investigation showed no significant effect of vitamin D deficiency on ATG16L1 mRNA expression in colon or ileum epithelial cells (Figure 14A).

From the 6 weeks Teklad® diet animals, RT-qPCR was performed on total RNA isolated from colon and ileum epithelial cells isolated from whole intestinal tissue. There was no significant effect of vitamin D deficiency on ATG16L1 mRNA expression in either colon or ileum epithelial cells, although there was a trend towards decreased ATG16L1 in VDD colon epithelial cells compared to CTL (Figure 14B). In the ileum epithelial cells, vitamin D status had no effect on ATG16L1 mRNA expression (Figure 14B).

Although vitamin D deficiency does not affect miR-142-3p expression in the intestine, data indicated a trend towards decreased ATG16L1 mRNA expression in VDD mice colon epithelial cells.
Figure 14. Vitamin D deficiency does not affect ATG16L1 mRNA expression in the colon. Mice intestine ATG16L1 mRNA expression was assessed by RT-qPCR. (A) VDD diet did not significantly affect ATG16L1 expression in intestinal whole tissue. (B) VDD diet did not significantly affect ATG16L1 expression in intestinal epithelial cells; however there was a trend towards decreased expression in colon. Unpaired t-test was used to determine statistical significance.
3.4 Vitamin D deficiency promotes reduction of ATG16L1 protein expression in the colon

Intestine ATG16L1 protein expression was assessed next. Colon epithelial cells from the 5 weeks TestDiet® diet animals were lysed and proteins separated by SDS-PAGE. After normalizing to GAPDH values, immunoblotting demonstrated significant reduction of ATG16L1 levels by vitamin D deficiency in colon epithelial cells (Figure 15A).

Similar procedure was conducted for the 6 weeks Teklad® diet animals; however ileum epithelial cells were also included for study. In contrast to findings from the TestDiet® animals, no change in colon ATG16L1 protein expression (normalized to β-actin values) was observed in response to vitamin D deficiency (Figure 15B). Vitamin D deficiency also showed no effect on ATG16L1 levels in the ileum (Figure 15B).

Taken together, results from the TestDiet® diet animal study suggest that vitamin D deficiency promotes reduction of ATG16L1 protein expression in the colon, disrupting autophagy. However this was not reconciled by results from the Teklad® diet animal study, which showed no effect of vitamin D deficiency on ATG16L1 protein expression. However, as described above, two of the animals died and three of the remaining animals developed a significant illness.
A

**5 weeks TestDiet® diet, Epithelial Cells**

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67 kDa

38 kDa
Figure 15. Vitamin D deficiency promotes reduction of ATG16L1 protein expression in the colon. Mice intestine ATG16L1 protein expression was assessed by immunoblotting. (A) ATG16L1 protein expression was significantly reduced in VDD colon epithelial cells. (B) Vitamin D deficiency had no effect on ATG16L1 protein expression in colon and ileum epithelial cells. Unpaired t-test was used to determine statistical significance. ** indicates P < 0.01.
3.5 Vitamin D deficiency does not affect miR-142-3p and ATG16L1 mRNA expression in murine-derived intestinal organoids

To characterize the influence of vitamin D deficiency on miR-142-3p and autophagy in a physiological model of the intestine, an intestinal organoid model was used. Intestinal crypts containing multipotent Lgr5\(^+\) stem cells were isolated from the Teklad® diet mice after sacrifice and cultured into organoids that recapitulated the physiological intestinal epithelium. For experiments, organoids were harvested before first passage on day 5 (P0) and after three passages on day 21 (P3), permitting analysis of miR-142-3p and ATG16L1 mRNA levels over time.

Using RT-qPCR, there was no significant treatment effect of vitamin D depletion on expression of miR-142-3p in colon intestinal organoids, although there was a trend towards increased expression in VDD (Figure 16A). There was no significant treatment effect of vitamin D depletion on expression of ATG16L1 in colon intestinal organoids (Figure 16B).

Taken together, observations indicate that vitamin D deficiency does not affect miR-142-3p and ATG16L1 mRNA expression in murine-derived intestinal organoids.
Figure 16. Vitamin D deficiency does not affect miR-142-3p and ATG16L1 mRNA expression in murine-derived intestinal organoids. Following generation and culture of mice colon organoids, miR-142-3p and ATG16L1 mRNA expression were assessed by RT-qPCR. (A) VDD diet did not significantly affect miR-142-3p expression; however there was a trend towards increased expression at P0 and P3. (B) VDD diet did not significantly affect ATG16L1 expression; however there was a trend towards increased expression at P0 and no change at P3. Two-way ANOVA was used to determine statistical significance.
Chapter 4
Discussion

4.1 Limitations

Several limitations were encountered during the experiments conducted in this thesis. First, due to challenges obtaining TestDiet® vitamin D deficient diet in a timely fashion, two different companies’ diets were used: TestDiet® for the initial experiments and Teklad® for the replicate experiments. While mice vitamin D levels fed TestDiet® or Teklad® diet were comparable, the diets differ in ingredient and macronutrient composition. This is of concern because the composition differences could have varying effects on the vitamin D-miRNA-ATG16L1 pathway being investigated, creating a confounding variable. Furthermore, whole tissue was assessed for mir-142-3p expression in the TestDiet® diet fed mice, while isolated epithelial cells were assessed in the Teklad® diet fed mice. Second, the Teklad® feeding was conducted for 1 week more than the TestDiet® feeding because of a timing conflict with a conference. Third, in the Teklad® mice cohort, 2 animals died unnaturally during the 6 weeks feeding duration, and another 3 animals were close to death at time of sacrifice. The sick mice presented signs of severe weight loss, ruffled fur, hunched posture, lethargy, abdominal distension, and diarrhea. These 5 animals were all on VDD diet, suggesting that vitamin D deficiency exacerbated symptoms. In addition, since 2 VDD mice were lost to premature death, this affected VDD sample size, decreasing statistical power. Fourth, the organoid studies were limited in sample size and organoid yield, dampening statistical power. Fifth and lastly, the intestinal organoid model may have exhibited cell heterogeneity across passages; organoid passaging may have inadvertently selected for propagation of a subset of intestinal cells. Due to these limitations, data from the Teklad® and organoid experiments must be interpreted with caution.
4.2 Vitamin D deficient diet reduces mice serum vitamin D levels

To generate a vitamin D deficient animal model, mice were fed for 5 weeks on TestDiet® VDD diet for the first round of experiments in accordance with protocols in previous literature (Assa et al., 2014, 2015). In the TestDiet® diet cohort, serum vitamin D levels were 0.53-fold lower in VDD-fed mice (26.0 ± 1.5 ng/mL [n = 23]) compared to CTL-fed mice (55.3 ± 1.9 ng/mL [n = 22], P < 0.0001) (Figure 12A). These values were consistent with those found from published data from other groups (Assa et al., 2014, 2015). For the replicate round of experiments, mice were fed 6 weeks on Teklad® VDD diet. In the Teklad® diet cohort, serum vitamin D levels were 0.58-fold lower in the VDD group (27.1 ± 3.0 ng/mL [n = 8]) compared to the CTL group ((64.1 ± 3.1 ng/mL [n = 10], P < 0.0001) (Figure 12B). Comparing the TestDiet® and Teklad® diet cohorts, VDD mice in both cohorts had almost identical mean vitamin D levels, whereas the TestDiet® CTL mice had 0.14-fold lower vitamin D levels compared to the Teklad® CTL mice. Thus, the VDD mice were validated to be vitamin D deficient compared to CTL controls.

4.3 Vitamin D deficiency does not affect miR-142-3p expression in the colon

Having generated a vitamin D deficient mouse model, effect of vitamin D deficiency on miR-142-3p expression in the intestine was assessed. Although not statistically significant, in the TestDiet® diet cohort, there was a trend towards increased miR-142-3p expression in VDD mice colon whole tissue (1.293 ± 0.135 fold change [n = 22]) compared to CTL mice colon whole tissue (1.000 ± 0.096 fold change [n = 21]) (Figure 13A). Ileum whole tissue miR-142-3p expression was identical for both groups (1.015 ± 0.166 fold change [n = 22] in VDD mice versus 1.000 ± 0.154 fold change [n = 21] in CTL mice) (Figure 13A).
In the Teklad® diet cohort, there was a non-significant trend towards increased miR-142-3p expression in VDD mice colon epithelial cells (1.449 ± 0.124 fold change [n=8]) compared to CTL mice colon epithelial cells (1.000 ± 0.201 fold change [n = 10]) (Figure 13B). Expression of miR-142-3p in the ileum epithelial cells was similar for VDD and CTL (0.974 ± 0.118 fold change [n = 8] versus 1.000 ± 0.163 fold change [n = 10], respectively) (Figure 13B). However, as two of the VDD mice died and three were significantly sick at the time of sacrifice, these results must be interpreted with caution.

Since data indicated a trend towards increased miR-142-3p expression in VDD colon and no effect in the ileum, this suggests that vitamin D signalling of miR-142-3p takes place predominantly in the colon. In addition, greater upregulation of miR-142-3p expression was observed in colon epithelial cells (1.449-fold increase) compared to whole tissue (1.293-fold increase), which suggests that vitamin D signalling of miR-142-3p may be primarily mediated in colon epithelial cells. There are a couple of possible explanations for the lack of alteration of miR-142-3p by vitamin D deficiency. First, miR-142-3p may not be a true target of vitamin D signalling. Second, there may be other miRNAs involved and are altered by vitamin D deficiency to a greater extent. Second, a prolonged time for vitamin D deficiency may be needed to observe alteration of miR-142-3p expression.

4.4 Vitamin D deficiency does not affect ATG16L1 mRNA expression in the colon

In response to miR-142-3p upregulation, intestine ATG16L1 mRNA expression was assessed. In the TestDiet® diet cohort, vitamin D deficiency had no impact on ATG16L1 expression in colon whole tissue (0.937 ± 0.105 fold change [n = 22] in VDD mice versus 1.000 ± 0.067 fold change [n = 21] in CTL mice) (Figure 14A). ATG16L1 expression in ileum whole tissue was also unaffected (1.206 ± 0.132 fold
change [n = 22] in VDD mice versus 1.000 ± 0.133 fold change [n = 19] in CTL mice) (Figure 14A).

Although not statistically significant, there was a trend towards decreased ATG16L1 mRNA expression in VDD mice colon epithelial cells (0.775 ± 0.138 fold change [n = 8]) compared to CTL mice colon epithelial cells (1.000 ± 0.154 fold change [n = 10]) (Figure 14B). Ileum ATG16L1 mRNA expression was unaffected by vitamin D deficiency (1.099 ± 0.182 fold change [n = 8] in VDD mice versus 1.000 ± 0.157 fold change [n = 10] in CTL mice) (Figure 14B).

Thus, data suggest that vitamin D deficiency does not affect ATG16L1 mRNA expression. Since miRNAs can also act by translation inhibition in addition to mRNA cleavage, this may account for the no effect on ATG16L1 mRNA by miR-142-3p in vitamin D deficiency.

4.5 Vitamin D deficiency promotes reduction of ATG16L1 protein expression in the colon

Intestine ATG16L1 protein expression was assessed to investigate potential translation inhibition by increased miR-142-3p in vitamin D deficiency. Analysis of the TestDiet® diet cohort demonstrated 0.55-fold reduction of ATG16L1 levels in VDD mice colon epithelial cells (0.452 ± 0.065 fold change [n = 17]) compared to CTL mice colon epithelial cells (1.000 ± 0.177 fold change [n = 18], P < 0.01) (Figure 15A).

However in the Teklad® diet cohort, vitamin D deficiency had no effect on colon ATG16L1 protein expression (1.005 ± 0.067 fold change [n = 8] in VDD mice versus 1.000 ± 0.090 fold change [n = 10] in CTL mice) (Figure 15B) nor in ileum (1.055 ± 0.186 fold change [n = 7] in VDD mice versus 1.000 ± 0.314 fold change [n = 10] in CTL mice) (Figure 15B).
Due to the limitations discussed earlier, it is difficult to make a conclusion from the Teklad® diet cohort. From the TestDiet® diet cohort however, data suggest that ATG16L1 protein expression in colon epithelial cells is repressed by ATG16L1 mRNA cleavage and translation inhibition, a consequence mediated by miR-142-3p elevation in vitamin D deficiency.

4.6 Vitamin D deficiency does not affect miR-142-3p and ATG16L1 mRNA expression in murine-derived intestinal organoids

Finally, murine-derived intestinal organoids were used to evaluate the effects of vitamin D deficiency on miR-142-3p and ATG16L1 mRNA expression in a physiological model. Analysis showed no significant treatment effect of vitamin D deficiency on miR-142-3p expression (F (1, 3) = 4.580, P = 0.1218). There were non-statistically significant trends towards increased miR-142-3p in the VDD mice colon organoids compared to CTL mice colon organoids at P0 (1.677 ± 0.104 fold change [n = 2] versus 1.000 ± 0.416 fold change [n = 3], respectively) and at P3 (0.125 ± 0.070 fold change [n = 2] in VDD mice organoids versus 0.060 ± 0.033 fold change [n = 3] in CTL mice organoids) (Figure 16A).

Analysis showed no significant treatment effect of vitamin D deficiency on ATG16L1 mRNA expression (F (1, 2) = 0.2021, P = 0.6971. Non-statistically significant trends towards increased ATG16L1 mRNA expression were observed in the VDD mice colon organoids compared to CTL mice colon organoids at P0 (1.558 ± 0.267 fold change [n = 2] versus 1.000 ± 0.048 fold change [n = 3], respectively) and comparable ATG16L1 mRNA expression at P3 (0.3.742 ± 0.149 fold change [n = 2] in VDD mice organoids versus 3.944 ± 1.054 fold change [n = 2] in CTL mice organoids) (Figure 16B). Due to major limitations of the organoid studies outlined
previously, replication of these experiments with an expanded sample size is warranted to draw a conclusion.
Chapter 5
Conclusions

IBD is a family of severe gastrointestinal diseases with lifelong symptoms and significant burden on patient quality of life. There is no cure and treatment is limited. Canada has one of the highest rates of IBD in the world, with more than 1 in every 150 Canadians affected. Rates of IBD have been increasing across all ages over the past decade, however most dramatically in children - emphasizing the emergence of environmental factors in disease pathogenesis. In light of the wide-spanning burden of IBD, vast efforts are ongoing to understand disease causality and pathogenesis.

Interplay of genetics, microbiome, immune response, and the environment is suspected to give rise to IBD. While numerous genetic polymorphisms have been linked to IBD, genetic-environmental interactions remains poorly characterized. Elucidation of these interactions is important to drive advances in disease prevention and patient therapy. Among several environmental risk factors implicated in IBD, a growing body of animal and human studies has highlighted vitamin D deficiency. Due to higher occurrence of vitamin D deficiency in Canada than in southern latitude countries largely due to lack of sunlight exposure, this provides additional rationale for studying the role of vitamin D. Currently, the mechanism by which vitamin D deficiency promotes disease is unclear. However evidence consistently implicates dysregulation of autophagy, a key cellular homeostasis pathway, as a potential mechanism.

Thus, we sought to explore the effects of vitamin D deficiency on autophagy, with a focus on epigenetics as a bridge between environmental and genetic risk factors. We are just beginning to understand the potential role of miRNAs in disease; scientists have already linked aberrant miRNA expression to IBD. Several studies have associated the miRNA, miR-142-3p, with increased susceptibility to colitis in animal colitis models and in IBD patients. In our laboratory, we have shown that
miR-142-3p directly targets ATG16L1, a critical autophagy protein, *in vitro*. Dysregulation of ATG16L1 has been linked by numerous studies to disruption of autophagy and increased inflammatory response, contributing to development of IBD. Thus in this thesis, we investigated our hypothesis that vitamin D deficiency alters miRNAs that target the autophagy gene ATG16L1, causing increased susceptibility to IBD.

To our knowledge, ours is the first investigation of effect of vitamin D deficiency on autophagy in the intestine via miRNA regulation. Our results showed no effect of vitamin D deficiency on miR-142-3p expression in the intestine of a mouse model. However downstream, ATG16L1 protein expression was significantly reduced. Data showed no alteration of ATG16L1 mRNA expression, indicating translational inhibition of ATG16L1 in vitamin D deficiency. We also demonstrated no effect of vitamin D deficiency on miR-142-3p and ATG16L1 mRNA expression in an intestinal organoid model over time. Herein we propose a potential mechanism by which the environment may disrupt autophagy. In this model, we propose that vitamin D deficiency represses ATG16L1 protein expression, possibly through miR-142-3p regulation, leading to increased susceptibility to IBD (Figure 17). Because of multiple limitations encountered in our experiments for this thesis and the promising data obtained so far, we will pursue further study of this intriguing pathway, which will deepen our understanding of the complex genetic-environmental interactions in the pathogenesis of IBD.
Figure 17. Proposed mechanism of vitamin D deficiency induced dysregulation of autophagy in IBD. Vitamin D deficiency suppresses ATG16L1 protein expression, possibly through miR-142-3p regulation, causing autophagy dysregulation leading to increased susceptibility to IBD.
Chapter 6

Future Directions

Based on our findings, we propose that vitamin D deficiency suppresses ATG16L1 protein expression causing increased susceptibility to IBD. However having encountered several limitations of the experiments conducted in this thesis, replication of these experiments is warranted to confirm our findings. We will replicate using TestDiet® diet, which produced promising data on miR-142-3p and ATG16L1 expression, and increase mice/organoids sample size to increase statistical power. In addition we will focus analysis on isolated epithelial cells versus whole tissue. With these changes and based on our preliminary data, we expect to observe statistically significant miR-142-3p increase in the colon epithelium, ATG16L1 mRNA decrease, and ATG16L1 protein decrease. We will also assess autophagy in paraffin-embedded intestinal tissue by immunohistochemistry for ATG16L1 and LC3, whose methodology we are currently optimizing. As well, we will determine the effect of miR-142-3p in VDD organoids by immunoblotting for ATG16L1 protein. These studies will delineate the effect of vitamin D deficiency on the autophagy pathway via miR-142-3p regulation. If we do not identify change in miR-142-3p expression despite reduction in ATG16L1, then we will assess expression of other miRs using NanoString technology as we have done previously. These studies will characterize the role of miRs, which we predict to target ATG16L1, on the autophagy pathway in vitamin D deficiency.

In addition to studying vitamin D deficiency induced by diet, we are interested in investigating vitamin D deficiency by VDR. Thus we wish to compare two models: WT C57BL/6 mice employed in this thesis and mice with intestine epithelial VDR KO. We will feed mice VDD or CTL TestDiet® diet for 5 weeks and then sacrifice with isolation of the colon and ileum tissues. RT-qPCR, immunoblotting, and immunohistochemistry will be used to investigate effects on miR-142-3p, ATG16L1,
and autophagy. We expect the VDR KO mice fed VDD diet to show the most
dramatic biological changes because of depleted vitamin D signalling compared to
VDD diet mice with functional VDRs. These studies will compare the effects of
vitamin D deficiency induced by diet or VDR KO on autophagy in the intestine via
miR-142-3p regulation.

We also wish to investigate vitamin D intervention. Current literature has shown
mixed results for vitamin D intervention in IBD patients, suggesting that vitamin D
intervention alone is probably not sufficient for suppressing disease symptoms
(Grunbaum et al., 2013; Jørgensen et al., 2010). Thus to investigate vitamin D
intervention, we will treat diet-induced VDD mice with different dosages of vitamin D
and assess effects on miR-142-3p, ATG16L1, and autophagy, and compare against
data obtained from VDD and CTL mice. This could also be extended to organoid
studies, with vitamin D supplementation of medium for organoids derived from VDD
mice. Based on findings from our thesis, we expect that vitamin D treatment would
decrease miR-142-3p expression, increase ATG16L1 expression, and reverse
autophagy disruption. These studies will assess the therapeutic potential of vitamin
D intervention on miR-142-3p levels and intestinal autophagy in mice with vitamin D
deficiency.

Another future direction is to assess the effect of vitamin D deficiency on autophagy
regulation in the context of NOD2 mutation, a genetic risk factor strongly associated
with CD (Economou et al., 2004). As mentioned earlier, evidences suggest
convergence of NOD2 and ATG16L1 in susceptibility to IBD (Travassos et al.,
2010). We are interested in characterizing the effect of vitamin D deficiency on
ATG16L1 in NOD2 mutated mice, which has been so far uncharacterized. We will
feed WT littermate control mice and NOD2 frameshift mutation (NOD2fs) mice either
VDD or CTL diet for 5 weeks and then sacrifice for isolation of the colon and ileum
tissues. RT-qPCR, immunoblotting, and immunohistochemistry will be used to
investigate effects on miR-142-3p, ATG16L1, and autophagy. We will also generate
intestinal organoids from the NOD2fs mice and assess miR-142-3p, ATG16L1, and autophagy compared to controls. Since autophagy is impaired in NOD2 mutation alone without influence of vitamin D deficiency, we expect exacerbated autophagy dysregulation and more advanced colitis in VDD diet NOD2fs mice compared to NOD2fs mice fed CTL diet. These studies will characterize the interplay between vitamin D deficiency and NOD2fs on autophagy in the intestine.

Since our findings indicate regulation of ATG16L1 and autophagy by miR-142-3p, we are interested in employing anti-miR-142-3p oligonucleotides (antagomirs). Compelling animal and human studies demonstrated therapeutic usefulness of antagomirs in a wide range of applications, including treating hepatitis C virus, cardiac remodelling, chronic inflammatory diseases, fibrosis, cardiac regeneration, angiogenesis, and glucose metabolism (van Rooij et al., 2012). In particular, miR-155 antagonism in murine macrophages has been shown to suppress granulocyte colony-stimulating factor expression, suggesting potential for miRs in alleviating chronic inflammatory diseases (Worm et al., 2009). For our proposed studies, we will feed WT and NOD2fs mice VDD or CTL diet as described previously, with additional delivery of miR-142-3p antagomirs via daily intraperitoneal injection. After 5 weeks, mice will be sacrificed and intestinal tissues isolated. RT-qPCR, immunoblotting, and immunohistochemistry will be used to investigate effects on miR-142-3p, ATG16L1, and autophagy. We expect antagomir inhibition of miR-142-3p would reduce miR-142-3p, increase ATG16L1 expression, and rescue autophagy in VDD WT mice, reducing their susceptibility to colitis. We expect similar rescue of phenotype in VDD NOD2 mice as well. These studies will determine the therapeutic potential of inhibition of miR-142-3p on intestinal autophagy in WT and NOD2fs mice under conditions of vitamin D deficiency.

Finally, we wish to translate our studies to clinical applications. In collaboration with Dr. Anne Griffiths and Dr. Daniel Roth, we have been collecting samples from paediatric patients 2-17 years old who are enrolled in the SickKids IBD Program. We
will investigate the relationship of vitamin D, miR-142-3p and ATG16L1 in samples from newly diagnosed patients using many of the same techniques employed in this thesis: ELISA analysis of serum vitamin D levels, RT-qPCR analysis of intestinal miR-142-3p and ATG16L1 levels, and immunohistochemistry analysis of intestinal ATG16L1 and LC3-II. Since we observed a trend towards vitamin D deficiency-induced increase of miR-142-3p and decrease of ATG16L1 in our mice studies, we anticipate similar increased miR-142-3p and decreased ATG16L1 and autophagy in correlation with reduced vitamin D, along with more severe symptoms. These studies will delineate the impact of vitamin D status on miR-142-3p and autophagy in paediatric patients with IBD.
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