Drug Response And Metabolism In Crohn's Disease

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Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Abstract

Inflammatory bowel disease (IBD) is an illness of chronic intestinal inflammation comprised of Crohn's disease (CD) and ulcerative colitis (UC). Specialists rely heavily on drugs that target a dysregulated immune system. There is a staggering degree of variation in drug response in CD. Our understanding of drug metabolism and response in IBD is limited. Gaining new insights into IBD-specific modifications of drug metabolism may allow for improved drug efficacy and reduced toxicity.

Cytochrome P450 (CYP) 3A4 is the most relevant determinant of drug metabolism and exposure for medications prescribed today. CYP3A4 is highly expressed in the liver, but is also important to intestinal drug metabolism. Little is known about CYP3A4 activity in disease states. We tested the hypothesis that CD affects the activity, expression and regulation of CYP3A4.

Acute, non-hepatic inflammatory states are reported to reduce hepatic CYP3A4 activity. Using midazolam pharmacokinetics and the cholesterol metabolite, 4β-hydroxycholesterol as in vivo probes of CYP3A4 activity, we were able to demonstrate and confirm that CYP3A4 activity is lower in CD. Conversely, we were unable to show, using in vitro modeling, that differences in CYP3A4 activity were due to differential nuclear receptor-signaling in CD.

CYP3A4 plays a key role in hepatic and intestinal first-pass metabolism, likely in concert with the xenobiotic exporter, P-glycoprotein (P-gp). The intestinal and colonic
expression of CYP3A4 in CD has not been well characterized. Using an immunobloting technique, we were able to demonstrate that the intestinal and colonic expression of CYP3A4 are reduced in CD.

Lastly, nuclear receptors such as FXR and PXR are important regulators of CYP3A4. Both are down-regulated in IBD. This may have important consequences for drug response in IBD. We confirm that a novel single nucleotide polymorphism in FXR results in a reduction in its downstream products in vivo and reveal a link between genetic variation in FXR and outcomes of CD severity, such as risk and time to surgery, particularly relevant to women affected by CD.

Ultimately, these studies demonstrate the impact of CD on drug metabolism pathways and offer insight into the overlap between CD pathogenesis and drug metabolism.

Keywords

Crohn's disease, inflammatory bowel disease, drug metabolism, nuclear receptor, farnesoid X receptor, pregnane X receptor, cytochrome P450 3A4, pharmacokinetics, single nucleotide polymorphism; biomarker
Co-Authorship Statement

Chapter 1:


AW, RGT, SW, MKD, RAH, and RBK were involved in patient selection and data collection. AW, WAT, and RBK contributed to the research design. AW, BLM and WAT carried out TMAO level analysis and FMO3 genotyping. YC and AW contributed to the statistical analysis of the data. AW wrote the final paper with assistance from WAT, YC, and RBK.


AW wrote the paper with assistance from CM and RBK.

Chapter 3:

Wilson A, Tirona R, Kim RB. CYP3A4 is markedly lower in patients with Crohn's disease. Inflammatory Bowel Diseases 2017; 23(5); 804-813.

AW was involved in patient selection and data collection. AW, RGT, and RBK contributed to the research design. AW and RGT carried out budesonide, midazolam, and fexofenadine plasma concentration analysis. AW performed the statistical analysis of the data. AW wrote the final paper with assistance from RBK. All authors had full access to all the data. All authors reviewed and approved the final version of this manuscript.
Chapter 4:

Wilson A, Urquhart B, Ponich T, Chande N, Gregor JC, Beaton M, Kim RB. The negative effect of Crohn's disease on intestinal first-pass metabolism. Drug Metabolism and Disposition 2018

AW, BU, TP, NC, JCG, and MB were involved in patient selection and data collection. AW and RBK contributed to the research design. AW performed all immunoblot analyses. AW performed the statistical analysis of the data. AW wrote the final paper with assistance from RBK. All authors had full access to all the data. All authors reviewed and approved the final version of this manuscript.

Chapter 5:


RBK supervised the study. AW was involved in data acquisition. AW, WAT and RBK contributed to the study concept and design. AA, LJ and AW carried out all sample analyses. RBK, AW and WAT were involved in data interpretation. Statistical analyses were performed by AW and YC. AW drafted the manuscript. Critical revisions were carried out by RBK, WAT. All authors had full access to all the data. All authors reviewed and approved the final version of this manuscript.
Dedication

To my husband Jon and my mother Susan. And mostly gratefully to my children Ava and Harry, who inspire me to lead by example.
Acknowledgments

I never thought I would be someone who completed a PhD; certainly not a PhD in a basic science. At my first advisory committee meeting, when Dr. Michael Rieder suggested I think about making "a go" at a PhD instead of a Master's degree, I felt giddy. Not the I'm-so-happy-you-see-how-much-work-I've-done-giddy, but the giddiness of having something so seemingly implausible suggested that I could hardly imagine it being a reality. And now, here I am, on the other side of that "feeling": that implausible, almost impossible-to-imagine sensation of having completed my PhD thesis. It's funny to say that one of the things I have learned most in this time is that a PhD takes a lot of hard work. And not just on the part of the candidate. My supervisor, my mentors, my colleagues and most importantly my family have worked, contributed and invested in this goal right along side of me. Some in ways that can be seen and are tangible: my approach to experiments, the analysis of my data, the description of my findings. Others have contributed in ways, unseen, but deeply felt: the provision of time, the sacrifice of time, the listening ear, the helping hand and the wise words. In the end, beyond the results and the conclusions and the many revisions, I am left with a time capsule of the last 5 years of my life. There is something uniquely privileged about being able to reflect back on what you’ve done and to say "look what I have learned" and to have it in hard copy. I am humbled by this experience.

I would like to use this moment to deeply thank my supervisor and mentor, Dr. Richard Kim. He has invested in my growth as a researcher and provided the platform to achieve my goals. His drive for success and to make a difference in the provision of health care have shaped my outlook on research and make me truly want to make a meaningful contribution to our understanding of inflammatory bowel diseases. I would like to make special mention of my Advisory Committee: Drs. Rommel Tirona, Ute Schwarz and Michael Rieder for their insights, feedback and support through this process. To the Richard Kim Personalized Medicine Laboratory members past and present, I am wholly grateful. Wendy Teft, Crystal Schmerk, Cameron Ross, Sara Lemay-Gallien, Heidi Liao, Laura Russell, Ahmed Almousa, Cheynne McLean, Markus Gulilat, Adrienne Borrie, Michelle Kim, Mandy Li, Laura Jansen, Sarah Woolsey, and Michael Knauer have all generously shared their knowledge, time and advice in a way that made this an experience to remember. Through all of my training, I have never had such a sense of community, support and friendship. In particular, I would like to recognize Drs. Wendy Teft and Crystal Schmerk, two strong women who have become great friends and know much more about basic and translational science than I and were kind enough to make time, on an almost-daily basis, to help me. Additionally, Dr. Yun-hee Choi and Rhiannon Rose generously contributed to data analyses for many projects.
I owe a big "thank you" to the division members of Western University Gastroenterology who have supported me from trainee to clinician to scientist. In particular, to Dr. Jamie Gregor who has been involved in more than a decade of my training, who always asks questions I do not know the answer to, and greatly contributed to my interest in pursuing research. In addition, Dr. Alan Thomson is a mentor who challenges my thinking and inspires me to ask tough questions about myself, what I want and how I'm going to get there. My colleague, mentor, role model and friend, Dr. Melanie Beaton has provided invaluable support over many years. Whenever I'm struggling with what to do or am feeling overwhelmed, I think "What would Melanie do?"

In addition, I am grateful to my patients and the patients of others who have kindly participated in my studies and donated their time. Their generosity is recognized and very much appreciated.

Lastly, I would like to acknowledge the contribution of my family. My mother Susan has been my ultimate mentor. She has inspired me by example and has provided invaluable guidance. My husband, Jon, has been an invaluable partner who has lived every moment of this experience with me. His confidence and approach to life have motivated me to move outside my comfort zone. Most of all, I want to recognize my children Ava and Harrison. As I see them learning, I've gained a new appreciation for learning. The transformative thing for me about having children has been the opportunity to look at myself in a new light: to see myself as my children see me - as a person of great worth, beyond what I have, what I know or who I know. The work herein and the things I have learned are part of what I want to give back to my children: my dedication to a goal, my commitment to a cause, and my refusal to sell myself short (even if it's something that seems ridiculous, like Aze Wilson completing a basic science PhD).

~ Aze Wilson, 2018 ~
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<tr>
<td>ABC</td>
<td>ATP-binding cassette protein</td>
</tr>
<tr>
<td>ADR</td>
<td>Adverse drug reaction</td>
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<tr>
<td>ANCA</td>
<td>antineutrophil cytoplasmic antibodies</td>
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<tr>
<td>APC</td>
<td>antigen-presenting cell</td>
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<tr>
<td>5-ASA</td>
<td>5-aminosalicylates</td>
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<td>ASCA</td>
<td>antibodies against <em>Saccharomyces cerevisiae</em></td>
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<td>ATG16L1</td>
<td>Autophagy-related protein 16-1</td>
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<td>ASBT</td>
<td>apical sodium-bile acid transporter</td>
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<td>AUC</td>
<td>area under the concentration-time curve</td>
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<td>AZA</td>
<td>azathioprine</td>
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<td>BSEP</td>
<td>bile salt export pump</td>
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<td>CA</td>
<td>cholic acid</td>
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<td>CAR</td>
<td>constitutive androstane receptor</td>
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<td>CARD9</td>
<td>Caspase recruitment domain-containing protein 9</td>
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<td>CD</td>
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<td>CDAI</td>
<td>Crohn's disease Activity Index</td>
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<td>CDCA</td>
<td>chenodeoxycholic acid</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>CKD</td>
<td>chronic kidney disease</td>
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<td>Abbreviation</td>
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<tr>
<td>CL/F</td>
<td>oral drug clearance</td>
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<td>CL&lt;sub&gt;s&lt;/sub&gt;</td>
<td>systemic clearance</td>
</tr>
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<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum plasma drug concentration</td>
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<td>CRP</td>
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<td>cytochrome P450</td>
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<td>ELISA</td>
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<td>erythrocyte sedimentation rate</td>
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<td>Fc Gamma Receptor 2A</td>
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<td>FGF</td>
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<tr>
<td>FMO</td>
<td>FAD-containing mono-oxygenase</td>
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<td>FXR</td>
<td>farnesoid X receptor</td>
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<td>GCA</td>
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<td>interferon-γ</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>ke</td>
<td>terminal elimination rate constant</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LCA</td>
<td>lithocholic acid</td>
</tr>
<tr>
<td>LHSC</td>
<td>London Health Sciences Centre</td>
</tr>
<tr>
<td>Ln</td>
<td>natural log</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MAAdCAM-1</td>
<td>mucosal addressin cell adhesion molecule-1</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>Mcp</td>
<td>macrophage attractant protein</td>
</tr>
<tr>
<td>MDR1</td>
<td>multi-drug resistance protein 1</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MRP</td>
<td>multidrug resistance protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAFLD</td>
<td>non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NOD2</td>
<td>Nucleotide-binding oligomerization domain-containing protein 2</td>
</tr>
<tr>
<td>NR</td>
<td>nuclear receptor</td>
</tr>
<tr>
<td>ns</td>
<td>non-significant</td>
</tr>
<tr>
<td>NTCP</td>
<td>sodium-taurocholate co-transporting polypeptide</td>
</tr>
<tr>
<td>NUDT</td>
<td>Nudix hydroxylase</td>
</tr>
<tr>
<td>OATP</td>
<td>organic anion transport polypeptide</td>
</tr>
<tr>
<td>OCP</td>
<td>oral contraception</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>TLCA</td>
<td>taurolithocholic acid</td>
</tr>
<tr>
<td>TUDCA</td>
<td>tauroursodeoxycholic acid</td>
</tr>
<tr>
<td>$t_{max}$</td>
<td>time to the maximum plasma drug</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
</tr>
<tr>
<td>TNBS</td>
<td>trinitrobenzenesulfonic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>TPMT</td>
<td>thiopurine S-methyltransferase</td>
</tr>
<tr>
<td>$t_{1/2}$</td>
<td>half-life</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
<tr>
<td>UDCA</td>
<td>ursodeoxycholic acid</td>
</tr>
<tr>
<td>UGT</td>
<td>UDP-glucuronosyltransferases</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

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1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is an illness of chronic intestinal inflammation that follows a remitting and relapsing course. It is comprised of two distinct entities: Crohn's disease (CD) and ulcerative colitis (UC). Classically, CD involves the full thickness of the bowel wall and follows a discontinuous path along the gut's full length from mouth to anus. UC is characterized by continuous and superficial mucosal inflammation confined to the colon and rectum (Table 1.1). The pathogenesis of IBD has not been fully elucidated. The most widely held hypothesis suggests that there is an overly aggressive immune response to luminal microbial antigens and other adjuvants that occurs in genetically susceptible individuals facilitated by certain environmental factors.
Table 1.1: A comparison of Crohn's disease and ulcerative colitis (adapted from Sleisenger & Fordtran's Gastrointestinal and Liver Disease 10th Edition)\(^1\)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Crohn's Disease</th>
<th>Ulcerative colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset (years)</td>
<td>15-30</td>
<td>15-30</td>
</tr>
<tr>
<td>Pattern of disease</td>
<td>Small intestine, large intestine, rarely stomach</td>
<td>Large intestine</td>
</tr>
<tr>
<td>Inflammatory lesion</td>
<td>Skip lesions/ulcers; cobble-stoning</td>
<td>Continuous erythema and friability</td>
</tr>
<tr>
<td>Depth of injury</td>
<td>Transmural</td>
<td>Mucosal</td>
</tr>
<tr>
<td>Presence of diarrhea</td>
<td>Common</td>
<td>Common</td>
</tr>
<tr>
<td>Presence of hematochezia</td>
<td>Less frequent</td>
<td>Frequent</td>
</tr>
<tr>
<td>Presence of abdominal pain</td>
<td>Mild to severe</td>
<td>Mild to severe</td>
</tr>
<tr>
<td>Complications</td>
<td>Fistulae, abscesses, strictures</td>
<td>Hemorrhage, toxic megacolon</td>
</tr>
<tr>
<td>Extra-intestinal manifestations</td>
<td>Anemia, ankylosing spondylitis, pyoderma gangrenosum, cholelithiasis, vitamin deficiency</td>
<td>Anemia, ankylosing spondylitis, pyoderma gangrenosum, primary sclerosing cholangitis</td>
</tr>
</tbody>
</table>
1.1.1 Intestinal Immunity

The intestinal immune system is complex. Firstly, it consists of the innate immune system that offers an initial rapid response to foreign antigen via immune cells bearing pattern-recognition receptors. Secondly, there is the adaptive immune system that provides "immunologic memory" via antigen-specific recognition. The intestinal epithelium acts as another layer of defense. It is a physical barrier between foreign and host microbiota and the lymphoid tissue of the gut and host circulation, preventing continuous stimulation of the mucosal immune system. Under normal conditions, the lamina propria beneath the intestinal epithelium hosts a complex network of immune cells. These cells preserve the balance between the need for immune tolerance of luminal microbiota with the need to defend against foreign pathogens and the disproportionate entry of microbiota. In a healthy individual, tight junctions seal the spaces between adjacent epithelial cells, while interspersed goblet cells and paneth cells provide mucous and antimicrobial proteins, giving additional defense. In IBD, disruption of the mucus layer, loss of epithelial tight junctions and increased intestinal permeability all serve to increase the intestinal immune system's exposure to bacteria allowing the dissemination of an immune response via its innate and adaptive arms. There is also a shift in production from anti-inflammatory proteins to pro-inflammatory cytokines. Activation of the nuclear transcription factor nuclear factor κB (NFκB) plays a role in this shift.

NFκB refers to a family of nuclear transcription factors consisting of p65, c-Rel, RelB, p50 and p52 that activate the transcription of target genes. In the unstimulated cell, NFκB proteins remain within the cell's cytoplasm. Activation of NFκB proteins leads to their release from bound inhibitors and translocation to the nucleus to exert a regulatory...
effect. Regulation of NFκB activity occurs via two pathways: one mediated by the inhibitor IκBα/β/ε, which aids in the retention of NFκB proteins within the cytoplasm, and the other mediated by the activating kinases IKKα/β, which when stimulated by specific cytokines led to the degradation of co-repressors and the activation of NFκB proteins. Genes regulated by NFκB include: 1) inflammatory and immunoregulatory genes 2) cell cycle regulating genes 3) anti-apoptotic genes 4) negative regulators of NFκB. Activation of NFκB can be mediated via bacterial wall products such as lipopolysaccharides as well as interleukin (IL)-1, tumor necrosis factor (TNF)-α and some viruses. NFκB activity has been observed in the mononuclear cells (macrophages and lymphocytes) of the lamina propria as well as in the epithelial cells of the inflamed gut. A key function of the NFκB pathway is the up-regulation of pro-inflammatory cytokines including, TNF-α, IL-1, IL-6, IL-12, IL-23, and inducible nitric oxide synthase (iNOS), all important in the dissemination of the immune response. Unchecked NFκB activity is a key component of the dysregulated inflammatory response seen in IBD. IBD medications such as prednisone, methotrexate and anti-TNF-α exert their effects partly by targeting the NFκB pathway.

1.1.2 Barrier Function

Defects in the epithelial barrier are well-documented in UC and CD. Intestinal permeability is regulated by changes in the cell cytoskeleton and tight junctions. A number of factors likely play a role in epithelial barrier dysfunction. Inflammatory cytokines such as TNFα, interferon (INF)-γ and IL-13 are involved in the
initiation and perpetuation of the dysregulated immune response in IBD \(^2\). These cytokines have also been implicated in the disruption of the epithelial barrier function \(^3\). This provides a plausible reason as to why inflammatory cytokines have been the target of pharmacological therapy in IBD.

1.1.3 The Intestinal Microbiome and Inflammatory Bowel Disease

In the healthy individual, the composition of the gastrointestinal (GI) microbiome is unique and consists of hundreds to thousands of species of bacteria, the majority of which can be categorized into four phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria\(^{18-22}\). Gut bacteria are closely intertwined with many human biological processes and modulate the processing of many nutrients and drugs, including lipids and amino acids\(^{23}\). Factors such as age and diet are known to influence the composition of the gut microbiome\(^{24}\). For example, a study by Wu \(et.al.\) (2011) showed that long-term diet was strongly associated with the composition of the gut microbiota, while Yatsunenko \(et.al.\) (2012) convincingly demonstrated a similar association with age\(^{25,26}\). Disease state has also been linked to alterations in the bacterial composition of the gut. Multiple studies demonstrate that diversity in the intestinal microbiota is reduced in IBD compared to non-IBD controls\(^{27,28}\). There is loss of members of multiple genera including \(Bacteroides\) species, \(Clostridia\) species, \(Eubacterium\) species, and \(Lactobacillus\) species\(^{29,30}\). Moreover, microbiota varies with disease activity and distribution\(^{31,32}\). Willing \(et.al.\) (2010)\(^{32}\) showed that the microbiota in inactive CD was different than that of non-IBD controls, while there was little difference between the
microbiota in inactive UC in comparison to non-IBD controls. Whether this loss of
diversity is a consequence or a precipitator of disease is unknown. It is speculated by
some that alterations in the gut microbial profile play a role in the pathogenesis and
phenotype of IBD. This is supported by the increased detection of invasive Escherichia
(E.) coli in inflamed ileal samples from patients with active CD. Other pathogens
have been associated with disease-onset; however, no causal link has been clearly
identified. Additional evidence supporting the IBD-gut bacteria hypothesis is the
modest benefit seen with the use of antibiotics, such as ciprofloxacin and metronidazole
in CD colitis. Furthermore, many of the genetic defects associated with IBD are found
in genes such as NOD2 and ATG16L1 which are important for innate immunity and the
ability to sense and clear intracellular bacteria. Lastly, many strains of genetically-
susceptible mice predisposed to the development of a spontaneous immune-mediated
colitis, such as the multi-drug resistance protein (MDR)-1 or IL-10 knock out murine
models, fail to develop such colitis when maintained under germ-free conditions.

1.1.4 Other Environmental Factors

In addition to the influence of the gut microbiome, other factors within the
environment likely influence an individual's susceptibility to IBD. Cigarette smoking is
repeatedly shown to be protective against the development of UC, but conversely
increases the risk of CD in Western populations. In Western countries, childhood
antibiotic exposure increases the risk of both UC and CD, while breastfeeding appears to
be protective. In two large administrative database studies, oral contraceptives were
shown to increase the risk and severity of CD\textsuperscript{41,42}. Similarly, low plasma concentrations of vitamin D increases CD risk, while appendectomy is protective against the development of UC\textsuperscript{43,44}. The mechanisms by which these factors impact disease susceptibility are not well-defined.

Another important consideration is the influence of diet. There is a rising incidence of CD and UC in nations not previously affected by IBD. This may be attributable to improvements in health care technologies and accessibility; however, it has been hypothesized that the "westernization" of food products has contributed to this epidemiologic shift. In a review by Kaplan and Ng (2017), it is discussed that "The increasing incidence of IBD in Europe and the United States since the 1950s, and in Asia in the early 1990s, coincided with the introduction and promotion of packaged food and fast food chains, as well as increased use of antibiotics in human beings and livestock."\textsuperscript{45} In North America, there has been an expansion in the production of specialty starches and high fructose corn syrup. This has necessitated the development of additives and novel methods for processing these products to ensure their shelf-life. Interestingly, this has coincided with significant increases in CD and UC cases\textsuperscript{46}. In support of this, murine models have linked dietary additives such as sweeteners and emulsifiers to the development of intestinal inflammation\textsuperscript{47,48}.

1.1.5 The Genetic Landscape of Inflammatory Bowel Disease

IBD is a polygenic disease: 241 risk loci within the human genome are associated with its onset\textsuperscript{49}. These associations tend to be weak, with odds ratios less than 2 and
explain only a small portion of an individual's risk for developing the disease\textsuperscript{50}. This is somewhat discordant with the high rates of heritability amongst individuals with affected family members as well as what has been reported in studies of monozygotic twins\textsuperscript{51}.

Variation in the \textit{NOD2} gene was the first and most consistently replicated susceptibility locus in CD\textsuperscript{49, 52-54}. \textit{NOD2} codes for the nucleotide oligomerization domain 2 (NOD2) protein, an intracellular protein found in epithelial cells, paneth cells, dendritic cells, and endothelial cells as well as macrophages\textsuperscript{55}. \textit{NOD2} contains a leucine-rich repeat region that acts as a receptor for bacterial muramyl dipeptide and allows the intracellular detection of bacteria by the innate immune system and the activation of the NF\kappa B pathway\textsuperscript{56}. Three single nucleotide polymorphism (SNP)s within the leucine-rich receptor have been documented as conferring an increased risk of CD as high as two-fold in heterozygous carriers and twenty-fold in homozygous carriers\textsuperscript{49}. Interestingly, despite these findings, the role of \textit{NOD2} in IBD pathogenesis is unclear. Thirty percent of individuals of Caucasian ancestry carry one of the risk SNPs and the majority do not develop CD. Moreover, \textit{NOD2} deficient mice do not develop a spontaneous colitis, despite increased presence of intestinal bacteria\textsuperscript{57}. Taken together, it would appear that defects in \textit{NOD2} are insufficient to cause CD.

Other risk loci associated with the onset of IBD include genes implicated in innate immune pathways such as \textit{CARD9}, \textit{FCGR2A}, and \textit{ATG16L1}\textsuperscript{58}. Genetic variation in various aspects of the adaptive immune system including SNPs in \textit{IL10} (T-cell tolerance) and \textit{IL23R} (T-cell differentiation) also contribute to the genetic landscape of IBD and confer an increased risk of disease susceptibility\textsuperscript{58}. 
The predictive value of genetics in IBD has also become of interest as a tool for determining a patient's response to their IBD medications, for example, azathioprine (AZA). Variation in the TPMT gene, a determinant of AZA metabolism and bioavailability is associated with an increased risk of AZA-induced myelotoxicity. Individuals homozygous for a reduced function variant have preferential formation of 6-thioguanine which can result in life-threatening bone marrow suppression \(^{59}\). The utility of pre-emptive TPMT genotyping is widely accepted. Canadian, American and British Gastroenterology Society guidelines, all advocate for pre-emptive TPMT genotyping prior to the introduction of thiopurine-based therapies in IBD \(^{60-63}\). Variation in other genes such as HLA-DQA1 and HLA-DRB1 as well as NUDT15 are associated with an increased risk of AZA-induced pancreatitis (homozygous variant carriers, odds ratio 15.83) and myelotoxicity (homozygous variant carriers, odds ratio 16.2) respectively\(^{64-67}\). These data highlight the role of genetic variation in IBD pathogenesis as well as in drug response.

1.1.6 Clinical Presentation, Diagnosis and Biomarkers

The clinical manifestations of CD and UC are largely determined by the intestinal distribution of the disease. Early in the disease course, both conditions are remitting and relapsing, with periods of activity punctuated by debilitating symptoms of abdominal pain, diarrhea with or without hematochezia, and weight loss as well as biochemical and endoscopic findings of inflammation. Patients may also develop extra-intestinal manifestations of IBD such as inflammatory arthropathy, dermatological or ocular
abnormalities or disorders of the hepatopancreatobiliary system. This can affect approximately 25–40% of patients. As the disease progresses over time, permanent damage to the intestinal structure may result, leading to an irreversible impairment of intestinal function, significant morbidity and long-term disability. Disease severity is marked by the need for and time to surgery, failure of multiple medical therapies, need for hospitalization and the presence of complications such as fistulae or strictures.

The need for surgical intervention is relatively common amongst individuals affected by CD. A recent systematic review and meta-analysis highlighted that the risk of surgery 1, 5, and 10 years after a diagnosis of CD is 16.3% (95% CI, 11.4%–23.2%), 33.3% (95% CI, 26.3%–42.1%), and 46.6% (95% CI, 37.7%–57.7%), respectively. The risk of surgery 1, 5, and 10 years after a diagnosis of UC is 4.9% (95% CI, 3.8%–6.3%), 11.6% (95% CI, 9.3%–14.4%), and 15.6% (95% CI, 12.5%–19.6%), respectively.

Endoscopic depiction of mucosal inflammation by ileocolonoscopy is the gold standard test for diagnosis of CD in combination with histological evidence of chronicity. Similarly, endoscopic re-evaluation of the colon including the terminal ileum is needed to assess drug response. Clinical and endoscopic scoring systems such as the Harvey-Bradshaw Index, the Crohn's Disease Activity Index, or the Simple Endoscopic Score for CD are used to evaluate CD activity to allow clinicians to appropriately titrate treatment. More recently, deep remission defined as histological mucosal healing, has become the coveted endpoint for clinical trials and real-world practice.
Table 1.2: Clinical indices for the evaluation of disease activity in Crohn's disease

<table>
<thead>
<tr>
<th></th>
<th>Size of ulcer 0-3</th>
<th>Ulcerated surface 0-3</th>
<th>Affected surface 0-3</th>
<th>Presence of narrowings 0-3</th>
<th>Daperno <em>et.al.</em> Gastrointestinal Endoscopy 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>The simple endoscopic score</strong></td>
<td><strong>for Crohn's disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Crohn’s disease activity index</strong></td>
<td>Number of liquid stools</td>
<td>Abdominal pain 0-3</td>
<td>General well-being 0-4</td>
<td>Presence of complications</td>
<td>Best <em>et.al.</em> Gastroenterology 1976</td>
</tr>
<tr>
<td></td>
<td>Taking lomotil</td>
<td>Presence of an abdominal mass 0-5</td>
<td>Hematocrit &lt;0.42 women; &lt;0.47 men</td>
<td>% deviation from standard weight</td>
<td></td>
</tr>
<tr>
<td><strong>Harvey-Bradshaw Index</strong></td>
<td>Number of liquid stools</td>
<td>Abdominal pain 0-3</td>
<td>General well-being 0-4</td>
<td>Presence of complications</td>
<td>Bradshaw Lancet 1980</td>
</tr>
<tr>
<td></td>
<td>Presence of an abdominal mass 0-5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*An additional weighting factor is applied to each variable to determine the final score*
Ileocolonoscopy is an invasive, and sometimes arduous test that may be associated with complications such as bleeding, perforation and rarely death\textsuperscript{79}. Given this, biomarkers of disease may also aid clinicians in the diagnosis and quantification of disease as well as in predicting the risk for complications. Such biomarkers include, but are not limited, to C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), fecal leukocyte markers (fecal calprotectin) and antibodies against \textit{Saccharomyces cerevisiae} (ASCA) and perinuclear antineutrophil cytoplasmic proteins (ANCA)\textsuperscript{80-83}. Unfortunately, these biomarkers are not without their own respective limitations: CRP and ESR often lack specificity for intestinal inflammation, while fecal calprotectin and serologic antibodies lack accessibility through non-tertiary and quaternary care centres and whose diagnostic accuracy may be confounded by other factors\textsuperscript{80, 81}. Therefore, the role of biomarkers in clinical practice has not been consistently defined.

1.1.7 The Therapeutic Landscape

CD and UC are largely incurable. Affected individuals are often committed to long-term drug therapy with immunosuppressive agents. Current treatments include the monoclonal antibodies known as biologics. These drugs target various proteins involved in the dissemination of the inflammatory response, such as TNF-\(\alpha\), a variety of interleukins (IL-12, IL-23) and gut integrin complexes. Other treatments include immunosuppressants, such as methotrexate and AZA, and anti-inflammatories such as the 5-aminosalicylate (5-ASA) compounds and glucocorticoids, (prednisone or budesonide)\textsuperscript{84, 85}. Despite their different immune targets, these therapies all focus on suppressing the
host intestinal immune response and serve to dampen the effect of inflammatory proteins (IL-6, IL-1, TNF-α, IFN-γ) released by effector cells (cytotoxic and helper T cells) on the intestinal mucosa.

The 5-ASA agents are largely ineffective in CD and are used predominantly for the management of UC. A systematic review evaluating the efficacy of oral 5-ASA for the maintenance of remission in CD found no evidence that 5-ASA agents were superior to placebo\textsuperscript{86}. It is hypothesized that the effect of the 5-ASA agents is limited to the mucosal surface of the intestine and are unlikely to benefit a trans-mural disease such as CD. 5-ASA compounds are metabolized by the ubiquitously expressed-N-acetyltransferase (NAT)\textsuperscript{187,88}. They are also metabolized by NAT2, though its intestinal expression is far less than what is seen in the liver. 5-ASAs are poorly absorbed and are largely excreted in the stool. Their mechanism of action is poorly described.

Glucocorticoid agents such as prednisone and budesonide are highly effective for the induction of remission in CD, though the original evidence for glucocorticoids in IBD was derived from a UC cohort\textsuperscript{89-91}. The utility of these drugs in CD are highlighted by their frequent and pervasive use: by their 10th anniversary of disease, more than two thirds of patients will have been exposed to a glucocorticoid, with more than 20% receiving greater than 3000mg within their first 5 years of diagnosis\textsuperscript{92}. Glucocorticoids diminish the inflammatory response via several pathways: they suppress the cytokine-mediated activation of T-cells, and antigen-presenting cells (APCs) including macrophages; they stabilize the liposomal membrane of neutrophils and prevent the release of catabolic enzymes; they inhibit phospholipase A2 and decrease the release of histamine, prostaglandins, leukotrienes and other pro-inflammatory chemicals; and they
promote vasoconstriction and reduce capillary permeability\textsuperscript{93}. Both budesonide and prednisone are rapidly metabolized by cytochrome P450 (CYP) 3A\textsuperscript{94-96}. CYP3A4 converts prednisone to its active metabolite, prednisolone. Prednisone has a significant \textit{systemic} anti-inflammatory effect\textsuperscript{97}. Conversely, budesonide undergoes substantial first-pass metabolism mediated by hepatic and intestinal CYP3A4\textsuperscript{98}. Budesonide, thus, has a greater local anti-inflammatory effect at the intestinal level compared to its systemic effect.

In the event that patients require prolonged or recurrent glucocorticoid exposure, clinicians have the opportunity to transition patients to one of two immunomodulators, AZA or methotrexate. AZA antagonizes purine metabolism and inhibits DNA synthesis. It has a limited benefit for the maintenance of remission in CD as well as a modest steroid-sparing effect\textsuperscript{99,100}. Similarly, methotrexate, a competitive inhibitor of dihydrofolate reductase, is effective for the maintenance of remission in CD and is used as a means for withdrawing patients from glucocorticoids\textsuperscript{101,102}.

Lastly, the monoclonal antibodies known as biologics have revolutionized the management of CD and UC. Biologics impair the function of the target molecule by inducing apoptosis of the target-expressing cell or by modulating the signaling pathway. In Canada, there are currently four biologic agents approved for the management of CD: infliximab, adalimumab, vedolizumab and ustekinumab.

Infliximab, the first biologic to be approved for the management of CD in Canada, is a chimeric human-murine monoclonal antibody directed against the pro-inflammatory cytokine, TNF-\textit{\alpha}. The efficacy of infliximab in CD was demonstrated in two landmark trials known as ACCENT 1 and ACCENT 2\textsuperscript{103,104}. Its sister drug,
adalimumab, is a humanized monoclonal directed against TNF-α that is delivered via subcutaneous route. The CHARM trial highlighted the benefit of long-term treatment with adalimumab in CD patients who responded to loading doses\textsuperscript{105}. Newer agents vedolizumab and ustekinumab target alternate pathways in CD. Vedolizumab binds to the α4β7 integrin expressed on T-lymphocytes and inhibits their interaction with the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) on endothelial cells within the gut, ultimately, preventing the trafficking of lymphocytes to the intestinal tissues\textsuperscript{106}. The GEMINI 2 randomized-controlled studies were the basis of approval for vedolizumab in CD\textsuperscript{107}. The Health Canada approval of ustekinumab for CD is based on three pivotal studies (UNITI-1, UNITI-2 and IM-UNITI)\textsuperscript{108,109}. These studies included more than 1,300 CD patients who were either new to, experienced with, or failed biologic therapy. Ustekinumab targets and binds the shared p40 subunit of interleukin-12 and interleukin-23, two pro-inflammatory cytokines produced by APCs involved in perpetuating the inflammatory response and suppressing regulatory T-cells\textsuperscript{110}.

Of note, the metabolism of the biologics is very different from the processes applied to small molecule drugs. Small molecules such as the glucocorticoids or immunomodulators are typically eliminated by hepatic metabolism or renal or biliary excretion. As monoclonal antibodies, the biologics are captured from the vascular space by endocytosis and subjected to proteolysis within the lysosome or salvaged by the neonatal Fc receptor (to which they owe their long half-life)\textsuperscript{111}.

1.1.8 Limitations of Treatment
Overall, CD patients are exposed to a heavy burden of medications over the course of their disease. Inter-individual responses to commonly used IBD medications vary significantly and rates of resistance, loss of response, and adverse drug reaction (ADR) remain high, despite a long history of use\textsuperscript{101-103, 112, 113}.

1.1.8.1 Adverse Drug Risks

The glucocorticoids, immunomodulators and biologics are associated with a number of adverse drug risks that in some cases are distinct and in others, overlapping. The glucocorticoids are associated with defects in bone metabolism as well as can negatively affect the cardiovascular, endocrine, psychiatric and immunologic systems\textsuperscript{114}. The immunomodulators like AZA are associated with hepato- and myelotoxicity, an increased risk of malignancy, infection and malaise\textsuperscript{61, 115}. The biologics may carry an increased risk of malignancy and infection as well as neurologic impairment and worsening of any underlying cardiovascular disease\textsuperscript{116, 117}.

1.1.8.2 Variability of Drug Response

Currently, there is a lack of predictably to drug response in CD. As an example, the corticosteroids have been used in the management of CD and UC for more than 70 years\textsuperscript{118}. Data derived from Olmstead County administrative databases revealed that within 30 days of treatment with a corticosteroid, 60% of CD patients will have a complete response to therapy, while 25% will have only a partial response and 15% will have no response\textsuperscript{118}. Similarly, in clinical trials, we see that rates of non-response are as high as 30% and 50% for budesonide and prednisone respectively\textsuperscript{91, 119}. Many groups
have attempted to uncover predictors of glucocorticoid response, but no predictor has made it to large-scale clinical practice\textsuperscript{120, 121}.

Likewise, response rates to biologic agents vary significantly between patients. Ben-Horin \textit{et.al.} (2014) summarized rates of biologic resistance seen in clinical trials as well as what was reported in select "real-world" case series. The authors found that approximately 40\% of clinical trial subjects and 20\% of "real-world" patients were resistant to the effects of infliximab or adalimumab\textsuperscript{122}. In a recent editorial by Jean-Francois Colombel, an international expert in IBD management, the author identified the crux of the IBD therapeutic paradigm: "One of the biggest challenges we are now facing [in the management of IBD] is the search for biomarkers predicting efficacy or failure of biologics and small molecule drugs in the hope of personalized therapy." The lack of predictability in drug response suggests a fundamental gap in our knowledge on how IBD impacts drug metabolism and exposure to large and small molecule drugs. Gaining new insight into CD-specific modifications of drug metabolism and response may allow for improved drug efficacy and reduced drug toxicity as well a better understanding of disease pathogenesis.

1.1.8.3 \textit{The Effect of Inflammation on Drug Metabolism}

The effect of inflammation on the CYP enzymes is well-established. \textit{In vitro} studies, \textit{in vivo} murine models and pharmacokinetic analysis in human subjects in an acute inflammatory state demonstrate that the expression and activity of CYP enzymes are down-regulated\textsuperscript{123}. Mechanisms of down-regulation have been suggested including inflammation-induced oxidative stress, the effect of inflammatory cytokines (TNF-\(\alpha\), IL-
1, IL-6) or changes in the activation of NRs such as PXR\textsuperscript{123}. This has not been studied in any significant way in the setting of IBD or specifically in CD. One small study conducted by Sanaee \textit{et.al.} (2011) evaluated the impact of CD on a patient's systemic exposure to the CYP3A4 substrate verapamil. The authors reported higher verapamil plasma concentrations in the CD cohort versus healthy controls. Plasma concentrations increased with increasing disease activity\textsuperscript{124}. This suggests a dampening effect of CD on CYP3A4 activity and emphasizes the paucity of available data pertaining to CD-related changes in drug metabolism.

1.2 Cytochrome P450

Drug metabolism is often divided into three phases: phase 1 metabolism characterized by reactions such as oxidation, reduction or hydrolysis that increase the hydrophilicity of a compound; phase 2 metabolism characterized by conjugation reactions that prepare compounds for excretion; and phase 3, where the products of phase 1 or 2 metabolism are recognized by membrane-bound transporters and are transported to the extracellular space\textsuperscript{125}. The reactions that make up phase 1 drug metabolism are catalyzed by a number of enzymes including, but not limited to, the following enzyme families: CYP, monoamine oxidase (MAO) and FAD-containing mono-oxygenase (FMO)\textsuperscript{125}.

The CYP superfamily is one of the most relevant determinants of drug metabolism in humans and is responsible for the metabolism and activation of a vast number of xenobiotics, including many of the drugs used today\textsuperscript{126}. CYP enzymes are
hemoproteins that are able to reversibly catalyze oxidation and reduction reactions using their heme group through the transfer of electrons\textsuperscript{127}. The CYP enzymes are sub-classified into families (1, 2, 3 and 4), sub-families and isoforms on the basis of their shared amino acid structures. In addition to variations in their amino acid sequences, CYP isoforms vary with respect to their catalytic activity and tissue localization\textsuperscript{126, 127}. CYP isoforms found in the hepatic parynchema are likely the most important enzymes for determining the disposition of drugs used in clinical practice today\textsuperscript{126} (Figure 1.1a).
**Figure 1.1a** Relative hepatic abundance of the cytochrome P450 enzymes (adapted from Xie et.al. 2009 Chapter 1, pg 4)\textsuperscript{125}

**Figure 1.1b** Relative intestinal abundance of the cytochrome P450 enzymes (adapted from Paine et.al. 2006)\textsuperscript{128}
1.2.1. Cytochrome P450 3A

The CYP3A subfamily is a key mediator of drug metabolism and disposition in humans\textsuperscript{129}. Overall, its isoforms are highly concentrated in organs essential to first-pass metabolism such as the liver and the intestinal tract and act as a barrier to the systemic exposure of its orally-ingested substrates\textsuperscript{130}. CYP3A accounts for 28\% and 80\% of the human hepatic and intestinal CYP content respectively (Figure 1.1a, 1.1b)\textsuperscript{125,128}. Three functional enzymes have been identified: CYP3A4, CYP3A5 and CYP3A7. CYP3A4 is the most abundant and comprehensively investigated of the CYP3A isoforms\textsuperscript{131}. It is responsible for the metabolism of more than 50\% of the drugs that are used in clinical practice\textsuperscript{132}. It is also important for the inactivation of pollutants and environmental chemicals known as xenobiotics as well as the metabolism of endogenous substances such as steroids, sterols, fatty acids and bile acids\textsuperscript{132}. CYP3A4 is localized to the liver, intestinal tract and kidneys, with the highest expression in the liver\textsuperscript{131}. In the liver, CYP3A4 is localized to the hepatocyte and biliary epithelium, while in the intestinal tract, CYP3A4 is found at the villous tip of individual enterocytes with the highest expression in the jejunum and ileum\textsuperscript{131,133-135}. CYP3A5 is similarly distributed, but to a lesser degree. Many of the CYP3A4 substrates are shared substrates of CYP3A5 due to their similar amino acid sequences (>85\% shared)\textsuperscript{136}. It is often impossible to distinguish between the contributions of either enzyme to a specific substrate's disposition. CYP3A7 is present only in the fetal liver and plays a minimal role in drug metabolism\textsuperscript{125}.

The expression of CYP3A4 is highly variable. It is inducible by a wide selection of compounds including drugs and endogenous substances; however, its inter-individual variability may also be independent of inducers. Studies by Rogers \textit{et.al.} (2003)\textsuperscript{137} and
Floyd et al. (2003)\textsuperscript{138} have found up to a 10-fold variation in inter-individual clearance of CYP3A substrates in healthy subjects, while other groups cite variation as high as 20-fold\textsuperscript{139}. Different factors influence CYP3A4 expression to a varying degree. This includes inducers and inhibitors such as a wide selection of drugs and endogenous compounds. Signaling pathways, in particular the nuclear receptor superfamily\textsuperscript{136, 140, 141} as well as sex, age, disease states and the presence or absence of inflammation are also important for determining its activity\textsuperscript{142}.

1.2.2. Regulation of CYP3A4

Complex signaling pathways mediated by nuclear receptors (NR) and other transcription factors appear to play an important role in the regulation of CYP3A4. Constitutive transcriptional regulation is mediated through CCAAT-enhancer binding proteins-\(\alpha\) and -\(\beta\), hepatocyte nuclear receptor (HNF)-1\(\alpha\), -3\(\gamma\), and -4\(\alpha\)\textsuperscript{143} while inducible transcriptional regulation is mediated through ligand-induced activation of the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), the farnesoid X receptor (FXR)\textsuperscript{144}, the liver X receptor (LXR), the vitamin D receptor (VDR) and the peroxisome proliferator-activated receptor, (PPAR\(\alpha\))\textsuperscript{136}. Specifically, in the case of PXR and CAR, the ligand-activated nuclear receptors migrate to the nucleus, bind to response elements on \textit{CYP3A4} and increase mRNA transcription significantly\textsuperscript{145}.
1.2.3 Role of Age, Sex and Genetics in CYP3A4 Variability

The role of age as a contributor to CYP3A4 variability is controversial. Several animal studies have described an age-dependent effect on CYP expression\textsuperscript{146}. The evidence for a similar effect in humans is less clear and studies have been contradictory. One study by Sotaniemi \textit{et al.} (1996) showed that the metabolism of the CYP3A4-probe drug, lignocaine was significantly less in individuals older than 65 years of age compared to a healthy young cohort (ages less than 25 years)\textsuperscript{147}; however, other groups have not been able to demonstrate a similar finding using other modalities for assessing CYP3A4 activity such as the C\textsuperscript{14} erythromycin breath test\textsuperscript{148}. Furthermore, age-related declines in hepatic CYP3A4 content are not consistently shown\textsuperscript{149, 150}.

Sex appears to be a determinant of CYP3A4 expression. Wolbold \textit{et al.} (2003) published an analysis in 94 surgical liver samples, evaluating the CYP3A4 content as well as the expression of P-gp, PXR and CAR. Higher CYP3A4 expression was seen in the women compared to the men, with a 50\% increase in the CYP3A-mediated metabolism of verapamil\textsuperscript{151}. This has been supported by other human studies using endogenous probes of CYP3A4 activity as well as in studies evaluating the pharmacokinetics of CYP3A4 substrates\textsuperscript{152, 153}. The reasons for sex-specific differences in CYP3A4 expression and/or activity are unclear. The role of estrogen in the activation of CYP3A4 pathways has been evaluated in a limited fashion. Estradiol (E2) may interact with regulators of CYP3A4 such as PXR or CAR\textsuperscript{154, 155}. However, with respect to PXR, E2 only occupies a small portion of the receptor, making binding weak and estradiol-induced PXR activation negligible\textsuperscript{154}. Furthermore, the E2-mediated repression of another CYP3A4 activator, FXR has been observed \textit{in vitro}, with
downstream consequences for FXR target genes (bile salt export pump, BSEP)\textsuperscript{156,157}. This has not been directly evaluated with respect to CYP3A4. At present, the mechanisms for sex-specific differences in CYP3A4 activity and expression as well as the role and degree of impact of estrogen on CYP3A4 are not known.

Genetic variability is also essential to CYP3A4 expression and in turn activity. To investigate this, Ozdemir \textit{et.al.} (2000) conducted a systematic review of 13 healthy-volunteer studies and 3 patient-volunteer studies\textsuperscript{158}. They assessed the inter- and intra-individual differences in the disposition of different CYP3A4 substrates based on genetic variability. They concluded that 90\% of inter-individual variability in CYP3A4 activity is genetically determined in healthy individuals. This was reported to be less of a factor in patient populations. No allelic variants were identified in this study; however, it prompted further investigation into the contribution of genetic variation in CYP3A4 activity.

A variant within \textit{CYP3A4} intron 6 (C>T, rs35599367), \textit{CYP3A4*22} has been associated with diminished CYP3A4 mRNA expression in cultured cells. It also accounted for a small fraction of the variability in mRNA expression in a cohort of healthy human livers\textsuperscript{159}. Otherwise, no common \textit{CYP3A4} genotypes have been identified that explain the variability in CYP3A4 expression\textsuperscript{160}. It has thus been hypothesized by Lamba \textit{et.al.} (2010) that CYP3A4 expression may be related to genetic variation in the transcription factors regulating CYP3A4, because its activity and protein expression is highly correlated with its mRNA expression, as shown in previous studies\textsuperscript{161-164}. Lamba \textit{et.al.} (2010) found that variation in CYP3A4 mRNA expression was related to genetic variation in the following nuclear transcription regulators: pregnane X receptor (PXR),
hepatic nuclear factor 3β (HNF3β, FOXA2), hepatic nuclear factor 4α (HNF4α), and hepatic nuclear factor 3γ (HNF3γ, FOX A3) as well as to the \textit{MDR1} polymorphism 3435C>T\textsuperscript{161}. They concluded that SNPs in these factors contributed to up to 24.3% of the variation seen in CYP3A4 expression. Similarly, another group showed that SNPs in \textit{VDR}, another transcriptional regulator of CYP3A4 influenced the activity of intestinal CYP3A4 \textsuperscript{161, 165}.

\textbf{1.2.4. Epigenetic Modification of CYP3A4 Expression}

"Epigenetics" refers to "the heritable changes in gene expression that are not coded in the DNA sequence itself."\textsuperscript{166} Epigenetic mechanisms such as histone modification by acetylation or methylation, DNA methylation and RNA-mediated gene silencing by non-coding RNAs, all play a significant part in, most-often, silencing DNA transcription.

DNA methylation is the system most-studied in the regulation of drug metabolizing genes.\textsuperscript{167} Peng \textit{et.al.} reported that 90\% of the "identified epigenetic regulation" in genes involved in drug metabolism pertains to DNA methylation\textsuperscript{168}. However, in the case of CYP3A4, it is the post-transcriptional changes mediated through non-coding RNAs such as various microRNAs (miRNAs) that appear to be the most impactful on its expression. As examples, the binding of miRNA-27b and -206 to the 3' untranslated region (UTR) of \textit{CYP3A4} results in a decrease in \textit{CYP3A4} mRNA levels\textsuperscript{169, 170}. Epigenetic mechanisms mediated through alterations in the expression of NRs such as PXR and VDR also contribute to the variability in CYP3A4 expression\textsuperscript{169, 171}. 
1.2.5. The Impact of Disease on CYP3A4

Several non-hepatic diseases appear to affect hepatic CYP3A4 expression and activity. Patients with chronic kidney disease (CKD) are exposed to a wide number of CYP3A-metabolized drugs. Down-regulation of hepatic CYP3A has been documented in this patient population in human studies as well as in *in vivo* models\textsuperscript{172, 173}. Velenosi *et al.* (2014) suggested that changes in CYP3A activity in CKD may be due to changes in "NR binding and histone acetylation"\textsuperscript{174}. Similarly, down-regulation of CYP3A has been documented in individuals with non-alcoholic fatty liver disease (NAFLD). Woolsey *et al.* (2015) demonstrated that individuals with biopsy-proven non-alcoholic steatohepatitis had a near 3-fold increase in midazolam (CYP3A4 probe drug) plasma concentrations versus healthy controls\textsuperscript{175}. This was validated by quantifying the plasma concentrations of the CYP3A-endogenous probe, 4β-hydroxycholesterol (4β-OHC). The plasma concentration of 4β-OHC was significantly lower in the NAFLD population. Decreased CYP3A4 expression and activity were also demonstrated using *in vivo* murine and *in vitro* cell models by the same group\textsuperscript{175}.

Other animal models of inflammation such as lipopolysaccharide-exposed mice demonstrate down-regulation of hepatic CYP3a11 mRNA (equivalent to human CYP3A4)\textsuperscript{176}. Similarly, the 3% dextran sulfate sodium (DSS) mouse model of colitis shows reduced CYP3a11 expression which is reversed with pre-treatment with metronidazole\textsuperscript{177}. In acute inflammation, such as with surgery or infection, CYP3A4 activity is diminished \textsuperscript{178, 179}. Several mechanisms by which inflammation alters CYP3A4 activity have been posited and are outlined by Aitken *et al.* (2006)\textsuperscript{123}. Increases
in oxidative stress and inflammatory cytokines as well as changes in NR signaling are highlighted as important.

The impact of IBD on CYP3A4 activity is less clear and has not been extensively studied. A study in 21 subjects with CD, versus 10 with UC and 26 healthy volunteers found a significant reduction in the 4β-hydroxycholesterol plasma concentrations of the individuals with CD, suggesting a total body decrease in CYP3A4 activity in this cohort\textsuperscript{180}. Conversely, a case-control study in a small pediatric CD population evaluating CYP3A4 mRNA expression in non-inflamed duodenal pinch biopsies found an increase in CYP3A4 expression\textsuperscript{181}. Ultimately, further work is needed to clarify the impact of CD on CYP3A4-mediated metabolism as this may impact non-IBD drugs prescribed to CD patients as well as commonly used CYP3A4 substrates such as the glucocorticoids prednisone and budesonide.

1.2.6. Important Drug-Drug and Food-Drug Interactions and CYP3A4

Interactions with other compounds, either prescribed drugs, food stuffs or herbal medicines taken at a patient's own discretion, influence CYP3A4 activity and likely account for a significant portion of the inter-individual variability seen within patient populations\textsuperscript{182}. A list of CYP3A4 substrates, inhibitors and inducers are included in Table 1.3. Substances interact with CYP3A4 either to enhance or impair its catalytic activity or to serve as a substrate for CYP3A4-induced metabolism. This is the basis for many drug-drug or food-drug interactions that may lead to varying degrees of patient toxicity or loss of drug efficacy. For example, the co-administration of ketoconazole, a
potent inhibitor of CYP3A4, with terfenadine, a non-sedating anti-histamine approved for the treatment of allergic rhinitis, led to reports of a deadly drug-drug interaction mediated through changes in CYP3A4 metabolism\textsuperscript{183}. In addition to its effects as an anti-histamine, terfenadine is also a cardiac potassium channel blocker with low systemic bioavailability due to extensive metabolism by CYP3A4. Unfortunately, ketoconazole-mediated inhibition of CYP3A4 activity led to increased plasma concentrations of terfenadine and, in past, has precipitated fatal ventricular arrhythmias\textsuperscript{184}. Similarly, the reduced systemic exposure and lack of efficacy of various CYP3A4 substrates such as benzodiazepines when co-administered with CYP3A4 inducers such as carbamazepine or of tacrolimus when co-administered with rifampin illustrates the importance of CYP3A4 to clinically relevant drug-drug interactions\textsuperscript{185, 186}. 
Table 1.3: Selected CYP3A4 substrates, inducers and inhibitors (adapted from United States Food and Drug Administration – Drug development and drug interactions)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Inducers</th>
<th>Inhibitors</th>
</tr>
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<tbody>
<tr>
<td>Tyrosine kinase inhibitor:</td>
<td>Rifamycin antibiotics:</td>
<td>Tyrosine kinase inhibitor:</td>
</tr>
<tr>
<td>Imantinib</td>
<td>Rifampin</td>
<td>Imantinib</td>
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<tr>
<td>Dasatinib</td>
<td>Rifabutin</td>
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<tr>
<td>Benzodiazepines:</td>
<td>Azole antifungals:</td>
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<tr>
<td>Midazolam</td>
<td>Ketoconazole</td>
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<tr>
<td>Triazolam</td>
<td>Itraconazole</td>
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<tr>
<td>Alprazolam</td>
<td>Fluconazole</td>
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<tr>
<td>Glucocorticoids:</td>
<td>Macrolide antibiotics:</td>
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<tr>
<td>Budesonide Prednisone</td>
<td>Erythromycin</td>
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<td>Non-sedating antihistamines:</td>
<td>Troleandomycin</td>
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<td>Terfenadine</td>
<td>Clarithromycin</td>
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<tr>
<td>Astemizole</td>
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<tr>
<td>Immunosuppressive agents:</td>
<td>Non-dihydropyridine calcium channel blockers:</td>
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<tr>
<td>Tacrolimus</td>
<td>Verapamil</td>
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<tr>
<td>Cyclosporine</td>
<td>Diltiazem</td>
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<tr>
<td>Sirolimus</td>
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<tr>
<td>HIV protease inhibitors:</td>
<td>Proton pump inhibitor:</td>
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<tr>
<td>Saquinavir</td>
<td>Omeprazole</td>
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<tr>
<td>Indinavir</td>
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<tr>
<td>Nelfinavir</td>
<td>Other:</td>
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<tr>
<td>Ritonavir</td>
<td>Hypericum perforatum (St.John’s wort)</td>
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<tr>
<td>HMG-COA reductase inhibitor:</td>
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<td>Simvastatin</td>
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<td>Atorvastatin</td>
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<tr>
<td>Lovastatin</td>
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<tr>
<td>Non-dihydropyridine calcium channel blockers:</td>
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<td>Verapamil</td>
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<td>Diltiazem</td>
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Other non-traditional medicines, such as *Hypericum perforatum* also known as St. John's wort, interact with CYP3A4 and have important consequences for other concomitantly administered CYP3A4 substrates. St. John's wort, a commonly used herbal therapy for treatment of depression, is a potent inducer of CYP3A4 metabolism via PXR\(^{187}\). A number of interactions with CYP3A4 substrates have been identified and reported\(^{125, 188}\).

Lastly, aspects of an individual's diet likely play an important role in inter-individual CYP3A4 variability. Bailey et al. (1998) discovered that grapefruit juice is an inhibitor of CYP3A4 metabolism\(^{189}\). This group and others have demonstrated significant increases in the bioavailability of orally administered drugs such as felodipine, cyclosporine and midazolam when administered concurrently with grapefruit juice\(^{190-192}\).

### 1.2.7. Quantifying CYP3A-Mediated Metabolism *In Vivo*

Given the breadth of CYP3A substrates and the potential for numerous drug interactions, several techniques are available for evaluating CYP3A activity *in vivo* using a variety of "probe" substances. A probe substance is defined as a compound whose pharmacokinetics are determined by a single elimination process and from which a enzyme's catalytic activity can be inferred\(^{125}\). Criteria for a valid probe are outlined in a review by P. Watkins in a 1994 review invited by Pharmacogenetics\(^{193}\). *In vivo* measures of CYP3A4 catalytic activity include the assessment of midazolam or quinine pharmacokinetics, the \(14\)C -erythromycin breath test, and the measurement of endogenous metabolites such as 4βOHC in plasma or 6β-hydroxycortisol (6βHC) in urine\(^{131}\).
Certain limitations exist for each of these modalities: conflicting reports regarding the accuracy of urinary 6βHC concentrations for estimating CYP3A activity exist; C14-erythromycin breath testing is only useful for the estimation of hepatic (not intestinal) CYP3A activity and requires the administration of a radiolabeled carbon\textsuperscript{193}.

Currently, the assessment of midazolam pharmacokinetics appears to satisfy all the tenants of a CYP3A probe drug outlined by Watkins\textsuperscript{193}. Midazolam is rapidly oxidized by CYP3A4 to its 1- and 4-hydroxymidazolam metabolites, detectable in blood by liquid chromatography-tandem mass spectrometry (LC-MS/MS)\textsuperscript{194,195}. It can be administered intravenously and orally to capture the contribution of hepatic and intestinal CYP3A activity\textsuperscript{131}. Furthermore, studies have validated the substitution of midazolam microdosing (in comparison to standard dosing) for the evaluation of hepatic and intestinal CYP3A activity\textsuperscript{196,197}. This eliminates concerns around a subject's exposure to the sedative and amnestic properties of midazolam.

The cholesterol metabolite, 4βOHC has also been proposed as a biomarker of CYP3A activity. Cholesterol undergoes transformation to form oxysterols, of which 4βOHC appears to be the product of CYP3A-mediated oxidation\textsuperscript{198}. Plasma concentrations of 4βOHC appear to correlate with CYP3A activity: patients exposed to increasingly potent CYP3A inducers had similarly matched increases in their 4βOHC plasma concentrations\textsuperscript{199}. Furthermore, \textit{in vitro} data support a mechanistic role for CYP3A in the formation of 4βOHC versus other metabolites\textsuperscript{198}. The utility of plasma concentrations of 4βOHC for evaluating CYP3A activity may be limited by its relatively slow rate of elimination and in conditions where cholesterol concentrations may be altered.
1.3 Nuclear Receptors

CYP3A4 expression and activity are regulated by the NRs, FXR and PXR. Both of these NRs are implicated in CD and UC pathogenesis. Disease-dependent changes in FXR and PXR activity may have important consequences for drug metabolism sequences in general and CYP3A4-mediated metabolism specifically. This section will provide an overview of IBD-related changes in PXR and FXR activity as well as the link to IBD pathogenesis.

1.3.1 Nuclear Receptors: a Brief Overview

NRs are a group of transcription factors that share a common structure: a ligand-binding domain at the C-terminus and a DNA-binding domain at the N-terminus. NRs regulate the transcription of genes that are important to the development of, metabolism and disease processes occurring within humans. They are activated by lipophilic substrates binding to a ligand-binding domain. Substrates include endogenous hormones, vitamins A and D, and xenobiotics as well metabolic intermediates such as fatty acids, bile acids and sterols. The binding of a ligand to the ligand-binding domain permits the dissociation of a bound co-repressor (Figure 1.2). Subsequent recruitment of a co-activator allows NRs to bind to specific DNA sequences in the regulatory domain of target genes and regulate their transcription.

To date, more than 48 members of the NR family have been identified. This is including, but not limited to, FXR, PXR, LXR and the glucocorticoid receptor (GR). Many NRs have an identified ligand, while others are termed "orphan receptors" with no
recognized ligand. PXR and FXR are important to drug metabolism and transport pathways and are increasingly recognized as being important to IBD pathogenesis. Herein, PXR and FXR will be discussed in greater detail.
Figure 1.2  Model of nuclear receptor molecular structure as well as signaling sequence. A represents the inactive cytoplasmic state bound to a corepressor. B represents binding of the ligand, loss of the corepressor, and recruitment of a coactivator as well as the heterodimerization with another nuclear receptor. There is translocation to the nucleus (C) and binding of the nuclear receptor complex DNA-binding domains to the target gene to induce transcription. Activation domain, AD; hinge region, HR; ligand-binding domain, LBD; DNA-binding domain, DBD; corepressor, CoR; coactivator, CoA; nuclear receptor, NR.
1.3.2 Pregnane X Receptor

PXR (gene NR1I2), also known as the steroid and xenobiotic-sensing NR, plays an important role in the "detoxification" of the human body\textsuperscript{203}. Similar to CYP3A4, it is abundantly expressed in the liver and intestine\textsuperscript{204}. It is activated by a variety of endogenous and exogenous compounds and in turn heterodimerizes with the retinoid X receptor (RXR) to up-regulate the transcription of target genes that code for proteins necessary for the degradation and clearance of toxins from the body\textsuperscript{203}.

PXR is activated by a number of different ligands including, but not limited to rifaximin\textsuperscript{205}, lithocholic (LCA) and deoxycholic acid (DCA)\textsuperscript{206} in humans and pregnenolone 16-carbonitrile (PCN)\textsuperscript{207} in rodents, from which its name is derived. PXR regulates the expression of a variety of genes including, but not limited to the following: UDP-glucuronosyltransferases (UGT)\textsuperscript{208}, glutathione-S-transferases (GST)\textsuperscript{209}, sulfotransferases (SULT)\textsuperscript{210} as well as drug efflux transporters from the ATP-binding cassette (ABC) exporter families including P-gp (gene MDR1) and the multidrug resistance proteins 2 and 3 (MRP2, MRP3). It also regulates members of the solute carrier organic anion transporter superfamily like organic anion transporter (OATP)\textsuperscript{1B1}\textsuperscript{211}. Most important to the discussion at hand, is the PXR-mediated regulation of CYP3A4\textsuperscript{212}. This relationship has been confirmed using \textit{in vivo} murine models, whereby disruption of the PXR gene impedes CYP3A4 activation via established PXR ligands\textsuperscript{206,213}. In humans, \textit{CYP3A4} mRNA expression as well as the protein's activity are increased in response to known PXR activators; it is felt that most drugs that induce \textit{CYP3A4} are acting via PXR\textsuperscript{203}.
1.3.3 Farnesoid X Receptor

FXR (gene symbol NR1H4) is a NR that acts as a sensor of intracellular bile acid concentrations within the liver and intestine and plays a key role in the regulation of the enterohepatic circulation \(^{214, 215}\). Activation of FXR by bile acids leads to significant changes in bile acid homeostasis as well as in the transcription of genes responsible for bile acid synthesis \(^{214}\). Similarly to PXR, FXR binds in tandem with RXRα to response elements within the promoter regions of target genes. This leads to the up-regulation of bile acid export systems in the ileum (organic solute transporter, OST- α/β) and liver (BSEP) and down-regulates the expression of bile acid import systems (apical sodium-bile acid transporter, ASBT, ileum; sodium-taurocholate co-transporting polypeptide, NTCP, liver) \(^{216-220}\). Bile acid-induced activation of FXR has also been found to reduce the transcription of genes encoding bile acid synthesis enzymes such as CYP7A1 (via the small heterodimer partner, an orphan nuclear receptor) thus reducing bile acid synthesis \(^{221}\). Other target genes of FXR include CYP3A4 and PXR\(^{144, 222}\). Gnerre et al. (2004)\(^{144}\) demonstrated an increase in CYP3A4 mRNA in HepG2 cells in response to the FXR-specific agonist, GW4064. A gel-mobility shift assay was used to highlight the capacity of FXR to bind to a 345-base pair sequence FXR-responsive unit within the CYP3A4 gene and confirmed the importance of this binding by introducing various mutations into different binding sites\(^{144}\). They subsequently confirmed the PXR-independent nature of FXR-mediated CYP3A4 activation by showing that GW4064 administration in PXR null mice did induce the mRNA expression of murine Cyp3a11, while GW4064 did not increase Cyp3A11 mRNA expression in FXR null mice. Similarly, Jung et al. (2006) confirmed the FXR-mediated regulation of PXR\(^{222}\). Wild type mice fed with GW4064 or
cholic acid (CA) had a robust induction of PXR detected by changes in PXR and SHP mRNA that was abolished when repeated in FXR null mice. Furthermore, FXR binding sites were detected in the murine PXR gene.

In addition to using detectable changes in the mRNA expression of downstream gene targets, in vivo changes in FXR activity can be estimated by plasma concentrations of the ileal hormone, fibroblast growth factor (FGF) 19 in humans and its murine ortholog, FGF15\textsuperscript{223-225}. FXR is found in small and large bowel enterocytes. It induces the expression of FGF19 which in turn represses the transcription of CYP7A1 and inhibits bile acid synthesis\textsuperscript{226}. Its plasma concentrations are affected by loss of viable ileal epithelium.

1.3.4 A Role for Bile Acids in Differential Nuclear Receptor Signaling in IBD

PXR and FXR are activated by bile acids, though not all bile acids exert equal effects. As previously stated, PXR is most robustly activated by free or conjugated LCA and DCA, while FXR is most robustly activated by free or conjugated CDCA and CA. There is limited data describing the pattern of bile acids in IBD; however, Duboc et.al. (2013) used LC-MS/MS technology to evaluate plasma and fecal bile acids in IBD subjects as well as healthy controls. They detected fundamental differences in fecal bile acids and linked these apparent disease-dependent changes to alterations in the gut microbial profile\textsuperscript{227}. Similarly, Gnewuch et.al. (2009) described differences in the ratio of primary to secondary bile acids using LC-MS/MS in subjects with UC versus non-IBD
controls. Authors hypothesized that this finding was likely due to described IBD-dependent differences in the gut microbial profile leading to differential de-hydroxylation of primary bile acids. Differences in bile acid patterns may be an underappreciated determinant of NR signaling in CD; however, this has not been yet explored.

1.3.5 Nuclear Receptors and a Role in IBD Pathogenesis

The role of NRs in CD and UC pathogenesis and, as a consequence, therapeutics has come under intense scrutiny within the last two decades. This is unsurprising given their pervasive role as master regulators of a number of biological processes. As discussed in earlier sections, NRs are important to drug metabolism sequences, specifically to the regulation of CYP3A4-mediated metabolism. In the following section, the current evidence for NR involvement in IBD susceptibility will be presented.

1.3.5.1 The Role of PXR in IBD:

PXR is down-regulated in the setting of CD and UC, independent of active inflammation. This finding was revealed in 2004, when Langmann et al. demonstrated a concomitant reduction in the intestinal mRNA expression of PXR and its downstream targets such as UGT, GST, and MDR1, key genes involved in the detoxification and processing of xenobiotics in subjects with CD and UC. Moreover, using three distinct intestinal model cell lines, they showed that TNF-α exposure blocked the PXR-mediated expression of CYP3A4 and MDR1 in LS174T cells. A subsequent study by Shah et al.
(2007) highlighted a protective role for PXR in the setting of an inflammatory insult. Ligand-induced activation of PXR in a DSS-mouse model of colitis showed symptomatic and histological improvement in the study animals\textsuperscript{211}. Though without established certainty, PXR-mediated amelioration of colitis has been hypothesized to occur through several different pathways including: the up-regulation of efflux transporter proteins such as P-gp or the suppression of the NFκB pathway.

As previously discussed, PXR is important to xenobiotic metabolism and up-regulates the expression of several exporter proteins\textsuperscript{203, 210}. Intestinal efflux transporters such as P-gp contribute to the integrity of the epithelial barrier by increasing the export of toxic environmental substances and bacterial toxins from within the enterocyte\textsuperscript{210}. This impedes the exposure of the host immune system to "foreign" antigen and reduces the risk of activating the host inflammatory cascade. A correlation between intestinal inflammation and a reduction in P-gp expression has been shown in a cohort of individuals with inflammatory gastrointestinal diseases including IBD\textsuperscript{142}.

Secondly, PXR-mediated suppression of NFκB proteins and in turn its target genes has been convincingly demonstrated in mouse models of colitis\textsuperscript{211, 229}. Shah et al. (2007) were able to show that the administration of the PXR ligand, (PCN) caused a significant reduction in the colonic mRNA expression of NFκB target genes, TNF-α, iNOS, IL-1β, IL-10. This was confirmed in \textit{in vitro} studies using HCT116 colon cancer cells\textsuperscript{211}. 
1.3.5.2 Genetic variation in PXR and its Influence on IBD

There is much interest in, not only the role of PXR in IBD pathogenesis, but in the link between IBD susceptibility and genetic variation in PXR (NR1I2). To date, no consensus has been reached regarding variations in the PXR gene and their association with IBD susceptibility. Of the existing studies, results remain conflicting. In 2006, Dring et.al. reported that the SNPs, -23585 and -24381 in PXR were predictive of susceptibility to both UC, CD and IBD overall in an Irish cohort of 422 IBD patients and 350 matched controls. They hypothesized that these SNPs, which have also been associated with a decrease in CYP3A4 activity, confirm a PXR-mediated contribution to the pathogenesis of IBD.

However, in the same year, Ho et.al. (2006) carried out a similar study in a Scottish cohort of 725 IBD patients and 328 controls and refuted the findings of the Irish study. They were unable to show an association between 5 different SNPs in PXR or in a variety of constructed haplotypes. They concluded that the positive findings of the study by Dring et.al. (2006) were reflective of genetic heterozygosity amongst controls, unclear population stratification and type 1 error.

In 2007, Martinez et.al. were able to reproduce the data reported in the study by Dring et.al. (2006) to a limited extent in a Spanish cohort of 696 IBD patients and 550 controls. They reported that the SNP, -23585, while not more common in IBD overall, was found more frequently in the subphenotype of UC-pancolitis. Carriers of the T/T genotype also had a different distribution of P-gp compared to non-carriers.

Subsequently, Glas et.al. (2011) carried out the largest genetic analysis in NR1/2 in a cohort of over 2800 individuals, over 1300 of which had IBD. They analyzed 8
SNPs, including -23585 and 24381, as well as several haplotypes. They found a weak association between the SNP 8055 and UC and more notably a haplotype consisting of multiple SNPs, including -23585 and -24381 that was associated with CD susceptibility in a sub-cohort of patients. They concluded that further investigation is needed to clearly define the relationship between PXR variation and the functional role of PXR in IBD.

1.3.5.3 PXR as a Target for IBD Therapy: Rifaximin Studies In Vivo

The activation of PXR activation by a variety of ligands has been shown to be an effective treatment of inflammation in mouse models of colitis. These studies have also demonstrated that the beneficial effects are mediated through the down-regulation of NFkB target genes. Studies by Cheng et al. (2010) and Ma et al. (2007) used PXR-humanized and PXR null mice to show the protective effect that PXR agonists, such as the rifamycins, have on inflammatory changes in the intestine induced in a DSS model of IBD. The rifamycin antibiotics include rifaximin and rifampicin, with the former stimulating intestinal PXR target genes alone due to its poor absorbability and low systemic bioavailability and the latter stimulating both intestinal and hepatic PXR target genes. Some success in CD and UC patients has been seen in short, open-label trials involving small cohorts of patients as well as in slightly larger blinded and randomized studies. The largest study to date in CD (n=402) showed that at the end of a 12-week treatment period, 62% of CD patients who received an 800-mg dose of extended release rifaximin were in remission, compared with 43% of patients who received placebo, a result that reached statistical significance. To date, this has not been applied to clinical practice.
1.3.5.4 The Role of FXR in IBD

It has been shown that bile acid-induced activation of FXR plays a role in regulating several genes that protect against intestinal inflammation, increased intestinal permeability and bacterial overgrowth \(^8, 244, 245\). Limited data suggest that FXR activity is repressed in CD\(^1\)\(^\text{80}, 246, 247\). While, FXR deficiency in mice and humans is not associated with the development of a spontaneous colitis, FXR deficiency appears to portend a more severe disease phenotype in the setting of a primary insult\(^244, 248\). These concepts will be discussed herein.

Researchers have highlighted that FXR-regulated pathways are important to intestinal integrity. Raimondi *et.al.* (2008) demonstrated that bile acids modulate intestinal paracellular permeability: CA, chenodeoxycholic acid (CDCA), DCA increased intestinal permeability, DCA- and CDCA- induced phosphorylation of the epidermal growth factor receptor, occludin dephosphorylation, and occludin redistribution, all key components of intestinal permeability\(^249\). Stojancevic *et.al.* (2012) and Luettig *et.al.* (2015) showed FXR regulates other proteins, such as keratin-13 and claudin-1 and claudin-2 involved in maintenance of the intestinal barrier\(^250, 251\).

Vavassori *et.al.* (2009) investigated the role of FXR activation in the regulation of inflammatory responses in a murine model of colitis\(^244\). At baseline, it was noted that increased cellular infiltrate and collagen deposition as well as increased expression of inflammatory genes (TNF\(\alpha\), IFN\(\gamma\), IL-1\(\beta\)) were seen in *FXR*-null mice in comparison to wild type mice. Acute and chronic colitis were then induced in wild type and *FXR*-null mice using rectal administration of trinitrobenzenesulfonic acid (TNBS) in 40% ethanol.
Colitis was prevented in 60% of the wild type mice receiving synthetic chenodeoxycholic acid (INT-747) and a reduction in the transcription of pro-inflammatory genes was seen. No effect was seen in FXR-null mice, demonstrating the role of FXR in the intestinal immune response.

A complimentary study by Gadaleta et al. (2011), linked FXR agonism to improvements in intestinal permeability in vivo and in vitro. It was further demonstrated that FXR agonism counteracts proinflammatory cytokine expression and secretion by enterocytes. In wild type mice, INT747 treatment induced FXR target genes in both ileum (small heterodimer partner, SHP) and colon (Fgf15). FXR null-mice expressed very low levels of SHP and Fgf15 at baseline and with INT747 administration. In wild type mice treated with 2.5% DSS, INT747 reduced wt loss, improved rectal bleeding scores, and prevented colonic shortening. At baseline, chemically-induced colitis was associated with complete disruption of the epithelial layer and acute inflammatory infiltrates in wild type and FXR-null mice. INT747-treated wild type mice showed less intestinal morphological alteration and decreased inflammatory infiltrates and less goblet cell loss. INT747 had no effect in FXR-null mice. Measures of increased permeability were markedly elevated in wild type and FXR-null mice after induction of colitis, though this was abolished in INT747-treated wild type mice. Furthermore, in DSS-treated wild type mice, INT747 significantly decreased colonic mRNA expression of pro-inflammatory genes IL-1β, IL-6 and macrophage attractant protein (Mcp)-1. Also, antimicrobial proteins, iNOS and cathelicidin in the colon and angiogenin 1 (Ang1) in the ileum, were significantly induced by INT747 in wild type but not FXR-null mice. It was concluded that FXR normalizes chemically-induced pro-inflammatory gene expression.
and reduces inflammatory infiltrates in the intestine. It also induces the expression of several antibacterial defense genes.

Moreover, Nijmeijer et al. (2011) evaluated the converse: is FXR activation repressed in the setting of chronic colitis? mRNA expression of FXR and its target gene, SHP were analyzed in ileal and colonic samples taken from IBD and healthy subjects. FXR activation in the ileum was found to be reduced in subjects with CD colitis, while no difference was seen in subjects with UC versus healthy subjects. Subsequent studies by Lenicek et al. (2011) and Iwamoto et al. (2013) support the findings of Nijmeijer et al. (2011) using the FXR activity surrogate marker, FGF19.

Ultimately, these studies highlight the potential importance of FXR to IBD pathogenesis. FXR activation contributes to the maintenance of intestinal epithelial integrity as well as down-regulates the expression of certain pro-inflammatory genes. This correlates with reduced intestinal inflammation in mouse models of colitis and human subjects with IBD.

1.3.5.5 Genetic Variation in FXR and its Influence on IBD

Furthermore, it should be mentioned that genetic variation in FXR has been linked to IBD pathogenesis. A study by Attinkara et al. (2012) evaluated the association between IBD and 5 variants (2 common, 3 rare) of the FXR (NR1H4) gene. They found that one of the rare variants (rs3863377) was less common in the IBD population versus the healthy controls. It was proposed that this variant confers a protective effect.
against the disease. The rare variant, FXR-1G>T (rs56163822) was more prevalent in the IBD group. Our group demonstrated that this variant is associated with reduced activation of downstream FXR gene targets\textsuperscript{252}. Nijmeijer \textit{et.al.} (2011) did not find any \textit{FXR} polymorphisms associated with the CD or UC susceptibility\textsuperscript{246}. Genetic variation in \textit{FXR} and the link to UC and CD remains poorly defined.

\subsection*{1.3.5.6 \textit{FXR} as a Target for IBD Therapy}

Success has been seen with the use of obeticholic acid (INT-747), a semi-synthetic and potent ligand of FXR in chronic liver diseases. A recent randomized controlled study in the New England Journal of Medicine revealed the utility of obeticholic acid for patients with primary biliary cholangitis (PBC)\textsuperscript{253}. Phase 2 trials in diabetic subjects with NAFLD have shown promise with reduced liver inflammation and fibrosis seen over the short term\textsuperscript{254}. \textit{In vitro} and \textit{in vivo} models of FXR agonism demonstrated down-regulation of stellate cell activity with reduced collagen deposition and up-regulation of SHP\textsuperscript{255,256}. As stated above, INT-747 has also been used with success in animal and \textit{in vitro} models of colitis\textsuperscript{8,244}. This has not been evaluated in human subjects with CD or UC.

\section*{1.4 Summary}

CD is an important disease due to its high prevalence, debilitating symptoms, associated complications and incurability. Patients are exposed to a high burden of
medications in addition to other treatments they may be receiving for co-morbid illnesses. CD pathogenesis is complex and multi-factorial with overlapping roles for the immune system, the human genome and the environment in the onset of disease. Though substantial advances have been made in the last two decades regarding our understanding of its pathophysiology, we are still unable to prevent its onset, predict those at imminent risk or select the most efficacious treatment for affected individuals. Disease severity based on patient demographic data or previous disease behavior often dictates the most appropriate therapy, but does not necessarily predict drug response. Small and large molecule drugs have found varying degrees of success in the management of CD; however, one of the unfortunate, overarching themes in CD therapeutics is a lack of inter-individual predictability of drug exposure and drug response.

Significant overlap is seen in CD pathogenesis and drug metabolism pathways. CYP3A4-mediated metabolism is one of the most important systems for the processing of xenobiotics including human drugs. Its activity is highly variable between individuals and, in the setting of other acute and chronic inflammatory illnesses, may be associated with additional variation in function. Its intestinal location and PXR- and FXR-mediated regulation make its expression and activity plausible targets for CD-induced changes; however, CD-related alterations in drug metabolism, as a whole, are largely unexplored. Experts in the field recognize that our inability to predict drug response in both CD and UC and to personalize therapies for patients is a huge deficiency in a field dependent on long-term drug use. Furthermore, CD-related changes in CYP3A4 activity may have important consequences for additional treatments patients receive for non-CD-related
diseases. Ultimately, a better and more complete understanding of drug metabolism sequences in CD is needed.
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2  HYPOTHESES & AIMS
2.1 Hypothesis

The interplay between genes, nuclear receptors (NR), cellular transporters, metabolizing enzymes and the gut microbial profile affect drug metabolism pathways and ultimately drug disposition in humans. The cytochrome P450 (CYP) family in general and CYP3A4 specifically play a principal role in an individual's systemic exposure to a drug. CD is a primary intestinal illness where variation in the aforementioned factors forms the basis of a still-incompletely defined pathophysiology.

We believe the presence of CD will negatively affect CYP3A4 expression and activity in such patients and that genetic variation in key NRs, such as the farnesoid X receptor (FXR) regulating CYP3A4 may have important consequences for disease severity and are an indirect determinant of drug response.

2.2 Aim 1:

a) To assess the impact of CD on the activity of intestinal and hepatic CYP3A4 using exogenous and endogenous in vivo probes.

b) To propose a molecular mechanism by which detectable changes in CD-specific CYP3A4 activity occur.

The activity of the enzyme, CYP3A4, is subject to significant inter-individual variation. Diet, concomitant drug exposures, age, sex, genetic variation, and NR regulation are a short, but incomplete, list of factors that may alter its activity\textsuperscript{1-6}. Acute
and, to a certain extent, chronic inflammatory states appear to affect CYP3A4 activity\textsuperscript{7,8}. Any changes in the activity of CYP3A4, a key enzyme involved in phase 1 drug metabolism, are thought to have important consequences for the disposition of substrates \textit{in vivo}. Little is known regarding the \textit{in vivo} influence of CD on this protein. Given that the intestine, in addition to the liver, is the site of highest expression of CYP3A4, one could assume a primary, inflammatory intestinal illness may have a substantial impact on CYP3A4 activity.

We hypothesize that CD negatively impacts CYP3A4 activity \textit{in vivo}, specifically in the intestinal compartment and such changes are due to a CD-specific effect on the activity of the NR, pregnane X receptor (PXR).

2.3 Aim 2:

To evaluate the impact of CD on the intestinal expression of CYP3A4 compared to a healthy population.

A hallmark of CD is disruption of the intestinal mucosal layer\textsuperscript{9,10}. The intestinal mucosa consists of the following: the epithelium, made up of enterocytes as well as goblet, paneth and neuroendocrine cells; the lamina propria, home to a variety of immune cells; and the muscularis mucosae\textsuperscript{11,12}. The onset of CD induces disruption of the epithelial cell tight junctions, epithelial cell apoptosis, expansion of the lamina propria with pro-inflammatory cells and recruitment of pro-fibrotic fibroblasts\textsuperscript{13-15}. As previously discussed, CYP3A4 is predominantly expressed in the liver and intestine, with
the highest expression of intestinal CYP3A4 on the enterocytes found at the villous tip of the small intestine\textsuperscript{16}, a prime target of the inflammatory lesions of CD. It likely acts in concert with the enterocyte-bound xenobiotic exporter P-glycoprotein (P-gp) to limit the oral bioavailability of several shared substrates\textsuperscript{17}.

Thus, we hypothesize that individuals with CD have reduced intestinal expression of CYP3A4.

2.4 AIM 3:

To explore the role of genetic variation in FXR, a key regulator of CYP3A4 metabolism, and its impact on CD severity, a surrogate marker of drug metabolism and response.

Genetic variation in key CYP3A4 transcriptional regulators, such as FXR and PXR may have important consequences for the function of the NR protein product and ultimately CYP3A4 expression and activity. Defects in key functions carried out by FXR are closely linked to CD pathophysiology and genetic variation may further impair these pathways and portend a more severe CD phenotype\textsuperscript{18-20}.

\textbf{We hypothesize that the }\textit{FXR -tg> T }\textbf{polymorphism confers a greater risk of severe CD phenotype and is associated with a reduction in the downstream FXR target, fibroblast growth factor (FGF) 19 and an expansion of the total bile acid pool.}
2.5 REFERENCES:


3 EVALUATION OF CYP3A4 ACTIVITY IN CROHN'S DISEASE

A version of this chapter has been published. It is reprinted with permission from Wilson A, Tirona RG, Kim RB. CYP3A4 Activity is Markedly Lower in Patients with Crohn's Disease. Inflammatory Bowel Diseases. 2017; 23(5):804-813.
3.1 Introduction

Inflammatory bowel disease (IBD) is a disease of chronic intestinal inflammation punctuated by episodes of active disease and quiescent remission. IBD can be differentiated into ulcerative colitis (UC) and Crohn's disease (CD), which can follow markedly different clinical courses: UC affects the mucosal layer of the large intestine while CD causes transmural inflammatory lesions that may affect any part of the intestinal tract. The treatment of IBD focuses on effecting and maintaining remission at the level of the intestine as well as inducing symptomatic response. CD therapeutic options have significantly improved over the past decade. Immunosuppressants such as azathioprine, methotrexate and glucocorticoids as well as biologic treatments such as infliximab, adalimumab, vedolizumab and ustekinumab are important therapies for the optimal treatment of CD.

The Cytochrome P450 (CYP) 3A subfamily of CYP enzymes is a key mediator of drug metabolism and disposition in humans. Three functional enzymes have been indentified: CYP3A4, CYP3A5 and CYP3A7. CYP3A4 is thought to be involved in the disposition of approximately 40-50% of drugs in clinical use, including many used to treat CD or UC. Specifically, CYP3A4 has been shown capable of metabolizing glucocorticoids used in CD, such as prednisone and budesonide. Based on limited budesonide pharmacokinetic (Pk) data from patients with CD as well as Pk data from healthy populations demonstrates that budesonide exposure after oral administration is highly variable. This variability may be due to inter-individual differences in total CYP3A4 activity and may account for differences seen in clinical outcomes amongst CD patients treated with budesonide.
CYP3A4 is the most thoroughly investigated of the CYP3A isoforms. CYP3A4 is expressed predominantly in the liver and intestinal tract. In the intestinal tract, CYP3A4 is found at the villous tip of individual enterocytes and aids in the catabolism of specific drug substrates. The expression of CYP3A4 mRNA decreases longitudinally along the intestinal tract, from small intestine to colon, with the highest expression in the jejunum and ileum. The interplay between intestinal CYP3A4 and intestinally-expressed importers and exporters is considered to be of particular relevance to intestinal drug metabolism and transport and to significantly impact drug exposure and response.

Intestinal CYP3A4 may act in concert with other transporters, such as P-glycoprotein (P-gp), and play a key role in first-pass metabolism in addition to the role played by hepatic CYP3A4. P-gp bound to the cell membrane of enterocytes may regulate the exposure of specific drugs to metabolism by CYP3A4 via an active "counter transport" mechanism. The influence of diseases such as infection and more chronic conditions such as cancer, organ transplantation and liver disease on in vivo CYP3A4 activity have been well-documented in the literature. Interestingly, to our knowledge, in vivo human CYP3A4 activity has only been reported in one other study in the setting of IBD. This study limited its evaluation of CYP3A4 activity to single-point determination of 4β-hydroxycholesterol (4βOHC) plasma concentrations.

Moreover, the mechanisms by which disease-dependent changes in CYP3A4 activity occur are not clearly defined. One existing hypothesis is that circulating inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α or interferon (IFN)-γ interfere with the transcription of CYP3A4 mRNA; however, data are limited to animal models and results have been conflicting. Interestingly, key
regulators of CYP3A4 activity, the nuclear receptors (NR), farnesoid X receptor (FXR) and pregnane X receptor (PXR) are both down-regulated in IBD and are thought to contribute to the pathogenesis of the disease<sup>21, 23, 24</sup>. Bile acids are important endogenous ligands of PXR and FXR, though not all bile acids are created equal<sup>25, 26</sup>. Secondary bile acids, lithocholic acid (LCA) and deoxycholic acid (DCA) are potent activators of PXR and the primary bile acid, chenodeoxycholic acid (CDCA) is a potent activator of FXR. The composition of the bile acid pool is highly dependent on the gut microbial profile<sup>27, 28</sup>. Studies have demonstrated a loss of microbial diversity in the intestinal tract of those with IBD, particularly amongst the Firmicutes and Bacteroidetes phyla, key phyla involved in the processing of bile acids (deconjugation, dehydroxylation)<sup>27-29</sup>; thus, one could surmise that changes in NR activation due to changes in the bile acid pool may result in differential CYP3A4 activity.

Accordingly, we sought to assess the <i>in vivo</i> activity of CYP3A4 in CD using well-known <i>in vivo</i> probes midazolam<sup>30</sup> and 4βOHC<sup>31</sup> and to investigate the role of differential PXR activation on CYP3A4 activity <i>in vitro</i>. 
3.2 Materials & Methods

3.2.1 All Subjects

This study was conducted in two parts: in the first part, a detailed Pk analysis of oral and intravenous midazolam metabolism was carried out in 8 patients with CD to evaluate the \textit{in vivo} activity of CYP3A4 in CD. In the second part, a cross sectional study in an independent cohort of patients with CD (n=74) and non-CD controls (n=74) was carried out to evaluate the \textit{in vivo} activity of CYP3A4 using the endogenous biomarker 4βOHC in plasma. Written, informed consent was obtained from each patient. Both parts of the study received approval by the Western University Health Sciences Research Ethics Board (104914, 105930).

3.2.1.1 Subjects Included in the Pharmacokinetic Study

Patient recruitment took place from March 2014 to September 2014. Eligible subjects were 18 years of age or older with a history of CD who were starting on or currently taking the oral medication, budesonide. Subjects receiving concurrent medical treatment for their CD (n= 8) were eligible for this study. Subjects were excluded from the study for the following reasons: having a hypersensitivity to budesonide; having a hypersensitivity to midazolam; having a serum creatinine concentration greater than 1.7 mg per deciliter; being pregnant or breastfeeding; having a pre-existing liver disease (examples include primary sclerosing cholangitis, autoimmune hepatitis, alcoholic liver disease, viral hepatitis, cirrhosis); taking other, concurrent benzodiazepines; being
unwilling to comply with the study protocol; having an estimated survival of less than one year; abusing or misusing alcohol; being an active smoker; receiving on-going therapy or treatment in the last 3 months with drugs known to be potent CYP3A inhibitors or inducers. These are as follows: macrolide antibiotics, ‘azole’ antifungals, nefazodone, verapamil, diltiazem, cyclosporine A, antiretrovirals, carbamazepine, phenytoin, lamotrigine, rifampin, rifabutin, dexamethasone, St. John’s Wort, bosentan. Subjects were screened for eligibility 4 weeks prior to enrollment. Body weight and routine blood tests (blood count, biochemistries, liver enzymes and creatinine) were measured for each patient. All female subjects were screened for pregnancy with a urine pregnancy test. At baseline, demographic data, including age, sex, year of CD diagnosis, disease distribution, and current medications were collected from each patient. Clinical parameters to assess for disease activity (Harvey-Bradshaw Index), and subjective response to budesonide, including efficacy and toxicity were noted.

Subjects were instructed to avoid citrus fruit products for one week prior to their study day due to the inhibitory effect on CYP3A4 activity. They were also instructed to avoid alcohol or caffeine one day prior to their study day. Subjects were instructed to take nothing by mouth the morning of their study day. At the commencement of each study day (time 0), subjects were administered an oral dose of budesonide (3-9mg). The dose of budesonide was chosen based on the standard practice of care carried out at London Health Sciences Centre (LHSC) for inducing remission in individuals with CD. Subjects were also given a prepared 100µg dose of oral midazolam. At the 8-hour time interval, subjects were given a prepared 50µg dose of intravenous (IV) midazolam. The dose of oral and IV midazolam were selected based on previous studies conducted to
assess the in vivo activity of CYP3A4 and P-gp.\textsuperscript{30, 32, 33} Blood samples were collected from each subject at 19 pre-specified time points measured in hours (0, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 8.08, 8.25, 8.5, 9, 9.5, 10, 11, 12). Samples were spun down using a centrifuge and plasma was extracted and stored at -80°C.

3.2.1.2 Subjects Included in the Cross Sectional Study

A cross sectional cohort was derived from patients being seen as part of the Personalized Medicine Program at Western University, London, Canada as well as healthy individuals being seen for colorectal screening consultation between March 2013 and March 2017. Each subject provided two blood samples for analysis. Subjects from the CD arm had a histopathological diagnosis. CD subjects were excluded if there was missing information pertaining to their CD medical history. Individuals were included in the control arm if there was no history of cardiovascular, neurological, pulmonary, renal, hepatic, malignant or auto-immune disease. Controls were required to be never-smokers, to consume less than 5 alcoholic beverages weekly and to have had no prior history of surgical intervention pertaining to the cardiovascular, neurological, pulmonary, renal, hepatic, or intestinal systems. All subjects were required to be 18 years of age or older and to not be taking a concomitant CYP3A4 agonist or antagonist (macrolide antibiotics, ‘azole’ antifungals, nefazodone, verapamil, diltiazem, cyclosporine A, antiretrovirals, carbamazepine, phenytoin, lamotrigine, rifampin, rifabutin, dexamethasone, St. John’s Wort, bosentan).
Data collected from the CD cohort included age, weight, sex, smoking history, CD phenotype, activity (assessed by the HBI\textsuperscript{34}), disease duration, medication use and history of surgical resections or hospitalizations. Data collected from the control cohort included age, weight, sex, smoking history and alcohol consumption, medical and/or surgical history and any prescription or non-prescription drug use.

3.2.2 Clinical Study Objectives and Outcomes:

The objective of this study was to test the hypothesis that CYP3A4 activity is decreased in CD. The primary endpoint was the midazolam AUC\textsubscript{0-∞} in individuals with CD compared to reported healthy cohorts. Secondary outcomes included the intestinal and hepatic extraction ratios for midazolam as well as other Pk parameters outlined in the following section. Pk parameters were also assessed for budesonide and the contribution of CYP3A4 to inter-individual differences in budesonide AUC\textsubscript{0-∞} was evaluated. Other endpoints included the 4βOHC plasma concentrations in an independent of cohort of CD patients compared to non-CD controls as a means of confirming or refuting the primary outcome. Any detectable differences in the bile acid pools of the controls versus the CD population were also assessed.

3.2.3 Pharmacokinetic Analysis

Pk analysis was performed by the non-compartmental method. Pk parameters were calculated based on its concentration-time curve. Collected parameters included the
following: maximum plasma drug concentration ($C_{\text{max}}$); time to the maximum plasma drug concentration ($t_{\text{max}}$); total drug exposure estimated by the area of under the plasma concentration-time curve ($\text{AUC}_{0-\infty}$); the drug half-life ($t_{1/2}$); oral bioavailability ($F$); systemic ($\text{CL}_s$) and oral drug clearance ($\text{CL}/F$). Estimates of CYP3A4-mediated hepatic and intestinal extraction ratios were calculated.

$C_{\text{max}}$ and $t_{\text{max}}$ were obtained by visual inspection. The $\text{AUC}_{0-\infty}$ for each patient was calculated using the trapezoidal rule (numerical integration) to the last measured time point and extrapolated to infinity using the terminal elimination rate constant ($k_e$), estimated by log-linear regression of last 4-6 data points on the concentration-time curve. The $t_{1/2}$ was obtained by dividing the constant 0.693 by $k_e$. Oral clearance was calculated as the ratio of the oral dose divided by the oral $\text{AUC}_{0-\infty}$. $F$ was calculated by dividing the product of the oral $\text{AUC}_{0-\infty}$ and IV dose by the product of the IV $\text{AUC}_{0-\infty}$ and oral dose. CYP3A4-mediated extraction in the liver and intestine were estimated by the hepatic and gut extraction ratios ($E_h$ and $E_{gi}$), where $E_h = \text{CL}_s/Q_{h, \text{plasma}}$ and $E_{gi} = 1-[F/(1-E_h)]$. $Q_{h, \text{plasma}}$ represented the liver plasma flow and was equal to $(1-\text{hematocrit}) \times Q_{h, \text{blood}}$. $Q_{h, \text{blood}}$ or liver blood flow was assumed to be 25.7 ml/min per kg of body weight.

### 3.2.4 Quantification of Midazolam Plasma Concentrations

Midazolam plasma concentrations were determined by LC-MS/MS using a previously described method by Gong et al. (2012) Supplemental Data\textsuperscript{32}. The lowest limit of quantification was 0.025 ng/mL.
3.2.5 Quantification of Budesonide Plasma Concentrations

For measuring budesonide, a five hundred microlitre plasma sample was acidified using 50µl 4% formic acid (diluted in water) containing the internal standard, d6-budesonide (10 µl, 500ng/ml in methanol; Toronto Research Chemicals, Toronto, ON). Standards made with drug free plasma were similarly spiked with budesonide internal standard as described above. Protein was precipitated from samples and standards with the addition of 500 µl of 30% ethanol in water and incubation at 4°C for 15 minutes. Precipitated proteins were pelleted by centrifugation at 14000g at 4°C for 10 minutes. Supernatants were applied to OASIS HLB 96-well plates (5mg/well, 30 µm particle size, Waters, Milford, MA) pre-treated with 1ml of methanol and water each. Wells were washed with 1ml of 20% methanol and eluted using 0.5ml of 100% methanol. The eluate was dried with heat (70°C) and reconstituted using the mobile phase (150µl of acetonitrile/5mM ammonium acetate in water [25%/75%]). One hundred microlitres of sample was injected into the liquid chromatograph (Agilent 1200, San Jose, CA).

Analytes were separated using reverse phase chromatography on a Thermo Hypersil Gold C18 Column (50 x 5mm; 5µm) using gradient elution with Mobile Phase A (5mM ammonium acetate in water) and Mobile B phase (acetonitrile) over minutes: 1 minute of 75:25 (A:B), from 75:25 to 20:80 (A:B) over 5 minutes, back to 25:75 (A:B) over 1 minute then held for 1 minute. The retention time for budesonide and d6-budesonide is 4.5 minutes.

Mass detection occurred on a Thermo TSQ Vantage triple quadrupole mass spectrometer, set in positive mode, with a quantitative and qualitative mass transition
measured for budesonide (431.2 →147.1 m/z). The standard curve was linear over 0 to 10ng/ml concentration range.

3.2.6 Quantification of 4β-hydroxycholesterol Plasma Concentrations

Plasma concentrations of 4βOHC were determined by LC-MS/MS following sterol isolation by saponification and enhancement of the analyte product by picolinic acid derivatization as described by Honda et.al. (2009)\textsuperscript{35} and Woolsey et.al. (2016)\textsuperscript{36}. A 4βOHC-standard curve from 0 to 200ng/ml was created in Krebs-Henseleit Bicarbonate buffer. Fifty microlitres of subject plasma and 100µl of standard were spiked with 1µl of 1mg/ml internal standard (d7-4βOHC) obtained from Avanti Polar Lipids (Alabaster, Alabama). Each aliquot was saponified in 500µl of 1M ethanolic potassium hydroxide at 37°C for one hour followed by the addition of 150µl of water. Sterols were twice extracted into hexane and further isolated by the evaporation of the hexane layers to dryness. Following this, 250µl of a derivatization mixture (2-methyl-6-nitrobenzoic acid, 4-dimethylaminopyridine, picolinic acid, pyridine, triethylamine) was added to each sterol extract and incubated at 80°C for one hour. Hexane (1ml) was added to each aliquot and, following centrifugation at 14,000 rpm, the supernatant was collected and evaporated to dryness at 80°C. Following re-constitution in acetonitrile and sodium chloride, a 20µl-aliquot was injected into the LC-MS/MS system. An Agilent C18 Zorbax Eclipse Plus column (100 x 2.1mm, 1.8µm) was used with mobile phases of 0.1% formic acid in water and 50:50 acetonitrile in methanol with 0.1% formic acid. The retention times for d7-4βOHC and 4βOHC were 8 minutes and 8.1 minutes respectively.
with each analyte detected in positive mode (mass transitions of 642.4>146.5m/z, d7-4βOHC and 635.4>146.5m/z, 4βOHC). The lower limit of detection of plasma 4βOHC was 2.5ng/ml.

3.2.7 Quantification of Bile Acid Plasma Concentrations

Plasma stored at -80°C was used for quantification of 12 bile acids (cholic acid, CA; chenodeoxycholic acid, CDCA; deoxycholic acid, DCA; glycocholic acid, GCA; glycochenodeoxycholic acid, GCDCA; glycodeoxycholic acid, GDCA; lithocholic acid, LCA; taurocholic acid, TCA; taurodeoxycholic acid, TDCA; taurolithocholic acid, TLCA; tauroursodeoxycholic acid, TUDCA; and ursodeoxycholic acid, UDCA) using high performance liquid chromatography/mass spectrometry (LC-MS/MS). Taurocholic acid-d5 (TCA-D5) was used as an internal standard (I.S). A standard curve of 1nM to 40µM was generated. Patient samples were homogenized by vortexing for 15 minutes at 4 °C @1400 RPM and incubated at -20°C for 20 minutes. Samples were centrifuged at maximum speed at 4°C for 30 minutes. Finally, 100µL of supernatant was transferred to vials and 80µL was injected into the LC-MS/MS system.

A two-dimensional LC system was used (Agilent HILIC plus column (4.6*50) 3.5µm followed by Phenomenex 00B-4462-Y0 Kinetex 2.6u C18 100A (3.0*50)). A gradient elution technique was employed using the Agilent 1290 system. Mobile phase A consisted of 2mM ammonium acetate (pH 4.15) with 10% acetonitrile. Mobile phase B consisted of acetonitrile: isopropyl-alcohol with water. The columns' temperatures were maintained at 60°C. Samples were eluted at a flow rate of 0.6ml/min to 1.4ml/min (0.1-
12 minutes). Before injection of the subsequent sample, columns were cleaned with 100% mobile phase B for one minute and returned to a linear equilibrium for 7 minutes for a total run of 20 minutes for each sample. Mass detection occurred on a TSQ-Quantum Ultra mass spectrometer equipped with HESI source and operated in negative mode (4500 v spray voltage, 350°C vaporizing temperature, 45 sheath gas pressure, 15 auxiliary gas pressure and 350 °C capillary temperature).

3.2.8 *In vitro* CYP3A4 reporter activity

Human hepatocarcinoma (HepG2) cells, obtained from Cedarlane (Burlington, Ontario) were cultured in DMEM (VWR, Radnor, Pennsylvania) supplemented with 10% fetal bovine serum, penicillin and streptomycin. Cell viability in the setting of bile acid exposure was assessed using the CellTiter-Glo Luminescent Cell Viability Assay purchased from Promega Corporation (Madison, Wisconsin) at serial concentrations and time points. Cells were then cultured in 12-well plates to 80% confluency in 3 days. DNA was transiently transfected by lipofection method. The ratio of plasmid used was 0.5µg of CYP3A4-XREM-Luc, 0.5µg of hPXR-pEF. Additionally, 0.5µg of renilla plasmid (pRL-CMV, Promega, Madison Wisconsin, USA) was co-transfected as an internal standard of transfection efficiency. Plasmids, CYP3A4-XREM-Luc and hPXR-pEF were previously prepared as described by Tirona *et al.* (2003)\(^37\). A corresponding "control" model was generated, with 0.5µg of pGL3 basic (Promega, Madison Wisconsin, USA) and pEF (Invitrogen, Carlsbad California, USA) control vectors and 0.5 µg of pRL-CMV as a transfection a control. Cells were treated with one of the
following: a pool of bile acids (25µM) representative of CD disease states (active CD, inactive CD) as well as that of a healthy individual; 0.1% DMSO in Optimem; or 10µl of one of GDCA, DCA or LCA. Cohort-specific bile acid pools were derived from subject data presented in Table 3.7 and 3.8. Cells were incubated for 24 hours, washed with phosphate buffered saline and lysed with passive lysis buffer. Using the Promega Corporation Dual Luciferase Reporter Assay System (Madison, Wisconsin USA), luciferase activity was measured by Glomax 20/20 Luminometer (Promega, Madison Wisconsin, USA) and normalized by dividing the relative light units by the Renilla luciferase activity (firefly:renilla luciferase ratio, F/R ratio). The fold-change in luciferase activity was then estimated by dividing the F/R ratio for each cell group by the control (pGL3 basic or pEF control vector). Each experiment was performed in triplicate and repeated 2-3 times.

3.2.9 Statistical Analyses

All statistical analyses were carried out in Graphpad Prism and SPSS. For the midazolam Pk analysis, data are presented as the arithmetic mean with standard deviations. Linear regression analysis was used to assess the possible relationship between the Pk values of budesonide and midazolam. A one-way ANOVA with Student's t-test and Bonferroni post hoc test were used to compare 4βOHC plasma concentrations between CD (active, inactive disease, n=74) and control groups (n=74). A p-value ≤0.05 was considered significant. A multiple linear regression analysis was used to further evaluate the relationship between presence or absence of CD, other covariates
and the inter-individual variation in 4βOHC plasma concentrations (natural log-transformed). Other covariates assessed included the following: sex, age, weight, and disease activity. Mean plasma concentrations for the 12 aforementioned bile acids were compared between the control group and the CD group (sub-divided by disease activity) using a Student's t-test. A p-value ≤0.05 was considered significant. Similarly, for the in vitro experiments, a one-way ANOVA with Student's t-test identified any significant differences between cell groups. A p-value ≤0.05 was considered significant.
3.3 Results

3.3.1 Subjects

Seven females and one male completed the midazolam Pk study protocol (n=8). The demographic data and clinical characteristics for each subject are presented in Table 3.1. There were no adverse events. All subjects were on budesonide therapy for a minimum of 1 month prior to entry into the study. All subjects had not received treatment with a CYP3A4-inhibiting or -inducing drug in at least 1 year (with the exception of budesonide). Seventy-four subjects with CD and 71 controls were included in an independent cohort to evaluate in vivo CYP3A4 activity using the endogenous probe, 4βOHC. Table 3.2 summarizes the demographic data as well as the clinical parameters for both populations.
Table 3.1: Demographics (Pk Analysis)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Wt (kg)</th>
<th>Ds Type</th>
<th>Ds Location</th>
<th>Surgery</th>
<th>Additional Medications (oral)</th>
<th>Bude dose, mg (Duration of tx, months)</th>
<th>HBI pre-Bude</th>
<th>HBI on Bude</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>61</td>
<td>F</td>
<td>78.3</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Lansoprazole</td>
<td>9 (3)</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>F</td>
<td>70.1</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Rabeprazole, Paroxetine, Trazadone</td>
<td>6 (3.5)</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>F</td>
<td>75.0</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Pantoprazole</td>
<td>9 (4)</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>4</td>
<td>62</td>
<td>F</td>
<td>57.4</td>
<td>CD</td>
<td>ileocolic</td>
<td>N</td>
<td>Salofalk</td>
<td>3 (2)</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>F</td>
<td>70.0</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Cipralec</td>
<td>9 (4)</td>
<td>12</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>27</td>
<td>M</td>
<td>76.6</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Nil</td>
<td>9 (3)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>F</td>
<td>69.0</td>
<td>CD</td>
<td>Ileocolonic</td>
<td>N</td>
<td>Synthroid, methotrexate (SC)</td>
<td>3 (6)</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>F</td>
<td>104.0</td>
<td>CD</td>
<td>ileal</td>
<td>N</td>
<td>Metoprolol, cipralec, lansoprazole, methotrexate (SC)</td>
<td>6 (1.5)</td>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>

Disease, Ds; years, yrs; Budesonide, Bude; Harvey Bradshaw Index of disease activity, HBI; Crohn's disease, CD; subcutaneous, SC; treatment, tx
**Table 3.2: Demographics (Cross-sectional Study)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Crohn's disease (n = 74)</th>
<th>Healthy Volunteer (n = 71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean, range)</td>
<td>38.46 (18-72)</td>
<td>45.3 (19-71)</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>43 (58.1)</td>
<td>43 (60.6)</td>
</tr>
<tr>
<td>Weight, kg (mean ± std)</td>
<td>79.25 ± 17.88</td>
<td>81.65 ± 19.33</td>
</tr>
<tr>
<td>CD location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal (%)</td>
<td>32 (43.2)</td>
<td>-</td>
</tr>
<tr>
<td>Colonic (%)</td>
<td>6 (8.1)</td>
<td>-</td>
</tr>
<tr>
<td>Ileo-colonic (%)</td>
<td>36 (48.6)</td>
<td>-</td>
</tr>
<tr>
<td>Disease duration, years (mean ± std)</td>
<td>6.79 ± 7.90</td>
<td>-</td>
</tr>
<tr>
<td>Disease activity (%)</td>
<td>30 (40.5)</td>
<td></td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>17 (23.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Current medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-ASA (%)</td>
<td>0 (0.0%)</td>
<td>-</td>
</tr>
<tr>
<td>Glucocorticoid (%)</td>
<td>25 (21.6%)</td>
<td>-</td>
</tr>
<tr>
<td>MTX (%)</td>
<td>10 (8.6%)</td>
<td>-</td>
</tr>
<tr>
<td>Thiopurine (%)</td>
<td>62 (53.4%)</td>
<td>-</td>
</tr>
<tr>
<td>Anti-TNF (%)</td>
<td>64 (55.2%)</td>
<td>-</td>
</tr>
<tr>
<td>Combination therapy (%)</td>
<td>42 (36.2%)</td>
<td>-</td>
</tr>
<tr>
<td>Surgery (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Hospitalizations (mean ± std)</td>
<td>0.41 ± 0.93</td>
<td>-</td>
</tr>
</tbody>
</table>

Kilograms, kg; standard deviation, std; inflammatory bowel disease, IBD; Crohn's disease, CD; ulcerative colitis, UC; tumor necrosis factor, TNF; 5-aminosalicylate, 5-ASA; methotrexate, MTX
3.3.2 Pharmacokinetic Assessment

The Pk parameters for budesonide, IV and oral midazolam are presented in Table 3.3. Budesonide values are standardized to a 1mg-dose. Mean plasma concentration-time curves for budesonide, IV and oral midazolam are shown in Figure 3.1. Budesonide plasma concentration-time curves for individual subjects are shown in Figure 3.2. Panel A illustrates the budesonide plasma concentration-time curves standardized to a 1mg-dose and panel B illustrates the budesonide plasma concentration-time curves colour-coded by dose. There was significant inter-patient variability in budesonide exposure (Figure 3.2) (coefficient of variation = 0.626; p<0.001).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
<th>Subject 7</th>
<th>Subject 8</th>
<th>Mean ± s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CL</td>
<td>69.0</td>
<td>139.4</td>
<td>245.0</td>
<td>95.1</td>
<td>46.5</td>
<td>129.2</td>
<td>180.7</td>
<td>32.1</td>
<td>117.1 ± 71.8</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>3.7</td>
<td>1.6</td>
<td>1.5</td>
<td>5.5</td>
<td>3.0</td>
<td>0.4</td>
<td>1.9</td>
<td>2.0</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>C(_{\text{max}})(iv)</td>
<td>14.3</td>
<td>3.6</td>
<td>0.7</td>
<td>8.6</td>
<td>25.2</td>
<td>48.0</td>
<td>42.9</td>
<td>13.05</td>
<td>13.05 ± 14.5</td>
</tr>
<tr>
<td>CL/F (ml/min)</td>
<td>268.9</td>
<td>206.7</td>
<td>899.3</td>
<td>525.6</td>
<td>480.0</td>
<td>322.2</td>
<td>317.2</td>
<td>483.0</td>
<td>437.9 ± 218.6</td>
</tr>
<tr>
<td>F</td>
<td>0.26</td>
<td>0.67</td>
<td>0.27</td>
<td>0.18</td>
<td>0.10</td>
<td>0.40</td>
<td>0.57</td>
<td>0.07</td>
<td>0.31 ± 0.22</td>
</tr>
<tr>
<td>E(_H)</td>
<td>0.06</td>
<td>0.12</td>
<td>0.21</td>
<td>0.10</td>
<td>0.04</td>
<td>0.12</td>
<td>0.16</td>
<td>0.02</td>
<td>0.11 ± 0.06</td>
</tr>
<tr>
<td>E(_\text{GI})</td>
<td>0.73</td>
<td>0.23</td>
<td>0.66</td>
<td>0.80</td>
<td>0.90</td>
<td>0.54</td>
<td>0.32</td>
<td>0.93</td>
<td>0.64 ± 0.25</td>
</tr>
<tr>
<td>Budesonide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C(_{\text{max}})*(ng/ml)</td>
<td>0.39</td>
<td>0.41</td>
<td>0.09</td>
<td>0.51</td>
<td>0.20</td>
<td>0.05</td>
<td>0.44</td>
<td>0.26</td>
<td>0.29 ± 0.17</td>
</tr>
<tr>
<td>CL/F (ml/min)</td>
<td>6034.7</td>
<td>6839.6</td>
<td>39841.5</td>
<td>4465.0</td>
<td>10174.4</td>
<td>178140.2</td>
<td>5653.4</td>
<td>7630.8</td>
<td>32347.5 ± 12617.6</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>3.2</td>
<td>3.8</td>
<td>3.2</td>
<td>2.8</td>
<td>2.2</td>
<td>3.8</td>
<td>3.2</td>
<td>3.8</td>
<td>3.2 ± 1.7</td>
</tr>
</tbody>
</table>

*normalized to a 1-mg dose

Intravenous, iv; systemic clearance, CL; \( t_{1/2} \); maximum concentration, C\(_{\text{max}}\); oral clearance, CL/F; hepatic extraction ratio, EH; intestinal extraction ratio, E\(_\text{GI}\); standard deviation, s.d.
**Figure 3.1** Mean intravenous midazolam (50µg-dose) (A), oral midazolam (100µg-dose) (A), and budesonide (standardized to a 1mg-dose) (B) plasma concentrations versus time (hours) for subjects with Crohn’s disease (N= 8); error bars represent the standard deviation upper limit. Oral, PO; intravenous, IV; hour, hr.
Figure 3.2 Budesonide plasma concentrations (ng/ml) versus time (hours) for 8 subjects with Crohn’s disease. Panel A shows values standardized to a 1-mg dose of budesonide received by each subject. Panel B shows values separated by the dose of budesonide administered to each subject (light grey notched line = 3mg; solid black line = 6mg; dark grey dotted line = 9mg).
An individual's exposure to a drug is represented by the area-under-concentration-vs.-time curve extrapolated to infinity (AUC$_{0-\infty}$). The mean oral AUC$_{0-\infty}$ of budesonide and midazolam were $2.02 \text{ng/ml*hr} \pm 1.25$ and $4.57 \text{ng/ml*hr} \pm 2.08$ respectively (Table 3.4 & 3.5). Mean oral clearance (CL/F) was $32,347.5 \text{ml/min} \pm 12,617.6$ (budesonide), and $437.9 \text{ml/min} \pm 218.6$ (midazolam). The systemic clearance (Cl$_s$) of midazolam was $117.1 \text{ml/min} \pm 71.8$ (Table 3.3).
Table 3.4: Comparison of the pharmacokinetic parameters of oral budesonide in a cohort of subjects with Crohn's disease to healthy populations*

<table>
<thead>
<tr>
<th>Study</th>
<th>Population</th>
<th>N</th>
<th>BUDE po dose (mg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (hr)</th>
<th>AUC po (ng/ml*hr)</th>
<th>AUC po (ng/ml*hr)</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Current study</strong></td>
<td>CD</td>
<td>8</td>
<td>1</td>
<td>0.27</td>
<td>3.38</td>
<td>2.02</td>
<td>2.02</td>
<td>3.5</td>
</tr>
<tr>
<td>Dilger et al. 2007</td>
<td>Healthy</td>
<td>12</td>
<td>3</td>
<td>1.00</td>
<td>4.5</td>
<td>4.88</td>
<td>1.62*</td>
<td>2.6</td>
</tr>
<tr>
<td>Ufer et al. 2008</td>
<td>Healthy</td>
<td>18</td>
<td>9</td>
<td>2.03</td>
<td>5.47</td>
<td>11.82</td>
<td>1.31*</td>
<td>3.4</td>
</tr>
<tr>
<td>Seidegard et al. 2000</td>
<td>Healthy</td>
<td>8</td>
<td>3</td>
<td>0.39^</td>
<td>4.87</td>
<td>4.51^</td>
<td>1.50^*</td>
<td>4.9</td>
</tr>
</tbody>
</table>

* All data are presented as previously published, with the exception of values indicated by "*".

* Data values are normalized to a 1mg oral dose of budesonide by dividing the AUC po value by the reported dose in mg.

^ Data are converted from nmol to ng

Crohn's disease, CD; N, number; budesonide, BUDE; oral, PO; maximum concentration, C<sub>max</sub>; time to maximum concentration, T<sub>max</sub>; area under the concentration time curve, AUC
Table 3.5: Comparison of the pharmacokinetic parameters of oral and intravenous midazolam in a cohort of subjects with Crohn's disease to healthy populations.

<table>
<thead>
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<th>Cohort</th>
<th>N</th>
<th>MIDpo dose (µg)</th>
<th>MIDiv dose (µg)</th>
<th>Cmax (ng/ml)</th>
<th>Tmax (hr)</th>
<th>AUCpo (ng/ml*hr)</th>
<th>AUCpo (ng/ml*hr)</th>
<th>CLs/F (ml/min)</th>
<th>CLs (ml/min)</th>
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<th>EH</th>
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<td>0.64</td>
<td>0.31</td>
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<td>CD</td>
<td>117</td>
<td>0.64</td>
<td>0.31</td>
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<td>0.64</td>
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<td>CD</td>
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<td>0.61</td>
<td>0.33</td>
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</tr>
<tr>
<td></td>
<td>CD</td>
<td>117</td>
<td>0.64</td>
<td>0.31</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Data are normalized to a 100-µg oral dose of midazolam by dividing the AUCpo value by the reported dose in µg and multiplying by 100µg.

Crohn's disease, CD; N, number; midazolam, MID; oral, PO; maximum concentration, Cmax; time to maximum concentration, Tmax; area under the concentration time curve, AUC; not reported, NR; intestinal extraction ratio, EGI; hepatic extraction ratio, EH.
3.3.3 Comparison between drugs

The mean hepatic and intestinal extraction ratios for midazolam representing the proportion of CYP3A4 activity occurring were 0.11 ± 0.06 and 0.64 ± 0.25 respectively (Figure 3.3a, b). The mean oral bioavailability (F) of midazolam was 31% ± 22% (Figure 3.3c). There was no significant relationship between budesonide exposure (AUC\(_{0-\infty}\)) and the oral clearance or exposures of midazolam (Figure 3.4).
Figure 3.3. Hepatic (A), intestinal (B) extraction ratios and oral bioavailability (C) separated by subject. The mean value is presented following the final individual subject value.
Figure 3.4 Correlation between budesonide exposure (AUC₀–∞; normalized to a 1mg-dose) and oral midazolam exposure (AUC₀–∞, ng/ml*hr) (panel A), oral midazolam oral clearance (Clᵣ/F, ml/min) (panel B) using the Spearman correlation coefficient (Rₛ).

AUC, area under the curve.
3.3.4 4β-hydroxycholesterol Plasma Concentrations Are Decreased in Crohn's disease

A second cohort of subjects with CD (n=74) provided a blood sample for the quantification of 4βOHC in plasma as did a cohort of non-CD controls (n=71) to estimate CYP3A4 activity in vivo. The 4βOHC plasma concentrations were significantly lower in the total CD population compared to the non-CD controls (CD= 18.68ng/ml±13.02ng/ml, non-CD= 58.14ng/ml±61.76ng/ml, p≤0.0001) (Figure 3.5) on bivariate analysis or when adjusting for the covariates, age, sex, and disease activity (Table 3.6). The multiple linear regression analysis accounted for 33.6% of the inter-individual variation in 4βOHC plasma concentrations. No significant difference was seen in 4βOHC plasma concentrations between CD subjects stratified by disease activity (inactive CD= 19.75ng/ml±13.34ng/ml, active CD= 17.11ng/ml±12.60ng/ml, p=0.99) (Figure 3.5).
Table 3.6. Multiple linear regression model for the effect on Ln-transformed 4βOHC plasma concentrations for the total population (n = 145)

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
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<td>0.447</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Sex</td>
<td>0.548</td>
<td>0.151</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Age</td>
<td>0.130</td>
<td>0.005</td>
<td>0.008</td>
</tr>
<tr>
<td>Weight</td>
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<td>0.004</td>
<td>0.92</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>-0.832</td>
<td>0.162</td>
<td>≤0.0001</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.200</td>
<td>0.197</td>
<td>0.31</td>
</tr>
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</table>

Figure 3.5 The mean 4βOHC plasma concentrations for subjects with active and inactive Crohn's disease (CD) as well as non-CD controls. Median values (thick horizontal line), 25th and 75th percentile values (box outline), minimum and maximum values (whiskers), and outlier values (circles). 4β-hydroxycholesterol, 4βOHC.
3.3.5 Plasma Bile Acid Profiles Differ Between Individuals with and without Crohn's Disease

Plasma bile acid profiles consisting of 12 bile acids were determined for all subjects (n=145). Tables 3.7 and 3.8 summarize the plasma bile acid profiles as whole concentrations and as a percentage of the total bile acid pool respectively.
Table 3.7: Bile acid profiles presented as concentrations (ng/mL)

<table>
<thead>
<tr>
<th>Bile acid (ng/mL)</th>
<th>Control (n=71)</th>
<th>Inactive CD (n=44)</th>
<th>Active CD (n=47)</th>
</tr>
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<tr>
<td>su 0.8586</td>
<td>0.7487</td>
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<tr>
<td>su 43.7</td>
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</tr>
<tr>
<td>su 8.98</td>
<td>14.6</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>100%&gt; 8.98</td>
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<td>19.4</td>
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</tr>
<tr>
<td>su 9.7</td>
<td>17.7</td>
<td>11.3</td>
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</tr>
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<td>100%&gt; 47.3</td>
<td>25.3</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>su 44.7</td>
<td>4.9</td>
<td>17.8</td>
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</tr>
<tr>
<td>100%&gt; 44.7</td>
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<td>su 17.6</td>
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<td>20.0</td>
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p-value

Comparison of control to active and inactive CD.
Table 3.8: Bile acid profiles presented as a percentage of the total bile acid pool by group

<table>
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<tr>
<th></th>
<th>Primary Bile Acids</th>
<th>Secondary Bile Acids</th>
<th>Tertiary Bile Acids</th>
<th>Total Bile Acids</th>
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<tr>
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<td>Conjugates</td>
<td>acids plus</td>
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<tr>
<td></td>
<td>T-UDCA</td>
<td>T-DCA</td>
<td>T-LCA</td>
<td>T-CA</td>
</tr>
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<td>Primary Bile Acids</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>su</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>su</td>
<td>6.94%</td>
<td>0.03%</td>
<td>6.19%</td>
<td>0.02%</td>
</tr>
<tr>
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<td>6.9%</td>
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<td>6.17%</td>
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<tr>
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<td>7.05%</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>3.31%</td>
<td>0.0%</td>
</tr>
<tr>
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<td>1.47%</td>
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<tr>
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<tr>
<td>100'0&gt;</td>
<td>3.97%</td>
<td>0.0%</td>
<td>3.31%</td>
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</tr>
<tr>
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<td>2.19%</td>
<td>0.0%</td>
<td>1.47%</td>
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</tr>
<tr>
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<td>8.96%</td>
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<td>8.32%</td>
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</tr>
<tr>
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<tr>
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<td>9.32%</td>
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<tr>
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<td>0.0%</td>
<td>1.47%</td>
<td>0.0%</td>
</tr>
<tr>
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<td>3.96%</td>
<td>0.0%</td>
<td>3.87%</td>
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</tr>
<tr>
<td>su</td>
<td>4.7%</td>
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<td>4.4%</td>
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</tr>
<tr>
<td>su</td>
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*p-values* (n=30) (n=44) (n=44) (n=44)

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<thead>
<tr>
<th></th>
<th>Plasma Bile Acids</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>Inactive CD</td>
</tr>
<tr>
<td></td>
<td>Active CD</td>
</tr>
</tbody>
</table>

Comparison of control to active and inactive CD.
Subjects with CD had a higher ratio of conjugated to unconjugated bile acids compared to controls (active CD = 5.9; inactive CD = 6.9; control = 3.0, Figure 3.6). Significant differences in the composition of the bile acid pool were seen based on presence or absence of CD. Differences in the percent composition of GCDCA, GCA, TCA, GDCA, LCA were most significant (Figure 3.7). CDCA and DCA were different between controls and CD subjects with active disease. There was no difference in UDCA, TDCA, CA.
**Figure 3.6** The ratio of the plasma concentration of conjugated to unconjugated bile acids stratified by cohort (control, active CD, inactive CD). The 95%CI is represented by the vertical T-line. *, p<0.05; **, p<0.01; ***, p<0.001. Crohn's disease, CD.
Figure 3.7 Bile acid profiles. The mean individual bile acid profiles expressed as mean concentrations in ng/ml and as a percentage of the total bile acid pool, stratified by cohort (control, active CD, inactive CD). The 95%CI is represented by the vertical T-line. *, p<0.05; **, p<0.01; ***, p<0.001. Crohn's disease, CD; cholic acid, CA; chenodeoxycholic acid, CDCA; deoxycholic acid, DCA; glycocholic acid, GCA; glycochenodeoxycholic acid, GCDCA; glycodeloxycholic acid, GDCA; lithocholic acid, LCA; taurocholic acid, TCA; taurodeoxycholic acid, TDCA; taurolithocholic acid, TLCA; tauoursodeoxycholic acid, TUDCA; and ursodeoxycholic acid, UDCA.
3.3.6 Activation of PXR by Individual Bile Acids and Cohort-Specific Bile Acid Profiles

To explore the impact of CD-dependent alterations in the plasma bile acid profile on PXR-mediated CYP3A4 activity, a transient transfection and dual luciferase assay were used. We found that luciferase activity (a surrogate of CYP3A4 activity) was markedly elevated in cells exposed to known PXR activators, DCA (12-fold), GDCA (10-fold) or LCA (23-fold) (Figure 3.8). These data are in agreement with other reports of bile acid-induced activation of PXR\textsuperscript{38,39}. Conversely, no difference was seen in the luciferase activity amongst the transfected cells exposed to the cohort-specific bile acid pools (Figure 3.8).
**Figure 3.8**  Effect of known bile acid PXR agonists and patient-derived bile acid profiles on CYP3A4-pGL3 basic reporter activity in HepG2 cells co-transfected with hPXR. One of DCA, LCA, GDCA (10µM) a bile acid pool (25µM) representative of inactive or active CD or a healthy population were incubated with cells for 24 hours. All experiments were completed in triplicate and repeated 3 times. Error bars indicate the 95% confidence interval.

Deoxycholic acid, DCA; lithocholic acid, LCA; glycol-deoxycholic acid, GDCA; Crohn's disease, CD; dimethyl sulfoxide, DMSO; pregnane X receptor, PXR; cytochrome p450, CYP; hepatocarcinoma, Hep;
3.4 Discussion

CYP3A4 is considered to be one of the most clinically-relevant determinants of drug metabolism and exposure today. Although there is a significant amount of data in terms of CYP3A4 activity in human subjects, nearly all such studies have been conducted in healthy volunteers. In disease conditions, such as CD, remarkably little is known with regards to the extent of CYP3A4 activity. In this study, we evaluated subjects with active CD on stable doses of the glucocorticoid drug, budesonide, where pharmacokinetic analysis of an exogenous probe substrate of CYP3A4 was utilized to better delineate the difference in hepatic versus intestinal CYP3A4 activity. We were also able to evaluate the impact of CYP3A4 activity on budesonide exposure, a known CYP3A4 substrate. Furthermore, we were able to validate our findings of reduced CYP3A4 activity in CD in an independent cohort of CD patients as well as explore a possible mechanism for this difference. To our knowledge, this is the first study to assess CYP3A4 activity in a CD population using both in vivo probes midazolam and 4βOHC.

Of high clinical relevance, is the near 5-fold higher AUC\textsubscript{0-∞} for oral midazolam in our patient Pk population compared to what has been reported in healthy populations (Figure 3.9b, Table 3.5\textsuperscript{30, 32, 40, 41}). This suggests that total CYP3A4 activity is profoundly reduced in CD compared to healthy individuals. Interestingly, the decrease in CYP3A4 activity cannot necessarily be attributed to a reduction in intestinal CYP3A4 activity as one might suspect in a primary intestinal illness. A higher degree of midazolam extraction and subsequently CYP3A4 activity occurred in the gut (E\textsubscript{gi}= 0.64 ± 0.25) compared to the liver (E\textsubscript{h}= 0.11 ± 0.06) (Figure 3.3a, b). And while, to date, the degree
of extraction in the liver versus the intestine has been reported at varying levels, the
degree of difference seen between the liver and gut extraction in our CD population has
not been seen in healthy populations\textsuperscript{33, 41-43}. One potential limitation of our Pk study
might be the absence of a control population. However, in comparison to a cohort of
healthy volunteers who participated in a previous study conducted in our laboratory\textsuperscript{32}
using the same midazolam microdose-based phenotyping approach for CYP3A4 and the
same LC-MS/MS assay used in the current study, we observed a marked reduction in our
CD subjects (Figure 3.9b, Table 3.5).
Figure 3.9  A comparison of oral budesonide (Panel A), and oral midazolam (Panel B) exposure ($AUC_{po\to\infty}$, ng/ml*hr) between our Crohn's disease population and reported values for healthy populations standardized to the same drug dose. AUC, area under the curve; po, oral
In this study, we note that subjects with CD have a comparable exposure to budesonide, a known substrate of CYP3A4, to what has been reported in studies of healthy individuals when standardized to the same dose (Figure 3.9a, Table 3.4)\textsuperscript{3, 5, 44}. We also see that budesonide exposure is highly variable in subjects with CD (Figure 3.2); consistent with what has been shown in healthy populations as well as in a single adult CD population\textsuperscript{3, 6}. This may account for the some of the variability seen in response to budesonide treatment. Based on our data, CYP3A4 activity accounts for only a small fraction of the variability in budesonide exposure. This suggests that there are other factors determining budesonide exposure in CD and that CYP3A4 may not have a significant impact on its disposition in this population; however, our sample size may be too small to draw any firm conclusions.

Moreover, we independently confirm a reduction in CYP3A4 activity in CD using the CYP3A4-\textit{in vivo} probe, 4\textbeta-OHC. The evaluation of hepatic CYP3A activity using this endogenous marker has been well-documented\textsuperscript{31}. CYP3A4 and 3A5 regulate the production of 4\textbeta-OHC and it is useful for the evaluation of CYP3A induction by specific drugs. The plasma concentration of 4\textbeta-OHC is significantly lower in the CD population compared to a control population (Figure 3.5). The effect of CD presence on CYP3A4 activity remains even when age, sex, weight and disease activity (assessed by HBI) are taken into account (Table 3.6). Of note, some groups endorse the use of the ratio of the plasma concentrations of 4\textbeta-OHC to cholesterol\textsuperscript{31, 45, 46}. In some populations, plasma concentrations of cholesterol may differ based on treatment, which may impact the ability to form 4\textbeta-OHC from its parent compound. Though, plasma cholesterol was not measured in our population, no subjects included in our study were being treated for
dyslipidemia. This may have minimized confounding in our population, though does not entirely eliminate it. Furthermore, some groups suggest there is only a weak correlation between midazolam Pk parameters (total and liver CYP3A4 activity) and 4βOHC plasma concentrations. However, this may be partially explained by the fact that 4βOHC is a substrate of both CYP3A4 and 3A5, while midazolam is only a substrate of CYP3A4. Moreover, 4βOHC has a long half-life (1-3 weeks) and therefore cannot be used to assess rapid fluctuations in CYP3A activity. It has been concluded that 4βOHC may be most useful for assessing constitutive CYP3A4 activity. Midazolam Pk parameters are considered the gold standard for assessing CYP3A4 activity in vivo. Despite these possible limitations, we are able to show, using both modalities, that CYP3A4 activity is reduced in CD.

Reduced hepatic CYP3A4 activity in the setting of systemic or non-hepatic inflammation has been reported, although our study is the first to demonstrate such an effect in CD using multiple CYP3A4-phenotyping modalities. In vitro and in vivo animal studies both conclusively show that in the setting of acute non-hepatic inflammation, hepatic CYP3A4 expression is down-regulated. Studies in humans show an increase in the plasma drug concentrations of CYP3A4 substrates in the setting of acute inflammation. The effect of chronic inflammation on CYP3A4 activity and expression in humans is not well defined and there is a paucity of literature on this subject. One small study did assess CYP3A4 activity in IBD. Iwamoto et.al. (2013) evaluated single-point 4β-OHC plasma concentrations in subjects with IBD as well as healthy volunteers. They found a significant reduction in the 4β-OHC plasma concentrations of their CD cohort compared to a cohort of healthy controls; however, their sample size was
small (21 CD patients, 26 controls), limiting the generalizability of their findings. They also did not account for CD disease activity in their analysis nor any other factors that may have confounded CYP3A4 activity. It has been suggested that hepatic CYP3A4 is down-regulated due to the effects of IL-1β, IL-6, TNF-α, and IFN-α and γ20, 22; however, in our cross-sectional cohort, no difference is seen in the CYP3A4 activity of CD subjects with and without active disease, suggesting an alternate mechanism for this reduced activity.

We tested the hypothesis that changes in bile acid-mediated activation of PXR account for detectable differences in CYP3A4 activity in CD using a HepG2 cell model. We show that there are differences in the bile acid profiles of CD (active and inactive) and non-CD cohorts (Table 3.7, 3.8; Figure 3.7). These data provide a more detailed analysis of bile acid pool composition in CD; however, they re-iterate principles already established in the literature49, 50. Specifically, that individuals with IBD have a different pattern of bile acids compared to healthy controls, perhaps due to documented changes in the presence of bile acid-modifying gut bacteria29, 50. We then applied these cohort-specific bile acid pools to a HepG2 cell model and evaluated the effect of bile acid-induced PXR on CYP3A4 activity. Our findings confirm that glycine-conjugated and unconjugated DCA as well as LCA are potent PXR agonists. Conversely, we did not find an appreciable difference in the activation of CYP3A4 based on exposure to our cohort-specific bile acid pools (Figure 3.8). The reasons for this may be multi-factorial: 1) plasma bile acid composition is a relatively dilute compared to what is seen in the liver and bile51; thus small differences in individual bile acid concentrations in plasma may under-represent the ultimate impact of these changes in the liver or bile on downstream
target pathways 2) other bile acid-activated NRs, such as FXR, with impact on CYP3A4 activity, may need to be accounted for. Ultimately, more studies are needed.

In summary, this is the first study in CD populations evaluating CYP3A4 activity using dual *in vivo* probes. CYP3A4 activity is lower in the setting of CD based on midazolam Pk and single point-4βOHC plasma concentration measurements. Interestingly, this effect is mainly due to a reduction in hepatic CYP3A4 activity based on the midazolam data. Our findings suggest that a disease-dependent reduction in CYP3A4 needs to be taken into consideration when prescribing substrate drugs such as certain HMG-CoA reductase inhibitors, benzodiazepines or oral anticoagulants to a CD population. The molecular mechanisms responsible for these findings remain to be explored.
3.5 References


4 EVALUATION OF CYP3A4 EXPRESSION IN CROHN'S DISEASE
4.1 Introduction

The intestinal mucosa plays a vital role in human health: it is the key interface for the absorption of nutrients including electrolytes and water as well as an effective barrier against host exposure to environmental pathogens and toxins. It is composed of a monolayer of columnar epithelial cells known as enterocytes, as well as goblet cells, paneth cells and enteroendocrine cells. Cells are arranged in villi, superimposed over the lamina propria and inferiorly bound by the muscularis mucosae\textsuperscript{1,2}. The enterocytes, overlaying the lamina propria, are tightly joined by a series of junctional proteins (tight junctions, adherens junctions, desmosomes) and are arranged in a folded pattern, producing crypts and villi on a cytoskeleton framework\textsuperscript{3}. The lamina propria, the bedrock of the epithelium, houses a number of different cell types that carry out a host of important functions for maintaining the integrity of the mucosal layer and its immunological role in barrier function. Fibroblasts, lymphocytes, plasma cells, granulocytes, macrophages and dendritic cells form part of the rich cellular fabric of the lamina propria amidst an extensive vascular network\textsuperscript{4}.

An extension of the barrier function of the intestine is its contribution to first-pass metabolism. It is well established that an individual's exposure to an orally-dosed medication may differ considerably from their exposure to its intravenously-dosed counterpart\textsuperscript{5}. The concept of first-pass metabolism refers to the extensive processing applied to orally-dosed drugs in the hepatic parenchyma and now, increasingly recognized, in the gut mucosa that limits the delivery of active drug to the systemic circulation\textsuperscript{6,7}. The cytochrome P450 enzyme (CYP) 3A4 is an integral part of the "first-pass" phenomenon in humans given its abundance in both liver and intestinal tissue. It
accounts for up to 40% and 80% of their respective CYP burdens and is involved in the metabolism of up to 50% of drugs used in clinical practice\textsuperscript{8-10}. Within the intestine, CYP3A4 is localized to enterocytes found at the tips of villi and is expressed in highest concentrations within the jejunum and ileum\textsuperscript{7,11}. Intestinal CYP3A4 may act in a coordinated manner with key xenobiotic exporters, such as P-glycoprotein (P-gp), a member of the ATP-binding cassette protein (ABC) superfamily\textsuperscript{12}. This idea is reinforced by the findings of multiple shared substrates and inhibitors as well as co-localization within villous tip-enterocytes. P-gp bound to the cell membrane of enterocytes may regulate the exposure of specific drugs to metabolism by CYP3A4. It is thought that P-gp reduces the concentration of a drug within the enterocyte by actively moving the drug back into the intestinal lumen across the plasma membrane. This limits the saturation of intestinal CYP3A4 and leads to more effective and complete metabolism of drugs\textsuperscript{13}.

The impact of chronic liver and kidney disease on the expression of hepatic CYP3A4 has been evaluated and reductions in CYP3A4 expression are observed\textsuperscript{14-16}. Similarly, research pertaining to P-gp reveals that there is over-expression of P-gp and the gene by which it is coded, \textit{MDRI}, in a multitude of cancers\textsuperscript{17,18}. The effect of a primary intestinal illness on the intestinal expression of CYP3A4 and P-gp has only been explored in a limited capacity\textsuperscript{19-25}. Crohn's disease (CD) is a primary, chronic illness of the intestinal tract that produces transmural, discontinuous inflammatory lesions along the gut's longitudinal access. It is often confined to the small and large intestines, but can be found anywhere from the oral cavity to the most distal colon\textsuperscript{26}. Hallmarks of CD include disruption of the epithelial cell tight junctions, epithelial cell apoptosis, expansion
of the lamina propria with pro-inflammatory cells and the recruitment and activation of pro-fibrotic fibroblasts\textsuperscript{27-29}. Such changes in barrier function lead to the trademark clinical profile of CD including abdominal pain, profuse diarrhea, intestinal stricturing and fistulization. Disruption of the intestinal mucosa may also have important implications for epithelial-based processes such as phase 1 and 2 drug metabolism and drug transport. Specifically, the loss of mucosal surface area may result in the loss of intestinally-expressed CYP3A4 and P-gp protein. The impact of CD on the protein expression of both CYP3A4 and P-gp has not been well-evaluated, particularly in an adult population.

The aim of this study was to evaluate the intestinal expression of CYP3A4 and P-gp at two intestinal sites in adult patients with CD compared to non-CD controls.
4.2 Materials & Methods

4.2.1 Subjects

The study protocol was approved by the University of Western Ontario Health Sciences Research Ethics Board (13067) and was carried out in accordance with the Declaration of Helsinki. Written and informed consent was obtained from all participants. Twenty three subjects with CD and 37 non-CD controls were recruited from a single centre in London, Ontario between August 2009 and December 2014. Eligible CD subjects were 18 years of age or older with a history of CD confirmed by previous histopathologic examination. Eligible controls were 18 years of age or older with no clinical history of gastrointestinal pathology. Subjects in either group taking CYP3A4 or P-gp agonists or antagonists (with the exception of budesonide) were excluded. Clinical data pertaining to CD subject age, sex, weight, smoking history, medication history and drug response, diagnosis, disease location, disease activity were collected. Demographic data were collected for each non-CD control. All participant data is summarized in Table 4.1.

4.2.2 Study Objectives and Outcomes

The objective of this study was to test the hypothesis that individuals with CD have reduced expression of intestinal CYP3A4 and P-gp protein relative to a healthy population. The primary endpoints were the density ratios of ileal CYP3A4 and P-gp protein to villin in subjects with CD compared to non-CD controls. Secondary endpoints included the density ratios of colonic CYP3A4 and P-gp protein to villin in CD versus non-CD cohorts, as well as the relative densities of ileal and colonic CYP3A4 and P-g
protein stratified by one of age, sex, or CD activity (by Harvey Bradshaw Index, HBI) respectively for the CD cohort as well as by age and sex in the non-CD cohort.

4.2.3 Tissue Collection

Tissues samples from the terminal ileum and ascending colon were obtained from all subjects at the time of colonoscopy. Tissue sampling from the CD cohort was obtained exclusively from non-inflamed areas (based on endoscopic examination) in the presence or absence of active disease. All tissue samples were immediately frozen on dry ice after excision and stored at -80°C until further use.

4.2.4 Protein Quantification:

Total protein was isolated from the ileal and colonic tissue samples and quantified using bovine serum albumin as an internal standard. Twenty micrograms of isolated protein was separated on a 4-12% tris-acetate gel and transferred to a nitrocellulose membrane. P-gp-expressing caco2 cells and human liver samples were used as controls for P-gp and CYP3A4 respectively. Blots were blocked overnight at 4°C in 10% non-fat dried milk in Tris buffer containing 1% Tween 20. Blots were incubated with the primary antibody, 3H8 directed against CYP3A4 (Thermoscientific, Rockford IL, USA; dilution 1:500) and C219 directed against P-gp (Covance, Dedham MA, USA; dilution: 1:500) respectively. Villin (CWWB1) was used as the internal standard (Santa Cruz Biotechnology; dilution 1:500). Horse radish peroxidase conjugate-labeled goat anti-mouse IgG was used as a secondary antibody (dilution 1:10,000). Detection was achieved by chemiluminescence with enhanced chemiluminescence western blot
detecting reagents (GE Healthcare, Chicago Illinois, USA). Quantification by densiometry was performed using Image J software. Relative CYP3A4 and P-gp protein expression were represented as the ratios of densities of the target protein to villin.

4.2.5 Statistical Analysis:

All statistical analyses were carried out in Graphpad Prism and SPSS. The distribution of the protein expression data was assessed using the D'Agostino & Pearson omnibus normality test. The Mann-Whitney test was used to assess any statistically significant differences in protein expression between two groups, while the Kruskal-Wallis test was used to assess any statistically significant differences in protein expression between three or more groups. Dunn's multiple comparison test was used to conduct a post-hoc analysis of data evaluated by the Kruskal-Wallis test. A linear regression analysis was used to assess the relationship between one of sex or age and the relative densities of ileal and colonic CYP3A4 and P-gp respectively in both CD and non-CD cohorts. A multiple linear regression model was to evaluate the effect of multiple covariates (age, sex, disease presence, disease activity) on inter-individual variation in ileal and colonic CYP3A4 and P-gp relative densities.
4.3 Results:

4.3.1 The effect of disease presence and activity on intestinal CYP3A4 protein expression

Demographic data are presented in Table 4.1. The expression of intestinal CYP3A4 protein is presented as the density of CYP3A4 protein on Western Blot relative to the density of villin (Figure 4.1 and 4.2). The protein expression patterns of CYP3A4 relative to villin in ileum and ascending colon are presented for CD and non-CD populations in Figure 4.3. CYP3A4 expression in both the CD cohort and the controls mirrored physiological patterns previously reported in healthy individuals: CYP3A4 content was higher in the ileum than in the colon (CD, p<0.0001; control, p<0.0001). Ileal CYP3A4 expression was decreased in CD compared to controls (CD, 0.40±0.35; control, 0.72±0.33; p<0.01) (Figure 4.3). Similarly, colonic CYP3A4 expression was decreased in CD compared to controls (CD, 0.08±0.08; controls, 0.37±0.34; p<0.0001) (Figure 4.1). Significant inter-individual variation was observed in ileal (CD, 39-fold; control, 93-fold) and colonic (CD, 49-fold; control, 67-fold) CYP3A4 expression.
Table 4.1: Demographics

<table>
<thead>
<tr>
<th>Variables</th>
<th>CD (n=23)</th>
<th>Non-CD Controls (n=37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean, range)</td>
<td>48.87 (22-80)</td>
<td>63.77 (27-85)</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>16 (69.6)</td>
<td>()</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>6 (26.1)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal disease (%)</td>
<td>11 (47.8)</td>
<td>-</td>
</tr>
<tr>
<td>Ileo-colonic disease (%)</td>
<td>9 (39.1)</td>
<td>-</td>
</tr>
<tr>
<td>Colonic disease (%)</td>
<td>3 (13.1)</td>
<td>-</td>
</tr>
<tr>
<td>Active disease (%)*</td>
<td>9 (39.1)</td>
<td>-</td>
</tr>
<tr>
<td>Previous resection (%)</td>
<td>4 (17.4)</td>
<td>-</td>
</tr>
<tr>
<td>Duration of disease</td>
<td>10.71±8.54</td>
<td>-</td>
</tr>
<tr>
<td>Medication use</td>
<td>38 (95.0)</td>
<td>-</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>19 (82.6)</td>
<td>-</td>
</tr>
<tr>
<td>5-aminosalicylates</td>
<td>0 (0)</td>
<td>-</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>11 (47.8)</td>
<td>-</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>14 (60.9)</td>
<td>-</td>
</tr>
<tr>
<td>Biologics</td>
<td>11 (47.8)</td>
<td>-</td>
</tr>
</tbody>
</table>

Crohn's disease, CD

*Disease activity is based on the Harvey-Bradshaw Index for individuals with CD;
**Figure 4.1** Intestinal expression of CYP3A4 and P-gp by Western blot for 21 of 23 CD participants (ileal data for subject 6 is not included in this figure nor data for subjects 22 and 23). Cytochrome P450 3A4, CYP3A4; P-glycoprotein, P-gp; Crohn's disease, CD; +, positive control.
Figure 4.2 Intestinal expression of CYP3A4 and P-gp by Western blot for 37 non-CD controls (data for subjects 28 and 32 are not included in this figure). Cytochrome P450 3A4, CYP3A4; P-glycoprotein, P-gp; +, positive control.
**Figure 4.3** The relative protein expression of CYP3A4 to villin in the ileum and ascending colon of individuals with (n=23) and without (n=37) CD. Per the box plot, mean values (thick horizontal line), 25th and 75th percentile values (box outline), minimum and maximum values (whiskers), and outlier values (circles). ***, p <0.0001, **, p<0.01. Cytochrome, CYP; Crohn's disease, CD.
Neither, disease location nor disease activity, assessed by the Harvey-Bradshaw Index (HBI), accounted for any appreciable difference in ileal or colonic CYP3A4 expression in the CD cohort. Multiple linear regression was performed on the relative densities of ileal and colonic CYP3A4, adjusting for age, sex, weight, disease presence, and disease activity (Table 4.2 and 4.3). Disease presence had a persistently significant and negative impact on the relative expression of both ileal and colonic CYP3A4 protein.
Table 4.2: Multiple linear regression model for the effect on relative densities of ileal CYP3A4 for the total population (n = 60)

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.740</td>
<td>0.277</td>
<td>0.011</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.034</td>
<td>0.110</td>
<td>0.762</td>
</tr>
<tr>
<td>Age</td>
<td>0.000</td>
<td>0.004</td>
<td>0.991</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>-0.279</td>
<td>0.131</td>
<td>0.048</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.001</td>
<td>0.180</td>
<td>0.995</td>
</tr>
</tbody>
</table>

Table 4.3: Multiple linear regression model for the effect on relative densities of colonic CYP3A4 for the total population (n = 60)

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.596</td>
<td>0.206</td>
<td>0.006</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.091</td>
<td>0.008</td>
<td>0.309</td>
</tr>
<tr>
<td>Age</td>
<td>-0.002</td>
<td>0.003</td>
<td>0.392</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>-0.279</td>
<td>0.106</td>
<td>0.012</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.062</td>
<td>0.143</td>
<td>0.669</td>
</tr>
</tbody>
</table>
4.3.2 The Effect of Age and Sex on Intestinal CYP3A4 Expression

CYP3A4 expression varied significantly with age in both the CD and control cohorts (Figure 4.4). Ileal CYP3A4 expression decreased with advancing age in the control cohort (Figure 4.4a); however, this was not observed in the corresponding colonic samples (Figure 4.4b). Conversely in the CD cohort, ileal CYP3A4 expression increased with age (Figure 4.4c). This was not seen in the CD ascending colon samples (Figure 4.4d). Sex did not have significant impact on CYP3A4 expression in either cohort at either intestinal location.
Figure 4.4 The influence of age on the relative densities of ileal and colonic CYP3A4 protein in control (Panel A and B) and CD (Panel C and D) populations. The solid line represents the linear regression. Cytochrome P450, CYP; Spearman correlation, R; Crohn's disease, CD; non-significant, ns.
4.3.3 The Effect of Disease Presence and Activity on Intestinal P-Gp Protein Expression

The expression of intestinal P-gp protein is presented as the density of P-gp protein on Western Blot relative to the density of villin (Figure 4.1 and 4.2). The protein expression patterns of P-gp relative to villin in ileum and ascending colon are presented for CD and non-CD populations in Figure 4.5. No difference was seen in the relative ileal P-gp expression in CD compared to controls (CD, 0.095±0.10; control, 0.11±0.09; p=ns) (Figure 4.3). Conversely, the relative colonic P-gp expression was decreased in CD compared to controls (CD, 0.026±0.029; controls, 0.17±0.21; p<0.01) (Figure 4.3). Significant inter-individual variation was observed in ileal (CD, 18-fold; control, 13-fold) and colonic (CD, 22-fold; control, 207-fold) P-gp expression.
**Figure 4.5** The relative protein expression of P-gp to villin in the ileum and ascending colon of individuals with (n=23) and without (n=37) CD. Per the box plot, mean values (thick horizontal line), 25th and 75th percentile values (box outline), minimum and maximum values (whiskers), and outlier values (circles). ***, p<0.01.** P-glycoprotein, P-gp; Crohn's disease, CD.
Similar to CYP3A4 expression, neither disease location nor disease activity, assessed by HBI, affected P-gp expression in the ileum or ascending colon of the CD cohort. Multiple linear regression was performed on the relative densities of ileal and colonic P-gp, adjusting for age, sex, weight, disease presence, and disease activity (Table 4.4 and 4.5). Disease presence had a persistently significant and negative impact on the relative expression of colonic P-gp protein (Table 4.5), but not in the ileum (Table 4.4).
Table 4.4: Multiple linear regression model for the effect on relative densities of ileal P-gp for the total population (n = 60)

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.186</td>
<td>0.073</td>
<td>0.015</td>
</tr>
<tr>
<td>Sex</td>
<td>-0.052</td>
<td>0.031</td>
<td>0.099</td>
</tr>
<tr>
<td>Age</td>
<td>-0.001</td>
<td>0.001</td>
<td>0.479</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>-0.002</td>
<td>0.036</td>
<td>0.947</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.032</td>
<td>0.047</td>
<td>0.497</td>
</tr>
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Table 4.5: Multiple linear regression model for the effect on relative densities of colonic P-gp for the total population (n = 60)

<table>
<thead>
<tr>
<th>Variable</th>
<th>β-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.360</td>
<td>0.113</td>
<td>0.003</td>
</tr>
<tr>
<td>Sex</td>
<td>0.043</td>
<td>0.047</td>
<td>0.368</td>
</tr>
<tr>
<td>Age</td>
<td>-0.004</td>
<td>0.002</td>
<td>0.028</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>-0.179</td>
<td>0.055</td>
<td>0.002</td>
</tr>
<tr>
<td>Disease activity</td>
<td>-0.037</td>
<td>0.071</td>
<td>0.602</td>
</tr>
</tbody>
</table>
4.3.4 The Effect of Age and Sex on Intestinal P-Gp Expression

Colonic P-gp expression varied significantly with age in both the CD and control cohorts (Figure 4.6). Colonic P-gp expression decreased with advancing age in the CD and control cohorts (Figure 4.6b, d). This remained significant based on the multiple linear regression analysis (Table 4.5). Conversely, this was not observed in the corresponding ileal samples (Figure 4.6a, c). Sex did not have significant impact on P-gp expression in either cohort at either intestinal location.
Figure 4.6 The influence of age on the relative densities of ileal and colonic P-gp protein in control (Panel A and B) and CD (Panel C and D) populations. The solid line represents the linear regression. P-glycoprotein, P-gp; Spearman correlation coefficient, R; Crohn's disease, CD; non-significant, ns.
4.4 Discussion:

The intestine is essential for nutrient absorption, host defense and plays a key role in drug metabolism in concert with the liver. The interplay of numerous factors likely contributes to inter-individual variation in drug exposure and response. Key phase 1 drug metabolism enzyme, CYP3A4 has been proposed to act in a synchronized manner with xenobiotic exporter, P-gp to significantly affect the oral bioavailability of numerous shared substrates. This theory is further supported by their co-localization in enterocytes.

Disease-dependent decreases in hepatic CYP3A4 activity and expression are observed in a myriad of chronic hepatic and non-hepatic diseases\textsuperscript{14-16, 30}. Similarly, the endothelial expression of P-gp at the blood-brain barrier is noted to be decreased in Alzheimer's disease\textsuperscript{31, 32}. The impact of disease on the intestinal fractions of the highly abundant and relevant CYP3A4 and P-gp has only been evaluated in a limited capacity. Specifically, studies assessing the impact of a chronic, primary intestinal illness on intestinal CYP3A4 expression are few. Data are mainly derived from pediatric populations and are contradictory.

Two small studies in pediatric patients with celiac disease and inflammatory bowel disease (IBD, either CD or ulcerative colitis, UC) showed a decrease and an increase in duodenal \textit{CYP3A4} mRNA expression respectively\textsuperscript{19, 20}. Similarly, a more recent study in a pediatric CD population showed a relative reduction in ileal \textit{CYP3A4} mRNA compared with \textit{CYP3A4} mRNA taken from non-inflamed duodenal biopsies\textsuperscript{21}. In an adult population of individuals with UC, decreased \textit{CYP3A4} mRNA expression was seen in rectal samples taken from inflamed tissue\textsuperscript{22}. 
The effect of an inflammatory bowel disease on P-gp expression has received more attention, with a large focus on ulcerative colitis (UC). Of 4 studies identified to date evaluating the mRNA and/or protein expression of P-gp (gene *MDR1*), reductions in the colonic expression of P-gp protein or *MDR1* mRNA are documented predominantly in UC patients with active disease23-25, 33.

Our study is the first to evaluate the concomitant protein expression of intestinal CYP3A4 and P-gp in a CD population. We demonstrate a significant reduction in ileal and colonic CYP3A4 as well as colonic P-gp expression in subjects with CD compared to those without. Based on our findings, one could hypothesize that alterations in the intestinal expression of important drug disposition regulators may account for a portion of the heterogeneity seen in CD-oral drug response.

A limitation of our study is the absence of further experimentation to confirm the mechanism by which CYP3A4 and P-gp protein are decreased in the setting of CD. Other studies have shown a decrease in mRNA, suggesting that the change is reflective of changes in gene expression19, 21; however, further study is needed to evaluate the contribution of other factors such as RNA polymerase recruitment, histone acetylation and methylation, the binding of transcription factors as well as a diverse range of possible epigenetic modifications.

Our data re-enforce the principal that age and disease appear to affect CYP3A4 expression, but highlight the specific impact on intestinal CYP3A4 rather than on hepatic CYP3A4. We show that CD independently and negatively affects CYP3A4 expression in the ileum and colon. Moreover, we show that CYP3A4 varies with age. Our data support an age-related decline in ileal CYP3A4 expression in our control cohort. The
literature, though inconsistently, demonstrates a similar decrease in hepatic and total body CYP3A4 with age. Interestingly, we see an increase in ileal CYP3A4 expression with age in our CD cohort. The reasons for this remain unexplained.

Colonic P-gp expression declined with age in both the CD and non-CD cohorts. The effect of age on intestinal P-gp expression is not well-studied. Decreases in the endothelial expression of P-gp at the blood-brain barrier are documented in Alzheimer's disease. It is unclear if this is secondary to the aging process or related to pathophysiologic changes of the disease.

There are several hypotheses to explain the decrease in hepatic CYP3A4 activity and/or expression seen in disease states such as acute infection, cancer, liver and kidney disease. It is has been proposed that during an acute inflammatory process, hepatic CYP3A4 is down-regulated due to the effects of IL-1β, IL-6, TNF-α, and IFN-α and γ. Intestinal CYP3A4 may likewise be down-regulated. Other theories pertain to disease-dependent changes in the activation of nuclear receptor (NR) such as the farnesoid X receptor or the pregnane X receptor. These transcription factors and master regulators of homeostasis control transcription of CYP3A4 and are increasingly shown to be relevant to and changed in those affected by IBD. This may have important consequences for intestinal CYP3A4 expression. Specific to intestinal CYP3A4, disruption of the intestinal mucosa by the discontinuous, transmural inflammatory changes of CD may also be a contributor to altered CYP3A4 expression; however, our data do not support differential CYP3A4 protein expression based on CD activity or location.
Overall, reasons for the molecular changes in the intestinal epithelium of CD patients may extend beyond the presence or absence of active inflammation and may be due to the inherent changes in the host’s physiology that predispose them to this condition. Here, we see that CYP3A4 protein expression is appreciably different in the setting of endoscopically normal intestinal tissue irrespective of disease activity. Langmann et al. (2004) showed that MDR1 mRNA coding for P-gp was reduced to the same degree in inflamed and non-inflamed UC colonic samples. It was proposed that loss of P-gp function may be an initiating factor pre-disposing to the development of chronic intestinal inflammation, rather than being a consequence of it. P-gp is co-localized in the enterocyte with CYP3A4; it is concentrated at the enterocyte brush border with CYP3A4 found below in the cytoplasmic endoplasmic reticulum. CYP3A4 and P-gp share many substrates, inducers and inhibitors. They are hypothesized to act in a coordinated manner in the determination of the disposition of their shared endogenous and exogenous substrates, playing a key role in detoxification and intestinal barrier function. Therefore, one could surmise that changes in CYP3A4 expression (similar to P-gp) could predispose to CD rather than be a downstream effect of its presence. Further studies are needed to explore these theories.

In conclusion, these data emphasize the loss of expression of CYP3A4 and P-gp in the CD gastro-intestinal tract. This appears to be independent of disease activity and location, highlighting a possible role for CYP3A4 and P-gp in CD pathogenesis and disease predisposition. Replication of this study, as well as assessment of protein activity, in a larger patient population would be useful in further elucidating the impact of altered enzyme expression on drug response in CD as well as disease pathogenesis.
4.5 References


5 GENETIC VARIATION IN CYP3A4-REGULATOR FXR PREDICTS SEVERITY OUTCOMES IN CROHN'S DISEASE

A version of this chapter has been submitted for publication: Wilson A, Almousa AA, Jansen LE, Choi Y, Teft WA, Kim RB. Genetic variation in the Farnesoid X Receptor predicts Crohn's disease severity in female patients. Gastroenterology 2018
5.1 Introduction

Crohn's disease (CD) is an autoimmune inflammatory bowel disease defined by remitting and relapsing episodes of intestinal inflammation\(^1\). CD pathogenesis is complex. Dysregulation of the host immune response in the setting of specific environmental and genetic factors are hypothesized to serve as disease precursors\(^1\text{-}^5\). A key component of this inappropriate immune response is the disruption of the mucus layer, loss of epithelial tight junctions and increased intestinal permeability and thereby an increase in the intestinal immune system's exposure to bacteria, resulting in an immune response via its innate and adaptive arms\(^2\text{-}^4\). Furthermore, there is a shift in production from anti-inflammatory proteins to pro-inflammatory cytokines through the activation of nuclear transcription factors such as nuclear factor κB (NFκB)\(^6\). Early in its course, CD is remitting and relapsing, with periods of activity punctuated by debilitating symptoms of abdominal pain, diarrhea, and weight loss as well as biochemical and endoscopic findings of inflammation. As the disease progresses over time, permanent damage to the intestinal structure may result, leading to an irreversible impairment of intestinal function, significant morbidity and long-term disability. Disease severity is marked by the need for and time to surgery, failure of multiple medical therapies, need for hospitalization and the presence of complications such as fistulas or strictures\(^7\).

Many studies have attempted to link genetic variation in key genes associated with inflammation, xenobiotic metabolism and transport as well as gene regulators of such pathways to CD susceptibility. Several single nucleotide polymorphisms (SNPs) in the multi-drug resistance-1 (MDR1) gene, the pregnane X receptor (PXR, NR1I2) gene and to a lesser extent the farnesoid x receptor (FXR, NR1H4) gene have been evaluated
and some linked to IBD susceptibility with varying degrees of success; however, none have emerged as a clinically translatable marker of IBD presence, drug response or disease severity.

There is now an increasing appreciation of the bile acid-sensing nuclear receptor, FXR, as the master regulator of bile acid homeostasis and transport pathways, intestinal inflammation, intestinal permeability and response to bacterial overgrowth. FXR is also an important regulator of drug metabolism sequences, with the ability to activate key cytochrome P450 (CYP) family isoform 3A4 directly and indirectly via the pregnane X receptor. Interestingly, the expression and activity of CYP3A4 is altered in CD, though this has not been conclusively linked to CD-related changes in FXR activity.

*In vivo* models confirm that in the absence of FXR activation, there is an expansion of the bile acid pool and a more severe presentation of chemically-induced colitis, including increased intestinal cellular infiltrate, collagen deposition and expression of inflammatory genes. FXR activation modulates fibroblast growth factor (FGF) expression, and plasma FGF19 concentrations have been used as a surrogate marker of FXR activity. Moreover, FXR agonism in these same models improves intestinal permeability and attenuates the production of pro-inflammatory cytokines such as IL-1β and TNFα via the nuclear factor κB (NFκB) pathway. Defects in the epithelial barrier are well-documented in CD and may contribute to disease onset and progression. There is data to support a decrease in FXR activity and expression in CD.

Our group was the first to identify and demonstrate that a SNP adjacent to the ATG start codon, in a sequence known as the Kozak consensus motif, in *FXR (NR1H4)*, -1G>T, is linked to reduced transactivation of FXR gene targets. Conservation of the
Kozak consensus motif is necessary to ensure ribosomal binding to mRNA transcripts and efficient protein translation\textsuperscript{28}. Genetic variation in the Kozak motif is associated with decreased protein translation\textsuperscript{28}. Van Mil \textit{et al.} (2007) demonstrated that the \textit{FXR}\textsubscript{1}-\textit{IG}>\textit{T} SNP is associated with reduced FXR protein expression as well as decreased activation of its down-stream targets, citing translational inefficiency as the underlying cause\textsuperscript{29}. Thus, despite being in a non-coding region of the \textit{FXR} gene, the \textit{FXR}-\textit{1G}>\textit{T} SNP may have important functional consequences. Interestingly, SNPs in \textit{FXR} tend to be rare, although they have been documented in FXR-deficiency, which presents as a more severe form of progressive familial intrahepatic cholestasis (PFIC), associated with coagualopathy, jaundice and a rapid progression to liver failure \textsuperscript{30, 31}. These data suggest major loss of function mutations in \textit{FXR} as contributors to CD are unlikely; however, partial loss of function or expression of \textit{FXR} due to changes in translational efficiency may contribute to CD progression or severity. To date, the role of \textit{FXR}-\textit{1G}>\textit{T} SNP has not been evaluated in a CD population.

Accordingly, we hypothesize that changes in the intestinal barrier regulated through reduced FXR expression among those who harbor the \textit{FXR}-\textit{1G}>\textit{T} SNP are more likely to exhibit a severe CD phenotype including a more rapid progression to surgery. Alterations in FXR activity may in part be secondary to genetic variation in the \textit{FXR} gene. In this study, we demonstrate the impact of \textit{FXR}-\textit{1G}>\textit{T} SNP on a CD population in comparison to genetic variation in \textit{PXR} and \textit{MDR1}. 
5.2 Materials And Methods

5.2.1 Subjects

This study was conducted in two parts: in the first phase, a retrospective, single centre, cohort study was carried out in 198 patients with CD, being seen as part of the Personalized Medicine Program at Western University, London Canada between March 2013 and 2015. The aim was to evaluate the utility of FXR-1G>T as a genomic biomarker of severity in CD. In the second phase, a separate cohort of patients with CD (n=188) were retrospectively screened for the FXR-1G>T genotype between November 2015 and June 2017 to validate the findings of the first phase. The cohorts were then pooled for further analysis (n= 386). In addition, all subjects were screened for MDR1 3435C>T and PXR -25385C>T. A subset of subjects underwent plasma FGF19 and plasma bile acid profile determination. Subjects from each cohort provided written, informed consent. Eligible subjects were more than 18 years of age. All subjects had a histopathological diagnosis of CD. Subjects were excluded if information pertaining to their medical history was unavailable or unknown or if they had a diagnosis of ulcerative colitis. Each subject provided one blood sample. The study protocol was approved by the Western University Health Sciences Research Ethics Board (15586).

5.2.2 Demographic and Covariate Data Abstraction

Data collected on subjects from both cohorts included age, sex, weight, smoking history, medical history, duration of disease as well as CD medication exposures and
responses (adverse drug reactions (ADRs), induction of remission, resistance or loss of response). Data relevant to their CD diagnosis was also collected including disease phenotype, disease activity (based on the clinical scoring index, Harvey-Bradshaw Index, HBI) at the time of blood collection, hospitalizations, and history of and time to surgical resection. This information was collected from patient records between the date of diagnosis and the study end period (cohort 1, March 30, 2015 and cohort 2, June 1, 2017).

5.2.3 Genotypic Analysis

DNA was extracted from whole blood using a standard DNA extraction protocol (MagNA Pure Compact System, Pleasanton California, USA). Allelic discrimination using TaqMan assays and a 7500 RT-PCR System (Applied Biosystems, Carlsbad California, USA) was used to determine the presence of the variant, FXR (NR1H4) -1G>T (rs56163822) in CD subjects with available DNA (n=386) as well as the variants, MDR1 3435C>T (rs1045642) and PXR (NR1I2) -25385C>T (rs3814055). Genotyping experiments included three positive controls and one negative control. Five percent of samples were genotyped in duplicate. Congruency was seen amongst all duplicated genotypes.
5.2.4 Fibroblast Growth Factor 19 Quantification

Blood samples were drawn from study subjects and plasma was extracted by centrifugation. A commercial enzyme-linked immunosorbent assay (ELISA) kit (FGF19 Quantikine ELISA kit, category no. DF1900; R&D Systems, Minneapolis, MN, US) was used for the colorimetric detection and estimation of FGF19 plasma concentrations following the manufacturer's instructions for subjects with available plasma samples who underwent surgical intervention (n=137). All plasma aliquots as well as the standard curve (0pg/ml-1000pg/ml) were assayed in duplicate.

5.2.5 Bile Acid Profile Determination

Plasma stored at -80°C was used for quantification of 12 bile acids (cholic acid, CA; chenodeoxycholic acid, CDCA; deoxycholic acid, DCA; glycocholic acid, GCA; glycochenodeoxycholic acid, GCDCA; glycodeloxycholic acid, GDCA; lithocholic acid, LCA; taurocholic acid, TCA; taurodeoxycholic acid, TDCA; tauroliothicholic acid, TLCA; tauroursodeoxycholic acid, TUDCA; and ursodeoxycholic acid, UDCA) using high performance liquid chromatography/mass spectrometry (LC-MS/MS). Taurocholic acid-d5 (TCA-D5) was used as an internal standard (I.S). A standard curve of 1nM to 40µM was generated. Patient samples were homogenized by vortexing for 15 minutes at 4 °C @1400 RPM and incubated at -20°C for 20 minutes. Samples were centrifuged at maximum speed at 4°C for 30 minutes. Finally, 100µL of supernatant was transferred to vials and 80µL was injected into the LC-MS/MS system.
A two-dimensional LC system was used (Agilent HILIC plus column (4.6*50) 3.5µm followed by Phenomenex 00B-4462-Y0 Kinetex 2.6u C18 100A (3.0*50)). A gradient elution technique was employed using the Agilent 1290 system. Mobile phase A consisted of 2mM ammonium acetate (pH 4.15) with 10% acetonitrile. Mobile phase B consisted of acetonitrile: isopropyl-alcohol with water. The columns’ temperatures were maintained at 60°C. Samples were eluted at a flow rate of 0.6ml/min to 1.4ml/min (0.1-12 minutes). Before injection of the subsequent sample, columns were cleaned with 100% mobile phase B for one minute and returned to a linear equilibrium for 7 minutes for a total run of 20 minutes for each sample. Mass detection occurred on a TSQ-Quantum Ultra mass spectrometer equipped with HESI source and operated in negative mode (4500 v spray voltage, 350°C vaporizing temperature, 45 sheath gas pressure, 15 auxiliary gas pressure and 350 °C capillary temperature).

5.2.6 Study Objectives and Outcomes

The objective of this study was to test the hypothesis that the FXR-1GT genotype is a predictor of disease severity in CD. The primary endpoints were the rate of surgery and time to surgery in subjects with an FXR-1GG genotype versus subjects with a -1GT genotype. Secondary outcomes included other indicators of severity such as number of flares, number of hospitalizations and number of failed medications all standardized to per-year of CD diagnosis. Other endpoints included determination of the plasma concentration of FGF19 in these two genotypic populations suggesting a mechanism by which FXR-1G>T is influencing intestinal barrier integrity. Moreover, we evaluated the
rate of surgery, time to surgery, flares, hospitalizations and number of failed medications amongst wild type and variant carriers of the MDR1 3435C>T (rs1045642) and PXR -25385C>T (rs3814055) as well as evaluated the plasma bile acid profile in subjects with a the heterozygous variant genotype (FXR-1GT) versus sex-, age- and weight-matched controls (FXR-1GG, wildtype).

5.2.7 Statistical Analysis

Statistical analysis was performed using Graphpad Prism, R, and SPSS statistical software. Allele frequency distribution for the FXR -1G>T, MDR1 3435C>T and PXR 25385 genotypes were tested for Hardy-Weinberg equilibrium using a $\chi^2$ goodness-of-fit test. A Cox proportional-hazards regression model with or without adjusting covariates was used to assess the influence of FXR-1T, MDR1 3435T and PXR -25385T variant carrier status on the time to first surgical resection for the total population, males and females and hazard ratios (HR) were expressed with 95% confidence intervals (CI). Risk of surgery associated with FXR-1T, MDR1 3435T and PXR -25385T variant carrier status was evaluated using a logistic model with and without adjustment and are expressed as odds ratios (OR) with 95%CI. Covariates that were considered included the following: PXR-25385C>T genotype, MDR13435C>T genotype, exposure to combined therapy with an immunosuppressant and biologic, any biologic, methotrexate, glucocorticoid or thiopurine exposure, biologic failure32 (defined as development of an ADR requiring cessation of the biologic; primary non-response: a lack of improvement in clinical symptoms with induction therapy as defined by their treating physician; or loss of
response: a recurrence in disease activity during maintenance therapy despite an adequate response to induction dosing as defined by the treating physician), any drug failure, hospitalizations, smoking history, duration of disease, pre-operative biologic exposure, and time to biologic exposure. A final model was constructed for the total population as well as stratified by sex, adjusting for FXR-1G>T genotype, age, weight and other, aforementioned covariates.

To test the hypothesis of an association between the FXR -1G>T SNP and a decrease in downstream products of the FXR gene, a Welch's t-test was used to compare FGF-19 plasma concentrations between genotype groups (FXR -1G>T variant carriers versus wild-type) within the surgical cohort (n=137). A p-value ≤0.05 was considered significant. A multiple linear regression analysis was used to further evaluate the relationship between FXR-1T carrier status, other covariates and the inter-individual variation in FGF-19 plasma concentrations (natural log-transformed) in participants who underwent a surgical intervention. The analysis was performed for the total population as well as stratified by sex. Other covariates assessed included the following: age, weight, disease activity, and disease location. Lastly, mean plasma concentrations for the 12 previously referenced bile acids were compared between FXR-1G>T variant carriers (FXR-1GT, n= 22) and age- and sex-matched wild type individuals (FXR-1GG) in a 2:1 ratio using a Student's t-test. A p-value ≤0.05 was considered significant.
5.3 Results

5.3.1 Study Population

Baseline characteristics for all participants are presented in Table 5.1. Five hundred and eighty-seven individuals with a diagnosis of inflammatory bowel disease (IBD) were screened at the time of visit to the Personalized Medicine Clinic. Individuals with a diagnosis of UC were excluded (n=201). Three hundred and eighty-six participants with CD were included in the final analyses (cohort 1, n=198; cohort 2, n=188) of which 137 participants had undergone an intra-abdominal surgical intervention for their CD. The rate of surgery increased with duration of diagnosis (1-year, 11.14%; 5-year, 23.32%; and 10 years after diagnosis, 29.53%). The most common indication for surgery was stricturing disease (GG, n = 61; GT, n = 8) followed by fistulizing disease (GG, n = 34; GT, n = 4). Surgical interventions included small bowel or ileocolic resections with primary anastomosis and total colectomies with formation of an ileostomy. Table 5.2 summarizes the demographic data for subjects undergoing a surgical intervention. Table 5.3 summarizes the demographic data for subjects not undergoing a surgical intervention.
Table 5.1: Demographic Characteristics of Patients in Cohort 1 and Cohort 2

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cohort 1 (n = 198)</th>
<th>Cohort 2 (n = 188)</th>
<th>Total population (n = 386)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean, range)</td>
<td>43.04 (18-85)</td>
<td>41.55 (18-80)</td>
<td>42.31 (18-85)</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>119 (60.1)</td>
<td>115 (61.2)</td>
<td>234 (60.6)</td>
</tr>
<tr>
<td>Weight, kg (mean ± std)</td>
<td>77.06 ± 18.43</td>
<td>76.64 ± 20.38</td>
<td>76.81 ± 19.42</td>
</tr>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal</td>
<td>75 (37.9)</td>
<td>60 (31.9)</td>
<td>135 (35.0)</td>
</tr>
<tr>
<td>Colonic</td>
<td>36 (18.2)</td>
<td>39 (20.7)</td>
<td>75 (19.4)</td>
</tr>
<tr>
<td>Ileo-colonic</td>
<td>87 (43.9)</td>
<td>89 (47.3)</td>
<td>176 (45.6)</td>
</tr>
<tr>
<td>Disease duration, years (mean ± std)</td>
<td>9.63 ± 10.36</td>
<td>9.11 ± 10.01</td>
<td>9.37 ± 10.18</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>63 (31.8)</td>
<td>36 (19.1)</td>
<td>99 (25.6)</td>
</tr>
<tr>
<td>Biologic exposure (%)</td>
<td>81 (40.1)</td>
<td>102 (54.3)</td>
<td>183 (47.4)</td>
</tr>
<tr>
<td>Anti-TNF (%)</td>
<td>81 (40.1)</td>
<td>93 (49.5)</td>
<td>136 (35.2)</td>
</tr>
<tr>
<td>Anti-integrin (%)</td>
<td>0 (0)</td>
<td>13 (6.9)</td>
<td>13 (3.4)</td>
</tr>
<tr>
<td>Anti-IL12/23 (%)</td>
<td>0 (0)</td>
<td>10 (5.3)</td>
<td>10 (2.6)</td>
</tr>
<tr>
<td>Combination therapy (%)</td>
<td>68 (34.2)</td>
<td>83 (44.1)</td>
<td>151 (39.1)</td>
</tr>
<tr>
<td>Glucocorticoid exposure (%)</td>
<td>157 (79.2)</td>
<td>105 (55.8)</td>
<td>262 (67.9)</td>
</tr>
<tr>
<td>Immunomodulator exposure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX (%)</td>
<td>50 (25.2)</td>
<td>39 (20.7)</td>
<td>89 (23.1)</td>
</tr>
<tr>
<td>Thiopurine (%)</td>
<td>131 (66.1)</td>
<td>122 (65.0)</td>
<td>253 (65.5)</td>
</tr>
<tr>
<td>Surgery (%)</td>
<td>62 (31.3)</td>
<td>75 (40.0)</td>
<td>137 (35.5)</td>
</tr>
<tr>
<td>Mean number of surgeries (mean ± std)</td>
<td>0.51 ± 0.99</td>
<td>0.67 ± 1.22</td>
<td>0.59 ± 1.11</td>
</tr>
<tr>
<td>Hospitalizations (mean ± std)</td>
<td>0.92 ± 1.67</td>
<td>1.42 ± 2.64</td>
<td>1.17 ± 2.22</td>
</tr>
<tr>
<td>FXR -1GT carrier status (%)</td>
<td>7 (3.5)</td>
<td>15 (8.0)</td>
<td>22 (5.7)</td>
</tr>
<tr>
<td>FXR -1GG carrier status (%)</td>
<td>191 (96.4)</td>
<td>173 (92.0)</td>
<td>364 (94.3)</td>
</tr>
</tbody>
</table>

Kilograms, kg; standard deviation, std; Crohn’s disease, CD; tumor necrosis factor, TNF; interleukin, IL; methotrexate, MTX; farnesoid X receptor, FXR
Table 5.2: Demographic Characteristics of CD Patients Undergoing Surgical Intervention by FXR \( -1G>T \) Genotype

<table>
<thead>
<tr>
<th>Variables</th>
<th>FXR -1GG n = 124</th>
<th>FXR-1GT n = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (mean, range)</td>
<td>44.84 (19-85)</td>
<td>40.15 (20-70)</td>
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<tr>
<td>Female sex (%)</td>
<td>59 (47.6)</td>
<td>12 (92.3)</td>
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<tr>
<td>Weight, kg (mean ± std)</td>
<td>76.23 ± 17.46</td>
<td>71.09 ± 20.53</td>
</tr>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal</td>
<td>38 (30.6)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Colonic</td>
<td>22 (17.7)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Ileo-colonic</td>
<td>64 (51.6)</td>
<td>11 (84.6)</td>
</tr>
<tr>
<td>Duration of disease, years (mean ± std)</td>
<td>16.29 ± 12.10</td>
<td>14.67 ± 12.50</td>
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<tr>
<td>Smoking history (%)</td>
<td>39 (31.4)</td>
<td>0 (0)</td>
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<tr>
<td>Biologic exposure (%)</td>
<td>62 (50.0)</td>
<td>8 (61.54)</td>
</tr>
<tr>
<td>Anti-TNF (%)</td>
<td>51 (41.1)</td>
<td>5 (38.46)</td>
</tr>
<tr>
<td>Anti-integrin (%)</td>
<td>6 (4.8)</td>
<td>1 (7.69)</td>
</tr>
<tr>
<td>Anti-IL12/23 (%)</td>
<td>5 (4.0)</td>
<td>3 (23.08)</td>
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<tr>
<td>Pre-operative biologic exposure (%)</td>
<td>27 (21.8)</td>
<td>4 (30.77)</td>
</tr>
<tr>
<td>Combination therapy (%)</td>
<td>43 (34.7)</td>
<td>3 (23.08)</td>
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<tr>
<td>Glucocorticoid exposure (%)</td>
<td>104 (83.8)</td>
<td>13 (100)</td>
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<tr>
<td>Immunomodulator exposure</td>
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<td></td>
</tr>
<tr>
<td>MTX (%)</td>
<td>32 (25.8)</td>
<td>5 (38.46)</td>
</tr>
<tr>
<td>Thiopurine (%)</td>
<td>79 (63.7)</td>
<td>7 (53.85)</td>
</tr>
<tr>
<td>Number of surgeries (mean ± std)</td>
<td>1.60 ± 1.31</td>
<td>1.85 ± 1.07</td>
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<tr>
<td>Time to first surgical intervention</td>
<td>5.99 ± 6.94</td>
<td>1.56 ± 2.08</td>
</tr>
<tr>
<td>intervention, years (mean ± std)</td>
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</tr>
<tr>
<td>Hospitalizations (mean ± std)</td>
<td>2.31 ± 2.81</td>
<td>2.62 ± 1.85</td>
</tr>
</tbody>
</table>

Farnesoid X receptor, FXR; kilograms, kg; standard deviation, std; Crohn's disease, CD; tumor necrosis factor, TNF; interleukin, IL; methotrexate, MTX
Table 5.3: Demographic Characteristics of CD Patients Who Did Not Undergo Surgical Intervention by $FXR\,-1G>T$ Genotype

<table>
<thead>
<tr>
<th>Variables</th>
<th>FXR -1GG n=240</th>
<th>FXR-1GT n=9</th>
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<tr>
<td>Age, years (mean, range)</td>
<td>41.04 (18-80)</td>
<td>36.44 (18-60)</td>
</tr>
<tr>
<td>Female sex (%)</td>
<td>153 (63.8)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Weight, kg (mean ± std)</td>
<td>77.28 ± 20.31</td>
<td>88.41 ± 16.54</td>
</tr>
<tr>
<td>Disease location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ileal</td>
<td>90 (37.5)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Colonic</td>
<td>56 (23.3)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Ileo-colonic</td>
<td>94 (39.2)</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>Duration of disease, years (mean ± std)</td>
<td>5.63 ± 6.66</td>
<td>5.92 ± 6.92</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>37 (15.4)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Biologic exposure (%)</td>
<td>103 (42.9)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Anti-TNF (%)</td>
<td>94 (39.2)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Anti-integrin (%)</td>
<td>7 (2.9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Anti-IL12/23 (%)</td>
<td>2 (0.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Combination therapy (%)</td>
<td>30 (12.5)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>Glucorticoid exposure (%)</td>
<td>187 (77.9)</td>
<td>6 (66.7)</td>
</tr>
<tr>
<td>Immunomodulator exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTX (%)</td>
<td>44 (18.3)</td>
<td>3 (33.3)</td>
</tr>
<tr>
<td>Thiopurine (%)</td>
<td>159 (66.3)</td>
<td>5 (55.6)</td>
</tr>
<tr>
<td>Hospitalizations (mean ± std)</td>
<td>0.57 ± 1.64</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

Farnesoid X receptor, FXR; kilograms, kg; standard deviation, std; Crohn's disease, CD; tumor necrosis factor, TNF; interleukin, IL; methotrexate, MTX
5.3.2 \textit{FXR-1G>T} Predicts Surgery Risk and Early Progression to Surgery Most Significantly in Women

All subjects underwent genotyping for the SNP \textit{FXR (NR1H4) -1G>T}. The minor allele (-1T) frequency was 2.8\% in the total cohort. \textit{FXR-1G>T} was in Hardy-Weinberg equilibrium. Genotype frequencies classified by the presence or absence of surgery are displayed in Figure 5.1. There was a strong association between carriers of the variant \textit{T} allele and surgical intervention (odds ratio, OR=2.78, 95\%CI=1.16-6.69, p=0.02) (Figure 5.1a). However, this association did not remain (OR=2.21, 95\%CI=0.65-7.50, p=0.20) with adjustment for covariates such as age, sex, weight, smoking history, drug exposures, hospitalizations, use of combination therapy or drug failures.
Figure 5.1 Genotype frequency separated by the presence or absence of an intra-abdominal surgical intervention related to the CD diagnosis for all subjects (A), female subjects (B), and male subjects (C). Genotypes are expressed as a percentage of the total population of study subjects undergoing surgery (n=137) or not undergoing surgery (n=249). *, p <0.05, **, p <0.01, ***, p <0.001. Crohn's disease, CD; farnesoid X receptor, FXR; number, n.
FXR-IT variant carriers were more likely to go on to an early surgical intervention compared to wild type individuals (combined cohorts, 5.99 years ± 6.94 versus 1.56 years ± 2.08; hazard ratio, HR=2.75, 95%CI=1.55-4.90, p < 0.001) (Figure 5.2).
Figure 5.2 Time to first surgery following the diagnosis of CD stratified by \textit{FXR-1G>T} genotype expressed as wild type (GG) or variant (GT) for individuals in cohort 1 (A), cohort 2 (B), and the combined cohorts (C). *, p < 0.05, **, p < 0.01, ***, p < 0.001. Crohn's disease, CD; farnesoid X receptor, FXR.
*FXR-1T* remained significant (p=0.002) as an independent predictor of early progression to surgical intervention in CD in a Cox proportional-hazards regression model with adjusting covariates. Covariates included in the model were: age, sex, weight, smoking history, number of drugs or biologics failed, hospitalizations, glucocorticoid, thiopurine and methotrexate exposure, combination therapy (biologic with an immunomodulator) and pre-operative biologic exposure. (Table 5.4, Figure 5.3).
Table 5.4: Cox regression for the effects of covariates on time to surgical intervention in a CD cohort (n = 386)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Hazard Ratio</th>
<th>95% Confidence interval</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXR-1T carrier status</td>
<td>2.70</td>
<td>1.45 – 5.02</td>
<td>0.002</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.00</td>
<td>0.99 – 1.01</td>
<td>0.80</td>
</tr>
<tr>
<td>Sex</td>
<td>0.87</td>
<td>0.60 – 1.26</td>
<td>0.47</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>1.00</td>
<td>0.99 – 1.01</td>
<td>0.48</td>
</tr>
<tr>
<td>Smoking history</td>
<td>0.77</td>
<td>0.51 – 1.17</td>
<td>0.22</td>
</tr>
<tr>
<td>Hospitalizations</td>
<td>1.12</td>
<td>1.07 – 1.11</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Exposure to glucocorticoids</td>
<td>2.68</td>
<td>1.49 – 4.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Exposure to methotrexate</td>
<td>0.79</td>
<td>0.49 – 1.25</td>
<td>0.31</td>
</tr>
<tr>
<td>Exposure to thiopurines</td>
<td>0.87</td>
<td>0.58 – 1.31</td>
<td>0.51</td>
</tr>
<tr>
<td>Exposure to combination therapy</td>
<td>1.12</td>
<td>0.68 – 1.83</td>
<td>0.67</td>
</tr>
<tr>
<td>Number of drugs failed</td>
<td>0.69</td>
<td>0.53 – 0.89</td>
<td>0.004</td>
</tr>
<tr>
<td>Number of biologics failed</td>
<td>1.76</td>
<td>1.22 – 2.53</td>
<td>0.003</td>
</tr>
<tr>
<td>Exposure to biologics pre-operatively</td>
<td>2.10</td>
<td>1.33 – 3.30</td>
<td>0.001</td>
</tr>
</tbody>
</table>
**Figure 5.3** HR estimates with the corresponding 95% CI for the evaluation of demographic, medication exposures/responses and *FXR*-1G>T genotype and the impact on time to first surgery. The HR estimate of each variable is marked with solid black square. The 95% CI is represented by the horizontal T-line through the square. The horizontal axis is plotted on a log scale. Hazard ratio, HR; confidence interval, CI; farnesoid X receptor, FXR; number, No.; methotrexate, MTX.
Most significantly, when data were stratified based on sex, the association between carriers of the variant $T$ allele and risk of surgical intervention and early progression to surgical intervention was strongest and most striking in women on simple linear regression analysis (risk of surgery, OR=27.46, 95%CI=2.28-330.50, p=0.01; progression to surgery, HR=9.08, 95%CI=4.72-17.48, p < 0.0001) (Figure 5.1b, Figure 5.4) and with adjustment for covariates (risk of surgery, OR=23.65, 95%CI=1.83-304.74 p≤0.05; progression to surgery, HR=6.15, 95%CI=2.82-13.42, p < 0.0001) (Table 5.5). Of the FXR-1GT carriers going on to surgery, 92.1% were women compared to the FXR-1GG carriers (47.6%) (Table 5.2). Conversely in men, FXR genotype was not significantly associated with risk or time to surgery on simple linear regression analysis (risk of surgery, OR=0.18, 95%CI=0.02-1.5, p=0.11; progression to surgery, HR=0.27, 95%CI=0.04-1.94, p=0.19) or multiple linear regression analysis (risk of surgery, OR=0.27, 95%CI=0.03-2.85, p=0.28; progression to surgery, HR=0.41, 95%CI=0.05-3.21, p=0.40).

Other indicators of severity evaluated included: number of drugs failed, number of surgeries, hospitalizations, number of flares. There was no difference between FXR-1G>T genotypes in any of these parameters of severity, even when adjusting for weight, age, sex and disease duration.
Figure 5.4 Time to first surgery following the diagnosis of CD stratified by FXR-1G>T genotype expressed as wild type (GG) or variant (GT) and female sex in cohort 1 (A), cohort 2 (B), and the combined cohorts (C). *, p <0.05, **, p <0.01, ***, p <0.001. Crohn's disease, CD; farnesoid X receptor, FXR
Table 5.5: Cox regression for the effects of covariates on time to surgical intervention in a female CD cohort (n = 234)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Hazard Ratio</th>
<th>95% Confidence interval</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXR-1T carrier status</td>
<td>6.15</td>
<td>2.82 – 13.42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.00</td>
<td>0.98 – 1.02</td>
<td>0.85</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>1.00</td>
<td>0.98 – 1.01</td>
<td>0.79</td>
</tr>
<tr>
<td>Smoking history</td>
<td>1.01</td>
<td>0.58 – 1.76</td>
<td>0.98</td>
</tr>
<tr>
<td>Hospitalizations</td>
<td>1.15</td>
<td>1.06 – 1.25</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Exposure to glucocorticoids</td>
<td>2.90</td>
<td>1.22 – 6.89</td>
<td>0.016</td>
</tr>
<tr>
<td>Exposure to methotrexate</td>
<td>0.95</td>
<td>0.52 – 1.73</td>
<td>0.86</td>
</tr>
<tr>
<td>Exposure to thiopurines</td>
<td>1.05</td>
<td>0.61 – 1.82</td>
<td>0.86</td>
</tr>
<tr>
<td>Exposure to combination therapy</td>
<td>1.41</td>
<td>0.70 – 2.83</td>
<td>0.34</td>
</tr>
<tr>
<td>Number of drugs failed</td>
<td>0.61</td>
<td>0.43 – 0.88</td>
<td>0.008</td>
</tr>
<tr>
<td>Number of biologics failed</td>
<td>1.87</td>
<td>1.15 – 3.02</td>
<td>0.011</td>
</tr>
<tr>
<td>Exposure to biologics pre-operatively</td>
<td>1.24</td>
<td>0.68 – 2.26</td>
<td>0.47</td>
</tr>
</tbody>
</table>
5.3.3 Genetic Variation in \textit{PXR} and \textit{MDRI} is not Associated with a Severe CD phenotype

Additionally, all subjects were screened for the SNPs, \textit{MDRI} 3435C$>$T and \textit{PXR} -25385C$>$T. Minor allele frequencies were 53.7\% and 40.2\% respectively. Overall, there was no significant association between either SNP and risk of (Figure 5.5) or time to surgical intervention (Figure 5.6-5.8). In cohort 1, male carriers of \textit{PXR} -25385T progressed to surgery more quickly than wild type men (HR=3.95, 95\%CI=1.54-10.15); however, this was not confirmed in cohort 2 nor in analysis of the combined cohort. Furthermore, there was no significant association between either SNP and other indicators of CD severity (number of drugs failed, number of surgeries, hospitalizations)
Figure 5.5 Genotype frequency for *MDR1 3435C>T* (panel 1) and *PXR -25385C>T* (panel 2) separated by the presence or absence of an intra-abdominal surgical intervention related to the CD diagnosis for all subjects (A), female subjects (B), and male subjects (C). Genotypes are expressed as a percentage of the total population of study subjects undergoing surgery (n=137) or not undergoing surgery (n=249). Multi-drug resistance protein 1, MDR1; pregnane X receptor, PXR.
Figure 5.6 Time to first surgery following the diagnosis of CD stratified by \textit{MDR1} 3435C>T (panel 1) and \textit{PXR} -25385C>T (panel 2) genotype expressed as wild type (CC) or variant (CT or TT) for individuals in cohort 1 (A), cohort 2 (B), and the combined cohorts (C). Multi-drug resistance protein 1, MDR1; pregnane X receptor, PXR; wild type (WT).
Figure 5.7 Time to first surgery following the diagnosis of CD stratified by MDR1 3435C>T genotype expressed as wild type (CC) or variant (CT or TT) for women in cohort 1 (A), cohort 2 (B), and the combined cohorts (C) and for men in cohort 1 (D), cohort 2(E) and the combined cohorts (F). Multi-drug resistance protein 1, MDR1; wild type (WT).
Figure 5.8 Time to first surgery following the diagnosis of CD stratified by PXR -25385C>T genotype expressed as wild type (CC) or variant (CT or TT) for women in cohort 1 (A), cohort 2 (B), and the combined cohorts (C) and for men in cohort 1 (D), cohort 2(E) and the combined cohorts (F). Pregnane X receptor, PXR; wild type (WT).
5.3.4 *FXR-1G>T* is a Determinant of FGF19 Plasma Concentrations in Women

Another objective of this study was to evaluate FGF19 plasma concentrations in *FXR-1G>T* wild type and variant allele carriers. FGF19 plasma concentrations were lower in *FXR-1GT* variant carriers, though this did not achieve statistical significance on bivariate analysis (GG = 0.35pg/L±0.04pg/L; GT = 0.23pg/L ±0.05pg/L , p=0.215) (Figure 5.9a) or multiple linear regression analysis (natural log transformed FGF19 plasma concentrations, Table 5.6). Furthermore, the multiple linear regression analysis only accounted for 6.5% of the inter-individual variation in FGF19 plasma concentrations.

Interestingly, when stratified by sex, women with an *FXR-1GT* genotype had a two-fold lower FGF-19 plasma concentration (p≤0.05) compared to women with a wild type genotype on univariate and multivariate analyses (Figure 5.9, Table 5.7). This was not seen in the male population.
Table 5.6 Multiple linear regression model for the effect on Ln-transformed FGF-19 plasma concentration for all subjects (n = 137, adjusted $R^2=0.065$)

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.748</td>
<td>0.635</td>
<td>0.007</td>
</tr>
<tr>
<td>FXR -1T carrier status</td>
<td>-0.476</td>
<td>0.345</td>
<td>0.170</td>
</tr>
<tr>
<td>Age</td>
<td>-0.002</td>
<td>0.007</td>
<td>0.787</td>
</tr>
<tr>
<td>Female Sex</td>
<td>0.317</td>
<td>0.208</td>
<td>0.130</td>
</tr>
<tr>
<td>Weight</td>
<td>0.006</td>
<td>0.006</td>
<td>0.236</td>
</tr>
<tr>
<td>Small bowel resection or disease</td>
<td>-0.516</td>
<td>0.274</td>
<td><strong>0.062</strong></td>
</tr>
</tbody>
</table>

Table 5.7 Multiple linear regression model for the effect on Ln-transformed FGF-19 plasma concentration for females (n = 74)

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\beta$-coefficients</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-1.287</td>
<td>0.738</td>
<td>0.086</td>
</tr>
<tr>
<td>FXR -1T carrier status</td>
<td>-0.714</td>
<td>0.367</td>
<td><strong>0.050</strong></td>
</tr>
<tr>
<td>Age</td>
<td>-0.003</td>
<td>0.009</td>
<td>0.693</td>
</tr>
<tr>
<td>Weight</td>
<td>0.001</td>
<td>0.007</td>
<td>0.897</td>
</tr>
<tr>
<td>Small bowel resection or disease</td>
<td>0.054</td>
<td>0.274</td>
<td>0.062</td>
</tr>
</tbody>
</table>
**Figure 5.9** The mean FGF-19 plasma concentrations stratified by $FXR-1G>T$ genotype, GG or GT for the total population (A) and for women (B). Median values (thick horizontal line), 25th and 75th percentile values (box outline), minimum and maximum values (whiskers), and outlier values (circles). Fibroblast growth factor 19, FGF-19; farnesoid X receptor, FXR.
5.3.5 The Bile Acid Profile is not Significantly Altered in FXR-1GT Carriers

Plasma bile acid profiles were constructed for all subjects who carried the variant FXR-1T allele (n=22). Similarly, a plasma bile acid profile was generated for age-, sex- and weight-matched wild type (FXR-1GG) subjects (n=40) (Table 5.8). There was no significant difference in the total plasma bile acid pool, the ratio of primary to secondary bile acids or the ratio of unconjugated to conjugated bile acids (Figure 5.10) based on FXR-1G>T genotype. Furthermore, there was no significant difference in any of the individual bile acids between FXR-1G>T genotypes, with the exception of GDCA (Figure 5.11). FXR-1T carriers had an increased concentration of GDCA compared to wild-type subjects (p= 0.003). No difference was seen in bile acid profiles when stratified by sex.
Table 5.8: Bile acid profiles

<table>
<thead>
<tr>
<th>Plasma Bile acids</th>
<th>FXR-IGG (n=40)</th>
<th>FXR-IGT (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml</td>
<td>ng/ml</td>
</tr>
<tr>
<td><strong>Primary bile acids plus conjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA</td>
<td>436.3</td>
<td>421.6</td>
</tr>
<tr>
<td>G-CDCDA</td>
<td>2075.7</td>
<td>2665.3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2522.0</strong></td>
<td><strong>3086.9</strong></td>
</tr>
<tr>
<td>CA</td>
<td>103.6</td>
<td>195.4</td>
</tr>
<tr>
<td>G-CA</td>
<td>277.1</td>
<td>371.2</td>
</tr>
<tr>
<td>T-CA</td>
<td>39.2</td>
<td>43.6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>419.9</strong></td>
<td><strong>610.2</strong></td>
</tr>
<tr>
<td><strong>Secondary bile acids plus conjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DCA</td>
<td>168.2</td>
<td>118.4</td>
</tr>
<tr>
<td>G-DCA</td>
<td>1961.4</td>
<td>1335.9</td>
</tr>
<tr>
<td>T-DCA</td>
<td>141.6</td>
<td>131.2</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2271.2</strong></td>
<td><strong>1585.5</strong></td>
</tr>
<tr>
<td>LCA</td>
<td>108.6</td>
<td>57.1</td>
</tr>
<tr>
<td>T-LCA</td>
<td>12.8</td>
<td>31.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>121.4</strong></td>
<td><strong>88.2</strong></td>
</tr>
<tr>
<td><strong>Tertiary bile acids plus conjugates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDCA</td>
<td>354.5</td>
<td>492.7</td>
</tr>
<tr>
<td>T-UDCA</td>
<td>1.9</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>356.4</strong></td>
<td><strong>495.8</strong></td>
</tr>
<tr>
<td><strong>Total bile acids</strong></td>
<td><strong>5681.1</strong></td>
<td><strong>5866.4</strong></td>
</tr>
</tbody>
</table>

*Plasma bile acid are represented as a percentage of the total bile acid pool

Cholic acid, CA; chenodeoxycholic acid, CDCA; deoxycholic acid, DCA; glycocholic acid, GCA; glycochenodeoxycholic acid, GCDCA; glycdeoxycholic acid, GDCA; lithocholic acid, LCA; taurocholic acid, TCA; taudoxycholic acid, TDCA; taurolithcholic acid, TLCA; taursodeoxycholic acid, TUDCA; and ursodeoxycholic acid, UDCA.
Figure 5.10 The mean total plasma bile acid concentrations (ng/ml) (A), the ratio of primary to secondary mean bile acid concentrations (B) and the ratio of mean unconjugated to conjugated bile acids (C) stratified by FXR-1G>T genotype expressed as wild type (GG) or variant (GT) for the total population of subjects with CD. The 95%CI is represented by the vertical T-line. Crohn’s disease, CD; farnesoid X receptor, FXR; bile acid, BA.
Figure 5.11 The mean individual bile acid profiles expressed as mean concentrations in ng/ml (A) and as a percentage of the total bile acid pool, stratified by \( FXR-1G>T \) genotype expressed as wild type (GG) or variant (GT). The 95%CI is represented by the vertical T-line. Cholic acid, CA; chenodeoxycholic acid, CDCA; deoxycholic acid, DCA; glycocholic acid, GCA; glycochenodeoxycholic acid, GCDCA; glycdeoxycholic acid, GDCA; lithocholic acid, LCA; taurocholic acid, TCA; taurodeoxycholic acid, TDCA; taurolithicholic acid, TLCA; taouroursodeoxycholic acid, TUDCA; and ursodeoxycholic acid, UDCA.
5.4 Discussion

FXR is an important determinant of intestinal barrier function, inflammatory response and drug metabolism. In this study, we are able to demonstrate an important role for a genetic variation adjacent to the ATG start codon of the bile acid sensing nuclear receptor, FXR, as a predictor of CD progression to surgical intervention (Figure 5.2). Remarkably, \textit{FXR-1T} variant carrier status remains an independent predictor of time to first surgery even when other important factors are taken into account (Figure 5.3, Table 5.4).

Most striking, is the discovery that women with a CD diagnosis carrying the \textit{FXR-1GT} genotype are most at-risk for progressing on to surgery (OR=27.46 95%CI=2.28-330.50, \( p=0.01 \)) and progressing on to surgery earlier in their disease course (HR=9.08, 95%CI=4.72-17.48, \( p < 0.0001 \)) compared to men or women with the \textit{FXR-1GG} genotype. Sex-specific differences in CD are largely unexplored; there is a paucity of data on how sex differences impact CD diagnosis and disease management. Interestingly, many important sex-related differences have been identified in other, common, chronic diseases such as coronary artery disease, rheumatologic autoimmune diseases, and renal disease\textsuperscript{33}. Despite these findings, disparity is seen in the management of men and women who suffer these conditions. For example, despite better outcomes in females, women with congestive heart failure receive fewer guideline-based treatments and transplantations compared to men\textsuperscript{34}. Similarly, despite a greater risk of stroke, women are less likely to be anti-coagulated in the setting of atrial fibrillation compared to men\textsuperscript{35}. Such differences and potential disparities have not yet been addressed adequately in CD. There are data to support a slightly higher incidence of CD in women across
several populations. Oral contraception (OCP) use has been linked to the onset of CD in a large prospective cohort study of American women as well as to the risk of CD-related surgery in a Swedish registry. These findings allude to the importance of a female-specific factor to the susceptibility and severity of CD. Our work further highlights the need to consider sex-related differences in CD management.

Interestingly, estradiol (E2), a major female sex hormone and the dominant form of estrogen present during a woman's reproductive years and the main form of estrogen used in OCP, has been shown to inhibit FXR activity via the estrogen receptor (ER) $\alpha$ in vivo and in vitro. Authors examining the relationship of OCP use to CD susceptibility and severity were unable to explain the biological mechanisms for their data. Our work suggests a plausible molecular mechanism for their findings. More recent work by Goodman et al. (2017), using a murine model of colitis, showed that ER$\alpha$ loss-of-function resulted in protection from chemically-induced colitis in female mice, directly linking E2-signaling to pathways of IBD.

Conversely, we found that the IBD-linked SNPs, $MDR1$ 3435C>T and $PXR$ -25385 are not predictive of CD severity. To date, there has been a lack of consensus regarding the relationship between CD susceptibility and genetic variation in $MDR1$ and $PXR$. Brinar et al. (2013) and Juyal et al. (2009) both indicated a link between $MDR1$ genetic variation and IBD, while a larger meta-analysis of existing studies refuted the finding. Similarly, Dring et al. (2006) concluded that $PXR$ -25385C>T was a significant predictor of IBD susceptibility; however, more recent and larger genomic association studies have failed to confirm these original findings. Our study confirms a lack of association between polymorphisms in either of these genes and CD severity,
while emphasizing the association and possible mechanism of the FXR gene. A strength of our study is the replication of the primary objective (association of FXR-1GT genotype with time to and risk of surgery) in a second cohort of patients recruited at a separate time interval. During the second time period, IBD therapies had expanded to include a wider range of biologic treatments, yet the association between FXR-1GT carrier status and risk and time to surgery persisted.

Additionally, a statistically significant difference in the FXR downstream product, FGF19 was seen between individuals with a variant versus wild type FXR-1G>T genotype when assessed in female CD patients (Figure 5.9b, Table 5.7). An almost two-fold increase is seen in the FGF19 plasma concentrations of women with a wild type FXR-1G>T genotype compared to carriers of the variant allele. Our group has previously shown in vitro and in vivo that the FXR -1G>T SNP is associated with reduced activation of downstream products 27. Data by Van Mil et.al. (2007) demonstrated a concomitant decrease in FXR protein and several of its down-stream gene targets29. They concluded that variation in the Kozak consensus motif of the FXR gene is associated with inefficient protein translation, providing a plausible functional mechanism. Interestingly, our finding regarding the downstream FXR target, FGF19, does not extrapolate to the total population, but is of particular relevance to women, highlighting the idea that a female-specific factor(s), in addition to genetic variation in FXR, is influencing the function of the FXR protein. A limitation of this data may be that FGF19 quantification was carried out in individuals with small bowel disease who underwent a small bowel resection of varying, non-quantifiable lengths. This may have impacted FGF19 plasma concentrations and may have confounded our findings.
Conversely, we found that plasma bile acid concentrations, another downstream product of FXR activity, are not increased in individuals with a $FXR-1GT$ genotype. This remained unchanged even with the stratification of bile acid profiles by $FXR-IG>T$ genotype and sex. This is in discordance with murine models of FXR deficiency where $FXR$ null mice had an increase in the total bile acid pool$^{15}$. Due to the low $FXR-1T$ allele frequency, only a small subset of study subjects ($FXR-1GT$, $n = 22$; $FXR1GG$, $n = 40$) underwent plasma bile acid quantification. This may have impacted our ability to demonstrate a significant difference between $FXR-1G>T$ genotypes.

We note that two previous studies have evaluated polymorphisms in $FXR$ and the link to IBD$^{13, 14}$. Nijmeijer et.al. (2011) did not find an association between $FXR-1T$ variant carrier status and IBD disease presence, location or disease type among 2355 IBD patients. Similarly, Attinkara et.al. (2012) failed to show an association between $FXR-1T$ variant carrier status and IBD susceptibility in a cohort of 1138 individuals, half of whom had an IBD diagnosis. However, no assessment was performed to evaluate the link between $FXR-1G>T$ and parameters of disease severity.

In conclusion, we outline important new data in a largely neglected area of CD management: genetic variation in $FXR$ has important clinical consequences, particularly for women with CD. Screening female CD patients for $FXR-1T$ variant carrier status may be useful for identifying female patients at risk for early, poor outcomes. Female carriers of the variant allele may benefit from earlier, more aggressive medical management. Further evaluation of sex-specific differences in CD management is needed to better personalize therapy and avoid the perpetuation of any unidentified disparities.
5.5 References


8. Zintzaras E. Is there evidence to claim or deny association between variants of the multidrug resistance gene (MDR1 or ABCB1) and inflammatory bowel disease? Inflamm Bowel Dis 2012;18:562-572.


6 DISCUSSION AND CONCLUSIONS
6.1 Summary and Discussion

The overall findings discussed in this work are summarized in Figures 6.1 and 6.2.

Figure 6.1 The proposed effect of Crohn's disease on CYP3A4 drug metabolism. Total CYP3A4 activity is decreased in CD. In health, secondary bile acids such as LCA are potent activators of PXR, a known regulator of CYP3A4 activity. Bile acid-induced activation of PXR leads to the translocation of PXR to the nucleus and binding of PXR to its response element contained within the CYP3A4 gene. This results in downstream CYP3A4 expression and activity. Bile acid patterns are altered in CD. There is a greater proportion of unconjugated bile acids as well as primary bile acids compared to individuals without CD (Panel A). This may lead to reduced bile acid-induced activation of PXR, with a resultant decreased in hepatic CYP3A4 activation. There is decreased expression of CYP3A4 protein in the intestine in CD (Panel B). Crohn's disease, CD; cytochrome P450 3A4, CYP3A4; lithocholic acid, LCA; pregnane X receptor, PXR.
Figure 6.2 The proposed effect of genetic polymorphism $FXR-1G>T$ on Crohn's disease. In health, FXR expression regulates bile acid homeostasis by repressing bile acid synthesis. Production of the ileal hormone, FGF-19 is a direct result of FXR activation and inhibits CYP7A1. FXR activation is also important for attenuating the NFκB pathway and the downstream production of pro-inflammatory cytokines as well as enhancing the integrity of the intestinal epithelial barrier (Panel A). In CD, FXR activity is attenuated with documented reductions in FGF19 production, an expansion of the bile acid pool, increased NFκB activity and disruption of the intestinal epithelial barrier (Panel B).
Figure 6.2 (continued) The presence of the genetic polymorphism, *FXR-1G>T* is associated with translational *inefficiency* of the FXR protein. We have documented that female carriers of the *FXR-1GT* genotype with CD have a more severe CD phenotype. This suggests that genetic variation in *FXR* and possibly the presence of estrogen further impair FXR activity in addition to changes already documented in CD (panel C and D). Farnesoid X receptor, FXR; Crohn's disease, CD; fibroblast growth factor 19, FGF19; cytochrome P450 7A1, CYP7A1; nuclear factor κB, NFκB;
6.1.1 Chapter 3

The cytochrome p450 (CYP) 3A family is the cornerstone of drug metabolism and drug disposition in humans\textsuperscript{1,2}. Of the 3A family, the 3A4 isoform is the most well-studied and makes up the highest CYP content in the liver and intestine, key organs of drug metabolism. CYP3A4 activity is highly variable between individuals due to a myriad of patient-intrinsic (sex, age, genetics) and extrinsic (diet, medications) factors\textsuperscript{3-10}. The impact of disease on CYP3A4 activity is still incompletely defined. Given the importance of CYP3A4 to the disposition of a wide range of clinically-important substrates and its high concentration in the intestine, understanding the effect of Crohn's disease (CD), an exceedingly prevalent, chronic, primary intestinal illness, on CYP3A4 activity is highly relevant. In Chapter 3, we aimed to assess the impact of CD on CYP3A4 activity using two \textit{in vivo} modalities in real-time (exogenous probe, midazolam and endogenous probe 4β-hydroxycholesterol, 4βOHC) as well as to propose a molecular mechanism for any detectable differences from non-CD controls. Due to the previously documented effect of acute and non-CD chronic inflammatory states on CYP-mediated metabolism, we hypothesized that CD would negatively impact CYP3A4 activity \textit{in vivo}, particularly intestinal CYP3A4. We posited that a decline in CYP3A4 activity would be related to CD-specific changes in pregnane X receptor (PXR) signaling. In fact, we observed that exposure to CYP3A4 substrate, midazolam, as represented by the oral area under the concentration-time curve (AUC\textsubscript{po}), was significantly increased in our CD cohort compared to reports in healthy controls\textsuperscript{11-14}; thus, we inferred there was a reduction in CYP3A4 activity in CD. Moreover, the greatest reduction in midazolam extraction by CYP3A4 appeared to be at the liver rather than the intestine. Similarly, we
were able to confirm a reduction in CYP3A4 activity in an independent CD cohort compared to non-CD controls using the endogenous biomarker, plasma 4βOHC. However, we were unable to provide a plausible mechanism for differential CYP3A4 activity in CD. Despite detectable differences in plasma bile acids, known PXR-CYP3A4 pathway inducers, in individuals with CD versus non-CD controls, we were unable to show an appreciable impact on PXR-signaling and downstream activation of CYP3A4 \textit{in vitro}.

Ultimately, we were able to identify CD-specific changes to CYP3A4 activity \textit{in vivo}. Despite being a primary intestinal illness, there appeared to be more of an impact on hepatic CYP3A4 activity based on oral and systemic midazolam pharmacokinetics. In other conditions, inflammatory cytokines are posited to decrease CYP3A4 activity\textsuperscript{8,15}; however, we did not see an appreciable difference between subjects with active and inactive CD. This suggests changes in CYP3A4 activity in CD may be a precursor to disease rather than a consequence of inflammation. Despite this observation, we were unable to provide a sound mechanism to explain CD-specific changes in CYP3A4 activity. PXR is an important regulator of CYP3A4 activity that is also known to be altered in CD\textsuperscript{16-18}. It is activated by bile acids, such as lithocholic acid (LCA) and deoxycholic acid (DCA)\textsuperscript{19,20}. We observed differences in the plasma bile acid profiles in our CD cohort, perhaps due to alterations in the gut microbiome\textsuperscript{21}. However, these differences did not translate to differential PXR signaling or downstream CYP3A4 activity in an \textit{in vitro} hepatocarcinoma (Hep) G2 model. Higher concentrations of bile acids reflective of hepatic concentrations (rather than blood) may be needed to further assess the impact of PXR on CYP3A4 activity in CD. All in all, our data demonstrate a
CD-specific decrease in CYP3A4 activity across two separate CD cohorts. This may have important implications for the disposition of CD-related and non-CD-related CYP3A4 drug substrates used in this patient population. Though differences in PXR signaling, due to changes in the plasma bile acid profile, did not account for differences in CYP3A4 activity, bile acid concentrations reflective of hepatic concentrations as well as the effect of other nuclear receptors, such as the farnesoid X receptor (FXR), may need to be considered.

6.1.2 Chapter 4

The concept of first-pass metabolism is well-established. A selection of orally-ingested substrates are subject to extensive processing within the intestinal tract as well as in the liver. CYP3A4 and P-glycoprotein (P-gp) play an important in this process. It is a long-held notion that CYP3A4 and P-gp, both concentrated within enterocytes at the intestinal villous tip, act in a coordinated manner to limit drug and xenobiotic oral bioavailability. According to Zhang and Benet (2001), this theory is built upon the findings of a common intestinal location, shared substrates and the poor oral bioavailability of such substrates\(^1\). Given that the inflammatory bowel disease (IBD), CD introduces a transmural intestinal insult that disrupts the intestinal barrier function, one could assume that this has important consequences for intestinally-expressed proteins such as CYP3A4 and P-gp. Thus, in Chapter 4, we aimed to evaluate the intestinal expression of CYP3A4 and P-gp protein in a cohort of individuals with and without CD. We hypothesized that individuals with CD have reduced intestinal expression of
CYP3A4 and P-gp. We observed a significant reduction in ileal and colonic CYP3A4 protein expression in subjects with CD compared to those without. Similarly, a reduction in colonic P-gp protein expression was seen in the CD cohort. Age appeared to play a role in CYP3A4 expression in both CD and non-CD cohorts, though with opposing effects. Our data highlight important and novel findings pertaining to CD-dependent changes in the intestinal barrier function, first-pass metabolism with consequences for drug and xenobiotic exposure. Further studies are needed to explore the mechanisms of these findings.

6.1.3 Chapter 5

Nuclear receptors (NR) are key regulators of development, metabolism and disease in humans\textsuperscript{22,23}. Several NRs, such as PXR and FXR, play a detoxification role by regulating the expression and activity of CYP3A4 and P-gp\textsuperscript{24}. \textit{In vivo} and \textit{in vitro} models have demonstrated a decrease in PXR and FXR signaling in IBD, with a concomitant improvement in intestinal inflammatory lesions with PXR and FXR up-regulation\textsuperscript{18,25-27}. Interestingly, knocking out \textit{PXR} and \textit{FXR} in murine models does not induce a spontaneous colitis as is seen in the \textit{MDR1a }\textasciitilde\textendash (codes for P-gp) murine model. Rather, it facilitates the onset and increases the severity of an inflammatory condition, suggesting that these NRs may contribute to, but are not wholly responsible for, IBD pathogenesis\textsuperscript{25}. Genetic variation in \textit{FXR} has been linked to deficits in FXR expression as well as a decrease in the activation of downstream targets\textsuperscript{28}. In Chapter 5, we aimed to explore the role of genetic variation in \textit{FXR}, a key regulator of CYP3A4 metabolism, and its impact
on CD severity. We hypothesized that the novel $FXR\, -1G>T$ polymorphism confers a greater risk of a severe CD phenotype and is associated with a reduction in the downstream FXR target, fibroblast growth factor (FGF) 19 and an expansion of the total bile acid pool. Indeed, we confirmed that the $FXR\, -1GT$ genotype predicts surgery risk and early progression to surgery most significantly in women. We also observed a significant reduction in FGF19 plasma concentrations in female carriers of the $FXR\, -1T$ allele; however, no appreciable differences were seen in the plasma bile acid pool of the $FXR\, -1GT$ carriers. Our data highlight the importance of drug metabolism sequences to IBD pathogenesis. Specifically, genetic variation in $FXR$, a key regulator of CYP3A4, appears to have important consequences for outcomes of disease severity in CD, indicating a role for more aggressive medical management in these individuals. Most interestingly, our data point to a sex-specific difference in CD, whereby women with CD are most adversely affected by genetic variation in FXR as it pertains to CD outcomes. This has gone largely unexplored. Our data offer a basis for exploring sex-specific differences in CD as they pertain to disease pathogenesis, outcomes and management. Ultimately, NRs such as FXR may be a key overlapping factor that links drug metabolism sequences and CD pathogenesis.

6.2 Implications

To date, there is limited data evaluating the effect of CD on major drug metabolism pathways. Ours is the first data set to confirm a decrease in CYP3A4 activity in CD using multiple modalities and to suggest that said decline may be driven by
decreases in hepatic CYP3A4 activity, beyond the impact of CD on the intestinal expression of CYP3A4. Neither across in vivo biomarkers of activity, nor in gene expression, did we find that CD activity (defined by the Harvey-Bradshaw clinical index) impacted on CYP3A4. This suggests that factors beyond inflammatory cytokines are contributing to CD-specific changes in CYP3A4. One could even construe that changes in CYP3A4 expression and/or activity may be an antecedent event in CD onset rather than a byproduct of disease progression.

These findings also have important implications for CYP3A4 substrates used in patients with CD. The glucocorticoids, prednisone and budesonide, are substrates of CYP3A4 and are widely used for inducing disease remission in CD\textsuperscript{29-32}. There is significant inter-individual variation in CD patient response to prednisone and budesonide\textsuperscript{33}. Our data show that budesonide exposure is highly variable amongst individuals with CD and mirror the variability seen in healthy volunteer populations\textsuperscript{34,35}. Conversely, we did not observe a correlation between CYP3A4 activity and budesonide oral bioavailability, suggesting that CYP3A4 activity did not contribute significantly to budesonide variability in our population; however, our sample size may have been too small to draw definitive conclusions. Additionally, there is little, if any, data evaluating the impact of CD on an individual's exposure to non-CD-related CYP3A4 drug substrates taken concomitantly for co-morbid illnesses. In a single study by Sanaee et al. (2011), the authors observed a marked increase in the plasma concentrations of CYP3A4-substrate, verapamil compared to healthy volunteers\textsuperscript{36}. Further study is needed to assess the clinical impact of CD-related decreases in CYP3A4 activity.
Moreover, we draw attention to the overlap between drug metabolism sequences and CD pathogenesis. Nuclear receptors, such as PXR and FXR are important regulators of drug metabolism. They influence human exposure to xenobiotics including clinically-used drugs by regulating the activity and expression of proteins involved in the different phases of drug metabolism; however, they are also increasingly recognized for their importance in the mechanisms of CD pathophysiology. In addition to contributing to barrier function and limiting drug absorption through the regulation of CYP3A4, FXR is important to the integrity of the intestinal epithelium. Our data demonstrate a novel and important implication for genetic variation in FXR as it pertains to CD outcomes. Specifically, novel polymorphism FXR-1G>T confers a greater risk of severe CD phenotype (as represented by risk and time to CD-related surgery) in women with CD and is associated with reduced expression of downstream FXR targets. These findings are important to drug disposition and exposure in CD for two reasons: 1) they link drug metabolism pathways to CD pathogenesis and suggest that derailments in the expression of key enzymes and transporters may be a co-incident or antecedent event in IBD pathogenesis rather than a consequence of disease presence 2) they identify an at-risk group within the larger CD community who may benefit from more aggressive "top-down" CD medical management. The latter reinforces concepts championed by experts in the field of CD therapeutics: that our approach to CD management needs to be tailored to the individual and that there needs to be a re-structuring of our current provincial approach to CD drug access.
6.3 Future Directions

The clinical relevance of CD-specific changes in CYP3A4 activity remains to be determined. Given the prevalence of CD worldwide, the clinical consequences of its impact on a major drug metabolism pathway is highly relevant to drug development and patient safety. Observational studies and pharmacokinetic analyses are needed in CD populations to evaluate the impact of derangements in CYP3A4 activity and expression on drug exposure and drug response.

In addition, our data evaluated changes in the hepatic and intestinal activity of CYP3A4 in CD as well as changes in the intestinal protein expression. Interestingly, in the midazolam pharmacokinetic analysis, it was observed that the decline in CYP3A4 activity was primarily related to hepatic CYP3A4. There are no studies evaluating the impact of CD on the hepatic expression of CYP3A4. Logistically, this may be a difficult feat to accomplish in human studies, as the gold standard-liver biopsy would likely be difficult to carry out in the setting of a non-hepatic disease. In its place, the use of intravenous, exogenous, systemic probes in larger CD cohorts to assess CYP3A4 activity may provide an adequate alternative to further explore this finding.

We also examined the role of bile-acid induced PXR signaling on CYP3A4 activity in vitro as a means for better understanding differential CYP3A4 activity in CD. We observed CD-specific differences in the bile acid profiles of CD and non-CD populations. Specifically, differences in the percent composition of potent PXR ligand, LCA were noted between groups. However, despite this, no difference was observed in CYP3A4 activity in an in vitro cell model with exposure to a disease-specific bile acid
pool. Of note, other CD-specific differences observed in the bile acid pools included an increase in glycine-conjugated deoxycholic acid (GDCA) and cholic acid (GCA) amongst the CD population as well as a relative decline in taurine-conjugated cholic acid (TCA) and glycine-conjugated chenodeoxycholic acid (GCDCA). Activation of PXR by GDCA was noted and may have compensated for any declines in CYP3A4 activity due to differences in LCA. In future, evaluating the effect of the CD-specific bile acid pool on FXR activation may also shed light on disparate CYP3A4 activation in CD.

One of the most interesting findings to emerge from this work is that of a link between drug metabolism pathways and FXR to disease severity in women with CD. We have identified a novel genomic biomarker of disease severity in women with CD with evidence of an impact on its downstream targets. Beyond the predictive value of $FXR-1G>T$ in women with CD for poor outcomes, we have identified an area in need of further investigation pertaining to CD pathophysiology. Validation of our retrospective study in an independent and external CD population would reinforce its utility as well as the application of this genomic test to a prospective cohort of woman to further characterize its impact on clinical outcomes. Furthermore, using molecular technologies to better understand the relationship between FXR, estrogen and CD, concepts not currently well-explored in CD, may offer additional insights into a complex disease.

6.4 Conclusions

The effect of CD on drug metabolism pathways has only been considered to a limited extent, despite a significant overlap between metabolism sequences, CD
mechanisms and disease location. The aim of this work was to systematically consider the impact of CD on key phase 1 enzyme CYP3A4, including its activity, expression and regulation. We were able to show that CYP3A4 activity and protein expression are down-regulated in CD. We observed a significant reduction in hepatic CYP3A4 activity in vivo, despite our initial hypotheses of CD having the greatest influence on intestinal CYP3A4. We also identified a significant relationship between CYP3A4 regulator, FXR and outcomes of disease severity in women with CD. We observed a reduction in downstream targets of FXR in female carriers of the \textit{FXR-1T} allele, highlighting an area of future research in CD pathogenesis as it may pertain to women. As a whole, these studies highlight the effect of CD on an important drug metabolism pathway and offer new insights into the overlap between CD pathogenesis and drug metabolism sequences.
6.5 References


Appendix A: Ethics Approval
Principal Investigator: Dr. Richard Kim
File Number: 104914
Review Level: Full Board
Protocol Title: Sudesonide Metabolism and Disposition Pathways in Inflammatory Bowel Disease
Department & Institution: Schulich School of Medicine and Dentistry/Medicine-Dept of, London Health Sciences Centre
Sponsor:
Ethics Approval Date: March 07, 2014
Ethics Expiry Date: November 30, 2015

Documents Reviewed & Approved & Documents Received for Information:

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This is to notify you that the University of Western Ontario Health Sciences Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this HSREB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request form.

Member of the HSREB that are named as investigators in research studies, or declare a conflict of interest, do not participate in discussions related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.
Dear Richard Kim,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

Kelly Patterson
Principal Investigator: Dr. Richard Kim
File Number: 4198
Review Level: Delegated
Approved Local Adult Participants: 500
Approved Local Minor Participants: 0
Protocol Title: Variability of intestinal drug transport and/or drug metabolism in health and disease (REB# 13097)
Department & Institution: Schulich School of Medicine and Dentistry/Medicine-Dept of London Health Sciences Centre
Sponsor:
Ethics Approval Date: July 11, 2013 Expiry Date: January 31, 2015
Documents Reviewed & Approved & Documents Received for Information:

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This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.
Western University Health Science Research Ethics Board  
HSREB Full Board Initial Approval Notice

Principal Investigator: Dr. Richard Kim  
Department & Institution: Schulich School of Medicine and Dentistry\Medicine-Dept of London Health Sciences Centre

HSREB File Number: 105930  
Study Title: Trimethylamine-N-oxide: a link between bile acid dysmetabolism and inflammatory bowel disease pathogenesis  
Sponsor: Canadian Institutes of Health Research

HSREB Initial Approval Date: December 10, 2014  
HSREB Expiry Date: December 10, 2015

Documents Approved and/or Received for Information:

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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review. If an Updated Approval Notice is required prior to the HSREB Expiry Date, the Principal Investigator is responsible for completing and submitting an HSREB Updated Approval Form in a timely fashion.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000040.
Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: November 10, 2015
Principal Investigator: Dr. Richard Kim
Department & Institution: Schulich School of Medicine and Dentistry\Medicine-Dept of, London Health Sciences Centre

Review Type: Full Board
HSREB File Number: 105930
Study Title: Trimethylamine-N-oxide: a link between bile acid dysmetabolism and inflammatory bowel disease pathogenesis
Sponsor: Canadian Institutes of Health Research

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2016/11/30
Expiry Date -2016/12/10

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.
Appendix B: Copyright Approval
Title: Trimethylamine-N-oxide: a link between the gut microbiome, bile acid metabolism, and atherosclerosis

Author: Aze Wilson, Cheynne McLean, and Richard Kim

Publication: Current Opinion in Lipidology

Publisher: Wolters Kluwer Health, Inc.

Date: Apr 1, 2016

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339 Windermere Road |
DR. AZE SUZANNE ALIU WILSON
Gastroenterologist

Assistant Professor - Department of Medicine
PERSONAL SUMMARY

Name: Aze Suzanne Aliu Wilson

Languages: English, Understood, Spoken, Read, Written
EDUCATION AND QUALIFICATIONS

Degrees and Diplomas

2014 - present  Doctor of Philosophy, Western University, Pharmacology  Toxicology, Doctor of Philosophy - Graduate, Physiology and Pharmacology
2011 - 2013  Gastroenterology Fellowship, Western University, Medicine, Postgraduate, Gastroenterology
2008 - 2011  Internal Medicine Residency, Western University, Medicine, Postgraduate, Internal Medicine
2004 - 2008  Doctor of Medicine, Western University, Medicine, Undergraduate
2000 - 2004  Bachelor of Science, Queen’s University, Life Sciences, Bachelor’s - Honours, Life Sciences

Research Training

2013 - 2016  Clinician Investigator Program, Western University, Medicine

Qualifications, Certifications and Licenses

2014 - present  Fellow, Royal College of Physicians and Surgeons of Canada, License
2014 - present  Fellow, Royal College of Physicians and Surgeons of Canada, License
2009 - present  Member, Medical Council of Canada, Membership
2008 - present  Member, Canadian Medical Protection Association, Membership
2008 - present  Independent License, College of Physicians and Surgeons of Ontario, License
APPOINTMENTS

Academic Appointments

2016 - present  Assistant Professor, Department of Medicine, Gastroenterology
2016 - present  Assistant Professor, Department of Medicine, Program of Experimental Medicine
POSITIONS HELD & LEADERSHIP EXPERIENCE

Academic Positions

2015 - present  Health Sciences Research Ethics Board Member, Western University
2015 - present  Pharmacogenomics of Gastroenterology Research Member, Western University
2012 - 2013  Gastroenterology Division Chief Resident, Western University
2012 - 2013  Gastroenterology Division Senior Resident Representative, Western University
2012  Residents in Research Review Panel Member, Western University
2011 - 2013  Gastroenterology Division Program Committee Member, Western University
2011 - 2012  Gastroenterology Division Junior Resident Representative, Western University
2008 - 2009  Social Support Committee Member, Western University
HONOURS AND AWARDS

Honours

Received

2014 Western University Internal Medicine Program Mentorship Award, Western University

Trainee


2013 Western University Gastroenterology Training Program Hepatology Research Award, Western University


2012 Western University Gastroenterology Training Program Inflammatory Bowel Disease Research Award, Western University
SERVICE AND ADMINISTRATION

Professional Affiliations and Activities

Professional Associations

2016 - present  Member, Ontario Medical Association
2014 - present  Member, Ontario Association of Gastroenterology
2011 - present  Member, Canadian Association of Gastroenterology
2011 - present  Member, American College of Gastroenterology

Peer Review Activities

Reviewer for research journal

2016 - 2017  Manuscript Reviews, Clinical Pharmacokinetics
2015  Manuscript Reviews, Journal of Bioequivalence Studies
2014  Manuscript Reviews, British Journal of Pharmacology
2014  Manuscript Reviews, Journal of Clinical Studies and Medical Case Reports
2013  Manuscript Reviews, Drug Design, Development and Therapy

Administrative Committees

Local

London Health Sciences Centre

2017 - present  Member, Drug Therapeutic Committee

Robarts Clinical Trials

2016 - present  Member, Robarts IBD Clinical Trials Steering Committee

University of Western Ontario

2016  Member, Health Sciences Research Ethics Board (Western University)

Western University, Department of Medicine

2017 - present  Member, Gastroenterology Research Committee
2017 - present  Member, Gastroenterology Competency Committee
RESEARCH AND SCHOLARLY ACTIVITIES

Grants

Peer Reviewed

Active Grants

2018 Delivery of safe azathioprine in IBD, Funding Source: AMOSO Innovation Fund, Principal Investigator: Aze Wilson, Grant Total: 75,000, Role: Co-Principal Investigator

2018 Optimizing infliximab in UC, Funding Source: AMOSO Innovation Fund, Principal Investigator: Aze Wilson, Grant Total: 77,000, Role: Co-Principal Investigator

Applied Grants

2018 - 2021 Enhancing Safety and Benefit from Azathioprine Therapy for Our Inflammatory Bowel Disease Patients, Funding Source: Crohn’s Colitis Canada, Principal Investigator: Aze Wilson, Grant Total: 259,851 CAD, Role: Principal Applicant

2018 - 2021 Genetic variation in MRP4 may predict azathioprine-induced myelotoxicity in inflammatory bowel disease, Funding Source: Crohn’s Colitis Canada, Principal Investigator: Aze Wilson, Grant Total: 50,000 CAD, Role: Principal Applicant

2018 - 2021 Optimizing infliximab in UC, Funding Source: Physician Services Incorporated, Principal Investigator: Aze Wilson, Grant Total: 229,000, Role: Co-Principal Investigator

Past Grants

2015 - 2017 CIHR/CAG, Funding Source: CIHR; Canadian Association of Gastroenterology, Principal Investigator: Richard B. Kim, Grant Total: 110,000 CAD, Role: Principal Applicant

Non-Peer Reviewed

Past Grants

2014 - 2015 Janssen IBD/Clinical Pharmacology Research Grant, Funding Source: Janssen Inc, Principal Investigator: Richard B. Kim, Grant Total: 20,000 CAD, Role: Principal Applicant

2010 - 2011 Western University Gastroenterology Division Grant Competition, Principal Investigator: James C. Gregor, Grant Total: 5,000 CAD, Role: Principal Applicant

Clinical Trials

Peer Reviewed

Active Clinical Trials

2017 - 2019 Individualization of Anti-TNF Therapies for IBD, Principal Investigator: Aze Wilson, Grant Total: 97,000 CAD, Role: Principal Investigator


**PUBLICATIONS**

**Peer Reviewed Publications**

**Journal Article**

*Published*


3. **Wilson A**, Tirona RG, Kim RB. CYP3A4 Activity is Markedly Lower in Patients with Crohn’s Disease. Inflamm Bowel Dis, 2017 Mar 15


*Submitted*

1. Aze Wilson, Ahmed A. Almousa, Laura E. Jansen, Yun-hee Choi, Wendy A. Teft, Richard B. Kim. Genetic variation in the Farnesoid X-Receptor Predicts Crohn’s Disease Severity in Female Patients. Gastroenterology, 2018 May 1
Book Chapter / Review Article

Published


Letter

Published

ABSTRACTS

Abstracts Presented
1. Aze Wilson, Trimethylamine-N-oxide and inflammatory bowel disease: differential role of intestinal microbiota in Crohn’s disease vs ulcerative colitis - Presenter at the “CAG/CCC Student Prize Paper Presentations”, 2015 Mar 1, Canadian Association of Gastroenterology, LONDON, Canada

Posters Presented
1. Aze Wilson, FXR-1G>T predicts deleterious outcomes in Crohn’s disease, 2018 Feb 16, European Congress of Crohn’s and Colitis (ECCO), Wien, Austria
3. Aze Wilson, HLADQA1*01/HLADRB1*07 is a major predictor of azathioprine-induced pancreatitis in inflammatory bowel disease, 2018 Feb 10, Canadian Association of Gastroenterology, Toronto, Ontario, Canada
5. Seana Nelson, Aze Wilson, Plasma biomarker may detect disease-dependent alterations in gut microbiota in IBD: a pilot study, 2018 Feb 10, Canadian Association of Gastroenterology, Toronto, Ontario, Canada
7. Aze Wilson, Trimethylamine-N-oxide and Inflammatory Bowel Disease: the differential role of the intestinal microbiome, 2015 Oct, American College of Gastroenterology, LONDON, Hawaii, United States
9. Aze Wilson, Trimethylamine-N-oxide and Inflammatory Bowel Disease: the differential role of the intestinal microbiome, 2015 Apr, Western University, LONDON, Canada
11. Aze Wilson, Trimethylamine-N-oxide and Inflammatory Bowel Disease: the differential role of the intestinal microbiome, 2015 Feb, Canadian Association of Gastroenterology, LONDON, Canada
17. Pari Basharat, Transient elastography for monitoring of liver fibrosis in methotrexate-treated patients with inflammatory disorders: Systematic Review, 2013, Canadian Association of Rheumatology, Canada
19. Aze Wilson, Pharmacokinetic profiles for oral and subcutaneous methotrexate in patients with Crohn’s disease, 2012 Oct, American College of Gastroenterology, Las Vegas, Nevada, United States
25. Aze Wilson, Patterns of Food Allergy in Eosinophilic Esophagitis, 2011 Feb, Canadian Association of Gastroenterology, Toronto, Ontario, Canada
27. Aze Wilson, Patterns of Food Allergy in Canada
PRESENTATIONS

Invited Lectures
1. Invited Lecturer, Is my PPI killing me and other tough questions, Clinical Pharmacology, Presenters: Aze Wilson, 2018 Mar 21, London, Ontario, Canada
2. Invited Lecturer, Biologics for inflammatory bowel disease: a focus on ustekinumab, Janssen, Presenters: Aze Wilson, 2017 Nov 15, London, Ontario, Canada
3. Presenter, Personalized Medicine in Inflammatory Bowel Disease, Division of Clinical Pharmacology, Presenters: Aze Wilson, 2016 Feb 4, LONDON, Canada
4. Presenter, Pharmacogenomics in IBD, Division of Gastroenterology, Presenters: Aze Wilson, 2015 Nov 3, LONDON, Canada
5. Presenter, Trimethylamine-N-oxide and IBD: the differential role of the intestinal microbiome, Division of Clinical Pharmacology, Presenters: Aze Wilson, 2014 Nov, LONDON, Canada
6. Presenter, IBD: From bench to bedside - new understanding in the molecular biology of the disease, Division of Gastroenterology, Presenters: Aze Wilson, 2014 Mar 4, LONDON, Canada
7. Presenter, Methotrexate and Inflammatory Bowel Disease: Let’s Get Personal, Division of Clinical Pharmacology, Presenters: Aze Wilson, 2013, LONDON, Canada

Student Presentation
1. Presenter, Trimethylamine-N-oxide and inflammatory bowel disease: differential role of intestinal microbiota in Crohn’s disease vs ulcerative colitis - Presenter at the “GI Topics in Research” course, Canadian Association of Gastroenterology, Presenters: Aze Wilson, 2015 Feb 25, LONDON, Canada
2. Presenter, Pharmacokinetic Profiles for Oral and Subcutaneous Methotrexate in Patients with Crohn’s Disease, Canadian Association of Gastroenterology, Presenters: Aze Wilson, 2012 Feb 1, LONDON, Canada

Symposia
2. Presenter, Drug Metabolism in Inflammatory Bowel Disease, Western University Clinician Investigator Program, Presenters: Aze Wilson, 2016 Jan 4, LONDON, Canada

GI Rounds
1. Presenter, Translational Research in IBD, GASTRO UWO, Presenters: Aze Wilson, 2017 Apr 11, London, Ontario, Canada
Grand Rounds
1. Presenter, "Is my PPI killing me?" and other tough questions, Clinical Pharmacology UWO, Presenters: Aze Wilson, 2018 Mar 21, London, Ontario, Canada

PhD Seminar
1. Presenter, Drug Response and Metabolism in IBD, Department of Physiology and Pharmacology, Presenters: Aze Wilson, 2018 Apr 2, Canada

Research
1. Presenter, Translational research and you: a clinical approach to basic science, LHSC Medicine Department, Presenters: Aze Wilson, 2017 May 11, London, Ontario, Canada

Research PhD

Resident IM Rounds
1. Presenter, Evidence Based Approach to CD MX, LHSC Medicine Department, Presenters: Aze Wilson, 2017 Feb 27, London, Ontario, Canada
**TEACHING RESPONSIBILITIES**

**Postgraduate Medical Education**

**Courses**

*Consultant - Subspecialty Clinical Teaching Unit*

- 2016 Feb 16 - 2016 Feb 21
- 2015 Sep 8 - 2015 Sep 15
- 2015 Jun 22 - 2015 Jun 28
- 2014 Sep 29 - 2014 Oct 5
- 2014 Jul 28 - 2014 Aug 4

*Examiner - OSCE PGY1 or PGY4*

- 2018 Feb 22

*Host - Journal Club*

- 2015 Sep 29 Clinical Pharmacology Evening Journal Club

*Instructor - Academic Half Day Seminars*

- 2016 Mar 22

*Participant - Journal Club*

- 2016 Feb 10
- 2016 Jan 21 Clinical Pharmacology Evening Journal Club

*Judge DOM Resident Research Day*

- 2018 May 11

**Undergraduate Medical Education**

**Courses**

*Digestive System & Nutrition*

- 2015 Sep 22 Small Group Activity
- 2012 Sep 11 Small Group Activity

*Instructor - Clinical Methods - Year 1 Advanced Interviewing*

- 2015 Nov 12

*Instructor - Clinical Methods - Year 1 Primary Physical Skills Part 1*

- 2017 Nov 2 - 2017 Dec 7
- 2016 Nov 3 - 2016 Dec 8
2016 Feb 3
2015 Feb 11
2014 Dec 5
2014 Feb 14
2013 Nov 29

_Instructor - Clinical Methods - Year 2 Gastroenterology_

2017 Nov 15   London, Clinical Methods - Small Groups

_Supervisor / Examiner - OSCE Year 2 or Year 4_

2018 Jan 11   Evaluating Year 4 Medical Student Clinical Exam Skills
2017 Feb 9   Evaluation Year 2 Medical Students at practice OSCE
2017 Jan 12   Evaluating Year 4 Medical Student Clinical Exam Skills

_Teaching OSCE Year 2_

2017 Nov 16
SUPERVISION AND MENTORING

Mentorship

Resident Research

2017 - present  Seana Nelson PGY1, Supervisor