1-1-2013

Genetic risk profiling to predict surgical outcome in inflammatory bowel disease patients.

Sehgal Rishabh
Royal College of Surgeons in Ireland

Genetic Risk Profiling to Predict Surgical Outcome in Inflammatory Bowel Disease Patients

Rishabh Sehgal, MB, BCh, MRCSI

Department of Surgery
Division of Colon & Rectal Surgery
Penn State Milton S. Hershey Medical Center
Penn State College of Medicine
Hershey, Pennsylvania, USA

A thesis submitted to the Royal College of Surgeons in Ireland for the degree of Doctor of Medicine (MD)

Royal College of Surgeons in Ireland
The Pennsylvania State University
2013

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of Doctor of Medicine (MD) is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

Signed Rishabh Sehgal

RCSI Student Number _____

Date _____

Supervised by

Walter A. Koltun, MD, FACS, FASCRS
Chief, Colon and Rectal Surgery
Peter and Marshia Carlino Chair in Inflammatory Bowel Disease
Professor of Surgery, Milton S. Hershey Medical Center Penn State College of Medicine, Hershey, PA

and

Professor Arnold Hill, MCh, FRCSI
Professor of Surgery
Royal College of Surgeons in Ireland
Beaumont Hospital, Dublin 9, Ireland

TABLE OF CONTENTS

TABLE OF CONTENTS
(continued)

TABLE OF CONTENTS
(continued)

TABLE OF CONTENTS
(continued)

TABLE OF CONTENTS
(continued)

TABLE OF CONTENTS
(continued)

ABSTRACT

BACKGROUND: Severe pouchitis (SP) and Crohn's disease (CD)-like complications confound the success of the ileal pouch anal anastomosis (IPAA) in patients with ulcerative colitis. Furthermore, there are no clear criteria for judging the severity of disease in patients with CD. Yet classification of patients into low- and high-risk severity groups would benefit both medical and surgical management. To date, approximately 83 single nucleotide polymorphisms (SNPs) within 55 genes have been associated with IBD.

OBJECTIVE: (1) To determine whether the NOD2 gene correlated with post operative IPAA complications (CD-like/pouchitis) (2) To identify SNPs that correlate with complications after IPAA that could be utilized in a gene signature fashion to predict IPAA postoperative complications aid in preoperative surgical decision making. (3) To identify genetic determinants (SNPs) that could be markers of CD severity by the use of frequency of ileocolic surgery as a surrogate for disease severity.

DESIGN: IPAA patients were retrospectively sub-classified into the following groups: 1) IPAA with CD-like complications (perianal fistula, pouch inlet stricture/upstream small-bowel disease, or biopsies showing granulomata) occurring at least 6 months after ileostomy closure; 2) IPAA with mild pouchitis ($\leq$3 episodes/y for 2 consecutive years); 3) IPAA with SP ($\geq$4 episodes/y for 2 consecutive years or need for continuous antibiotics); 4) IPAA without complications or pouchitis; The severity of CD was quantified by dividing the total number of ileocolectomy procedures by the time between IBD diagnosis and the patient's last clinic visit, the rationale being that more severe disease would be associated with a more frequent need for surgery. The 3 NOD2 SNPs (rs2066844, rs2066845, and rs2066847) were genotyped using polymerase chain reaction. Genotyping for 83 IBD associated SNPs was performed on a customized Illumina Veracode genotyping platform.

RESULTS: NOD2 mutations were significantly higher in the SP group (67%) compared with both asymptomatic (5.4%, $P < .001$) and CD-like pouch groups (14.3%, $P = .008$) groups. The top 2 SNPs for CD-like complications were in the 10q21 locus and the gene for PTGER4 ($p = 0.006$ and 0.007), whereas for SP it was NOD2 and TNFSF15 ($p = 0.003$ and 0.011). Probability equations suggested that the risk of these 2 complications greatly increased with increasing number of risk alleles, going as high as 92% for SP and 65% for CD-like complications. The average number of ileocolectomies per patient was 1.7 (range, 1-5) with an average duration of disease of 14.7 years. SNP rs4958847 in the IRGM gene was the most significant SNP as being associated with ileocolectomy. Patients carrying the "at-risk" allele for this SNP ($n = 20$) had an average of 1 surgery every 6.87 ± 1.33 years in comparison with patients carrying the wild-type genotype ($n = 46$) who averaged 1 surgery in 11.43 ± 1.21 years ($p = 0.007$).

CONCLUSIONS: In this IPAA patient cohort, asymptomatic IPAA patients have a low incidence of NOD2 mutations not significantly different from patients with mild pouchitis or healthy controls. Patients with SP had the highest incidence of NOD2 mutations vs. CD-like, suggesting a different genetic makeup in these 2 patient groups. Preoperative genetic analysis and use of gene signatures hold promise for improved preoperative surgical patient selection to minimize these IPAA complications. SNP rs4958847 in the IRGM gene correlated very significantly with frequency of surgery in patients with ileocolonic CD. This SNP may be a marker for disease severity and/or early recurrence after ileocolectomy and may assist in surgical and medical decision making.

FUNDING BODY

This work has been supported by The American Society of Colon and Rectal Surgeons (ASCRS) Research Foundation **"International Research Fellowship Award"** for grant submission: "Correlation of Genotype with Complications after IPAA"
$50,000 for 1 year (September 1, 2010 – August 31, 2011) – **Rishabh Sehgal**

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

Sehgal R, Berg A, Polinski JI, Hegarty JP, Lin Z, McKenna KJ, Stewart DB, Poritz LS, Koltun WA. Genetic risk profiling and gene signature modeling to predict risk of complications after IPAA. *Dis Colon Rectum.* 2012 Mar;55(3):239-48.
PMID: 22469789. Impact Factor: 3.33

Sehgal R, Berg A, Polinski JI, Hegarty JP, Lin Z, McKenna KJ, Stewart DB, Poritz LS, Koltun WA. Mutations in IRGM are associated with more frequent need for surgery in patients with ileocolonic Crohn's disease. *Dis Colon Rectum.* 2012 Feb;55(2):115-21.
PMID: 22228152. Impact Factor: 3.33

- **Winner of the American Society of Colon & Rectal Surgeons (ASCRS) Harry E. Bacon Foundation Award: Best Basic Science Podium Presentation 2011**

- **Received an EDITORIAL: Kennedy GD. Surgical genomics: using new technology to answer age-old questions. *Dis Colon Rectum.* 2012 Feb;55(2):113-4.**

Sehgal R, Berg A, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA. NOD2/CARD15 mutations correlate with severe pouchitis after ileal pouch-anal anastomosis. *Dis Colon Rectum.* 2010 Nov;53(11):1487-94.
PMID: 20940596. Impact Factor: 3.33

Sehgal R, Koltun WA. Scoring systems in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol.* 2010 Aug;4(4):513-21.
PMID: 20678023

Tyler AD, Milgrom R, Stempak JM, Xu W, Brumell JH, Muise AM, **Sehgal R**, Cohen Z, Koltun W, Shen B, Silverberg MS. The NOD2insC polymorphism is associated with worse outcome following ileal pouch-anal anastomosis for ulcerative colitis. *Gut.* 2013 Oct;62(10):1433-9.
PMID: 22879519. Impact Factor: 10.73

LIST OF ORAL PODIUM PRESENTATIONS

Sehgal R, Berg A, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA. (2013, March) *NOD2/CARD15 mutations correlate with severe pouchitis after Ileal Pouch Anal Anastomosis (IPAA)*. Oral Presentation at **Plenary Session** presented at: 2013 Sylvester O' Halloran Surgical Scientific Meeting; Limerick, Ireland.

Sehgal R, Berg A, Polinski JI, Hegarty JP, Lin Z, Poritz LS, Koltun WA. (2012, September) *Mutations in IRGM are associated with more frequent need for surgery in ileocolonic Crohn's disease (CD) patients.* Oral Presentation presented at: Sir Peter Freyer Surgical Symposium at University College Galway, Ireland.

Sehgal R, Berg A, Polinski JI, Hegarty JP, Lin Z, Stewart DB, Poritz LS, Koltun WA. (2012, September) *Gene Signature Modeling to Predict Risk of Crohn's Disease-like Complications or Severe Pouchitis after Ileal Pouch Anal Anastomosis.* Oral Presentation presented at: Sir Peter Freyer Surgical Symposium at University College Galway, Ireland.

Sehgal R, Berg A, Polinski JI, Hegarty JP, Lin Z, Poritz LS, Koltun WA. (2011, May) *Mutations in IRGM are associated with more frequent need for surgery in ileocolonic Crohn's disease (CD) patients.* American Society of Colon and Rectal Surgeons (ASCRS) Annual Meeting; Vancouver, Canada.

- **Winner of the Harry E. Bacon Foundation Award**
  Best Basic Science Podium Presentation

Sehgal R, Berg A, Polinski JI, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA. (2011, April) *Mutations in IRGM are associated with more frequent need for surgery in ileocolonic Crohn's disease (CD) patients.* Oral Presentation presented at: *Pennsylvania Society of Colon and Rectal Surgeons.* (PSCRS) Annual Resident Research Night Meeting; Pennsylvania, PA.

Sehgal R, Berg A, Polinski J, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA. (2011, February) *Correlation of Gene Haplotypes With Complications After Ileal Pouch Anal Anastomosis.* Oral Presentation presented at: 6th Annual Academic Surgical Congress; Huntington Beach, CA.

- **Top 10 highest scored abstract in the Oral Presentation category.**

Sehgal R, Berg A, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA.(2010, May) *NOD2/CARD15 mutations correlate with severe pouchitis after Ileal Pouch Anal Anastomosis (IPAA)*. Oral Presentation presented at: American Society of Colon and Rectal Surgeons (ASCRS) Annual Meeting; Minneapolis, MN.

Sehgal R, Berg A, Hegarty JP, Kelly AA, Lin Z, Poritz LS, Koltun WA.(2010, April) *NOD2/CARD15 mutations correlate with severe pouchitis after Ileal Pouch Anal Anastomosis (IPAA)*. Oral Presentation presented at: *Pennsylvania Society of Colon and Rectal Surgeons.* (PSCRS) Annual Resident Research Night Meeting; Pennsylvania, PA.

ACKNOWLEDGEMENTS

This study was performed in the Division of Colon and Rectal Surgery at the Department of Surgery of Penn State Milton S. Hershey Medical Centre during the years 2008-2011. I am grateful for the Carlino Fund for IBD Research and The American Society of Colon & Rectal Surgery (ASCRS) Research Foundation for providing financial support of my work by way of an International Research Fellowship award for grant submission 'Correlation of Gene Haplotype with Complications after Ileal Pouch Anal Anastomosis' (2010).

I am deeply indebted to my supervisors Dr.Walter A. Koltun and Dr. Lisa S. Poritz for their outstanding supervision, support and contribution to my success. Thank you for providing me with such a mentally stimulating environment to grow. Your constant guidance and critical criticism throughout my journey will always have a special place in my heart. In addition, I would like to thank the other colorectal surgeons in the Division: David Stewart, Kevin McKenna, and Evangelos Messaris.

I would like to extend my heartfelt gratitude to all my colleagues in the Department: John P. Hegarty and Leonard Harris for showing me the ways of the lab and always being there for me when things went pear shaped. Your immense knowledge of science is awe-inspiring. Thank you for all your support and for the great laughs over the years. I will forever cherish our friendship. Thanks to Dr. Zhenwu Lin, Wei Yu, and Gerrit John for providing me with a much deeper understanding of pathway analysis and various basic science techniques. Furthermore, thank you to Sue Deiling for helping me with patient consents, data gathering, and storage.

A big thank you to Arthur Berg for all the statistical analyses and for being patient with my millions of questions and requests. In addition, a special thank you to Kimberly Walker for keeping me on track and helping me with grant writing and formatting.

Last but not least, I would like to thank my parents, family and friends for all their love, help, support, patience, and encouragement.

ABBREVIATIONS

AIEC	Adhesive and invasive Escherichia *coli*
5-ASA	5-aminosalicylic acid
ASCA	Anti-*Saccharomyces cerevisiae* antibody
ATG16L1	Autophagy 16-Like 1
bp	Base pair
CARD15	Caspase recruitment domain-containing protein 15
CCR6	Chemokine receptor 6
CD	Crohn's disease
CDAI	Crohn's disease activity index
CMV	Cytomegalovirus
CRP	C-reactive protein
EIM	Extra intestinal manifestations
EUA	Examination under anesthesia
FAP	Familial Adenomatous Polyposis
GI	Gastrointestinal
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
HRQL	Health related quality of life
IC	Indeterminate colitis
IBD	Inflammatory Bowel Disease
IBDU	IBD-Unclassified
IECs	Intestinal epithelial cells
IFN-γ	Interferon-gamma
IRGM	Immunity Related GTPase Family M
IL23R	Interleukin 23 receptor
IPAA	Ileal Pouch Anal Astomosis
JAK2	Janus kinase 2
LCLs	Lymphoblastoid cell lines
LD	Linkage disequilibrium
LN	Liquid nitrogen
LOD	Logarithm of the odds
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAP	Mitogen-activated protein
Mb	Megabase
MDP	Muramyl dipeptide
MRI	Magnetic resonance imaging
MyD88	Myeloid differentiation primary response gene (88)
NBD	Nucleotide binding domain
NF-κB	Nuclear Factor kappa B
NLR	NOD-like receptor
NOD2	Nucleotide-binding oligomerization domain protein 2
NAC	N-acetyl-L-cysteine
NSAIDs	Non-steroidal anti-inflammatory drugs
pANCA	Perinuclear anti-neutrophil cytoplasmic antibody
PSC	Primary Sclerosing Cholangitis
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction

PDAI	Pouchitis disease activity index
PRR	Pattern recognition receptor
RIP2	Receptor interacting protein-2
SCID	Severe combined immunodeficiency
STAT3	Signal transducer and activator of transcription 3
SNP	Single Nucleotide Polymorphism
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
TNFSF15	Tumor necrosis factor superfamily, member 15
UC	Ulcerative colitis
WTCCC	Wellcome Trust Case-Control Consortium

LIST OF TABLES AND FIGURES

TABLES

FIGURES

Chapter 1

Introduction

Idiopathic inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory condition affecting the gastrointestinal tract (GI) that most commonly affects young adults aged 15-30 years [Loftus EV 2002], and results in lifelong need for medical and surgical care. The etiology of IBD still remains largely unknown however the working paradigm incorporates the interplay between the gut microbiome, environmental and genetic factors that lead to a deregulated host immune response. The two major categories of IBD are Ulcerative colitis (UC) and Crohn's disease (CD), and each disease is distinct both clinically and histologically. CD is characterized by transmural, patchy, granulomatous inflammation that can affect any part of the gut in a skip-like fashion, but most commonly the terminal ileum and colon. In contrast, the inflammation in UC is limited to the rectum and colon, affects only the mucosa and sub-mucosal layers of the bowel, is continuous, and is not associated with granulomas.[Lennard-Jones 1989, Friedman 2008] Such differences between CD and UC produce the varied clinical symptoms seen in patients (Table 1.1). The diagnosis of CD versus UC is usually made using these clinical characteristics that imperfectly define the two diseases. In up to 16% of cases there are overlapping histologic features present that make the diagnosis uncertain, and the term Indeterminate Colitis (IC) is used.[Matsui T, 2003]

In the past two decades, genetic variants and their role in the pathogenesis of IBD have been the subject of intensive research. The essence of this exciting work is to identify the possible etiology of IBD by identifying and then correlating genetic mutations found in large populations of IBD patients compared to healthy controls. To date, much of this work has focused on identifying specific pathways of immune function and inflammation, looking to develop newer medicines. However little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making. At the time of this work approximately 80 single nucleotide polymorphisms (SNPs) in 55 genetic loci have been associated with IBD. Thus, it is clear that IBD is a 'complex-trait' disease, potentially involving many mutations in several different genes affecting a multitude of different biological pathways. This wide variability of genetic mutations presumably results in many variations in disease characteristics or clinical phenotype. Thus the old paradigm of IBD being made up of only CD vs. UC is now being refined into smaller categories of disease subtype, based on the presence or absence of individual mutations in various genes.

The goal of the present work was to correlate these 80 IBD specific genetic variants (Single Nucleotide Polymorphisms, SNPs) with specific UC and CD phenotypes in surgical patients. We sought to implement surgical relevancy to these recently identified genetic mutations associated with IBD. This work, which represents a largely retrospective study, will lay the basis for subsequent prospective trials that will seek to use genetic analysis to predict surgical outcome in the various forms of IBD that are treated with surgery.

1.1

Clinical Manifestations of Inflammatory Bowel Disease

The clinical presentation of IBD is characterized by a chronic relapsing and remitting spectrum of symptoms. Most patients symptoms are present for a variable amount of time, ranging from months to even years. The type of symptoms varies depending on the anatomical location affected by the disease (Table 1.1). Though CD can affect any region of the GI tract, it however most commonly affects the terminal ileum and cecum, accounting for up to 40% of cases, followed by the small bowel in 30% of cases, and the colon in 25% of cases [(Ruthruff B.2007)]. UC is usually restricted to the colon and rectum and propagates in a continuous fashion for a variable distance proximally. The inflammation in UC can involve the entire colon (pancolitis) and sometimes spreads as far back to involve the ileum, a condition known as backwash ileitis.

General symptoms that are common in both forms of colitis (CD and UC) include colicky abdominal pain, increased frequency of bowel movements, urgency, tenesmus, pyrexia, loss of appetite, weight loss and fatigue. Patients with CD may present with obstructive symptoms due to narrowing of the bowel lumen by stricture formation and present with symptoms such as abdominal distention, nausea and vomiting. CD patients are also more prone to penetrating (fistulizing) and perianal disease. UC patients can sometimes present as a surgical emergency with grossly dilated colon, a condition known as toxic megacolon, perforation, and hemorrhage. Several scoring and classification systems have been developed that utilize such features in order to aid in assessing disease severity, effectiveness of medication and predicting outcomes for this complex condition (see below).

IBD is a systemic disease and can involve other organ systems with a reported frequency ranging from 6-47%.[(Rothfuss KS 2006)] These extraintestinal manifestations (EIMs) most commonly involve the joints (eg: peripheral arthritis, axial arthropathies, ankylosing spondylitis and sacroiliitis), eyes (eg: episcleritis and uveitis), liver (eg: primary sclerosing cholangitis) and skin (eg: erythema nodosum, pyoderma gangrenosum and oral ulcerations). Development of one EIM carries an increased risk of developing other EIMs. [(Rothfuss KS 2006)]

Table 1.1
Comparisons of various clinical factors in Crohn's disease and ulcerative colitis

	Crohn's disease	Ulcerative colitis
Incidence	3.1–14.6 per 100 000	6–14.3 per 100 000
Socio-geographic	Higher rates in northern latitudes and in industrialized, urban and richer populations	Higher rates in northern latitudes and in industrialized, urban and richer populations
Terminal ileum involvement	Common	reflux ileitis
Colon involvement	Common	Usually
Rectum involvement	Possible	Always
Anal disease	Common	Rare
Bile duct involvement	Low	Higher rate
Distribution of Disease	Entire gut possible, skip lesions	Continuous inflammation from rectum proximal
Endoscopy	Geographic and serpiginous ulcers	Continuous mucosal inflammation
Depth of inflammation	May be transmural, deep into tissues	Shallow, mucosal
Fistulae	Common	Rare
Stenosis	Common	Rare
Granulomas on biopsy	Can have granulomas	Granulomas not seen
Crypt abscesses on biopsy	Seldom present	Commonly present
Surgical cure	Often returns following removal of affected part	Usually cured by removal of colon & rectum
Smoking	Higher risk for smokers	Lower risk for smokers

Table adapted from Lennard-Jones 1989 and Friedman 2008.

1.2

Scoring Systems in Inflammatory Bowel Disease

Historically, the lack of a single effective and sensitive test for IBD has posed a great challenge in assessing disease severity, effectiveness of medication and predicting outcomes for this complex condition. The chronic relapsing and remitting nature of IBD also adds to the dynamic clinical picture that physicians face; for example, the clinical picture that a patient initially presents with may not necessarily be the same after a 10-year period of disease. Several IBD scoring and classification systems have been developed over many years to classify and characterize IBD patients, with the goal of helping to better define the disease status and effectiveness of therapy. Recent genetic investigations have revealed the complexity of IBD at the pathophysiologic level, revealing numerous genetic mutations associated with the disease. Thus, these clinically based IBD classification systems can provide the basis for the eventual correlation between the underlying genotype with clinical expression of disease and lead to better characterization of disease subtypes in order to customize treatment regimens.

1.2.1 Rome/Vienna classification system

Since the publication of Dr Burrill B Crohn's 1932 landmark article 'Regional ileitis: a pathologic and clinical entity' (Crohn BB, Ginzburg L, Oppenheimer GD 1984), much work has gone into trying to classify the disease. Farmer *et al.* based the earliest classification on the anatomical location of disease in 1975. In his study entitled 'Clinical patterns in Crohn's disease: a statistical study of 615 cases' (Farmer RG, 1975), Farmer hypothesized that disease location may directly determine the clinical course and prognosis of the disease. Disease locations were segregated into ileocolic, small intestine, colon and anorectal. Farmer noted that patients who had colonic involvement required operations less frequently than those with ileocolic disease. However, it was the colonic group that most often presented with rectal bleeding, perianal fistulae, toxic megacolon and arthritis. Those with small bowel disease presented earlier with obstructive symptoms and, thus, had a more frequent need for surgery. With the ileocolic subgroup of patients, the main presenting symptoms were perianal and rectal fistulae, abscesses and intestinal obstruction. Building on this, in 1988, Greenstein *et al.* observed that surgical indications remained the same for repeated operations and influenced the speed with

which reoperation occurred [Greenstein AJ 1988]. The authors noted two different clinical patterns, independent of anatomic distribution: the perforating disease type, which was shown to be more aggressive; and the non-perforating type, which had a more 'indolent course'. This behavioral classification, in conjunction with Farmer's anatomic classification, allowed the Working Party of Gastroenterologists to introduce the 1991 Rome Classification, where further phenotypic characterization was carried out by looking at: location (i.e., stomach/duodenum, jejunum, ileum, colon, rectum or anal/perianal); behavior, defined as primarily inflammatory, primarily fistulizing or primarily fibrostenotic; extent of disease (i.e., localized or diffuse); and operative history (i.e., primary or recurrent). By using this classification, it was recognized that there could be as many as 756 subgroups of CD. Steinhard *et al.* found that there were insufficient definitions of disease behavior, which resulted in only a 'fair interobserver agreement' when used by multiple different caregivers [Steinhart AH, 1998]. The aim of the International Working Party became, therefore, to develop a simpler classification of CD based on objective and reproducible clinical variables.

The Working Party made many revisions and modifications to the Rome Classification and came up with their final draft in 1998, which is now known as the Vienna classification (Table 1.2.1) [Gasche C 2000]. This classification was created in order to standardize the description of study populations in clinical trials and to aid in correlating putative etiologic factors with particular clinical phenotypes. The final version incorporated three main disease descriptors: age of diagnosis (A1–2), location of disease along the GI tract (L1–3) and clinical behavior, such as stricturing, inflammatory or fistulizing disease (B1–3). Validation of this classification using population clusters from Europe, Scandinavia and the USA showed a high degree of interobserver agreement in classifying patients [Satsangi J 2006].

1.2.2 Montreal classification system for Crohn's Disease

The Montreal classification was introduced in 2005 (Table 1.2.1).[Satsangi J, 2006] This was a further refinement of the Vienna classification, in that there was no change in the three major categories, but modifications were made within each of the categories. An additional age subgroup allowed pediatric CD to be defined (below 16 years of age). In the location category, upper GI disease (L4) could now be added to each of the L1–3 subclasses, as it had been noted that upper GI involvement was relatively common, and could coexist with ileal and colonic disease. The behavior category was amended in that perianal disease ('p') was

added to the B1–3 classes as it was shown that perianal fistulizing disease can be variably associated with intestinal fistulizing disease. Patients with intestinal fistulizing disease, however, had a higher frequency of surgery compared with those with perianal fistulizing disease.

The Montreal classification system is still in its relative infancy and studies are underway to validate and assess this new scoring system among different populations. Chow *et al.* recently showed that the Montreal classification was more sensitive to behavioral phenotypic changes compared with the Vienna classification, after excluding perianal disease from the fistulising disease category.[Chow DK, 2008] Furthermore, it was useful in predicting the clinical course and the need for surgery. Their retrospective longitudinal study looked at a total of 109 patients with CD who were followed-up for an average of 4 years. Using the Montreal classification, CD behavior changed 3 years after diagnosis with an increase in stricturing and penetrating phenotypes, but this was only detected by the Vienna classification after 5 years, suggesting a better sensitivity to disease behavior characteristics. Disease location remained stable on follow-up in both classification systems. In total, 31% of patients with stricturing and penetrating phenotype underwent major surgery during the follow-up period, as determined by the Montreal classification.

Table 1.2.1 Vienna and Montreal classifications for Crohn's Disease		
	Vienna	**Montreal**
Age at onset	A1 < 40 years A2 ≥ 40 years	A1 below 16 y A2 between 17 and 40 y A3 above 40 y
Disease location	L1 terminal ileum L2 colon L3 ileocolon	L1 ileal L2 colonic L3 ileocolonic L4 isolated upper disease*
Disease behavior	B1 inflammatory B2 stricturing B3 penetrating	B1 non-stricturing, non-penetrating B2 stricturing B3 penetrating "p" perianal disease modifier
*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present. ''p'' is added to B1–B3 when concomitant perianal disease is present.		

Table adapted from: Sehgal R, Koltun WA. Scoring systems in inflammatory bowel disease. Expert Rev GastroenterolHepatol. 2010 Aug;4(4):513-21.

1.2.3 Crohn's Disease Activity Index

To assess disease severity, CD activity indexes have been developed. In 1976, Best *et al.* published their proposed Crohn's Disease Activity Index (CDAI), (Best WR 1976. Singleton JW 1987. Yoshida EM. 1999) which was ultimately adopted by the National Cooperative Crohn's Disease Study Group. To create the index, 18 proposed predictor variables were initially gathered from 112 patients with CD at each clinic visit. Clinic scores were compared with scores from previous visits and critiqued by a consultant gastroenterologist. By using multiple regression analysis, the 18-predictor variables were eventually consolidated down to eight clinical variables. Three of the eight variables were derived from a 1-week patient diary (Table 1.2.2). Each independent variable was coded so that 0 corresponded to good health and increasing positive values corresponded to greater degrees of illness. A formula assigning weighting factors to multiply each disease value by was devised. A CDAI value of 150 and below was defined as quiescent disease. Values above that indicated active disease, while those above 450 reflected extremely severe disease. Changes in CDAI values from each visit correlated well with physicians' assessments of change in patient status.

The CDAI has become the most commonly used clinical severity-scoring index, especially in therapeutic research protocols. For example, Loftus *et al.* evaluated the effects of adalimumab maintenance therapy on health-related quality of life (HRQOL) in patients with moderate to severe Crohn's disease. In this Phase III, randomized, double-blind clinical trial of moderate to severe Crohn's disease patients, HRQOL outcomes were compared between the adalimumab maintenance treatment groups (every other week and weekly injection) and the adalimumab induction-only group. The Zung Self-Rating Depression Scale, functional assessment of chronic illness therapy (FACIT)-Fatigue, visual analog pain scales, Inflammatory Bowel Disease questionnaire (IBDQ), and Medical Outcomes Study 36-item Short Form Health Survey (SF-36) were analyzed for 499 randomized responders (defined as a decrease of $\geq$ 70 points from baseline in the CDAI) at baseline and weeks 4, 12, 26, and 56. They found that at baseline, overall HRQL was reduced. However, following a 4-week adalimumab induction therapy, patients experienced statistically significant improvements in all HRQOL measures ($P < 0.0001$). When compared with patients who were assigned to placebo after induction therapy, patients who continued adalimumab at 40 mg every other week maintenance therapy reported less depression ($P < 0.01$), fewer fatigue symptoms ($P < 0.001$), greater improvements in the IBDQ ($P < 0.05$), greater SF-36 physical component summary scores ($P < 0.05$), and less abdominal pain ($P < 0.05$) from weeks 12 to 56. They also had greater SF-36 mental component summary scores at week 56 ($P < 0.05$). Patients who continued adalimumab at 40-mg weekly maintenance therapy reported less depression and fewer fatigue symptoms at week 56, greater improvement in IBDQ, and less abdominal pain from weeks 12 to 56 (all $P < 0.05$ vs. placebo). Therefore, adalimumab maintenance therapy provided sustained improvements in HRQOL for patients with moderate to severe Crohn's disease through week 56. (Loftus EV 2008)

Furthermore, Smith *et al.* performed an open-labeled pilot prospective trial, investigating the safety and efficacy of low-dose naltrexone, an opioid antagonist, in patients with active CD. Eligible subjects with histologically and endoscopically confirmed active CDAI score of 220-450 were enrolled in a study using 4.5 mg naltrexone/day. Infliximab was not allowed for a minimum of 8 weeks prior to the study initiation. Other therapy for Crohn's disease that was at a stable dose for 4 weeks prior to enrollment was continued at the same doses. Patients completed the IBDQ and the SF-36 quality of life surveys and CDAI scores were assessed pre-treatment, every 4 week on therapy and 4 week after completion of the study drug.

Seventeen patients with a mean CDAI score of 356 +/- 27 were enrolled. CDAI scores decreased significantly (P= 0.01) with low-dose naltrexone, and remained lower than baseline 4 weeks after completing therapy. Eighty-nine percent of patients exhibited a response to therapy and 67% achieved a remission (P < 0.001). Improvement was recorded in both quality of life surveys with low-dose naltrexone compared with baseline. No laboratory abnormalities were noted. The most common side effect was sleep disturbances, occurring in seven patients. The authors concluded that low-dose naltrexone therapy does appear to be effective and safe in subjects with active Crohn's disease. (Smith JP 2007)

Table 1.2.2. CDAI items and weighting factors

Item (cumulative 7 day score)	Weighting factors (multiply item)
• Number of liquid or very soft stools	2
• Abdominal pain score in one week (rating, 0-3)	5
• General well-being (rating, 1-4)	7
Sum of physical findings per week: • Arthritis/arthralgia • Mucocutaneous lesions (e.g. erythema nodosum, aphthous ulcers) • Iritis/uveitis • Anal disease (fissure, fistula, etc) • External fistula (enterocutaneous, vesicle, vaginal, etc) Fever over 37.8°C	20
Antidiarrheal use (e.g. diphenoxylate)	30
Abdominal mass (no = 0, equivocal = 2, yes = 5	10
47 minus hematocrit (males) or 42 minus hematocrit (females)	6
100 x (1- [body weight divided by a standard weight])	1

Remission of Crohn's disease is defined as a CDAI of less than 150. Severe disease was defined as a value of greater than 450. Most major research studies on medications in Crohn's disease define response as a fall of the CDAI of greater than 70 points.

1.2.4 Truelove–Witts Severity Index in UC

In 1955, Truelove and Witts published their landmark paper 'Cortisone in ulcerative colitis: final report on a therapeutic trial' in the *British Medical Journal* [Truelove SC, 1955]. In order to study the effects of cortisone versus placebo in their randomized controlled trial, the authors devised a method to segregate patients according to their disease severity. Six parameters were studied that included: number of bowel movements in 24 hours, blood in stool, core body temperature, heart rate, hemoglobin and erythrocyte sedimentation rate (ESR) (Table 1.2.3).

A total of 210 UC patients were studied, out of which 109 were treated with 100 mg of cortisone for 6 weeks and 101 patients received placebo. For every stage of disease severity, patients treated with cortisone did much better compared with the corresponding control patients.

The Truelove–Witts Severity Index was the first attempt to classify disease severity of UC patients into mild, moderate and severe disease (Table 1.2.3). This scoring system is popular in the clinical arena owing to its objectivity and ease of use. It has stood the test of time amongst other similar scoring systems that incorporate added clinical and biochemical parameters in their criteria. It continues to be used today in assessing response to therapy in the acutely flaring patient, because of its simplicity and clinical relevancy. For example, Guijarro *et al.* recently evaluate the effectiveness and safety of oral N-acetyl-L-cysteine (NAC) co-administration with mesalamine in UC patients. Thirty seven patients with mild to moderate UC were randomized to receive a four-week course of oral mesalamine (2.4 g/day) plus N-acetyl-L-cysteine (0.8 g/day) (group A) or mesalamine plus placebo (group B). Patients were monitored using the Truelove-Witts Severity Index (TWSI). The primary endpoint was defined as clinical remission (TWSI < or = 2) seen at 4 weeks. Secondary endpoints were clinical response (defined as a reduction from baseline in the TWSI of > or = 2 points) and drug safety. The serum TNF-alpha, interleukin-6, interleukin-8 and MCP-1 were evaluated at baseline and at 4 weeks of treatment. There was clinical remission of 63% and 50% after 4 week treatment with mesalamine plus N-acetyl-L-cysteine (group A) and mesalamine plus placebo (group B) respectively. Analysis of variance of data indicated a significant reduction of TWSI in group A ($P = 0.046$) with respect to basal condition without significant changes in the group B ($P = 0.735$) during treatment. Clinical responses were 66%

(group A) vs. 44% (group B) after 4 weeks of treatment. Clinical improvement in group A correlated with a decrease of IL-8 and MCP-1. Rates of adverse events did not differ significantly between both groups. In conclusion, group A (oral NAC combined with mesalamine) contrarily to group B (mesalamine alone), the clinical improvement correlated with a decrease of chemokines such as MCP-1 and IL-8. NAC addition did not produce any side effects.(Guijarro LG 2008)

Table 1.2.3. Truelove-Witts Ulcerative Colitis Severity Score

	Mild	Severe
Bowel Movements	≤4	≥6
Fever	Absent	>99.5°F. (37.5°C) x 2days
Heart Rate (beats/min)	<100	>100
Haemoglobin	Normal	≤75% of normal for sex
Blood in stools	Streaks	Grossly bloody
ESR (mm)	<30	>30

Table adapted from: Sehgal R, Koltun WA. Scoring systems in inflammatory bowel disease. Expert Rev Gastroenterol Hepatol. 2010 Aug;4(4):513-21.

1.2.5 Montreal Classification for Ulcerative Colitis

The Montreal Working Party decided to not only make improvements to the initial Vienna CD classification, but to also introduce a classification system for UC as well. (Satsangi J, 2006) The Working Party devised a classification system by first defining anatomical disease extent and then including disease severity characteristics (Table 1.2.4). Extent of disease categories defined proctitis (E1), left sided colitis (E2) and pancolitis (E3). Severity of disease category definitions took into account clinical parameters very akin to the Truelove-Witts criteria where S0 defined clinical remission while S3 had at least 6 bloody bowel movements a day, tachycardia, fever, anemia and elevated ESR. This system therefore utilized criteria that were very clinically relevant from both an anatomic and symptomatic perspective and which were past accepted features of disease severity found in other grading schema.

Table 1.2.4. Montreal classification of Ulcerative colitis

EXTENT	SEVERITY
E1: Ulcerative proctitis (involvement limited to the rectum) E2: Left sided UC (involvement limited to colorectum distal to the splenic flexure) E3: Extensive UC (involvement extends proximal to the splenic flexure)	S0: Clinical remission (asymptomatic) S1: Mild UC • passage of four or fewer stools/day with or without blood, absence of any systemic illness, and normal ESR) S2: Moderate UC • passage of more than four stools per day but with minimal signs of systemic toxicity S3: Severe UC • passage of at least six bloody stools daily • pulse rate of at least 90 beats/min • temperature of at least 37.5°C • Haemoglobin of less than 10.5g/100ml • ESR of at least 30 mm/h

Table adapted from: Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut. 2006 Jun;55(6):749-53.

1.3

Diagnosis and medical management of Inflammatory Bowel Disease

Making an accurate diagnosis of CD vs. UC is a crucial first step in order to appropriately manage these conditions. IBD patients should be cared for by a multidisciplinary team consisting of gastroenterologists, colorectal surgeons, clinical nurse specialists, a dietician, pharmacist, pathologist and GI radiologist.[Mowat C 2011] The diagnosis is usually made based on a thorough clinical history and examination, blood tests, endoscopic, radiographic, and histopathological findings that correlate with the disease pattern. In approximately 5% of cases, IBD affecting the colon is unclassifiable even after clinical, radiological, endoscopic and pathological criteria due to considerable overlap. This is now known as 'IBD, type unclassified (IBDU).' The term 'indeterminate colitis (IC)' is usually reserved for situations when the pathologist is unable to classify the disease after a through examination of a colectomy specimen. [Mowat C 2011]

1.3.1 Clinical history and examination

The salient points that one should cover in an IBD focused history include the presence or absence of any constitutional symptoms such as pyrexia, malaise, weight loss, and abdominal pain. Potential risk factors such as smoking, family history, previous appendectomy, any recent foreign travel (especially to rural areas) and recent episodes of infectious gastroenteritis should also be covered. A detailed history pertaining to bowel movements is important and should include stool frequency, consistency, urgency, tenesmus and rectal bleeding. A through medication history should be taken and the presence of any extraintestinal manifestations should be sought out. [Mowat C 2011.Stange EF, 2006. Stange EF 2008. Sands BE 2004)

A full physical examination should be conducted covering all organ systems and to assess the patient's health status globally. It is important to check the patient's weight, body mass index, signs of anemia, and hydration status. The abdomen should be formally examined and perineal evaluation conducted.

1.3.2 Blood, Fecal and Serological Investigations

So far there is a lack of a single effective blood or fecal test that is diagnostic for IBD. Blood tests aid in providing information regarding the patient's overall nutritional status and level of disease severity. Standard blood tests that should be performed include full blood count, urea and electrolytes, liver function tests, Vitamin B12, erythrocyte sedimentation rate (ESR) and C reactive protein (CRP). Elevated CRP levels have been shown to correlate with moderate to severe CD disease activity in approximately 45% of cases. However, when taking both CRP and endoscopic findings together, the correlation was shown to be as high as 65%. (Langhorst J, 2008, Benevento G, 2010,Vermeire S, 2006)

Fecal calproctectin and lactoferrin levels help gauge the amount of intestinal inflammation as they reflect the amount of polymorphonuclear leukocyte-derived proteins that have migrated within the inflamed intestinal mucosa.(Benevento G, 2010) These markers are sensitive for intestinal inflammation however lack specificity as other conditions of the bowel can present similarly (eg- neoplasias, polyposis, NSAID use and increasing age). (Benevento G, 2010.) Of the two fecal markers, calproctectin levels have been shown to be more stable and resistant to degradation therefore making calproctectin a more accurate and attractive marker for colonic inflammation. (Mowat C 2011.Benevento G, 2010,Vermeire S, 2006, Roseth AG, 1999)

Serological markers such as anti-Saccharomyces cerevisiae antibodies (ASCA) and perinuclear antineutrophil cytoplasmic antibodies (pANCA) have a 90% sensitivity for IBD overall. ASCA is more associated with CD whereas pANCA has a greater association for UC and both markers have been utilized to differentiate the two conditions. However, there is much variation of these markers amongst different ethnic and geographic populations as well as before and after surgery. Therefore, the current guidelines for the management of IBD do not recommend their routine use. (Mowat C 2011.Benevento G, 2010. Li X, 2008.)

1.3.3 Imaging Modalities

Radiological modalities play an important role for the clinician to appreciate the extent of the disease, type of disease behavior (eg- stricturing, penetrating, and abscess formation) and visualize any suspected complications. The mainstay of imaging include small bowel enteroclysis and small bowel series (or follow through), intestinal ultrasound, computer tomography (CT) and magnetic resonance imaging (MRI). Out of all these options,

ultrasound and MRI carry the least radiation risk and are safe in pregnancy. Ultrasound is advantageous for detecting disease of the terminal ileum and intra-abdominal abscesses in thin individuals; however, its use is limited by that fact that it is highly operator dependent. Some IBD patients will require numerous x-ray studies during their lifetime; a recent suggestion has been that MRI of the abdomen be used to detect intestinal disease, especially when studying youthful patients in order to minimize long term radiation risk. (Messaris E 2010) The main imaging modality for diagnosing IBD is the use of small bowel enteroclysis and follow through. Both modalities have been shown to carry similar sensitivity and specificity. The former carries a much higher radiation risk and is more expensive compared to the latter. Both CT and MRI are popular in appreciating transmural inflammation, extraluminal complications and accurately delineating fistulizing disease. The decision to use such scans is dependent largely on the availability and on institutional protocols. (Mowat C 2011. Benevento G, 2010)

1.3.4 Endoscopy

Upper and lower GI endoscopy allows the physician to directly visualize the colonic mucosa and objectively assess the degree of muscoal involvement and severity. Current guidelines recommend at least two biopsies to be taken from five sites including the distal ileum and rectum.(Mowat C 2011) Endoscopy is relatively contraindicated during times of active disease or possible toxic megacolon due to the increased risk of perforation and haemorrhage.

Histopathological features which are consistent with CD include the presence of transmural inflammation that is present in a skip like fashion (segmental colitis or enteritis), cobblestone mucosa, rectal sparing and ulcerations (aphthous, discrete, serpiginous). Furthermore, the presence of granulomata is suggestive of CD, however are infrequently found (15% to 36%) on biopsy specimens. These aforementioned features are not present in UC as the inflammation is restricted to the mucosa and submucosa, thus helping to differentiate the two conditions. (Table 1.1) (Leighton JA, 2006) Although when UC is particularly severe, i.e. "toxic," the gross and microscopic pathology can be difficult to differentiate and can in fact even appear Crohn's like.

1.3.5 Conventional Medical Management

The main objective of medical management for IBD is to maintain remission and prevent relapse of the disease. Conventional management options depend largely upon disease activity and extent. Patients are usually managed in a step up fashion, with more aggressive medications being used in each step until disease is controlled. UC patients are initially treated with 5-Aminosalicylic acid (5-ASA, mesalamine). There are several delivery options available which include oral tablets, sachets or suspensions by using prodrugs (eg- balsalazide, olsalazine, or sulfasalazine) in order to reduce systemic absorption and maximize colonic delivery; and rectal mesalamine formulations in the form of enemas or suppositories. Patients who fail to respond to 5-ASA are usually commenced on a 2-4 week course of oral corticosteroids. If after this time the patient responds favorably, the steroids should be tapered off (typically 5mg/week to complete withdrawal) and put back on 5-ASA maintenance therapy. If the patient is steroid dependent, i.e.- unable to taper off steroids or relapses within 3 months of stopping steroids, then such patients should be commenced on a 8-12 week course of immune modulators such as azathioprine (2-2.5mg/kg/day) or 6-mercaptopurine (1-1.5mg/kg/day) with regular full blood counts performed to monitor for the development of any potential toxic side effects such as myelotoxicity and leukopenia. Should such patients show improvements, then it is recommended that they continue on thiopurine maintenance. However in moderate-to-severe disease or if the steroid dependent patient is still noted to have refractory disease, then a trial with biologics such as anti-TNF antibodies Infliximab (5mg/kg at week 0, week 2, and week 6) or adalimumab can be used and/or surgical opinion should be sought. (Burger D, 2011. Baumgart DC, 2007. Mowat C 2011)

Similarly, CD patients who have mild-to-moderate ileocolonic disease are usually commenced on a 2-4 week course of oral Budesonide (9mg/day) and are discouraged to smoke tobacco. A Cochrane review showed 5-ASA (mesalamine) to have a limited beneficial effect in maintaining medically induced remission in CD patients.(Gordon M, 2011.) If a favorable response is observed, then such patients should be tapered off over the next 2-3 months and followed up routinely. However, in case of an inadequate response, patients should then be commenced on oral Prednisolone (40mg/day). If the patient is steroid responsive or dependent (ie- unable to reduce to ≤10mg/day), then an 8-12 week course of either Azathioprine (2-2.5mg/kg/day), 6-mercaptopurine (1-1.5mg/kg/day) or Methotrexate (25mg/kg) should be attempted with regular full blood counts performed to monitor for the

development of any potential toxic side effects such as myelotoxicity and leucopenia. If a favorable response is seen, then the patient should be maintained with such immunomodulators. However if they are refractory to immunomodulators or intolerant to steroids then objective surgical assessment for fibrostenotic and/or fistulizing disease is warranted followed by either active surgical management or consideration of anti-TNF therapy. (Burger D, 2011. Baumgart DC, 2007. Mowat C 2011)

1.4

Surgical Management of Inflammatory Bowel Disease

1.4.1 Surgical Management of Crohn's Disease

Although medical management helps to control symptoms, approximately 70% of patients with CD will require surgical resection of the affected bowel during the course of the disease.[Bernell O, 2000. Regueiro M, 2010] Surgery for CD is by no means curative and recurrence of symptoms is part of the natural history of the disease. Surgery for CD is usually considered as a last resort or performed only when complications of the disease have occurred. The indications for surgery can be subdivided into elective and emergent. Elective surgery can be performed for failed medical management, multiple bouts of sub-acute bowel obstruction (eg- secondary to stricture formation), the development of intra-abdominal fistula or abscess, failure to thrive/growth retardation (in the pediatric population) and the development of carcinoma. Emergent indications for surgery would include bowel perforation, haemorrhage and toxic-colitis. [Strong SA 2001]

Surgical options are individualized depending on the pre-operative indications and intra-operative finding. In general, ileocolectomy is the most commonly performed operation as ileocolonic disease is the most frequently affected location in CD. Resection is the preferred option especially when it is the first surgery as this provides an adequate sized colectomy specimen that the pathologist can examine in detail in order to confirm the diagnosis. Furthermore, bowel resection with primary anastomosis is the mainstay of surgical management for CD as recurrence rates are significantly higher with other available surgical options such as bypass procedures.[Strong 2001] The type of anastomotic technique used (ie- end-to-end vs. side-to-end anastomosis) does not play a role in disease recurrence.[Strong SA 2001. Scott NA, 1995. McLeod 2009]

In difficult circumstances where patients suffer frequent bouts of intestinal obstruction secondary to multiple CD associated strictures within the small bowel, alternative bowel conserving surgical procedures are performed in order to avoid short-gut syndrome. In 1982 Lee and Papaioannou published a study that followed 9 CD patients with extensive small bowel involvement that underwent 'minimal' surgery (i.e. strictureplasty) to simply relieve intestinal obstruction. Their long-term results showed a marked improvement in general health in a short period of time post-operatively with dramatic improvements in weight gain

and food tolerance and sustained patency of the bowel lumen despite diseased bowel being left in-situ.[Lee EC, Papaioannou N. 1982. Strong SA 2001. Hull TL 1999] Subsequent studies have shown comparable recurrence and reoperation rates following strictureplasty versus conventional small bowel resections.[Ozuner G, 1995. Ozuner G, 1996. Fazio VW, 1993] Therefore strictureplasty is considered to be a safe and appropriate procedure especially in the setting of diffuse involvement of the small bowel with multiple strictures; in patients who have undergone previous small bowel resections (>100cm); in patients with already established short gut syndrome and in the setting of nonphlegmonous fibrotic strictures. [Hull TL 1999. Strong SA 2001]

Depending on definition, 70-90% of patients have endoscopic evidence of recurrent CD within one year after intestinal resection.[Regueiro M, 2010] However, clinically relevant recurrence is often delayed and up to one-third of patients will not develop clinically significant disease for approximately 15-20 years.[Ritchie JK. 1990. Lennard-Jones JE, 1967. Bernell O 2000] Several risk factors that are associated with disease recurrence include an early age of diagnosis (younger than 25 years), active smoking (carries five-fold increase recurrence risk), the presence of granulomas on biopsy, duration of CD before resection, site of bowel involvement (ileocolic> small bowel > colonic), and type of disease (perforating CD > non-perforating type).[HJ Buhr 2001. Swoger JM, 2010] Anti-TNF-alpha medications such as infliximab (Remicade®) have been shown to delay recurrence rates however currently there is no way to accurately identify patients who would develop early disease recurrence and therefore warrant the institution of such potentially dangerous and expensive medications versus the patients who will have relatively quiescent disease. Radical resections do not protect against early recurrence, therefore whenever possible, the surgeon should perform bowel conserving operations especially in patients who are prone to multiple CD recurrences in order to preserve intestinal length and to always document the total length of the gut prior to and after resection.[HJ Buhr 2001]

1.4.2 Surgical Management of Ulcerative Colitis

1.4.2.1 Total Proctocolectomy with Ileal Pouch Anal Anastomosis

Despite advances in medical therapy, approximately one third of patients with UC eventually require surgery.[McLaughlin SD 2008] Early surgical management for UC involved performing a subtotal colectomy with completion protectomy and creating a permanent end ileostomy. However the fact that patients were left with a stoma was less than ideal. In 1947, Ravitch and Sabiston described an operation for benign lesions that involved restoring gastrointestinal continuity by performing a proctocolectomy with a straight ileoanal anastomosis. [Ravitch MM, Sabiston DC Jr 1947. Bach SP 2006.] This technique was further refined by Sir Alan Parks and John Nicholls in 1978 at St. Mark's Hospital, London, where they developed an operation that removed the inflamed colon and upper third of the rectum, stripped the remaining rectal mucosa down to the dentate line followed by bringing down the terminal ileum to create a 30-40cm ileal reservoir (pouch) and performing an ileoanal anastomosis using a hand sewn technique.[Parks AG, Nicholls RJ. 1978 McLaughlin SD 2008 Bach SP 2006] (see Figure 1.4.1). Now, the use of staplers have superseded the hand sewn technique, which leaves a 1-2cm cuff of residual rectum which improves continence but has the potential to become symptomatic or cancerous. The most common configuration of the pouch is the 'J' or two-loop pouch due to the ease of construction by utilizing less amount of intestine. Other designs include the S, H and W pouches, which however are more technically challenging to construct.[McLaughlin SD 2008] This operation can be performed in several stages (1, 2, or 3). The majority of surgeons perform this operation in 2 stages, i.e.- performing a total proctocolectomy with pouch reconstruction with a diverting ileostomy which is reversed in 6-8 weeks. This method allows the distal anastomosis to heal and avoids risk for potential pelvic contamination.[Bach SP 2006]

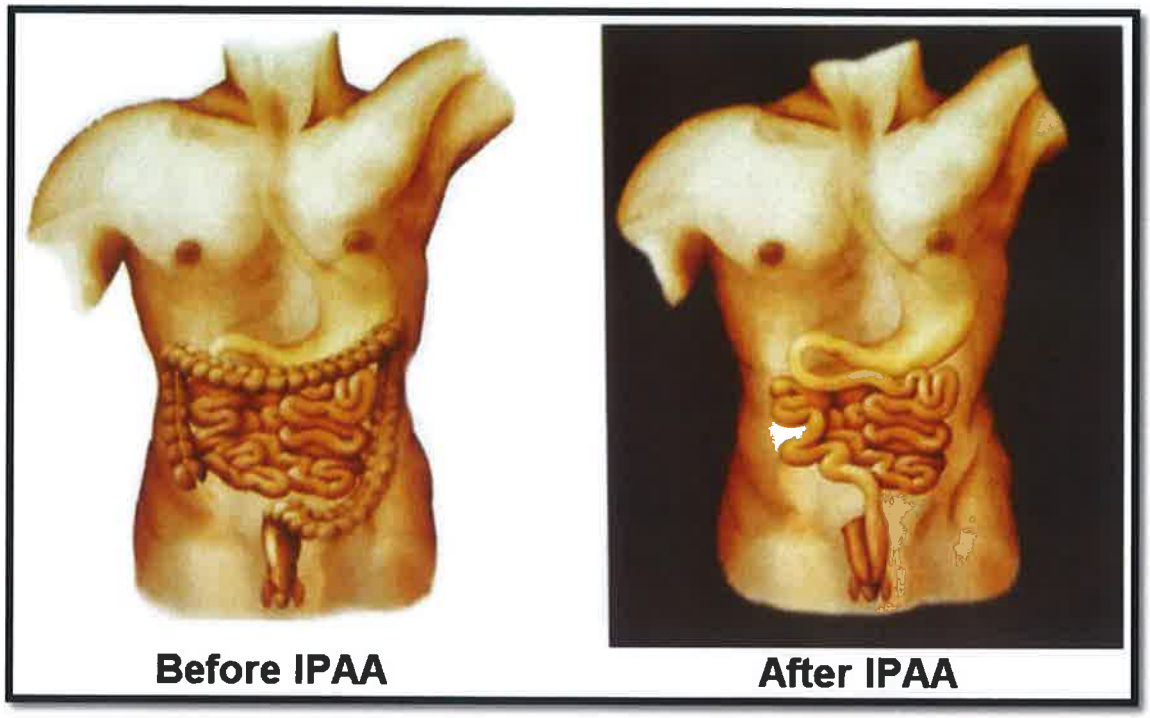

Figure1.4.1: Ileal Pouch Anal Anastomosis involves performing a total proctocolectomy followed by fashioning an ileal pouch reservoir that is anastomosed to the anal canal. The most common configuration is a J-pouch as shown here on the right. (Original illustration created by the artists at Hershey Medical Center)

1.5

Complications of IPAA

Total proctocolectomy with ileal pouch-anal anastomosis (IPAA) is now considered the gold standard for the surgical treatment of UC. The advantages of this procedure include: (1) the re-establishment of gastrointestinal continuity; (2) elimination of the symptoms of IBD and improvement of health-related quality of life; (3) the decreased need for UC-related medications, particularly immune modulators and biological agents with avoidance of their associated potential adverse effects, and (4) reduced risk for dysplasia and eventual cancer. However, adverse outcomes or complications can occur after IPAA surgery. These include early postoperative complications which usually are technically related such as breakdown of the ileoanal anastomosis leading to pelvic sepsis that are usually conservatively managed, or ones occurring later that can suggest recrudescent IBD. Such later complications include pouch-anal or pouch-intestinal fistuli, afferent limb stricture and mild or severe pouchitis. The severity of such complications can sometimes suggest a diagnosis of CD, which usually is a pre-operative contraindication for IPAA, because of its known high failure rate (typically 50%).[Braveman JM 2004] Both of these late complications (severe pouchitis and CD-like) are the leading causes of pouch failure, pouchectomy and permanent stoma.[Shen B 2009]

1.5.1 Pouchitis

Pouchitis, an idiopathic inflammatory condition, is the most common long term post operative complication of the ileal pouch.[Nicholls RJ 1998. Macafee DA, 2004] It was first described in 1977 as an inflammatory condition of the Koch continent ileal reservoir. The term soon gained widespread acceptance in 1978 to be associated with the IPAA.[Coffey JC, 2009] Depending on definition and duration of follow up, it can affect 15-70% of UC pouch patients [Beliard A, 2010] and on average develops within 34 months post operatively (range 2-102 months).[Macafee DA, 2004, Madiba TE, 2001] In contrast, the incidence of pouchitis in patients who have undergone the IPAA for other reasons such as Familial Adenomatous Polyposis (FAP) is rare and ranges from 3-14%.[Macafee DA, 2004]

1.5.1.1 Pouchitis: Etiology/Risk Factors

The exact aetiology of pouchitis still remains unclear. However, the fact that this condition is almost exclusively seen in UC patients and rarely in FAP, that it only occurs after the restoration of GI continuity (i.e. ileostomy closure), and responds to antibiotic or probiotic therapy, points towards a dysregulated immune response to overgrowth of certain commensal gut microflora in a genetically susceptible host.[Yu ED 2007 Shen B, 2004] This can sometimes suggest a recurrence of a UC-like disease in the ileal pouch in a subset of patients.[Yu ED 2007. Shen B, 2004]

Within weeks to a few months after ileostomy closure, the ileal pouch undergoes a number of nonspecific adaptive changes ("colonic metaplasia") in order to accommodate the fecal stream and gut microbes. Biopsies taken from functioning pouches commonly show partial villous atrophy, crypt hyperplasia, mucosal inflammation, increased crypt cell proliferation and change in mucin type from small bowel type sialomucin to a large bowel type sulphomucin.[Nicholls RJ, Banerjee AK. 1998. de Silva HJ 1991] When comparing pouches performed for UC to those created for FAP, increased intraepithelial lymphocyte counts, leucotriene B4 and prostaglandin E2 are found in patients with colitis compared with those with polyposis.[Nicholls RJ, Banerjee AK. 1998. De Silva, H.J.1991. Goldberg, P.A 1996. Gertner, D.J.1994]

So far, no single causative organism has been identified as an etiologic factor in pouchitis. However, a 100:1 increased anaerobe/aerobe ratio within pouches has been observed.[Nicholls RJ, Banerjee AK. 1998. Coffey JC, 2009] Duffy *et al* have shown the presence of sulfate-reducing bacteria exclusively within UC pouches when compared to FAP pouches and ileostomy effluent. This most probably is related to fecal stasis after ileostomy closure and may play a role in the pathogenesis of pouchitis. Furthermore, there was no significant difference in the levels of *Lactobacilli, Bifidobacterium, Bacteriodessp, Clostridium perfrigens,* enterococci, and coliforms in the UC and FAP pouch groups. [Duffy M, 2002. Coffey JC, 2009]

Several risk factors for developing pouchitis have been suggested however with conflicting and often limited data in the literature. Some proposed risk factors include: the presence of extraintestinal manifestations of UC, in particular primary sclerosing cholangitis (PSC), which has been shown to increase the risk by 1.5-2 fold; [Pardi DS, 2009. Penna C, 1996] a more severe preoperative disease course as judged by the use of preoperative steroids or the presence of

thrombocytosis; villous blunting of the ileal mucosa prior to the creation of the pouch; mucosalischemia; backwash ileitis; the use of nonsteroidal anti-inflammatory drugs (NSAIDs);Crohn's disease; the presence of perinuclear Anti-Neutrophil Cytoplasmic Antibodies (pANCA) and Anti-CBir 1 antibodies.[Pardi DS, 2009. Shen B 2004. Nicholls RJ, Banerjee AK, 1998] and current smoking is protective against developing pouchitis compared to nonsmokers or ex-smokers.

1.5.1.2 Pouchitis: Clinical Symptoms and Classification

Pouchitis typically presents clinically with a spectrum of symptoms which include, increased stool frequency, anal bleeding, urgency, abdominal cramping and pelvic discomfort.[Macafee DA, 2004] Depending on severity, patients can also present with fever, dehydration, and malnutrition which may warrant hospitalization.[Yu ED 2007] Such symptoms are thought to represent a recurrence of UC and is associated with an increased patient morbidity and negative health related quality of life. Therefore it is important to be able to precisely classify the degree of severity in order to tailor management accordingly. Classification systems also aid in comparing results among different medical centres and assess the response to therapy in both clinical practice as well as in clinical trials.[Heuschen UA 2002]

There is no one universally accepted classification systems for pouchitis. Shen *et al* proposed a clinical classification schema based on response to therapy.[Shen B 2003] He proposed three main sub-groups, (1) antibiotic-responsive pouchitis, a condition in which patients have infrequent episodes (< 4 episodes per year) responding to a 2-week course of a single antibiotic, (2) antibiotic-dependent pouchitis is a condition with frequent episodes (> 4 per year) or with persistent symptoms requiring long-term, continuous antibiotic or probiotic therapy, and (3) antibiotic-refractory pouchitis is a condition in which patients fail to respond to a prolonged course of antibiotics and require 5-ASA, corticosteroids or immunomodulator (Imuran, 6MP or Remicade) therapy (Figure 1.5.1). Some other examples of classification systems for pouchits include categorizing severity into mild, moderate or severe and/or based on the frequency of flare ups (infrequent-2 episodes vs. relapsing > 3 episodes vs continuous).[Macafee DA, 2004. Coffey JC, 2009]

Several scoring systems have been developed that incorporate clinical, endoscopic and histological parameters to assess the severity of pouchitis. Examples of such scoring systems

include the most commonly used Pouch disease activity index (PDAI) devised at the Mayo Clinic, Rochester, Minnesota; the modified PDAI; St Mark's (Moskowitz) criteria and Heidelburg pouchitis activity score.[Macafee DA, 2004. Heuschen UA 2002] The PDAI uses an 18 point scoring system which incorporates clinical (range, 0-6 points), endoscopic (range, 0-6 points) and histology (range, 2-6 points) parameters. A score of $\geq$ 7 has been shown to be associated with clinically significant pouchitis (Table 1.5.1). [Sandborn WJ, 1994. Heuschen UA 2002. Shen B, 2003] However, despite the wide acceptance of the PDAI as an objective assessment tool for severity of pouchitis, several criticisms have been made towards it such as the increased costs and delay in calculating the histological component which therefore hinders the routine use of this scoring system as a diagnostic modality.[Shen B, 2003] The modified PDAI proposed by Shen *et al* excludes the histopathological component and has been shown to be more economic and with comparable sensitivity and specificity to the PDAI [Shen B, 2003. Kohyama M, 2009] Furthermore, it has been suggested by Heuschen *et al* that a PDAI score of 7 still may be too high for making the diagnosis of pouchitis and it fails to predict the success of treatment in that antibiotics have been shown to be effective in patients with PDAI scores <7. [Heuschen UA 2002. Kohyama M, 2009]

The lack of a universally accepted clinical classification system for diagnosing pouchitis has made it challenging for both clinicians and researchers alike to speak the same language when it comes to this debilitating condition.

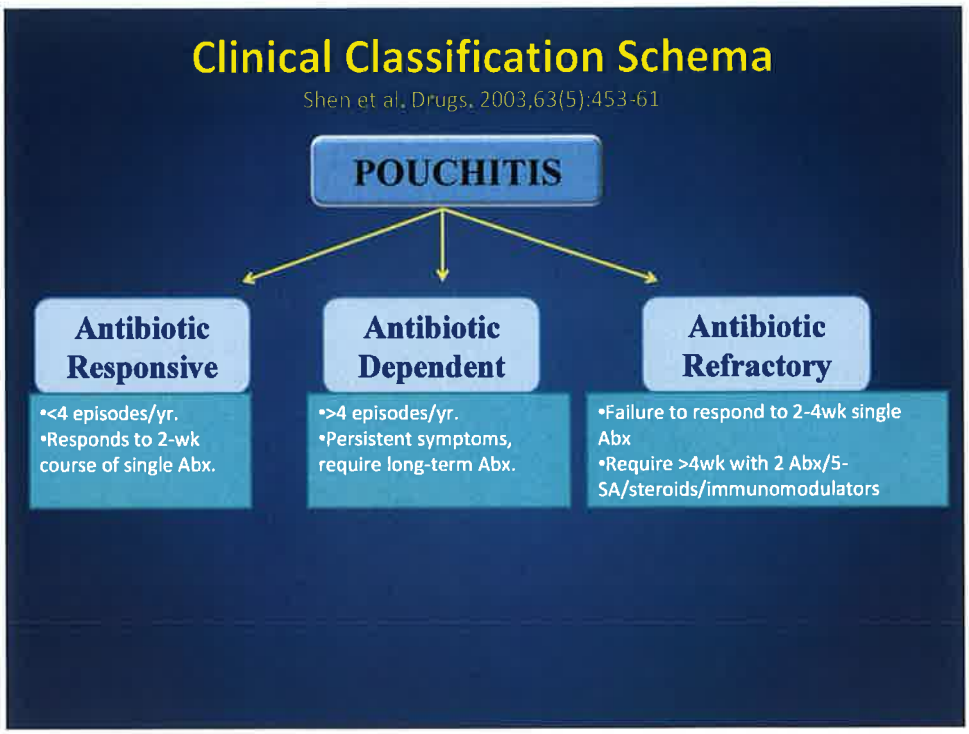

Figure 1.5.1: Pouchitis Clinical Classification Schema.

Table 1.5.1: The Pouchitis Disease Activity Index (Sandborn WJ, 1994)

Criteria	Score
Clinical:	
Stool frequency	
Usual postoperative stool frequency	0
1-2 stools/day > postoperative usual	1
3 or more stools/day > postoperative usual	2
Rectal bleeding	
None or rare	0
Present daily	1
Fecal urgency or abdominal cramps	
None	0
Occasional	1
Usual	2
Fever (temperature >37.8° C)	
Absent	0
Present	1
Endoscopic inflammation:	
Edema	1
Granularity	1
Friability	1
Loss of vascular pattern	1
Mucous exudates	1
Ulceration	1
Acute Histologic inflammation:	
Polymorphic nuclear leukocyte infiltration	
Mild	1
Moderate + crypt abscess	2
Severe + crypt abscess	3
Ulceration per low-power field (mean)	
>25%	1
25-50%	2
>50%	3

The PDAI uses an 18 point scoring system which incorporates clinical (range, 0-6 points), endoscopic (range, 0-6 points) and histology (range, 2-6 points) parameters. A score of ≥ 7 has been shown to be associated with clinically significant pouchitis

1.5.1.3 Pouchitis: Diagnostic Evaluation

Diagnosing pouchitis is based on a through assessment of clinical symptoms, endoscopic and histologic evaluation. It is important to use a multi-modal approach in order to distinguish pouchitis from other inflammatory disorders of the pouch such as *Clostridium difficile* or cytomegalovirus (CMV) infection, pouch stricture, cuffitis, or functional disorders such as Irritable Pouch Syndrome.(Yu ED 2007 Pardi DS, 2008, Coffey JC, 2009) In addition, endoscopy with biopsy is a powerful tool for dysplasia surveillance and can aid in therapeutic intervention such as stricture dilation.(Yu ED 2007)

Endoscopy is usually performed in the clinic setting by using a paediatric proctoscope or a flexible sigmoidscope. The characteristic features of pouchitis on endoscopy are similar to those for acute inflammation which include erythema, edema, increased granularity, loss of vascular pattern and friability with superficial ulcers.(Coffey JC, 2009, Pardi DS, 2008) Four to six biopsies from the body of the pouch are usually sufficient for making a histological diagnosis of pouchitis and assessing the degree of severity. It is not uncommon for pouches to have some degree of chronic and acute inflammation present as baseline secondary to adaptive changes of the pouch mucosa to fecal stasis and does not necessarily indicate active pouchitis. (Yu ED 2007,Pardi DS, 2008, Coffey JC, 2009) Histological features that are characteristic of active inflammation of the pouch include the presence of neutrophil infiltration with occasional crypt abscesses and mucosal ulceration. These findings are oftentimes present on a background of chronic changes such as villous atrophy, crypt distortion or hyperplasia and chronic inflammation.(Pardi DS, 2008)

Once the diagnosis of pouchitis is made, subsequent biopsies are not routinely performed unless there is a change in the clinical parameters and/or the patient stops responding to his/her routine course of antibiotics.(Pardi DS, 2008)

1.5.1.4 Pouchitis: Treatment

Pouchitis typically responds well to a short course of antibiotic therapy. Up to 80% of patients respond favorably to a seven day course of metronidazole (15-20 mg/kg three times daily) thus further strengthening the argument for a microbial component to the aetiology of pouchitis.[Coffey JC, 2009. Nicholls RJ, Banerjee AK. 1998] Apart from its bactericidal property against anaerobic microbes, metronidazole has been shown to inhibit superoxide associated free radical induced mucosal damage.[Coffey JC, 2009. Nicholls RJ, Banerjee AK. 1998. Levin KE, 1992] Metronidazole is usually used as a first line medication for pouchitis as it is cheaper than the other treatment options available and most commonly in the acute setting in order to avoid potential associated side effects such as nausea, dyspepsia, dysgeusia and neuropathy.[Coffey JC, 2009]

Alternatively, a 2 week course of Ciprofloxacin (500mg twice a day) has also shown to be efficacious against acute pouchitis. Shen *et al* performed a randomized clinical trial to directly compare metronidazole with ciprofloxacin and found that although both antibiotics were effective in treating acute pouchitis, ciprofloxacin was much better tolerated and had a larger reduction in the PDAI with greater improvements in clinical symptoms and endoscopic scores[Shen B, 2001. Yu ED 2007] In chronic cases, combination therapy with metronidazole and ciprofloxacin is recommended at monthly intervals so as to avoid associated adverse effects and to reduce antibiotic resistance.[Macafee DA, 2004] Other antibiotics that are acceptable alternatives to the aforementioned antibiotics include tetracycline, clarithromycin, amoxicillin/clavulanic acid, doxycycline, and rifaximin. Several enemas are also available such as budesonide enemas, and alicaforsen (an antisense inhibitor of intercellular cell adhesion molecule-1) enemas.[Yu ED 2007, Macafee DA, 2004. Miner P 2004]

As mentioned above, due to the fact pouchitis responds favorably to a course of antibiotics in the majority of cases, there most likely is a microbial component to its aetiology.[Coffey JC, 2009] Antibiotic refractory pouchitis usually requires long term maintenance therapy with antibiotics in the majority of cases and probiotics have been shown to be efficacious in a smaller subset. VSL#3® is a probiotic consisting of 4 strains of *Lactobacillus*, 3 *Bifidobacterium* species, and *Streptococcus salivarius* subsp.[Yu ED 2007] Probiotic use has been shown to delay the relapse rate and maintain clinical remission in antibiotic refractory pouchitis patients.[Yu ED 2007. Gionchetti P, 2003] However, maintaining remission can be challenging

and several other concomitant therapies are sometimes used which include steroids, NSAIDs, immunomodulators and biologics such as azathioprine/6MP and infliximab respectively. (Yu ED 2007. Coffey JC, 2009)

1.5.2 Crohn's Disease-Like Complications

The IPAA is generally considered a pre-operative contraindication in patients with known Crohn's disease because of its high failure rate (typically 50%, range 25-100%). (Sagar PM 1996. Shen B 2009) Patients who develop CD-like complications of the IPAA have an approximately 5-fold estimated increased risk of pouchectomy and permanent stoma. (Shen B 2009) Furthermore, CD of the pouch carries an increased risk for developing dysplasia or adenocarcinoma of the IPAA.(Kariv R, 2007) However, the development of *de novo* CD of the pouch which can occur weeks (early onset) to years (late-onset) has perplexed clinicians and surgeons alike for quite some time. Despite the fact that the IPAA has been a popular surgical choice since 1978, data pertaining to the pathogenesis, diagnosis and treatment options for CD-like complications of the pouch is still largely lacking. (Shen B 2009) The true incidence of CD of the pouch in patients who initially undergo surgery for pre-operative diagnosis of UC is not exactly known. Reported cumulative frequencies however range from 2.7% to 13%, depending on diagnostic criteria used, studied sample size and degree of follow up. (Shen B. 2009. Peyrègne V, 2000. Goldstein NS, 1997. Harley JE, 2004)

1.5.2.1 CD-like Complications: Etiology and Pathogenesis

The etiology and pathogenesis of post-IPAA CD still remains to be fully elucidated. Several studies have investigated risk factors that correlate with the development of this complication which include: pre-operative diagnosis of IC or CD, having a pouch for an extended period of time, a positive family history of CD, active smoking, and seropositive anti-*Saccharomyces cerevisiae* (ASCA)-IgA. (Melton GB, 2008.Wu H, 2009. Shen B, 2006. Delaney CP 2002. Melmed GY 2008. Shen B, 2009) Different phenotypes of CD of the pouch have been shown to correlate with different risk factors. For example, the development of fistulizing CD of the pouch correlated with younger age, female gender, a preoperative diagnosis of IC, without the use of nonsteroidal anti-inflammatory drugs.(Wu H, 2009. Shen B, 2006)

It is not clear as to how exactly *de novo* CD of the pouch arises. It has been speculated that post operative changes in bowel anatomy alters the luminal microbial environment (i.e.

dysbiosis) within the pouch thus creating a 'CD-friendly' environment. This is supported by the clinical observation that CD of the pouch most commonly originates at the anastomosis and the bowel segments proximal to it.[Wu H, 2009. Shen B, 2009] Furthermore, fecal stasis within the pouch combined with surgically-induced ischemic injury in a genetically susceptible host could potentially increase the risk of developing CD of the pouch. [Wu H, 2009]

1.5.2.2 CD-like Complications: Clinical Symptoms and Diagnosis

The features of CD of the pouch can be difficult to differentiate from that of chronic refractory pouchitis and thus makes diagnosing this condition challenging. CD-like complications of the pouch carries a 5-7-fold increased risk of pouch failure, resulting in pouch resection or permanent diversion.[Shen B, 2008. Shen B 2009] Similar to conventional CD and pouchitis, CD of the pouch is associated with a wide spectrum of clinical symptoms, endoscopic, histological features and prognosis. Several general, non-specific symptoms include an increased stool frequency, fecal urgency and/or incontinence, nocturnal seepage, abdominal and pelvic discomfort, and the development of extraintestinal manifestations such as arthralgias and pyoderma gangrenosum. [Wu H, 2009. Shen B, 2009] More specific symptoms that may indicate that a patient has developed CD-like complications include, an active smoking status, the development of pyrexia, weight loss, nausea and vomiting, bowel obstruction, iron deficiency anemia, perianal disease and fistulae (eg. pouch-cutaneous, pouch-vesicular and pouchvaginal) and/or abscess formation outside the pouch-anal anastomosis in the absence of postsurgical complications such as anastomotic leak, or sepsis. [Wu H, 2009. Shen B, 2009. Shen B 2005]

Pouch endoscopy is considered to be the first-line investigation of choice for the diagnosis of CD-like complications. Endoscopic features that may suggest CD of the pouch include (1) the presence of discrete small and large mucosal ulcers, loss of vascular pattern, hemorrhage, inflammatory pseudopolyps within the pouch, cuff, or neoterminal ileum; (2) ulcerated lesions in the afferent limb proximal to the pouch and/or strictures at the pouch inlet, in the absence of active NSAID use; and (3) the presence of granulomas, although rare (present in only 10-20%), on pouch or small bowel histology remote from any anastomosis. Typically, the presence of such clinical and endoscopic findings is considered to be consistent with the diagnosis of CD of the pouch if the patient is 6-12 months status post IPAA with full restoration of GI continuity, and especially if the patient is an active smoker due to the fact that ex-tobacco usage or having never smoked is protective for UC and/or pouchitis. [Wu H, 2009. Shen B, 2009. Shen B 2005. Hyman NH, 1991. Wolf JM, 2004. Shen B, 2004] Other modalities that can be used to

diagnose CD of the pouch include upper GI endoscopy and radiologic evaluation such as small bowel contrast studies, computer tomography (CT) and/or magnetic resonance imaging (MRI) enterography. The uses of push enteroscopy, capsule endoscopy, and serologic markers, however, have yet to be fully evaluated. If however the endoscopic and radiological modalities are inconclusive or if surgical intervention is indicated, a formal examination under general anesthesia (EUA) should be undertaken for both diagnostic and therapeutic purposes. (Wu H, 2009. Shen B, 2009. Shen B 2005.Melmed GY, 2008)

1.5.2.3 CD-like Complications: Treatment

Once diagnosed with CD of the pouch, patients are commonly managed medically to maintain remission and in order to retain their pouch. The use of NSAIDs and smoking tobacco is discouraged. The treatment for CD of the pouch depends on the type of predominant complications present, ie- Inflammatory CD vs. Fibrostenotic CD vs. Fistulizing CD of the pouch.

Patients with inflammatory CD of the pouch are often managed with topical or oral 5-aminosalicylates, topical or oral corticosteroids, oral antibiotics and immunomodulators. In refractory cases, the use of biologics such as the anti-tumour necrosis factor-alpha (anti-TNFα) agent infliximab and adalimumab has shown to be efficacious. (Wu H, 2009. Havaran L 2011)

Fibrostenosing CD of the pouch is often managed with a combination of medical and endoscopic modalities. Medical therapy is similar to the inflammatory CD complications subtype which includes the use of topical or oral 5-aminosalicylates, topical or oral corticosteroids, oral antibiotics, immunomodulators or biologics. Dilatation of the pouch stricture via endoscopic balloon dilation with or without infiltration of long-acting corticosteroids provides symptomatic relief especially for short length (<1 cm) strictures. Long and high grade strictures can be managed with endoscopic needle knife 'stricturoplasty' with or without intralesional injection of the long-acting synthetic corticosteroid triamcinolone. (Wu H, 2009. Shen B, 2009)

The mainstay for treating fistulizing CD of the pouch is by the use of antibiotics, immunomodulators and biologics. Several studies have shown the use of infliximab and adalimumab to be beneficial in both the short and long term maintenance of patients with an

IPAA performed for a pre-operative diagnosis of UC who subsequently develop CD-related fistulae. (Wu H, 2009. Shen B, 2009. Colombel J-F, 2003. Coburn LA, 2006. Shen B, 2009. Ricart E, 1999. Haveran LA, 2011)

Surgery is reserved for patients who fail to respond to medical management. Surgical options range from strictuloplasty, incision and drainage of pouch-related abscess with or without seton or mushroom catheter placement, fistulotomy/fistula repair, and diverting ileostomy with or without pouch excision. (Wu H, 2009. Shen B, 2009) It should be mentioned that pouch excision or permanent diversion performed for CD-like complications does not guarantee a cure for the disease as CD can recur in other bowel segments and/or present as perineal disease. Furthermore, patients who undergo pouch excision can develop persistent perineal sinuses which warrant further surgery. These patients will also require to be kept on life long medical therapy. (Wu H, 2009. Shen B, 2009)

1.6

Etiology of Inflammatory Bowel Disease

The overall incidence of IBD, especially the rates of CD, has steadily increased since the 1920s with approximately 100-200 per 100,000 of Western Caucasians living with the disease.[Binder, 2004. Crohn, 1932. Cho JH, 2008] Industrialized countries such as north America and Europe have the highest incidence of IBD. Within the United States, the prevalence of IBD varies amongst ethnic groups and reflects that seen globally with individuals of European ancestry having the greatest prevalence followed by African Americans, Hispanic and Asian subgroups. Individuals of Jewish decent have a 2-4 times greater prevalence of IBD when compared to any other ethnic group irrespective of geographic location.[Ahmad T. 2001] In particular the Ashkenazi Jews have the greatest prevalence for the disease when compared with Sephardic or Oriental Jews.[Ahmad T. 2001, Roth MP, 1989]

1.6.1 Etiological Clues

The etiology of IBD still remains largely unknown. However, the proposed mechanism of pathogenesis incorporates the interplay of environmental factors including most notably the gut microflora, and host genetic factors that cause a dysregulation of the immune response, leading to unsuppressed inflammation within the GI tract (Figure 1.6.1).

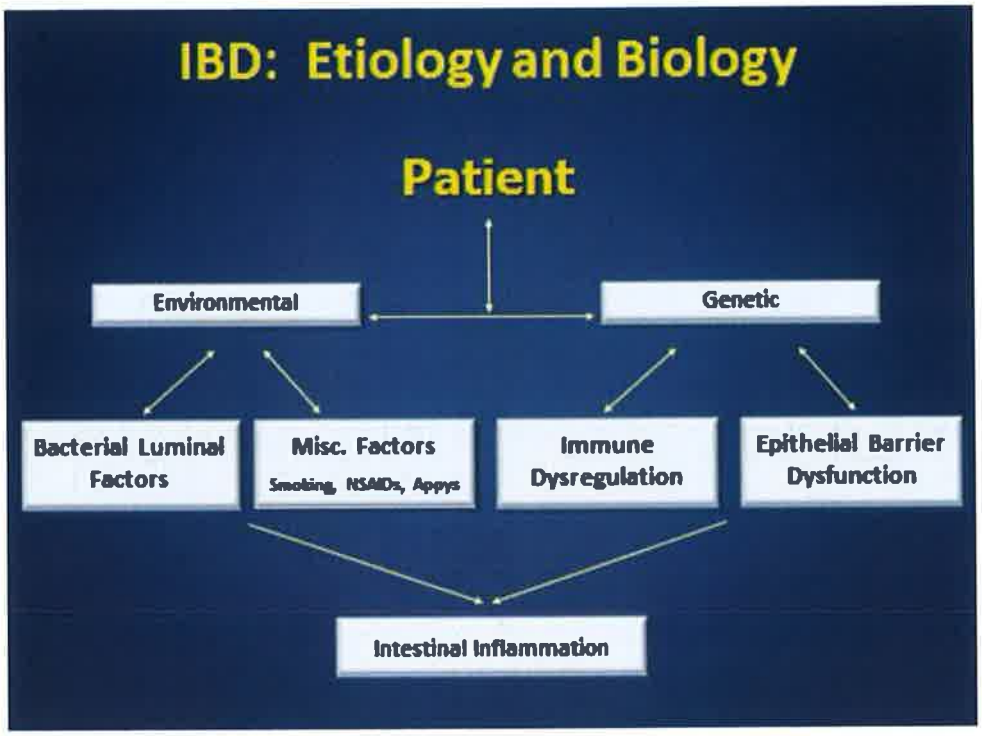

Figure 1.6.1: Current Proposed mechanism of IBD Pathogenesis incorporates Environmental, Microbial flora and Genetic components. There is this genetic predisposition to the illness that combines with some form of environmental insult that then combines to create intestinal inflammation that we interpret as IBD. We can make that paradigm a little more complicated or a little more clear if we sub-segregate the Genetic component into an immune deregulatory component, and an epithelial barrier dysfunction component. Similarly, the environmental half of that paradigm can be further sub-classified into the gut microbiome component and various miscellaneous factors that affect the prognosis of these diseases such as smoking. In some fashion genetic defects occur in either immune dysregulation or epithelial barrier dysfunction that then interact with luminal agents such as the gut microbiome or other factors that then result in intestinal inflammation.

1.6.2 Environmental Factors:

The prevalence of IBD has steadily increased in the latter half of the 20th century. Immigration studies have provided some clues towards an environmental cause in that individuals who migrate from low risk geographic areas to higher risk areas acquire an increased risk for developing IBD as that of the native population. (Binder 2004, Probert CS, 1992.Carr I, 1999. Montgomery SM, 1999) IBD tends to be more prevalent in industrialized, urban areas and amongst higher socioeconomic cohorts. (Mahmud N, 2001, Hou JK, 2009 Ahmad T. 2001) This has given rise to the 'hygiene hypothesis' that suggests the lack of exposure of the immune system to normal commensal antigens, including microbes, early in life leads to a lack of tolerance and thus an exaggerated immune response later in life.

Of the environmental factors, tobacco smoking is the strongest IBD associated risk factor. Patients who actively smoke are at an increased risk for CD while patients who are ex-smokers or have never smoked are at a greater risk for UC.[Abraham C, 2009] Active smoking has repeatedly been shown to be associated with more aggressive disease course, a higher rate of clinical recurrence and the need for surgery in CD patients. [Bernstein C, 2006. Cottone M 1994. Sutherland L 1990. Reese G 2008. Ahmed T 2011] Other factors such as the use of oral contraceptives [Godet PG 1995], various dietary factors,[de Hertogh G 2008] and vaccination have shown conflicting results and are inconclusive.

1.6.3 The Gut Microbiome

The commensal microflora within the gut play an important role in maturation of the immune system, assisting in metabolizing nutrients, and in maintaining overall gut homeostasis. Several studies have demonstrated that certain gut commensals possess anti-inflammatory properties such as the ability to regulate CD4+ T lymphocyte differentiation and function.[Ivanov II, 2008, Mazmanian SK, 2008] The diversity of the resident microflora is kept relatively stable in the normal healthy individual and are found in the greatest concentration ($>10^{12}$) in the distal ileum and colon which can partly be the reason for CD affecting these areas most commonly.[Ahmed T 2011] IBD patients however, have an imbalance in the composition of the microbiota within the gut, known as a *dysbiosis*, that leads to a disinhibited host immune response and the development of inflammation.[Cerf-Bensussan N, 2010] One of the best examples of such alterations in the gut flora is seen in the setting of the pseudomembranous colitis caused by an inappropriate overgrowth of *Clostridium difficile* secondary to overuse of broad spectrum antibiotics. Further clinical evidence of the role of bacteria in the development of IBD comes from the observation that diverting the fecal stream and thus the bacterial load away from the inflamed intestinal segment will lead to considerable reduction of the inflammation and improvement of symptoms especially if diseased bowel has been resected. However, disease tends to recur rapidly after restoration of GI continuity.[Rutgeerts P 1991] Some patients with IBD can have symptoms ameliorated with a course of antibiotics. Probiotic therapy has been shown to be of benefit in small studies of IBD patients, a finding much more consistent in well controlled animal studies.[Heilpern D, 2008]

There is strong evidence suggesting that there is a change in the symbiotic relationship of the gut flora and the host in the setting of IBD.[Baker PI, 2009] IBD patients have a 30-50% overall

reduction in the diversity of enteric bacteria.[Ahmed T 2011] Increased numbers of the *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of Johne's disease in cattle, a condition very similar to CD in humans, has been shown within the mucosa in CD patients when compared to healthy controls. [Baker PI, 2009, Friswell M, 2010] The LF82 strain of 'adhesive and invasive' *Escherichia coli* (AIEC) has also been shown to be present in markedly increased numbers within the mucosa of CD patients. This particular strain, as the name suggests, has the ability to adhere to and disrupt the intestinal barrier by the production of an alpha haemolysin. It is able to replicate within macrophages and is associated with granuloma formation and increased release of pro-inflammatory cytokines such as IL-8 and TNF-alpha. [Friswell M, 2010] The bacterium *Faecalibacterium prausnitzii* belonging to the Firmicutes phylum is a common gut commensal that has been shown to possess anti-inflammatory properties by way of secreting active metabolites that block nuclear-factor-kappa-B (NF-κB) activation and reduce IL8 secretion in murine models, as well as secreting increased anti-inflammatory cytokine IL10 and decreased IL12 levels in blood monocytes. Very low levels of *F. prausnitzii* have been strongly associated with ileal CD.[Friswell M, 2010] Furthermore, reduced inflammation has been seen upon oral administration of live *F. prausnitzii* in animals with active colitis.[Ahmed T 2011]

Overall dysbiosis of the gut microbiome fails to fully explain the entire pathogenesis of IBD. Not all patient symptoms respond favorably to a course of antibiotics due to disease heterogeneity, and symptoms tend to recur upon completing their course of antibiotics. It is not uncommon for IBD patients to require other concomitant regimens such as the use of steroids, aminosalicylic acid, and even anti-tumor necrosis factor alpha (TNF-α) medications.

1.6.4 Genetic Role:

A positive family history of IBD has been shown to increase an individual's overall risk of having the disease.[Grant SF 2008] The risk of developing IBD in first-degree relatives in Caucasian non-Jewish individuals has been estimated to be 5% for CD and 1.6% for UC. [Grant SF 2008] In contrast, the risk for developing IBD within the Jewish population increases to 8% and 5.2% respectively.[Grant SF 2008] However, patients with two family members with the disease have an approximately 30% risk for developing the disease themselves.[Grant SF 2008. Ahmad T, 2001. Orholm M, 1991] Therefore the relative risk for a sibling to develop IBD is approximately 30-40 for CD and 10-20 for UC when compared to the general population. [Grant SF 2008] In 75% of families only single types of colitis (CD only or UC only) runs within

them while in the remaining 25% of families have a mixed pedigree i.e. having both CD and UC affected members.[Binder V. 1998, Cho JH 2007, R.K. Russell, 2008] Such 'IBD families' are generally concordant for age of onset, location and behavior thus further strengthening the argument for the genetic basis of IBD pathogenesis. [Peeters M, 1996, Colombel J-F, 1996, Satsangi J, 1996, Bayless TM, 1996, Ahmad T, 2001]

The strongest evidence for the genetic basis for IBD however is provided by twin studies. Several studies have shown a strong disease concordance for both CD and UC in monozygotic twins when compared with dizygotic twins.[Grant SF 2008] There is an overall 37% concordance rate in monozygotic twins which changes to approximately 7% in dizygotic twins.[Lees CW, 2009, Halfvarson J, 2003, Orholm M 2000, Thompson NP 1996] Orholm*et al* showed disease concordance for CD to be as high as 58% in monozygotic twins and 18% in monozygotic UC twins, while for dizygotic twins the rate are 0% and 4.5% for CD and UC respectively.[Orholm M 2000] Furthermore, the majority of studies pertaining to monozygotic twins have shown observed concordance for the same type of IBD.[Grant SF 2008, Orholm M, 2000, Thompson NP 1996, Tysk 1988. Halfvarson J, 2003] However there are rare instances where this is not been borne out therefore suggesting that environmental factors may play a role in disease expression.[Grant SF 2008] Such familial association provides strong evidence for a genetic component playing a strong role in disease pathogenesis.

1.7

Overview of IBD Genotyping Methodology

Similar to most other immune mediated diseases such as multiple sclerosis, systemic lupus erythematosus, type 1 diabetes mellitus and rheumatoid arthritis, IBD is a 'complex multigenic' disease that does not follow a 'one gene, one disease' Mendelian pattern of inheritance. These disorders are difficult to classify due to variations in severity of symptoms, clinical features, variable prognosis and age of onset. 134(Holly K. 2002) Such complex diseases tend to involve an unknown number of multiple genes that affect various biological pathways and usually interact with a variable number of environmental factors.[Holly K. 2002, Motulsky AG. 2006, Schork NJ 1997] This wide genetic variation presumably results in the diverse clinical phenotype that is seen in IBD. (Figure1.7.1)

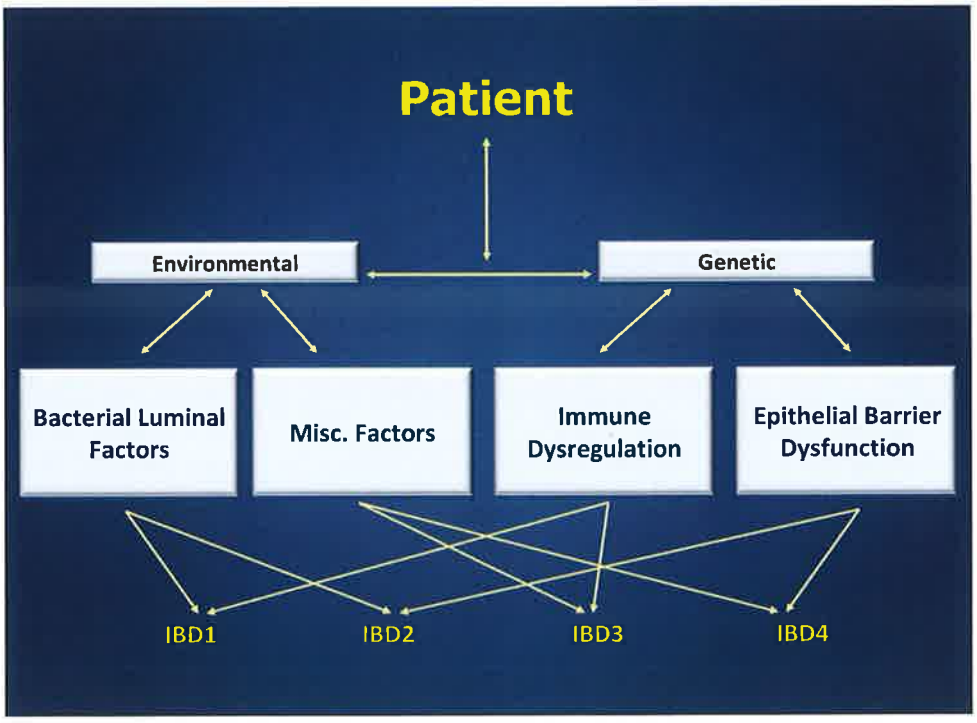

Figure 1.7.1: Complex diseases such as IBD have a variable phenotype owning to various combinations of environmental and genetic factors.

Several strategies have been used to identify and characterize the genes that are associated with IBD. Earlier methods, including linkage analysis and candidate gene studies showed

promise however were limited largely due to the relatively small patient numbers in studied cohorts and often leading to false-positive reports.(Hirschhorn JN, 2002. Xavier 2008) Such linkage studies also only identified notably large areas of the human genome associated with IBD, typically containing numerous genes. Ever more fine mapping using relatively tedious microsatellite markers was used to identify the NOD2/CARD15 gene in 2001 as being associated with CD, but the field stalled for several years due to these relatively tedious mapping techniques. These limitations of mapping were soon overcome by technological advancements that led to the development of high throughput commercially available genotyping microarray platforms (discussed in section 1.9) facilitated by the completion of the Human Genome Project in 2003 and International Haplotype Map (HapMap) Project (http://www.hapmap.org/) in 2005.(International HapMap Consortium 2005) These projects provided an open access database to more than four million of the most abundant and validated genetic variants (known as single nucleotide polymorphisms, SNPs) seen in the human genome across several populations including those from Nigeria (Yoruba), Japan, China and the United States (which included people of European ancestry).(Manolio TA 2008)Each SNP is assigned an identification number called a reference SNP (rs) number and cataloged in the National Center for Biotechnology Information's dbSNP database.(National Center for Biotechnology Information, National Library of Medicine. Database of Single Nucleotide Polymorphisms. http://www.ncbi.nlm.nih.gov/sites/entrez)

Currently the dbSNP database totals 12 million SNPs out of which up to 9 million have been annotated as validated (validation criteria are available online at http://www.ncbi.nlm.nih.gov/projects/SNP/snp_legend.cgi?legend=validation).(Espen 2009.)
This resource permitted companies to develop novel genotyping arrays that allowed researchers to study over a million SNPs in as little as 72 hours using automated equipment (see genotyping chapter). This rejuvenated interest in identifying the possible etiology of IBD by introducing the concept of genome wide association studies (GWAS) that attempt to identify genetic variants associated with disease by studying large populations of IBD patients compared to healthy controls without having any prior knowledge of gene function and have led to a myriad of studies of genetic association in IBD and other diseases. (Budarf ML, 2009. Xavier 2008. Ishihara S 2009.)

1.7.1 Genetic Linkage Studies

Genetic linkage mapping approaches were the first methods used to identify relatively large regions of the genome that most likely contained genes that predisposed to IBD. Linkage analysis is performed by studying defined areas along the genome that possibly have disease associated variants and statistically evaluating how these areas segregate (to affected or unaffected individuals) in multigenerational families.[Cho JH 2007. Dawn Teare M2005. Schork NJ 1997)]

Recombination (or 'shuffling') of genetic material occurs during the process of meiosis. If two genes are within close vicinity to each other on a chromosome, then they have a small chance to recombine or become separated by DNA fractures and cross over to the homologous strand of DNA. In contrast, two genes that are positioned far apart on the chromosome have a greater chance to recombine. Two genetic loci are said to be *linked* if they are transmitted together during meiosis from parent to offspring more often than is expected under independent inheritance. [Dawn Teare M, 2005. Cho JH 2007]If such linkage is observed across a population, the term *linkage disequilibrium (LD)* is used and reflects the association of that part of the genome with the studied phenotype or clinical characteristic.[Dawn Teare M, 2005)]

Linkage between two genomic sites is usually reported as a logarithm of the odds (LOD) score. In general, a large positive score correlates with linkage and a low or negative score is evidence against. [Dawn Teare M, 2005.] Landers and Kruglyak developed specific LOD criteria for interpreting and reporting linkage results. Areas of 'suggestive linkage' correspond to a LOD score of 2.2 with a P-values of less than 7×10^{-4}, areas of 'significant linkage' correspond to a LOD score of 3.6 and P-values of less than 2×10^{-5}, and areas of 'highly significant linkage' have a LOD score of 5.4 with P-values of less than 3×10^{-7}. Areas are said to have 'confirmed linkage' if significant linkage has been replicated in an additional larger cohort, preferably by an independent group of investigators with a nominal P value of 0.01.[Lander E, 1995)]

Early work in linkage analysis revealed several genetic loci (termed IBD1 through IBD9) within several chromosomes to be linked (Table 2 and Figure 3) and having an association with CD only, UC only or both. These initial studies that were conducted in the early 1990s have been replicated since in much larger cohorts.[148-150(Gaya DR, 2006. van Heel DA, 2004. Cavanaugh J

2001.)The landmark discovery of the first IBD susceptibility gene, *NOD2/CARD15* (found in locus *IBD1*) on chromosome 16q12 in 1996 is one of the best examples of applying linkage analysis with positional cloning techniques.[151](Hugot JP, 1996)

Table 1.7.1. IBD linkage regions

Linkage region	Chromosome	Linked Disease	Gene association	References
IBD1	16q12	CD	NOD2/CARD15	Hugot JP, 1996.Hugot J, 2001. Ogura Y, 2001.Hampe J, 1999.Williams CN, 2002.Paavola-Sakki P, 2003.
IBD2	12p13.2 – q24.1	UC > CD		Satsangi J, 1996. Hampe J, 1999.Ma Y, 1999. Paavola-Sakki P, 2003.Barmada MM, 2004.Parkes M, 2000.
IBD3	6p	CD, UC	HLA class II	Hampe J, 1999.Dechairo B 2001.Rioux JD, 2000. Williams CN 2002.Barmada MM 2004. van Heel DA, 2004.
IBD4	14q11–q12	CD		Ma Y, 1999.Duerr RH, 2000.Vermeire S, 2004
IBD5	5q31	CD	OCTN1, OCTN2	Ma Y, 1999.Peltekova V, 2004.Peltekova V, 2004.Williams CN, 2002.
IBD6	19p13	CD, UC		Rioux JD, 2000.Paavola-Sakki P, 2003.
IBD7	1p36	CD, UC		Cho JH, 1998.
IBD8	16p	CD		Hampe J, 2002.
IBD9	3p26	CD, UC		Satsangi J, 1996.Rioux JD, 2000.

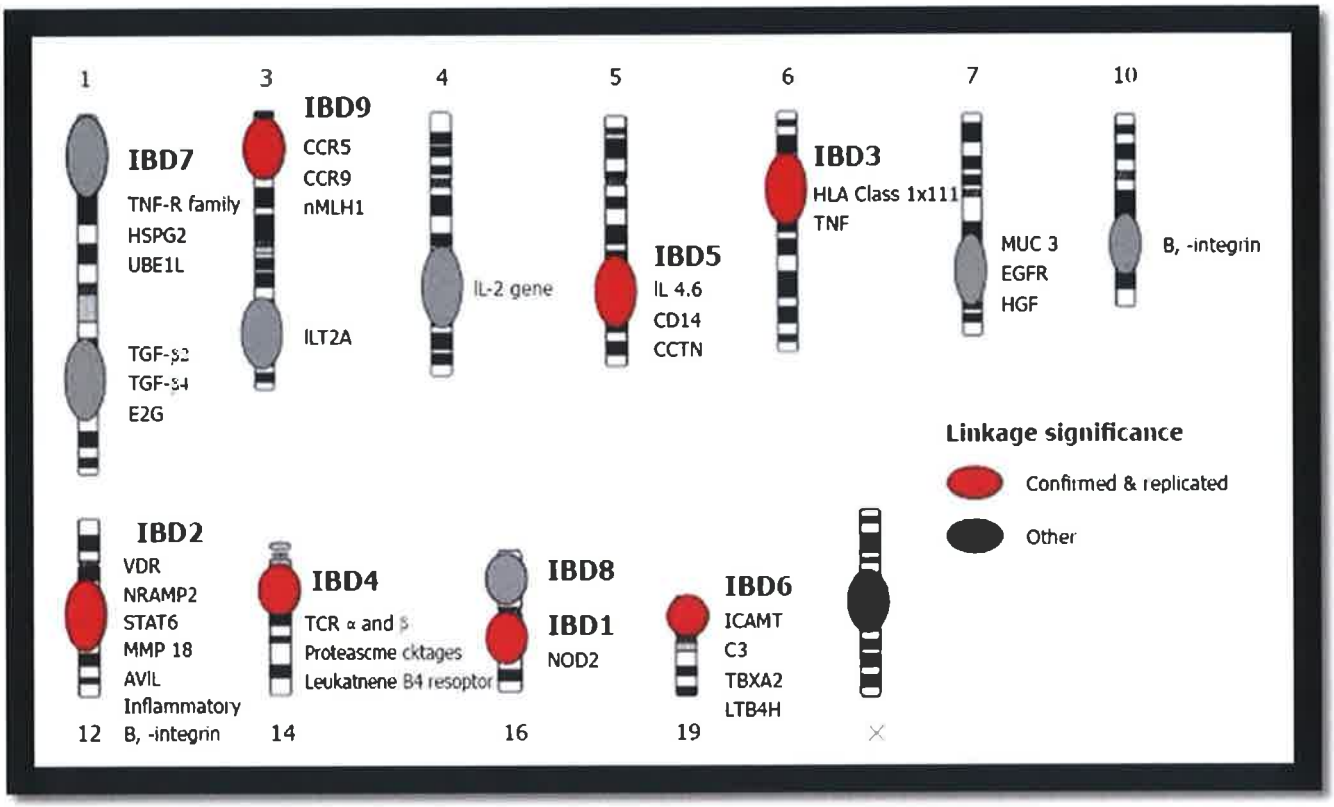

Figure 1.7.2: IBD susceptibility loci confirmed by non-parametric linkage analysis. (Van Limbergen J 2009. Colin Noble, 2006)

1.7.2 Candidate Gene Studies

In contrast to Linkage Analysis, the Candidate gene approach focuses on studying specific genes that are thought to mechanistically play an important role in key inflammatory pathways associated with disease pathogenesis. Such candidate genes should have been shown to reside within areas of linkage that are associated with disease and/or are selected based upon experimental animal models that suggest a role in IBD.[Cho JH. 2007] These hypothesis driven studies are population based and are conducted as case-control studies, comparing diseased cohorts with healthy controls. This is advantageous as such studies provide insight into gene function and potentially identify proteins responsible for disease pathogenesis and may provide targets for novel drug development. Furthermore candidate gene studies involve larger study samples that aid in statistical correlation between specific genetic variants and the disease phenotype.[Holly K. 2002] However, concerns regarding false positive reports, failure of data replication and poor choice of candidate genes due to inadequate experimental justifications are some of the criticisms of this approach.[Holly K. 2002]

The highly polymorphic human leucocyte antigen (HLA) complex on chromosome 6p21.3 (IBD3) is one of the most consistently linked regions associated with IBD. Candidate gene studies have been used to identify many genetic regions within the HLA region and help provide further insight into disease pathogenesis. [Ahmad T, 2006. Ishihara S, 2009]

1.7.3 Genome Wide Association Studies (GWAS)

The limitations associated with linkage analysis and candidate gene studies were overcome by (1) advancements made in microarray-based technologies that allowed for the testing of millions of SNPs in a very affordable single genotyping reaction and (2) the completion of the Human Genome Project in 2003 and the International Haplotype Map (HapMap) Project in 2005. These two resources provided researchers with powerful tools for identifying disease susceptibility genes by scanning large populations, either as case-control cohorts or in families, for millions of SNPs located throughout the human genome. By comparing genetic variants between diseased and non-diseased subgroups on such a grand scale, it was possible to detect genetic variations that correlated with the disease process and phenotype in question. Unlike candidate gene studies, GWAS does not require any previous knowledge about particular disease associated genes or mechanisms behind the phenotype and is thus much more suited for studying complex diseases.

Performing genotyping in such a large sample size requires a multi-step study design that incorporates stringent quality control and replication along the way in order to ensure the validity of the results. (Xavier RJ 2008.) GWA scans typically begin by scanning an initial group of individuals with the disease or trait of interest and an appropriate number of non-diseased matched controls for comparison. This is known as the discovery set. Before any kind of replication study can be undertaken, statistical thresholds are set to ensure strong association between the SNPs and the disease. The next step is to use the identified SNPs from the first stage and replicate them in a second and even third independent case-control group, undergoing further quality checks thus further narrowing down the most promising SNPs in each run. Those SNPs that are significantly detected on both the discovery set and replication sets are felt to have a true association with disease. (Xavier RJ 2008. Pearson TA2008. Witte JS. 2010)

To date there have been several GWA studies that have been conducted specifically for IBD (Table 1.7.2). A detailed discussion about each study is beyond the scope of this review. One study that warrants discussion however is the GWAS that was performed by The Wellcome Trust Case Control Consortium (WTCCC) (Wellcome Trust Case–Control Consortium 2007). The WTCCC is a group of 50 research groups across the United Kingdom which was established in 2005. By utilizing the Affymetrix 500K GeneChip Mapping Array Set they examined 14,000 individuals each for 7 common diseases (2000 individuals per disease) which included bipolar disorder, coronary artery disease, CD, hypertension, rheumatoid arthritis, type 1

diabetes, and type 2 diabetes. Control groups included ~3000 healthy individuals. By using the multi-step process as laid out above and a threshold of significance of $P<5x10^{-7}$, a total of 9 novel genetic risk factors were identified for CD which was more than the total number of IBD associated genes already known at the time and provided great insight into various pathways involved in disease pathogenesis. (Xavier RJ 2008) These results have been highly replicated since. The WTCCC study provided strong validation of the GWAS approach.

So far, GWA studies have identified approximately 80 SNPs in 55 genetic loci to be associated with CD and/or UC. Many (but not all) of these SNPs have had the genes within which they reside identified and then their IBD association confirmed by separate independent study of large groups of patients. However not all SNPs have been confirmed and since many such studies are done on patients from only a certain region or ethic or national background, what may be a disease-related SNP in one study may not be such in another. This large number of SNPs and genes nonetheless seems in a general way to relate to various categorical functions of the GI tract (Figure 1.7.3 and 1.7.4). *NOD2/CARD15*, *TLR4* and *CARD9* and the autophagy genes (*ATG16L1*, *IRGM*) relate to innate immunity against enteric commensal organisms. Transcription factors (*STAT3*, *NKX2-3*), HLA-region, *TNFSF15/TL1A*, and cytokine receptors (*IL23R*) are critical players in acquired immunity. *OCTN1* and *DLG5* promote normal function and integrity of a healthy gut mucosa. Thus these SNPs and their genes 'make sense' from the standpoint of pathophysiology of IBD and work is presently proceeding to identify the specific mechanisms that result in intestinal inflammation when one or more of these mutations are present in any one individual. What follows below is a discussion of some of the key genes and inflammatory pathways that have been implicated in IBD pathogenesis.

Table 1.7.2 . Genome-wide Association Studies in Inflammatory Bowel Disease (Lees CW, Satsangi J. 2010)

Study (year)	Country	Platform/SNP (n)	Discovery set	Replication set
Yamazaki *et al.* (2005)	Japan	73k	94 CD; 752 HC	484 CD
Duerr *et al.* (2006)	North America	Illumina® 300k	567 CD; 571 HC	401 CD; 433 HC; 833 families
Hampe *et al.* (2007)	Germany	Nonsynonymous SNP 20k	735 CD; 368 HC	498 CD; 1032 HC; 380 families
Franke *et al.* (2007)	Germany	Affymetrix™ 100k	393 CD; 399 HC	942 CD; 1082 HC; 375 trios
Raelson *et al.* (2007)	Quebec founder population; Germany	Perlegen® 165k	382 CD	521 trios; 752 CD; 828 HC
Liobelle *et al.* (2007)	Belgium; France	Illumina 300k	547 CD; 928 HC	1266 CD; 559 HC
WTCCC/Parkes *et al.* (2007)	UK	Affymetrix 500k	1748 CD; 2938 HC	1182 CD; 2024 HC
Barrett *et al.* (2008)	UK/North America/France/Belgium	Meta-analysis	3230 CD; 4829 HC	2325 CD; 1809 HC; 1339 trios
Fisher *et al.* (2008)	UK	Nonsynonymous SNP 14k	905 UC; 1465 HC	2082 UC; 3029 HC
Franke *et al.* (2008)	Germany	Affymetrix 500k	1167 UC; 777 HC	1855 UC; 3091 HC
Kugathasan *et al.* (2008)	North America; Italy	Illumina 550k	1011 IBD; 4250 HC	173 IBD; 3481 HC
Barrett et al (2009)	UK	Affymetrix 6	2361 UC; 5417 HC	2321 UC; 4818 HC
Silverberg *et al.* (2009)	North America	Illumina 550k and 300k	1052 UC; 2571 HC	1405 UC; 1115 HC
Asano et al (2009)	Japan	Illumina 550v3 Affymetrix 10k	752 UC; 2062 HC	265 UC; 665 HC
Imielinski et al (2009)	Europe/ North America	Illumina 550k	1636 CD; 724 UC; 53 IBD-U; 6158 HC	R1: 289 CD; 120 UC; 73 IBD-U; 1696 HC and R2: 531 CD; 4109 HC.
McGovern et al (2010)	Europe	Illumina 550k	2693 UC; 6791 HC	2009 UC; 1580 HC
Franke et al (2010)	Germany	Affymetrix 6	1043 UC; 1703 HC	2539 UC; 5428 HC

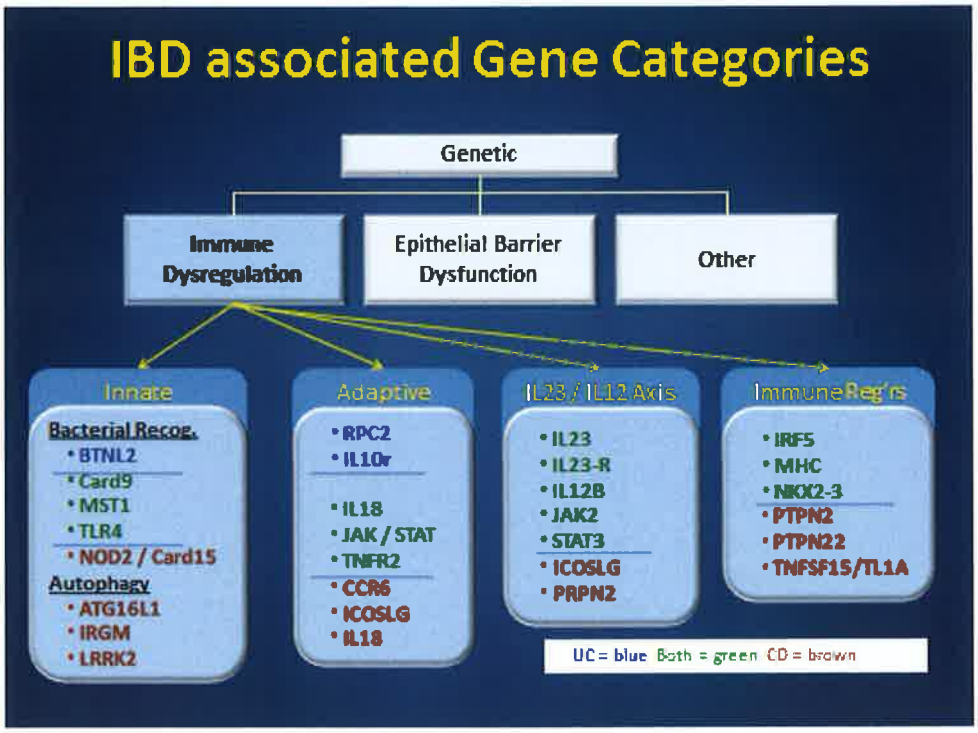

Figure 1.7.3: Genes associated with IBD can be subcategorized into several functional categories.

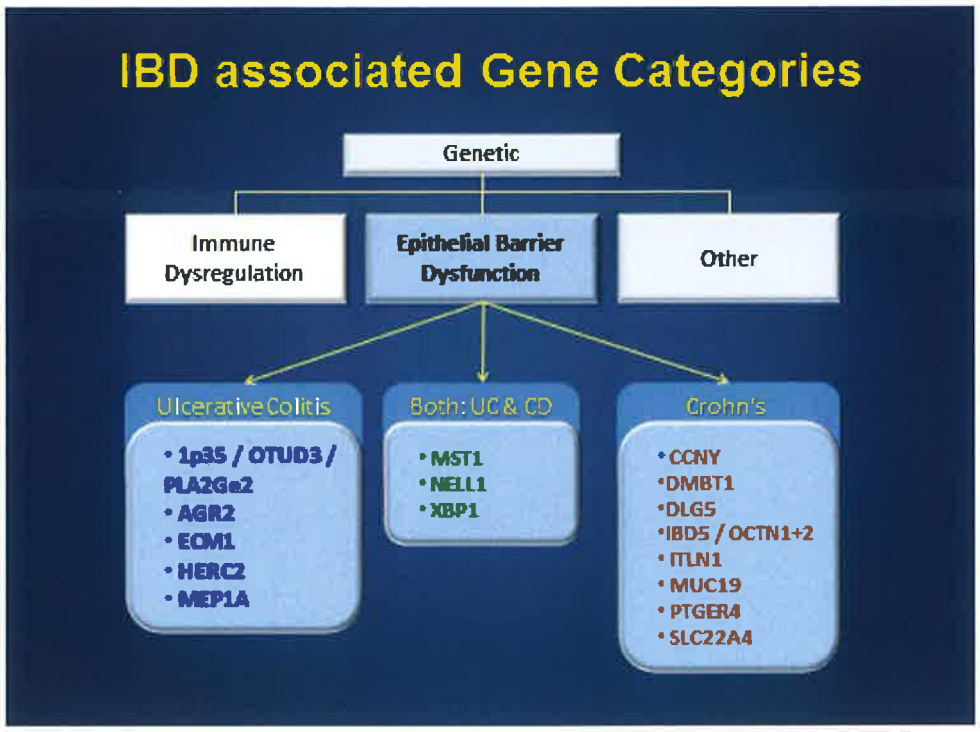

Figure 1.7.4. Genes associated with IBD can be subcategorized into several functional categories.

1.8

Genetics of Inflammatory Bowel Disease

1.8.1 INNATE IMMUNE SYSTEM: NOD2/AUTOPHAGY/TLR

1.8.1.1 IBD1: NOD2/CARD15

1.8.1.1.1 NOD2 Discovery

Hugot *et al* were the first to identify the pericentromeric region on chromosome 16 to be associated with CD by linkage analysis in 1996. [Hugot JP, 1996] This linkage association was initially not fully replicated by several other groups due to discrepancies in the number of affected family members studied, mixed ethnicities, and genetic markers used. [Cavanaugh J 2001.Ohmen JD 1996.Parkes M 1996, Mirza MM 1998, Brant SR 1998, Hugot J-P 1996, Rioux JD 1998,Vermeire S 2000] However, these discrepancies were laid to rest when in 2001 The IBD International Genetics Consortium [Cavanaugh J 2001] conducted a multicenter linkage trial involving 12 centers spanning three continents with a total of 613 white nuclear families that had greater than two affected sibling pairs with IBD and both parents available for genotyping. By using specific LOD criteria for interpreting and reporting linkage results laid out by Landers and Kruglyak, [Kruglyak L 1996.Lander E1995] chromosome 16 was shown to be in strong linkage overall with a LOD score of 4.96. When the families were segregated for CD only, a LOD score of 5.79 was observed. No such association was observed in the UC or mixed families. Thus, this study confirmed chromosome 16 to be associated specifically with CD. This study was soon followed by three landmark studies, two of which were published in the same issue of *Nature* conducted by Hugot *et al.* [Hugot JP 2001] and Ogura *et al.* [Ogura Y 2001] followed by a report a month later in *The Lancet* by Hampe J *et al.* [Hampe J 2001] that identified several CD susceptibility SNPs within the *NOD2/CARD15* gene on chromosome 16q12 by using positional-cloning strategies, based on linkage analysis followed by fine mapping technique.

1.8.1.1.2 *NOD2 Structure*

Within the gut, *NOD2* is expressed in several cell types such as monocytes, dendritic cells, and intestinal epithelial cells however is highly concentrated within Paneth cells of the ileum which is also the most commonly affected site in CD.[Inohara, 2005. Rosenstiel P 2003.Yuan Q 2004.Gutierrez O 2002] The structure of this cytoplasmic protein is comprised of three domains, an amino (N) terminus which contains caspase activating recruitment domains (CARD) that attach to a central nucleotide-binding domain (NBD) which facilitates self-oligomerization during activation and a carboxy- (C) terminus that contains leucine rich repeats (LRR) which is the main site of bacterial recognition (Figure 1.8.1). [(Ogura Y, 2001. Creagh EM 2006.Martinon F 2005)]

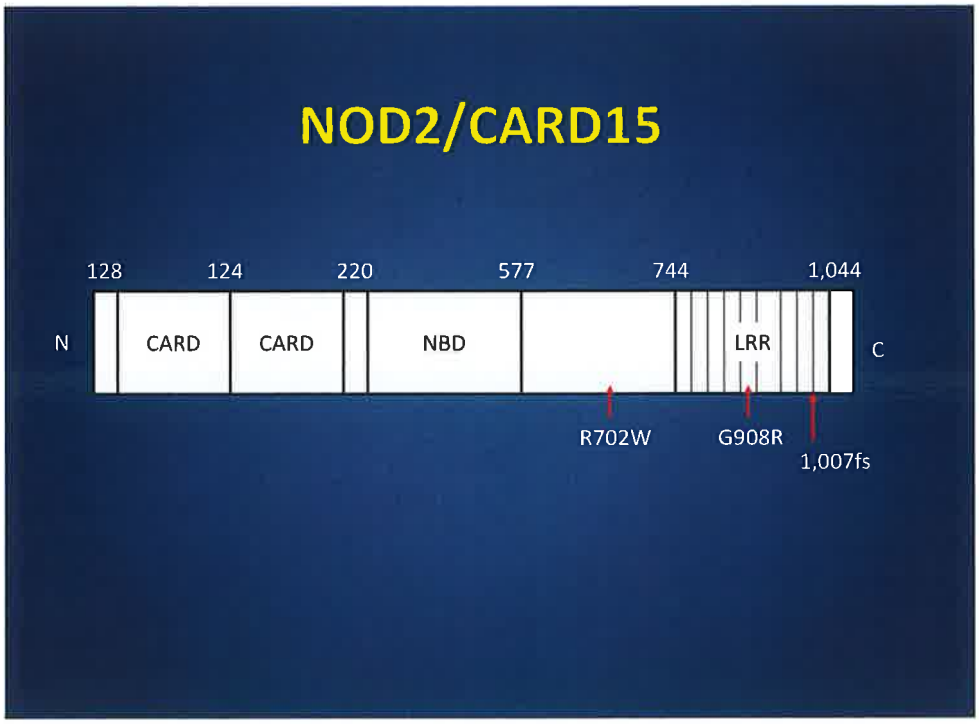

Figure 1.8.1: NOD2 cytoplasmic protein consists of a tripartite structure: 1) N-terminal CARD domains, a central nucleotide-binding domain (NBD) and a C-domain with multiple leucine rich domains (LRR). The 3 most common NOD2 polymorphisms (R702W and G908R are point mutations and L1007fsinsC leads to a frame-shift mutation) lie in and /or in close proximity to the LRR region shown.

1.8.1.1.3 *NOD2 Signaling Pathway*

The protein product of this gene acts as a sensor for bacterial cell wall component N-acetyl muramyl dipeptide (MDP, also known as MurNAc-L-Ala-D-isoGln) which is found on both Gram positive and Gram negative bacteria.[Meylan E 2006,Kanneganti TD 2007,Coulombe F2009,Bonen DK 2003, Chamaillard M 2003, Girardin SE 2003,Inohara N 2003] Once the LRR region is stimulated, NOD2 undergoes conformational changes and self-oligomerization that leads to the exposure of CARD domains which permits the activation and binding of the adaptor protein receptor interacting protein-2 (RIP2, also known as RICK or CARDIAK), a serine threonine kinase that binds with complimentary CARD-CARD regions.[Ogura et al., 2001,Lecat A 2010,Ting JP 2010,Inohara N2000, Abbott DW2004] The NOD2-RIP2 complex then activates the IκB kinase (IKK) complex through Lys^{63}-linked polyubiquitination of its main regulatory gamma subunit (IKK γ , also known as NEMO) [Carneiro LA 2007, Lecat A 2010. Ting JP, 2010] This is followed by the recruitment of the TAK1 complex which phosphorylates the IKKβ subunit. [Lecat A2010.] The phosphorylated IKKβ subunit leads to the release and activation of transcription factor Nuclear Factor-kappa-B (NF-κB, which consists of p50/RelA and p52/RelB dimers) that translocate into the nucleus and act on target genes. [Lecat A, 2010.] NF-κB plays a key role in the orchestration of the inflammatory and immune response and is involved in regulating genes responsible for producing several proinflammatory cytokines such as IL-1β, IL-8, IL-6, TNF-α and IL-12, adhesion molecules, chemokines, and growth factors. It also maintains normal gut homeostasis by expressing antimicrobial peptides such as α/β-defensins. [Spehlmann ME 2009,Wehkamp 2005]

1.8.1.1.4 *NOD2 Clinical Correlation*

The three most common CD susceptibility variants found within the *NOD2* gene lie within or in close proximity to the carboxy terminus LRR region (Figure 1.8.1). The two missense mutations (rs2066845/SNP12/G908R and rs2066844/SNP8/R702W) and one insertion mutation (rs2066847/SNP13/3020insC) that leads to a frameshift substitution at Leu1007 causing a premature stop codon of the LRR protein are associated with aberrant bacterial sensing ability and decreased activation of NFκB and inflammatory cytokines.[Bonen 2003.Chamaillard 2003.Yuan Q 2004.Lees CW 2009.] These three common mutations have been found in up to 40% of European and North American CD patients when compared with 10% to 15% of the healthy population. [Rogler G.2010] Patients carrying one risk conferring allele have a 2.39 fold (95% CI: 2.76–3.59) increased chance of developing CD. However the risk increases 17 fold (95% CI: 10.7–27.2) when two risk conferring alleles are present. [Economou M 2004.] Increasing numbers of these *NOD2* mutations have been shown to be correlated with ileal involvement, early age of onset, and stricturing and/or penetrating phenotype.[Abreu MT 2002.Cuthbert AP 2002.Hampe J 2002. Lesage S 2002. Radlmayr M 2002.]

NOD2 has been shown to be highly expressed within Paneth cells that are in the base of the crypts of Lieberkuhn of the small intestine and have been implicated in playing a role in degranulation of antimicrobial peptides such as cryptidins and α-defensins HD5 and HD6 [Ouellette AJ 2006. Simms LA 2008. Wehkamp J. 2005] and in regulating host-microbe homeostasis. [Yuan Q 2004]Several studies have shown NOD2's involvement against gut pathogens such as *Listeria, Pneumococci, Mycobacterium tuberculosis* and *Helicobacter pylori* most likely owning to this antimicrobial property.[Hisamatsu 2003.Till 2008.Billmann-Born S 2010] *NOD2* has also been shown to promote the process of autophagy mediated endocytosis and is involved in the modulation of Toll-like receptor (TLR) signaling.[Brain O 2010. Watanabe T 2004. Watanabe T 2005]

1.8.1.2 *Toll-like Receptors*

Similar to the NOD-like receptors (NLRs), Toll-like receptors (TLRs) play a key role in innate immunity by constantly sampling a variety of highly conserved molecular patterns (pattern recognition receptors, PRRs) of microbial pathogens in order to differentiate between 'self' and 'non-self'.[Cario E. 2010. Testro AG, 2009] There are a total of 13 different types of mammalian TLRs of which 10 (TLR 1-10) are found in humans. They all have an internal cytoplasmic component that has a highly conserved region which is similar in structure to the interleukin-1 receptor and thus known as the Toll/IL-1 receptor (T1R) domain [Testro AG, 2009]; a central transmembrane protein, and an extracellular component which consists of several variable leucine-rich repeats that constantly scan their environment for specific pathogen associated molecular patterns (PAMPs). [Cario E. 2010.] The various TLR subtypes in humans are programmed to detect specific PAMPs. For example, TLR2 detects bacterial lipoproteins, lipoteichoic acid, peptidoglycan and zymosan; TLR3 is specific for sensing double strand RNA, TLR4 and TLR5 are mainly responsible for detecting lipopolysaccharide (LPS, an outer membrane component of Gram-negative bacteria) and flagellin respectively.[Rhee SH 2011.] Once the intracellular T1R domain is activated, downstream signaling takes place mainly via the adapter protein myeloid differentiation factor 88 (MyD88) dependent pathway (TLR1, 2, 4, 5, 6, 7, 8 and 9) and the subsequent production of several proinflammatory cytokines. TLR3 on the other hand activates an alternative 'MyD88-independent' pathway and results in the production of type 1 interferons.[Levin A, Shibolet O 2008. Testro AG, 2009.] Mutations within intestinal TLRs have been shown to be associated with IBD however their function still remains to be fully deciphered.

TLR4 warrants special mention as it is the only receptor that is capable of activating both MyD88 dependent and independent pathways (i.e. the activation of proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and type 1 interferons respectively). The TLR4 gene is located on the long arm of chromosome 9 and plays an important role in the detection of Gram-negative outer membrane LPS. Human intestinal epithelial cells (IECs) normally express TLR3 and TLR5 and only minimally express TLR2 and TLR4. Functional studies have shown IBD patients to have a significantly higher expression of TLR4 within the intestine especially when comparing inflamed colonic mucosa with non-inflamed controls.[Cario E, Podolsky DK. 2000. Szebeni B.] Polymorphisms within the TLR4 gene (Asp299Gly and Thr399Ile) and its associated coreceptor CD14 have been shown to be associated with

aberrant sensing of LPS and reduced activation of the NF-κB inflammatory pathway.[Rallabhandi P, 2006. Corr SC, 2009.] Several studies have imperfectly linked these polymorphisms with UC, CD, or both most likely due to disease heterogeneity amongst IBD patients from different locations and limited study sample size. [Franchimont D, 2004. Török HP, 2004. Brand S, 2005.] A meta-analysis performed by Browning *et al* showed that IBD patients carry a significantly higher frequency for the Asp299Gly polymorphism that causes structural alterations of the TLR4 extracellular domain, when compared to controls (odds ratio 1.36, 95% CI 1.01-1.84). [Browning BL, 2007.]Furthermore, the 1-260C>T polymorphism within the CD14 promoter has been shown to be associated with UC but not CD in a German cohort. However this finding was inconsistent amongst the Hungarian population where the converse disease association was observed.[Baumgart DC, 2007. Levin A, Shibolet O 2008.]

1.8.1.3 *AUTOPHAGY: ATG16L1, IRGM*

Autophagy (literally meaning 'self-eating') is a highly conserved process which is stimulated during times of cellular stress and produces more energy in order to remove unwanted cytoplasmic waste materials such as damaged organelles, apoptotic bodies, intracellular viruses, bacteria and parasites (xenophagy).[Fritz T2011] In general terms, this process can be divided up into three distinct steps: (1) the formation of a crescent or cup shaped isolated membrane known as 'phagophore,' which is stimulated by the help of Beclin/VPS34 that is found within several membrane systems such as the endoplasmic reticulum (ER). These membrane systems are regulated by three key pathways: (a) the inhibitory target of rapamycin (mTOR), (b) the class I phosphatidylinositol-3-kinase (PI3K) cascade, and (c) the autophagy-promoting JNK1 (cJun N-terminal kinase 1) signaling pathway; (2) the elongation step involves the extension of the phagophore which eventually fuses into a spherical double membrane shaped structure known as the 'autophagosome' within which dangerous materials are sequestered. This is followed by (3) lysosome binding with the autophagosome to form an 'autolysosome' leading to the contents being degraded by acidic lysosomal hydrolases (Figure 1.8.2).[Billmann-Born S 2010. Deretic 2011.Bao 2010.Eskelinen and Saftig 2009.Glick 2010 Baker PI 2009.Mizushima N 2007.Rubinsztein DC 2005.Cecconi and Levin 2008.]

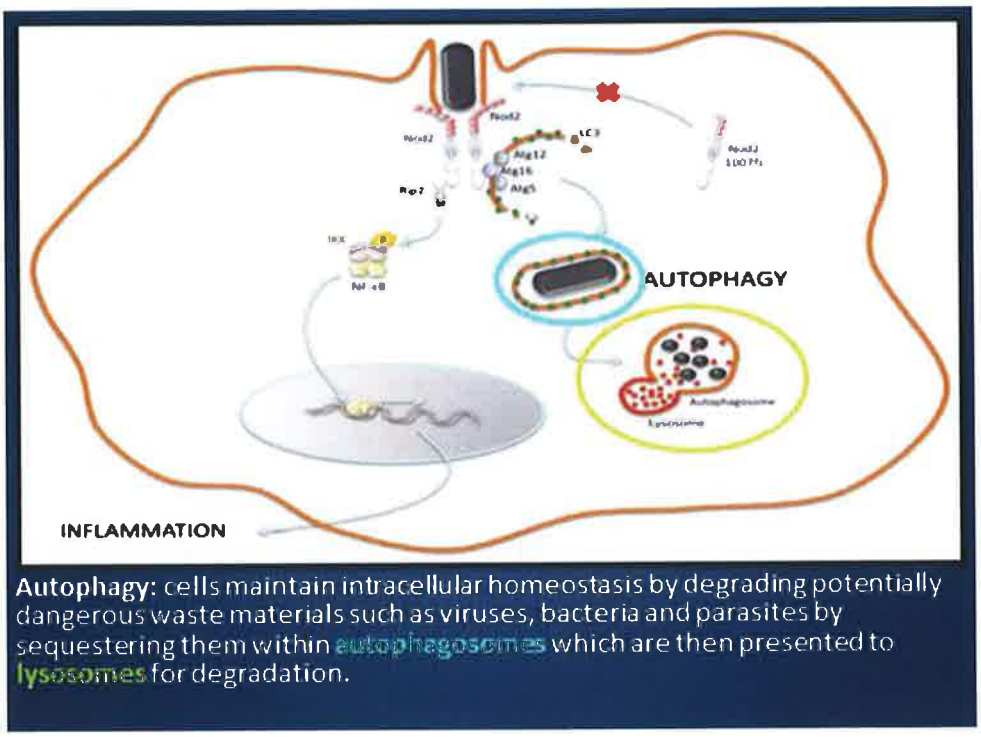

Figure 1.8.2.Autophagy overview.

Autophagy has been shown to be associated with a wide variety of conditions such as Alzheimer's disease[Lee JH 2010], Huntington's disease[Martinez-Vicente M 2010], Diabetes mellitus [Meijer AJ 2008], aging[Cuervo AM 2008] and cancer[Levine B. 2007, Deretic 2011].In 2007 several independent large GWA studies including the Wellcome Trust Case Control Consortium showed strong association of two AuTophaGy-related (ATG) genes, (1) autophagy-related 16-like 1 (ATG16L1) gene and (2) the immunity-related GTPase family M (IRGM) gene to be associated with CD susceptibility.[HampeJ 2007, Rioux JD. 2007, The Wellcome Trust Case Control Consortium. 2007.) ATG16L1 is directly involved with the basal autophagy apparatus, while IRGM is part of the immunity-related GTPases (IRGs) family which is responsible for innate immunity.[Deretic V, Levine B. 2009.)

1.8.1.3.1 *ATG16L1*

The ATG16L1 gene is found on chromosome 2q37.1 and codes for the Atg16L protein which is involved in the formation of the autophagosome. It does this by initially binding to two other autophagy proteins Atg12 and Atg5. The formation of this 800 kDa complex binds to the 'phagophore' that elongates to form a double membrane autophagosome. (Rubinsztein DC 2005. Massey DC 2007.) The human Atg16L protein consists of a central ATG16 domain in its N-terminus which is responsible for Atg5 binding and eight tryptophan-aspartic acid dipeptide (WD) repeat domains in the C-terminus whose role is as of yet still unknown (Figure 1.8.3). The ATG16L1 SNP rs2241880 within the ATG16L1 gene has most significantly been associated with CD susceptibility. This variant leads to a point mutation, replacing threonine for alanine at position 300 of the amino acid sequence (T300A).(Cheng JF, 2010. Hampe 2007.Billmann-Born S 2010) A deficiency in ATG16L1 has been shown to be associated with aberrant recruitment of the Atg5-Atg12 complex thus decreasing the ability of the cell to form autophagosomes and clear unwanted waste. 246(Hruz P 2010) Kuballa *et al* studied the role of this T300A variant in human intestinal epithelial cells (Caco-2) and showed the inability of these cells to form autophagosomes around internalized *Salmonella enteric serovar typhimurium.*(Kuballa P 2008. Hruz P 2010) Furthermore, IBD patients carrying this particular SNP are more prone to ileal CD with no association seen with UC patients.(Fowler EV 2008. Prescott NJ 2007.) Recent studies have shown that CD patients who are homozygous for the ATG16L1 risk allele (G) have Paneth cell granule abnormalities characterized by defective secretion of antimicrobial peptides therefore leading to an increased expression of acute phase reactants and cytokines such as IL-1β and Il-18 which potentiate the inflammatory response.(Cadwell K 2008. Saitoh T 2008.Márquez A, 2009.)

Recently, Cooney *et al* have shown a link between NOD2 and ATG16L1 associated susceptibility genes in a single functional pathway. In their study, the stimulation of NOD2 by MDP was shown to induce autophagy and subsequent bacterial clearing.(Cooney R, 2010.) This observation was also noted by Travassos *et al* (Travassos LH, 2010) CD patients who carried a gene variant within either the *NOD2* (3020insC) or *ATG16L1* (T300A) gene were shown to have an aberrant formation of autophagosomes, antigen presentation and thus decreased clearing of pathogens.(Netea MG, 2010)

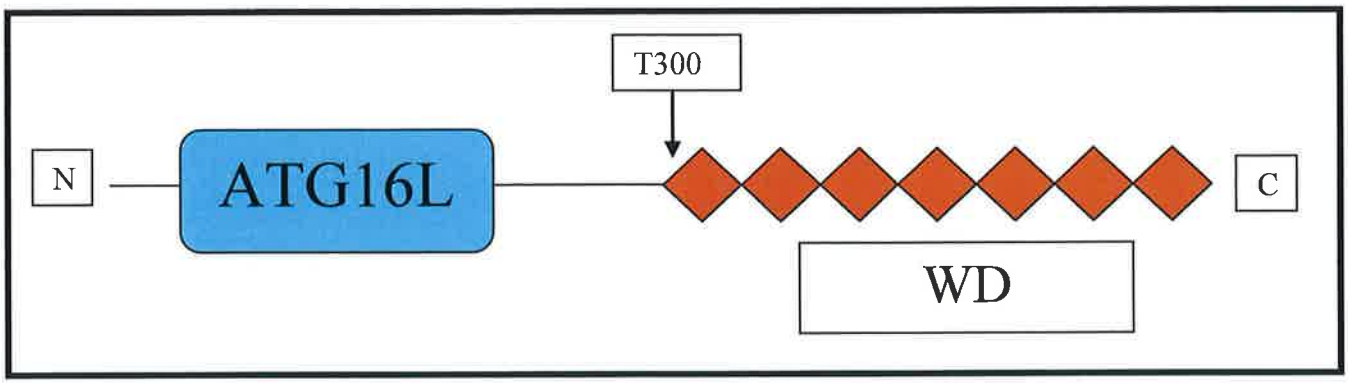

Figure 1.8.3: Structure of ATG16. The N-terminal domain of the ATG16 protein is essential for binding with ATG5 and ATG12 in order to form the autophagosome. The C-terminus contains 8 WD repeats whose function is not known. It is this area that contains the CD associated SNP (T300A).[Billmann-Born S 2010]

IRGM

The human immunity-related guanosinetriphosphatase (GTPase) gene on chromosome 5q33.1 was first shown to be associated with CD susceptibility in 2007 by the WTCCC GWA study. Although there are 23 Irg genes in the mouse that are regulated directly via interferon-γ (IFN- γ), there are only 3 IRG genes identified in humans and the functional role of any of these human IRG genes is unknown. Human IRG is not directly controlled by IFN- γ, however it has been shown to be responsible for IFN- γ induced autophagy and protecting against Mycobacterium tuberculosis in human macrophages.[Singh SB 2010. Singh SB2006.] siRNA knockdown of IRGM has shown defective autophagy and increased survival of *Toxoplasma gondii, Listeria monocytogenes* and *Mycobacterium tuberculosis*. [Singh SB 2006. 2009 Parkes M, 2007]

The initial WTCCC study reported two non-coding IRGM SNPs that most significantly correlated with CD, rs13361189 and rs4958847, however no causative mutation was shown when the IRGM exon was resequenced. Several subsequent studies have since confirmed the association of these IRGM SNPs with CD. [Huett A, 2008. Roberts RL 2008. Latiano A, 2009. Weersma RK 2009. Barrett JC 2008. Fisher SA 2008.Franke A 2008.] The SNP rs4958847 has been shown to be associated with fistulizing CD phenotype.[Latiano A, 2009.]Recently, McCarroll *et al* showed a 20-kb deletion polymorphism 1.6 kb upstream from the IRGM gene which was in perfect linkage disequilibrium with rs13361189.[McCarroll SA 2008. Lees CW 2009.]They followed up this finding by genotyping 685 North American case-controls for this polymorphism and found a 10% frequency in unaffected healthy individuals, and an elevated frequency in IBD patients. McCarroll *et al* also studied the other IRGM SNP reported in the WTCCC rs4958847 and

found only a partial correlation with rs13361189 and the upstream deletion polymorphism with CD susceptibility (P = 0.003).[McCarroll SA 2008] This probably suggests that there is an alternative mode by which these SNPs cause disease such as gene splicing and expression, or interacting with other yet to be identified SNPs found in linkage disequilibirium.[Baker PI 2009 19817674]

1.8.2 Genetic Variants Affecting Mucosal Transport and Integrity: OCTN 1, OCTN 2

The IBD5 gene found on chromosome 5q31 is the second most significant locus (after IBD1/NOD2 on chromosome 16) to be associated with CD. This locus was initially discovered in the year 2000 by Rioux *et al* when they performed a GWAS in 158 Canadian families and through higher-density mapping in the 5q31-q33 region revealed a locus of genome wide significance (LOD score 3.9) that contributed to CD susceptibility in families with early-onset disease.[Rioux JD, 2000] Peltekova *et al* identified two SNPs within the organic cation transporter cluster that are thought to be the main disease associated variants in the IBD5 gene loci. These two coding variants include (1) the solute carrier family 22, member 4 (SLC22A4) also known as an organic cation transporter 1 (OCTN1) and (2) solute carrier family 22, member 5 (SLC22A5), also known as organic cation transporter 2 (OCTN2).[Peltekova VD 2004] In the same study, Peltekova *et al* explained the functional defect that was caused by the two SNPs in OCTN1 (rs1050152) and OCTN2 (rs2631367). The SNP in OCTN1 causes a C1672T point mutation on exon 9, while the SNP in OCTN2 results in a G-207C transversion mutation at the promoter region, thus disrupting a heat shock element (HSE) upstream of the start codon.[Peltekova VD, 2004. Wang J, 2011.] Both SNPs have been shown to be in LD and the 'TC' is significantly increased in CD patients.[Peltekova VD2004] This association is compounded with the concurrent presence of NOD2/CARD15 variants with an odds ratio of 7.28-10.5. OCTN2 is responsible for transporting carnitine, a molecule responsible for transporting long chain fatty acid through the mitochondria.[Silverberg MS. 2006.] Mice that are deficient in OCTN2 have been shown to develop intestinal lymphocytic infiltration. [Silverberg MS. 2006]

Recently Wang *et al* performed a meta-analysis encompassing 26 studies to evaluate the association of the IBD5 locus to the predisposition of IBD. They confirmed that the SNPs of OCTN1/2 'TC' haplotype contribute to the susceptibility of CD in pediatric, adults and Caucasians IBD populations. The locus only contributed to the susceptibility of UC in a recessive manner in adults and Caucasian populations.[Wang J, 2011.] Further studies are warranted in order to unravel the functional effects of OCTN1/2.

1.8.3 Genetic Variants Affecting The Adaptive Immune System:IL23-Th17 Axis; TNFSF15/TLA1

1.8.3.1 Interleukin-23-Th17 axis

Duerr RH *et al* were the first to show an active role of the adaptive immune system in IBD.[Budarf ML 2009] In 2006 they performed a GWA study interrogating for 308,332 SNPs in a population consisting of 567 non-Jewish ileal CD patients compared with 571 non-Jewish healthy controls. This study identified three SNPs that were the only variants to remain significant after Bonferroni correction. Two of the three SNPs were found within the *NOD2/CARD15* gene and the third SNP rs11209026 (Arg381Gln) belonged to the IL-23 receptor (IL-23R) on chromosome 1p31.[Duerr RH 2006] Patients who are carriers of the glutamine allele are three times less likely to develop CD when compared to those carrying the arginine allele.[Duerr RH 2006] Duerr *et al* also demonstrated several other intergenic SNPs for IL23R and the adjacent IL-12 receptor beta-2 (IL12RB2) gene that were also shown to be significantly associated with non-Jewish, ileal CD.[Duerr RH 2006] The IL23R gene has since been shown to be significant in a number of other geographical CD populations including French[Libioulle C 2007], Canadian[Raelson JV 2007], Dutch[Weersma RK 2008, Weersma RK, 2008], Finnish[Lappalainen M, 2008] and British.[Cummings JR 2007, Tremelling M, 2007, Van LJ 2007, Lees CW 2009] Furthermore, this gene has been linked to other autoimmune conditions such as rheumatoid arthritis, psoriasis and anklosying spondylitis.[Shih DQ 2008]

IL23R plays an important role in the IL23/Th17 inflammatory pathway which involves several other important IBD associated genes such as STAT3, JAK2, CCR6 and TNFSF15.[Shih DQ2008. Abraham C, Cho JH 2009.Budarf ML 2009 Barrett JC 2008] It forms part of the membrane receptor for the IL23 cytokine. IL23 is made up of two subunits, the p19 subunit which is specific to IL23 and the p40 subunit that is shared with the IL-12 (IL12β) cytokine. IL23 is predominantly secreted by macrophages and dentritic cells.[Abraham C, Cho JH 2009] Stimulation of the IL23 receptor leads to the activation of the janus kinase 2 gene (JAK2) and signal transducer and activator of transcription 3 (STAT3) gene signaling pathway. JAK2 is closely related to IL23R and leads to autophosphorylation and tyrosine phosphorylation of IL23R. This subsequently leads to the recruitment, phosphorylation and homodimerization of STAT3 that translocates into the nucleus to transcribe pro- and anti-inflammatory cytokines.[Shih DQ 2008] Interestingly, Mannon *et al* recently performed a double-blind trial to evaluate the safety

and efficacy of a human monoclonal antibody against the p40 subunit of interleukin-12 (anti-interleukin-12) in 79 patients with active Crohn's disease. Patients who received anti-interleukin-12 therapy showed improvement compared to placebo group as evidenced by decreased levels of interleukin-12, interferon-gamma, and tumor necrosis factor alpha within mononuclear cells of the colonic lamina propria and an overall clinical improvement in the studied CD cohort of patients.(Mannon PJ, 2004)

The IL23 signaling pathway plays an important role in $CD4^+$ T helper cell differentiation via activation of retinoic acid-binding orphan receptor-γt (ROR- γt) signaling, and the production of IL-17A, IL-17F, IL6 and TNF-alpha cytokines.(Shih DQ2008. Abraham C, Cho JH 2009. Abraham C, Cho J 2009. Budarf ML 2009) Studies in mice have shown the development of fatal multi organ inflammation when IL23p19 is over expressed.(Wiekowski MT 2001. Lees 2009) Furthermore, Elson *et al* demonstrated an exaggerated inflammatory response in severe combined immunodeficient (SCID) mice after bacterial-reactive CD4+ Th17 cells were injected. Conversely, an improvement in active colitis as seen by the down-regulation of several inflammatory cytokines and chemokines was shown when monoclonal anti-IL-23p19 was administered in these SCID mice.(Elson CO, 2007) Other similar studies conducted in animals highlight the importance of IL23 in mediating inflammation.(Abraham C, Cho JH 2009. Hue S 2006.McGeachy MJ 2007. Yen D 2006.)In addition, increased levels of IL23 and Th17 cytokines have been demonstrated within the colonic mucosa in IBD patients.(Abraham C, Cho JH 2009.)

1.8.3.2 TL1A (TNFSF15)

Tumor necrosis factor superfamily member 15 (TNFSF15) gene also known as TNF superfamily ligand A (TL1A), is found on chromosome 9q32 and is the only gene to be associated with CD in both Asian[Yamazaki K, 2005] and Caucasian populations.[Tremelling M, 2008. Barrett JC, 2008 Picornell Y, 2007. Shih DQ 2011] TL1A is also associated with UC in British populations however not in Japanese cohort.[Yamazaki K, 2005.Kakuta Y 2006.] Haplotypes composed of 5 TL1A SNPs have been associated with an increased risk of CD (*haplotype* A) in Japanese populations however *haplotype* B is protective in British and American populations. Furthermore, *haplotype* B is associated with a more severe disease course in Jewish populations as evidenced by an increased frequency of small bowel surgery, fibrostenosis/perforation behavior, and perianal manifestations.[Michelsen KS, 2009, Shih DQ 2011.] The association of TL1A with IBD has now been confirmed by several genome wide association studies including the Wellcome Trust Case Control Consortium, NIDDK IBD Genetics Consortium and the Belgian-French IBD Consortium.[Barrett JC 2008]

TL1A is expressed in endothelial cells, lymphocytes, plasma cells, monocytes, and dendritic cells and acts as a ligand which binds to death domain receptor 3 (DR3).[Endo K, 2010] Microbial stimulation of Toll-like receptors causes an up-regulation of this complex that activates the NF-kappa B and MAP kinase pathways, thereby leading to the increased production of the antiapoptotic protein c-IAP2 and interferon gamma (IFN-γ) cytokine by human $CD4^+$T and NK cells, thus participating in the T-helper-1 (Th1) mediated immune response which is more commonly seen in CD.[Endo K, 2010. Shih DQ 2009] Several studies have shown increased expression of TL1A within the inflamed gut mucosa in CD patients[Shih DQ 2009, Endo K, 2010. Bamias G, 2003. Prehn JL, 2004. Takedatsu H, 2008.] Takedatsu *et al* have showed a significant increase in TL1A, DR3, IFN-γ and IL-17 in gut associated lymphoid tissue (GALT) after inducing colitis by using dextran sodium sulphate (DSS) in mouse models. The inflammation was shown to regress after the administration of anti-TL1A monoclonal antibodies.[Takedatsu H, 2008] In addition, Michelsen *et al* have demonstrated a synergistic effect of TL1A with IL-23 to induce IL-17 production in $CD4^+$ T cells in murine models.[Michelsen KS, 2009]

Furthermore, TL1A is associated with other inflammatory conditions such as rheumatoid arthritis, autoimmune encephalomyelitis, and asthma.[Fang L, 2008. MeylanF 2008. Michelsen KS, 2009. Pappu BP 2008)

1.8.3.3 *Human Leukocyte Antigen (HLA)*

The highly polymorphic gene dense human leukocyte antigen (HLA) complex on chromosome 6p21.3 (IBD3) is one of the most consistently linked regions associated with IBD.[Ahmad T, 2006)] Candidate gene studies have been used to identify many genetic regions within the HLA region that have provided further insight into disease pathogenesis. Based on family studies, it is estimated that the HLA region contributes approximately 10-33% of the total genetic risk of CD and between 64-100% of the total genetic risk of UC.[Ahmad T, 2006. Yang H, 1999.Satsangi J, 1996)

The HLA complex is sub-categorized into type I (A, B and C) and type II (DR, DQ, DP) genes that predominantly play a role in lymphocyte function. The majority of studies in IBD have focused on the role of HLA type II as they play an important role in presenting antigens to T-lymphocytes. This subsequently leads to B-cells to produce antibodies against that particular antigen.[Ahmad T, 2006.)]

Of the HLA type II subtypes, HLR-DRB1 is the most extensively studied gene in IBD. An example of a HLA type II complex gene that is associated with IBD severity is the HLADRB1*0103 allele. The overall frequency of the HLA-DRB1*0103 allele is less than 2% amongst European and white North American populations. This allele was initially shown to be associated with UC in 1996 and later confirmed as a CD susceptibility gene in 2000. [Ahmad T, 2006.)] Several studies since have repeatedly correlated this rare allele with isolated colonic CD phenotype with odds ratios ranging from 5.1 to 18.5.[Hancock L, 2008. Ahmad T2002. Newman B2004. Fernandez L, 2004.)] Recently, Hancock *et al* confirmed the association of isolated colonic (Montreal classification L2) CD with HLADRB1*0103 in their cohort of 675 patients. In addition the authors found a novel association of this allele with earlier time to first surgery.[Hancock L, 2008.)] Furthermore, this allele has also been shown to correlate with perianal and fistulizing CD phenotype most likely owning to its strong affinity to colonic disease.[Ahmad T, 2006. Ahmad T 2002. Fernandez L, 2004)

In addition, the association of this allele with UC amongst Spanish, British, North American and Mexican cohorts is well documented. In particular this allele has a strong correlation with extensive or severe disease as defined by the need for colectomy for failed medical management. Similar to CD, the frequency of this allele is not high enough for it to be clinically useful as a marker of disease severity.(Ahmad T, 2006.)

Other HLA Class II associations with IBD include the HLA-DRB1*07 allele with ileal involvement in CD patients; HLA-DRB1*04 especially in conjunction with NOD2/CARD15 and ileal (L1) CD phenotype; HLA-DRB1*1502 is unique in that it is common in both Eastern and Western UC populations. Furthermore several HLA Class II loci are associated with extra-intestinal manifestations of IBD. For example, HLA-DRB1*0103 has been shown to be associated with migratory large joint arthritis and uveitis.(Ahmad T, 2006. Orchard TR, 2000.Orchard TR, 2002.)

Even though there is strong correlation of disease susceptibility genes within the HLA complex with IBD phenotype, their use as genetic makers still remains elusive due to limited sensitivity and specificity.(Ahmad T, 2006.) However it is thought that such genetic markers could be used in conjunction with other genetic polymorphisms and serological markers in order to increase the statistical power to predict IBD phenotype.(Ahmad T, 2006.)

1.9

Mass-Throughput Genotyping Technology

The DNA sequence is coded by four nucleotides: adenine (A), cytosine (C), thymine (T), and guanine (G). Single Nucleotide Polymorphisms (SNPs, pronounced as 'snip') are genetic variations that occur within an individual's DNA sequence and account for >90% of all sequence polymorphisms with an estimated frequency of 1 out of every 1,000 bases in the human genome.(Syvanen AC 2001. Callegaro A 2006.) Only those polymorphisms that have a minor allelic frequency of at least 1% in a given population are considered as SNPs. (Nowak D, 2009.) The position at where such SNPs occur within the DNA sequence can potentially have an effect at the phenotypic level.(Syvanen AC 2001.) Those SNPs that lie in a coding region are further sub-divided into synonymous or non-synonymous based upon whether they cause a change in the amino acid sequence or not.(Nowak D, 2009.) The work done by the Human Genome and International HapMap Projects has provided great insight into the variations observed within the human genome across populations.(Syvanen AC 2001.)

Human genetic diversity is maintained by genetic recombination during meiosis. The important property of the genome is that it is organized into discrete blocks of information that are transferred together during human reproduction. 313(Gabriel SB 2002) There is very limited haplotype diversity within each block which means that SNPs that reside in a particular block tend to be correlated. Therefore, not all SNPs within each block need to be assayed. The number of SNPs needed to be assayed is dependent on the size of the genomic block and the strength of LD present. The stronger the LD, the fewer amounts of SNPs needed to represent that particular block and vice versa.(Maresso K 2008. Grant SF 2008.) This has substantially reduced the overall number of SNPs that need to be studied in order to capture the overall variation within the genome. This has led to the development of high-throughput SNP microarray technology which has revolutionized the field of genetics by allowing researchers to study such genetic variations in a highly efficient, reliable and cost effective manner.(Grant SF 2008)

At the moment, there are two high throughput SNP genotyping platforms commercially available for performing GWA studies; the Affymetrix Gene Chip® and Illumina Bead Chip®. An overview of both platforms is shown in Figure 1.9.1 and Figure 1.9.3. Each platform comes with its unique arrays, reagents, fluidics system, hybridization oven and scanner, and

data analysis software. These microarrays require a DNA concentration between 250-750ng per sample which is used to interrogate over 500,000 SNPs simultaneously. Processing time is different between the two platforms, with 5 days for the Affymetrix protocol compared to 3 days for Illumina. It should be mentioned that there is a lot of flexibility within these platforms and researchers are now able to choose from a variety of scalable and customizable platforms depending on the number of samples and number of SNPs of interest (Figure 1.9.2). A brief general overview of the protocol used by the two platforms is laid out below.

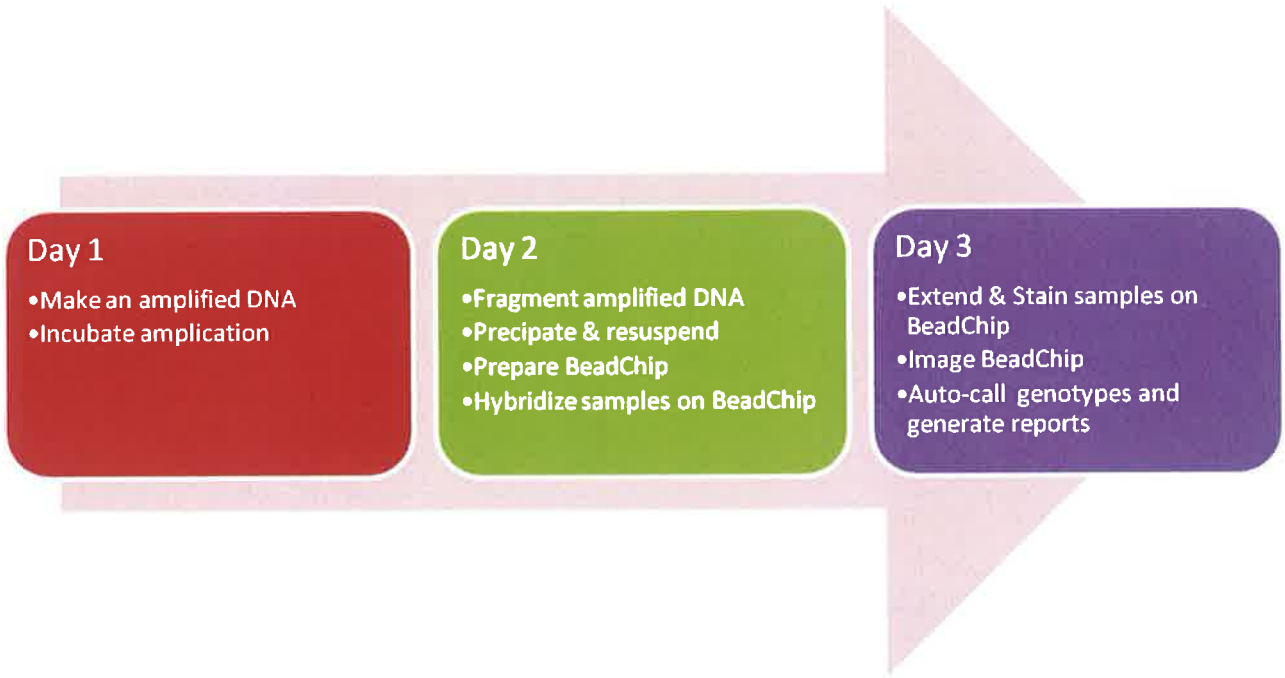

Figure 1.9.1. Illumina BeadChip Workflow.

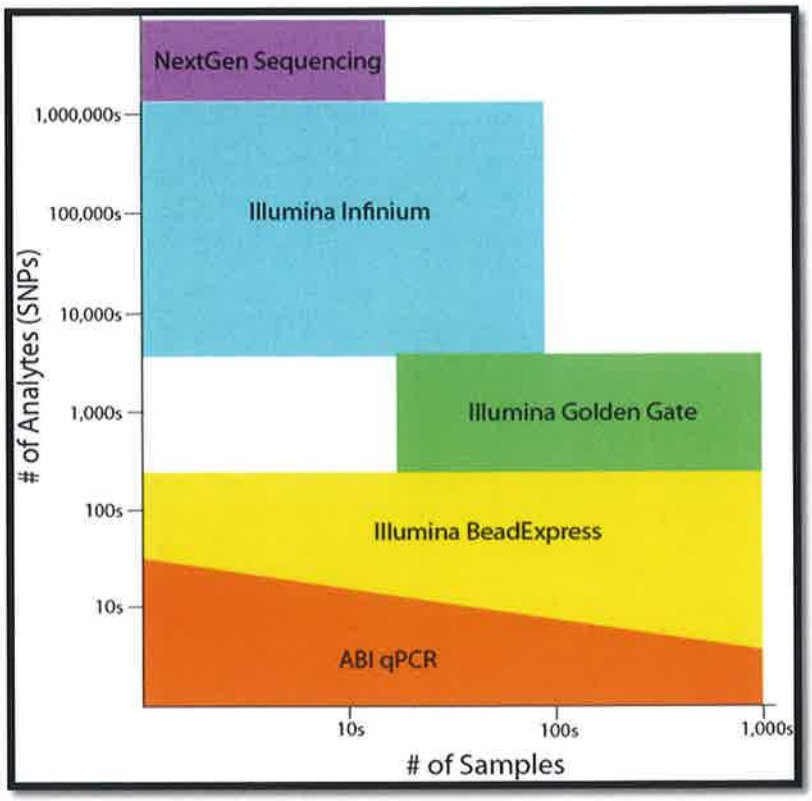

Figure 1.9.2: The choice of appropriate technology is a balance between the # of SNPs to be analyzed and the # of samples to be analyzed (and costs).

1.9.1 *Affymetrix (Santa Clara, CA, USA) GeneChip®*

Once DNA is appropriately quantified, it is digested by restriction enzymes which results in a 4 bp overhang fragment. Specific adaptor sequences then bind to these fragments and the complex is then amplified by the use of a generic primer. The PCR reaction is programmed so that only fragments that are of 200-1100bp in size are amplified in order to reduce genomic complexity. The amplified DNA is subsequently fragmented, labeled with a fluorochrome, and hybridized to millions of probes (oligonucleotides) which have been pre-synthesized on a 1.28 cm^2 area made of quartz(Figure1.9.3). In addition, a number of control probes and platform specific quality control measures are installed that act to ensure quality control. The samples are scanned and results are analyzed using the AffymetrixGeneChip Operating genotyping software provided. (Grant SF 2008.MaressoK 2008. Xavier RJ 2008.) Data is then reported as (1) intensity which provides information regarding the DNA copy number, and (2) SNP alleles that provide genotyping information.(Nowak D, 2009)

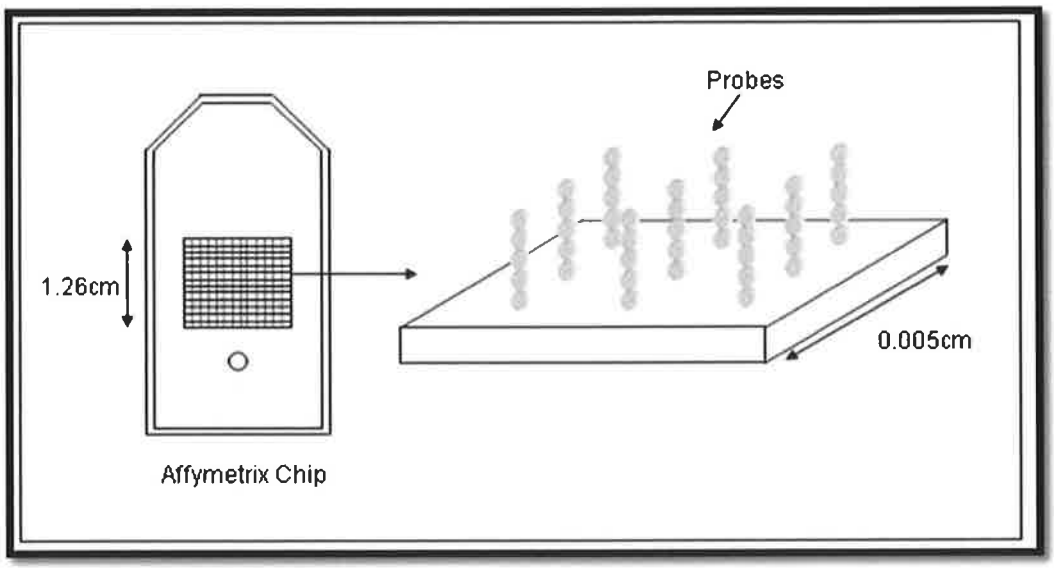

Figure1.9.3.AffymetrixGeneChip®: Once the DNA is digested into fragments by restriction enzymes, it is ligated and undergoes PCR to produce fragments of selected size (200-1,100bp). These are then labeled with a fluorochrome and hybridized to the microarray. Within the microarray the DNA fragments bind to their allele-specific perfect matched probes. The array is scanned by a laser which detects the fluorescent signals that are bound to the DNA. Data is then reported as (1) intensity which provides information regarding the DNA copy number, and (2) SNP alleles that provide genotyping information.(Nowak D, 2009)

1.9.2 Illumina

In contrast, the Illumina Bead Chip® platform utilizes 2 μm-sized silica beads that are coated with probe sequences. Once DNA samples have been adequately quantified (between 250-750ng), they are amplified 1000-2000x and subsequently fragmented to yield strands that are of approximately 300 to 600 bp in size. The samples then undergo alcohol precipitation, are resuspended and applied onto a BeadChip where they are left to hybridize overnight onto locus-specific 50-mer capture probes to SNP loci. Once hybridized, the SNP locus-specific primers undergo a base extension step in order to determine allelic specificity and are then fluorescently stained. The BeadChip is then put through a high-resolution confocal scanner that determines the intensity of the each of the fluorescently stained samples. Quality control probes are also inserted within the assay to ensure optimal reaction for every step. Genotype and control results are then automatically called and analyzed using Illumina's Genome Studio software.(Grant SF 2008. Maresso K. 2008. Gunderson KL 2006)

1.10
Study Aims

Specific Aim # 1: Chapters 3 and 4

1.1 (Studies #1 and 2)

To recruit and retrospectively sub-classify patients who have undergone the IPAA operation by the Division of Colon and Rectal Surgery at the Penn State Milton S. Hershey Medical Center into the following categories based on criteria set by Shen *et al*:

- **a.** IPAA without complications: no CD-like complications, no pouchitis for 2 consecutive years.
- **b.** IPAA with CD-like complications: defined as the presence of a peri-anal fistula, pouch inlet stricture, upstream small bowel disease, pouch biopsies showing granulomata, any and all occurring at least 6 months after closure of ileostomy.
- **c.** IPAA with Mild pouchitis: defined as 3 or fewer episodes per year for 2 continuous years.
- **d.** IPAA with Severe pouchitis: defined by the need for continuous medical therapy (antibiotics or immunosuppressants) OR 4 or more episodes per year for 2 consecutive years.
- **e.** Control populations will be identified and will include:
  - **i.** Ulcerative Colitis treated with colectomy + end ileostomy.
  - **ii.** Crohn's Colitis treated with colectomy + end ileostomy.

1.2 (Study #1)

To genotype IPAA patients for the three most common SNPs within the NOD2 gene using standard PCR gel electrophoresis. The hypothesis for this specific aim is to determine whether patients experiencing complications after IPAA (CD-like fistulae, strictures, granulomata, and/or pouchitis) carry a higher than expected rate of NOD2 mutations compared with IPAA patients without such complications.

1.3 (Study #2)

Perform DNA SNP analysis on IPAA patients so recruited, assaying for 80 previously identified SNPs shown to be associated with IBD (CD or UC) using a customized Illumina Bead Xpress Veracode technology array.

1.4 (Study #2)

To then statistically correlate genotype (single SNP and multi-SNP haplotypes) with the categories of IPAA patients in the attempt to define genetic correlates and/or predictors of surgical outcome after IPAA.

1.5 (Study #2)

Such identified specific SNPs then will be used to develop hypothetical predictive gene signatures which could possibly be utilized to predict post-operative complications and thus aid in preoperative surgical decision making.

Specific Aim # 2: Chapter 5

2.1 (Study #3)

To recruit CD patients who have previously undergone ileocolectomy for ileocolic disease by the Divison of Colon and Rectal Surgery at the Penn State Milton S. Hershey Medical Center and determine their surgical frequency.

2.2 (Study #3)

Perform DNA SNP analysis on CD patients so recruited, assaying for 80 previously identified SNPs shown to be associated with IBD (CD or UC) using a customized Illumina Bead Xpress Veracode technology array.

2.3 (Study #3)

To then statistically correlate genotype (single SNP and multi-SNP haplotypes) with the frequency of ileocolectomies in the attempt to identify genetic mutations that correlate with rapid recurrence of CD after Ileocolectomy.

Chapter 2

Methods

The following methods were used as necessary to perform the subsequent studies (1 - 3)

2.1 Patient Recruitment/creation of IBD Patient Registry

The Division of Colon & Rectal surgery care for a large group of IBD patients at The Milton S. Hershey Medical Center situated in Central Pennsylvania. This is a 500 bed medical center with attatched medical school. In excess of 800 surgical procedures are done per year with approximately 200 for IBD. There are in excess of 1,000 IBD patients seen in clinic per year. The medical center has a General Clinical Research Center where research patients receive education, and have clinical trials performed. Institutional Review Board (IRB) and Human Subjects Protection Office (HSPO) oversight is present.

In 1998, the Division of Colon & Rectal Surgery established an IBD Family Registry, which now has expanded to include any and all patients with IBD cared for at the Penn State Milton S. Hershey Medical Center. The core goal of this project is to enroll patients for the purpose of investigating the genetic basis for IBD. This registry stores the patients' demographics, personal medical history, and characteristics of illness and then a blood donation is used to create a DNA cell bank by immortalizing the patient's white cells using Epstein Bar Virus transformation. During the past years, the division have recruited over 167 families with IBD (defined by at least two family members with IBD) and harvested white cells from each diseased and healthy family member and created over 581 immortalized cell lines to perform genetic analysis. In addition, over the past 3 years the division have also recruited patients with sporadic IBD (142 UC, 108 CD), cancer associated IBD (18 UC) and a large group of otherwise healthy controls (344 individuals). This has created a DNA library with approximately 1000 cell lines for the study in perpetuity (by virtue of the EBV immortalization process) of these individuals' genetic make-up. This resource allows ongoing research into the genetics of IBD without the difficulties associated with having to repetitively draw blood from patients for each new experiment. This registry and the clinical patient population who have undergone the Ileocolectomy and IPAA procedure will specifically be used to investigate the research projects so defined.

2.2 Human Subjects

The subject recruitment process began by making telephone contact to potential candidates based on surgical log evaluation. The purpose, requirements, and risks and benefits of the study were explained during this initial contact and subjects were given the option to participate. A clinic appointment was then scheduled with one of the study investigators and the consent and release of medical record documents reviewed and signatures obtained. All documents had been reviewed by the institutional review board and approval granted. The study subjects received a copy of the consent document (see Appendix I). At this time registered specialists performed venipuncture and the blood samples were given to one of the study investigators for processing of DNA.

Once permission was granted, the medical charts from the Milton S. Hershey Medical Center were evaluated for the specific clinical characteristics listed below. If the medical record indicated relevant care been received at an outside institution, those institutions were contacted and the required records requested. Documentation of release of medical records were faxed to the appropriate institution and request made for the appropriate records to be mailed to the principal investigator. The information obtained was entered into a computer protected database with net-work security. Access was only available to those involved with the project. Upon entry into the study the subjects were assigned a code number and all future documentation referred to this code system. An official master list of study subjects and code numbers were kept in a locked file in the principal investigator's office.

The Milton S. Hershey Medical Center Institutional Review Board granted approval for this study. A copy of the certification of approval has been included in Appendix 2.

2.3 Resources

Three laboratories of 800 square feet in area each was available with all essential equipment to perform genetic assays, cell cultures, EBV transformation, PCR, and gel blots which include sterile hood, 38° incubator, PCR machines, UV projector, camera apparatus, -70° freezer, liquid nitrogen (LN) storage facilities, electrophoretic equipment, and gel densitometry.

2.4 Medical Record Chart Review

Patients' medical records were obtained including progress notes, hospital admission and discharge documents, surgical operative notes, endoscopy reports, and pathology reports. The review included patient demographics and documentation of clinical parameters of disease including: 1) sex, ancestry, family history, prior number of surgeries, smoking history, 2) extent of disease (defined by surgical pathology), 3) medications/dose including Imuran, Remicade, steroids, 4) Frequency of pouchitis attack and medications used in management, 5) presence or absence of primary sclerosing cholangitis, 6) presence or absence of arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. Data was saved on Penn State Hershey Medical Center, Division of Colon & Rectum's IBD registry database using Microsoft Infopath 2007 and SQL Server 2005. Please refer to the detailed clinical questionnaire that all IBD patients were required to complete upon recruitment to the IBD Registry in Appendix 3.

2.5 Mononuclear Cell Isolation:[(Splawski JB, 1991)]

The following procedure was performed within the Colon and Rectal Surgery research laboratory at Milton S. Hershey Medical Center. A standard lymphocyte cell isolation technique was followed as referenced in Current Protocols in Immunology. Briefly, in a sterile, laminar flow bio-safety cabinet, 10 ml blood is diluted 1:2 and mixed with sterile Dulbecco's phosphate buffered saline (DPBS) and slowly layered on top of 10 ml 1.077 g/ml Ficoll-Paque (Amersham Biosciences). The blood-Ficoll-Paque gradient was centrifuged at 400g for 30 minutes at room temperature. The mononuclear cell interface was collected and washed two times with DPBS and the cells collected by centrifugation at 250 g for 8 minutes.

2.6 Epstein Barr Virus Transformation:[Caputo JL, 1991]

The mononuclear cells were then used to create immortalized B-cell lines using Epstein Barr virus (EBV) to provide an indefinite source of DNA from each recruited individual. Epstein Barr Virus lymphoblastoid B cell lines are created by infecting cells with EBV obtained from the high-titer supernatant of the cotton-top marmoset lymphoblastoid cell line, B95-8 (CRL-1612, ATCC Manassas, VA). Briefly, B95-8 cells are grown to confluence and stimulated to produce virus by treatment with 40 ng/ml phorbol ester (phorbol 12-myristate 13-acetate). Following exposure, cells are washed with DPBS and the media replaced. Virus is allowed to accumulate in the culture media for 72 hours, whereupon the high-titer EBV supernatant is 0.2 um filtered and stored in 2.5 ml aliquots in liquid nitrogen.

Washed peripheral blood mononuclear cells (PBMC) were infected by re-suspension in RPMI-1640 media containing 12% fetal bovine serum (FBS) and 25% EBV supernatant then incubated in a 5% CO_2 incubator at 37°C. Media of the mononuclear culture was replenished as needed and transformation of B lymphocytes into lymphoblastoid cell lines (LCLs) typically achieved within 3 weeks as indicated by development of large, round, unattached clumps of cells. LCL B-cell lines were stored at $1x10^7$ cells/ml in FBS with 10% DMSO in secure liquid nitrogen tanks.

2.7 DNA Isolation

DNA was extracted from $1x10^6$ transformed B-cells using a DNA isolation kit (QIAAmp DNA Blood silica-membrane-based DNA purification Midi Kit, Qiagen, 51185) following the manufacturer's recommended protocol. Transformed B-cell vials were thawed out of liquid nitrogen and transferred to labeled 15ml tubes. Cells were pelleted at 1500 rpm for 5 min. The pellet was then resuspended in 2 ml Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen) and 200 µl Qiagen Protease added and tubes vortexed for 3 sec. 2 ml Buffer AL was then added and tubes quickly invert approximately 15 times (together in a rack), then vortexed again for 30 sec. Tubes were then incubated at 70°C for 30 minutes followed by the

addition of 2 mls of 200-proof EtOH. They were then inverted 10 times and vortexed for 1 min. Qiagen 15ml midi columns were labeled and half of the lysate added to the column. There were then centrifuged at 3300 rpm for 3 min. The filtrate was discarded and the remaining lysate added and centrifuged at 3300 rpm for another 3 minutes. Filtrate was discarded. 2mls Buffer AW1 was added to the column and centrifuged at 4200 rpm for 2 min. 2 mls of Buffer AW2 was then added and columns centrifuged at 4200 rpm for 20 min. The filtrate was discarded and the columns placed in new, labeled 15ml tubes. 300 µl Buffer AE was added to the column, capped and centrifuged at 4200 rpm for 5 minutes. DNA was then quantified using a spectrophotometer at 260nm. A working solution of DNA was then created by diluting the sample with Tris-EDTA to create stock of 10 ng/µl.

2.8 Polymerase Chain Reaction (PCR)

In order to study specific segments of DNA, many copies first need to be produced in order to have enough DNA to work with. The polymerase chain reaction (PCR) was developed by Dr. Kary Banks Mullis in 1983 while working as a DNA chemist at Cetus Corporation in Emeryville, California. This invention earned Dr. Mullis the Nobel Prize in chemistry in 1993 and is hailed as one of the monumental scientific techniques of the twentieth century.[Bartlett JM, Stirling D 2003)]

The process of PCR begins by first denaturing the double helix of DNA by applying heat (94–98 °C for 20–30 seconds) in order to yield two single-stranded DNA molecules. Next is the annealing step (temperature is lowered to 50–65°C for 20–40 seconds) whereby two sets of primers (short pieces of DNA) that are specific for sequences located on either end (flanking region) of the DNA segment of interest are attached to the single-stranded DNA via complementary base pairing (adenine with thymine and cytosine with guanine via hydrogen bonds). The temperature is increased to 72–80°C which is ideal for the enzyme DNA polymerase to function in the extension and elongation step. During this step two new strands of DNA are formed from the original template by the addition of complementary nucleotides (base pairs). This process results in the duplication of the original DNA, with each of the new copies comprised of one old and one new strand of DNA. Each of these strands can then be used to create another two new copies, and this cycle of denaturing and synthesizing new DNA is repeated as many as 30 or 40 times, leading to more than a billion exact copies of the original DNA segment of interest. This entire process takes place within a DNA Thermal

Cycler machine that is fully automated and is programmed to alter the temperatures needed for optimal denaturing and synthesis of DNA. The PCR product is then confirmed by visualizing the anticipated DNA bands by employing an agarose gel electrophoresis technique and comparing the size of the DNA fragment to a standard DNA ladder that acts as a marker for molecular weight.

2.9 Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate protein/DNA based on the charge or size of the fragment. The components of this procedure include: an electrophoresis chamber and power supply; gel casting trays; sample combs around which agarose forms the sample wells in the gel; electrophoresis buffer, usually Tris-acetate-EDTA (TAE); Ethidium bromide (EtBr) which is a fluorescent dye used for staining nucleic acids and transilluminator (an ultraviolet light-box), which is used to visualize ethidium bromide-stained DNA in gels.

To make a 2% gel, 2 grams of agarose powder is mixed into 100 ml 1x Tris-Acetate-EDTA buffer. This mixture is heated in the microwave until the agarose power is completely dissolved into solution. This is followed by the addition of 4µl of ethidium bromide, an aromatic intercalating agent that is commonly used as a nucleic acid stain. When exposed to UV light it will fluoresce with an orange color thereby intensifying almost 20-fold after binding to DNA. The solution is left to cool and quickly poured into a gel casting tray containing appropriate sized combs and left alone to solidify at room temperature. Once solidified, the combs are removed carefully and the gel is securely placed into the electrophoresis chamber and filled completely with TAE buffer solution. Samples of DNA plus loading dye (loading dye helps keep the DNA inside the wells and help to locate how far the fragment has travelled on the gel) are carefully pipetted into each well and the chamber lid closed and connected to an electric supply. The DNA fragments will travel towards the positive electrode (i.e. from the black lead towards the red). The electricity is switched on and the gel is run for approximately 30-40 minutes at 100 volts. Once the bands have travelled enough distance, the gel is placed under a UV light box which aids in visualizing the ethidium bromide-stained DNA. The DNA bands are compared to a standard DNA ladder that acts as a marker for molecular weight. A photograph of the DNA bands is usually taken soon after.

2.10 DNA Genotyping using Illumina® BeadExpress VeraCode Technology

In the past, genotyping patients for a large number of SNPs (dozens to hundreds) has proven quite challenging, time consuming, and expensive. However, since the development of DNA microarrays (also known as 'DNA chip') in the early 1990s, such robust genotyping is now possible.

With the help of Illumina®, a customized DNA microarray specific for 83 SNPs associated with IBD was developed (see Chapters 3-5). This customized DNA microarray uses Illumina's VeraCode technology and is capable of analyzing 96 patient DNA samples at a rate of 80 samples per hour. The VeraCode technology utilizes digital holographic codes to provide a robust detection method for multiplex assays. Comprised of cylindrical glass microbeads measuring 240 microns in length by 28 microns in diameter, VeraCode microbeads provide an ideal surface for numerous bioassays including genotyping, gene expression, and protein-based assays. Digital holographic elements are embedded within each microbead to create unique bead types. When excited by a laser, each VeraCode bead emits a unique code image, allowing for quick and specific detection by Illumina's BeadXpress Reader System. Depending on desired multiplex levels, assays are created by pooling microbeads with code diversities from one to several hundred. VeraCode beads are highly stable and the digital coding provides customizable tracking of not only the target(s) of interest, but also of critical identifiers such as sample ID, laboratory ID, and reagent kits. This allows for extremely efficient high-throughput genotyping. The steps for running this DNA microarray are laid out below:

- In order to obtain accurate DNA concentrations in our samples, an ultrasensitive fluorescent nucleic acid stain Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™) was used. The major advantage of using this reagent is that it can quantitate as little as 25pg/ml of dsDNA with a standard spectrofluorometer thereby being extremely sensitive for dsDNA and not taking into account other contaminants commonly found in nucleic acid preparations.

- Once dsDNA concentrations are optimized, they were diluted and loaded into 96 well plates. The samples were run on Illumina's BeadXpress Reader in Penn State Hershey Medical Center's Functional Genomics Core Facility.

- BeadExpress data was reported for risk and wildtype alleles based on the current literature and latest The National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/) and correlated with the clinical subgroups as detailed in Specific Aim 1 and 2. Single/multiple SNP statistical analysis was then performed.

Chapter 3

Study #1: *NOD2/CARD15* Mutations Correlate with Severe Pouchitis after Ileal Pouch-Anal Anastomosis

3.1 INTRODUCTION

Ulcerative colitis (UC) is an inflammatory condition of the colon that has no clear etiology or medical cure. Although medical therapy aids in controlling the inflammation, approximately 25-33%[Shen B, 2005] of patients will eventually require surgery in order to alleviate their symptoms and improve quality of life. Total proctocolectomy with ileal pouch-anal anastomosis (IPAA) is now considered to be the operative gold standard for the treatment of UC. This procedure avoids a permanent stoma, decreases the risk of dysplasia and cancer, and allows patients to discontinue steroids and other immune therapies that can have significant side effects.

Post-operative complications related to the IPAA can occur, however. These include early complications which are usually technically related, or ones occurring later that can suggest recrudescent inflammatory bowel disease (IBD), such as perianal fistuli, afferent limb stricture or severe pouchitis recalcitrant to conventional management with antibiotics. The severity of such complications can sometimes suggest a diagnosis of Crohn's Disease (CD), which is usually considered a pre-operative contraindication for IPAA, because of historic high failure rates.[Shen 2010]

In the past two decades, genetic variants and their role in the pathogenesis of IBD (both UC and CD) have been the subject of intensive research. The goal of this exciting work is to discover the possible etiology of IBD by identifying genetic mutations associated with IBD through studying large populations of IBD patients. One of the earliest genes in which mutations have been shown to be associated with Crohn's disease was the NOD2/CARD15 gene on chromosome 16.[Hugot JP 2001. Ogura Y 2001]This gene encodes a protein that functions as an intracellular sensor of muramyl-dipeptide (MDP), a component of bacterial cell walls. Three common mutations within this gene lead to a compromised host immune response to enteric bacteria. Pouchitis is in part, due to an over-growth of bacteria as evidenced by its responsiveness to antibiotics. Mutations in the NOD2/CARD15 gene could possibly contribute to pouchitis and/or be

predictive of Crohn's-like complications such as fistuli or strictures in patients thought to have UC. The present study therefore was undertaken to test the hypothesis that patients suffering complications after IPAA (CD-like fistuli, strictures, granulomata and/or pouchitis) have a higher than expected rate of NOD2 mutations compared to IPAA patients without such complications.

3.2 METHODS

3.2.1 Patients

A retrospective patient chart review was conducted looking at all patients who underwent a total proctocolectomy with IPAA reconstruction at The Milton S. Hershey Medical Center from July 1990 to July 2009. From a total of 382 IPAA patients, 107 patients were subsequently recruited into the study population presented here. All CD-like and severe pouchitis patients were fully recruited. Mild pouchitis and asymptomatic pouch patients were recruited without selection bias based on availability defined by follow up clinic visits during the study period (September 2008 – September 2009). Patients were classified into the following groups: (1) IPAA without complications or pouchitis; (2) IPAA with mild pouchitis; (3) IPAA with severe pouchitis; and (4) IPAA with CD-like complications. Control populations of patients were CD patients with colitis undergoing total proctocolectomy/ileostomy and otherwise healthy controls. Criteria defining these patient groups are presented in Table 3.1. Medical records, including progress notes, hospital admission and discharge documents, surgical operative notes, endoscopy reports, and pathology reports were reviewed.

Table 3.1. Definition for each Pouch Sub-group

Pouch Sub-groups	N	Definition
IPAA without complications or pouchitis	37	IPAA without complications or pouchitis for at least 2yrs after stoma closure.
IPAA with mild pouchitis	33	≤3 episodes/year for 2 consecutive years, effectively treated with 7-10 days of ciprofloxacin or metronidazole
IPAA with severe pouchitis	9	≥4 episodes/yr for 2 consecutive years OR the need for continuous antibiotics.
IPAA with CD-like complications*	28	the presence of any of the following: • peri-anal fistula (N=12) • pouch inlet stricture/proximal small bowel disease (N=11) • biopsies showing granulomata (N=3) • Antibiotic resistant pouchitis (N=5) Any or all occurring at least 6 months after ileostomy closure
CD colitis patients (Controls)	11	Treated with total proctocolectomy with end ileostomy
Healthy Patients (Controls)	269	No immunologic based disease, no gastrointestinal surgery

*Patients in IPAA with CD-like complications may have had more than 1 defining complication.

Individual episodes of pouchitis were defined by conventional clinical criteria including symptoms (increased frequency of bowel movements, diarrhea, tenesmus), pouchoscopy (gross inflammation) and biopsy showing inflammation superimposed on chronic inflammation. Patients were defined as having severe versus mild pouchitis according to criteria established by Shen et al.[Shen 2003]

Patient demographics and documentation of clinical parameters of disease were gathered and included: 1) sex, family history, date of IBD diagnosis, prior number of surgeries, smoking history, 2) extent of disease, defined by surgical pathology; 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab and steroid use; 4) frequency of pouchitis attacks and medications used in management, and 5) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. The study was approved by The Milton S. Hershey Medical Center Institutional Review Board (IRB).

3.2.2 DNA/Cell Bank

The identified IPAA patients were contacted and recruited into our IRB approved genetic IBD cell/DNA bank originally established in 1998. Informed consent was obtained and patients donated blood samples which were used to create immortalized B-cell lines using Epstein Barr virus (EBV) to provide an indefinite source of DNA from each recruited individual.[Zhang 2003]Briefly, blood was placed into sterile phosphate buffered saline (PBS), mixed, and slowly layered onto sterile Ficoll-Paque (Amersham Biosciences) solution. The blood-Ficoll mixture was centrifuged in a Sorvall RT centrifuge at 1000 RPM for 30 minutes at room temperature until the cellular gradient was achieved. The lymphocyte interface was carefully extracted. These cells were washed with PBS, mixed, and centrifuged at 1000 RPM for 10 minutes. The cellular pellet was resuspended in media containing Epstein Barr virus for transformation and incubated. Once transformed, cells were stored by placing $1x10^7$ cells/ml in 1 ml aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks till use.

3.2.3 DNA Isolation

DNA was extracted from $1x10^6$ transformed B-cells using a DNA isolation kit (QIAAmp DNA Blood silica-membrane-based DNA purification Midi Kit, Qiagen, 51185) following the manufacturer's recommended protocol. Transformed B-cell vials were thawed out of LN2 and transferred to labeled 15ml tubes. Cells were pelleted at 1500 rpm for 5 min. The pellet was then resuspended in 2 ml Dulbecco's Phosphate-Buffered Saline (DPBS, Invitrogen) and 200 µl Qiagen Protease added and tubes vortexed for 3 sec. 2 ml Buffer AL was then added and tubes quickly inverted approximately 15 times (together in a rack), then vortexed again for 30 sec. Tubes were then incubated at 70°C for 30 minutes followed by the addition of 2 mls of 200-proof EtOH. They were then inverted 10 times and vortexed for 1 min. Qiagen 15ml midi columns were labeled and half of the lysate added to the column. There were then centrifuged at 3300 rpm for 3 min. The filtrate was discarded and the remaining lysate added and centrifuged at centrifuged at 3300 rpm for another 3 minutes. Filtrate was discarded. 2mls Buffer AW1 was added to the column and centrifuged at 4200 rpm for 2 min. 2 mls of Buffer AW2 was then added and columns centrifuged at 4200 rpm for 20 min. The filtrate was discarded and the colums placed in new, labeled 15ml tubes. 300 µl Buffer AE was added to the column, capped and centrifuged at 4200 rpm for 5 minutes. DNA was then quantified using a spectrophotometer at 260nm. A working solution of DNA was then created by diluting the sample with Tris-EDTA to create stock of 10 ng/µl.

3.2.4 PCR Genotyping:

Once patients were classified into their respective clinical subgroups, PCR for the 3 NOD2/CARD15 mutations was performed after appropriate annealing temperatures and reaction conditions were optimized (Table 3.2, Figure 3.1). PCR products were separated by electrophoresis on a 2% Tris-Acetate-EDTA-Ethidium Bromide agarose gel at 100V for 30 minutes and visualized on a UV transilluminator. PCR data was further verified using a customized Illumina® BeadArray using VeraCode technology programmed for two of the three NOD2/CARD15 polymorphisms (rs2066844 p.Arg702Trp, and rs2066845 p.Gly908Arg) with 100% agreement between PCR data and BeadArray on all patients.

Table 3.2. NOD2 PCR reaction summary

SNPs	Mutation	Primer	Primer sequence	PCR reaction	Fragment length (bp)
rs2066844	R702W	Wildtype Forward: Mutant Forward: Reverse:	5'-ATC TGA GAA GGC CCT GTT CC-3' 5'-ATC TGA GAA GGC CCT GTT CT-3' 5'-CCC ACA CTT AGC CTT GAT G-3'	Denature 95°C 5min. 94°C 30 sec, 60°C 30 sec, 72°C 30sec. 30 cycles. Extension 72°C 2min.	438
rs2066845	G908R	Forward: Reverse:	5'-CCC AGC TCC TCC CTC TTC-3' 5'-AAG TCT GTA ATG TAA AGC CAC-3'	Denature 95°C 5min 94°C 30 sec, 56°C 30 sec, 72°C 30sec. 30 cycles. Extension 72°C 2min.	380
rs2066847	3020insC	Forward: Wildtype Reverse: Mutant Reverse:	5' CTT CAA CCA CAT CCC CAT TCC-3' 5'- AAG CCC TCC TGC AGG CCC T-3' 5'-AGC CCT CCT GCA GGC CCC-3'	Denature 95°C 5min 94°C 30 sec, 67.0°C 60 sec, 15 cycles. 94°C 15 sec, 63.0°C 30 sec, 72°C 30sec. 20 cycles Extension 72°C 2min	330

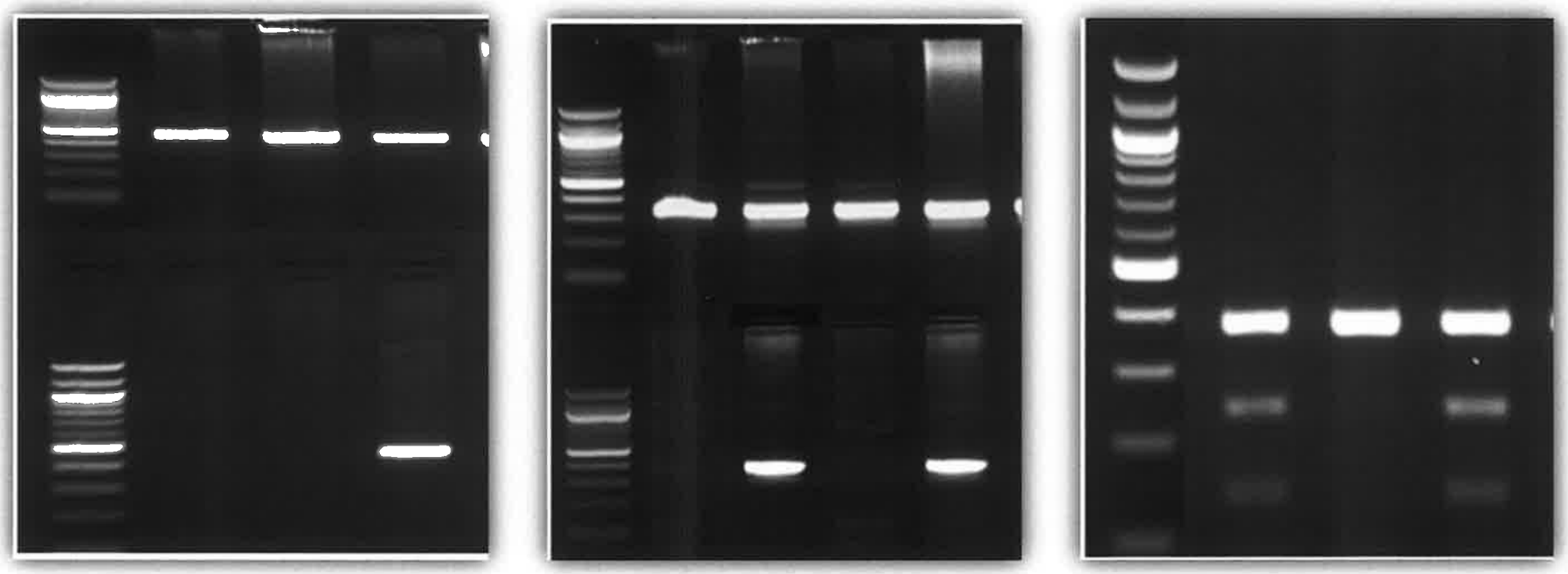

Figure 3.1. PCR gel electrophoresis for the three common NOD2 polymorphisms. Lane one corresponds to the standard 100bp ladder. Lane 2 onwards depicts the PCR product. For rs2066844 and rs2066847, the top row corresponds to the wildtype allele. The bottom row corresponds to the mutant allele. DNA product in both top and bottom rows corresponded to the heterozygous haplotype. Similarly, for the rs2066845 SNP, DNA product was spliced with a restriction enzyme to revealing the heterozygous haplotype. Spliced DNA produce only corresponded to a mutant however if no split produced was produced by the restriction enzyme, then this corresponded with the wildtype haplotype.

3.2.5 Statistical Analysis:

The R statistical software system (version 2.9.2, http://www.r-project.org/) was used to perform statistical analysis. Data (reported as mean ± standard deviation) was analyzed using Chi-square with Yates correction with statistical significance reported as $p < 0.05$.

3.3 RESULTS

107 IPAA patients were recruited into the various groups and their associated clinical data is summarized in Table 3.3. The overall mean follow-up for each group was over 5 years. The average time to the development of complications was over 3 years in the CD-like and severe pouchitis groups, and 2.8 years in the mild pouchitis group.

The incidence of NOD2/CARD15 mutations amongst the various study groups is shown in Figure 3.2. The mutation incidence amongst the healthy population cohort was 8.5% (23/269) while the known CD group was 45.5 % (5/11). The severe pouchitis group had the highest NOD2/CARD15 frequency which was significantly elevated when compared with the asymptomatic IPAA group and healthy controls (66.6% vs. 5.4% vs. 8.5% respectively, $p < 0.001$). There was no significant increase in NOD2/CARD15 mutations observed in the mild pouchitis or CD-like IPAA groups compared to healthy controls or asymptomatic IPAA patients (18.2% vs. 14.8% vs. 8.5% vs. 5.4%, respectively). The allelic distribution of NOD2/CARD15 mutational variants for each patient subgroup is summarized in Table 3.4. Though not statistically significant, all groups had the R702W (rs2066844) as the most common except in the severe pouchitis group, where the C-insertion mutation (rs2066847) was greatest in incidence.

Table 3.3: Patient Demographic/Operative Data

	Crohn's like (N=28)	Severe Pouchitis (N=9)	Mild Pouchitis (N=33)	Asymptomatic (N=37)
Sex M/F	16/12	5/4	17/16	26/11
FHx for IBD (Yes/No)	4/24	4/5	9/24	10/27
Smoking (Current/Ex-smoker/Never)	1/6/21	0/3/6	0/10/23	0/6/31
Extraintestinal Manifestations PSC Dermatologic Ocular Arthritis	 1 1 1 3	 1 0 0 0	 3 0 0 0	 3 0 0 1
Avg time to IPAA after Dx (yrs)	5.7±5.4	5.8±7.0	7.9±8.4	11.2±9.0
Average follow-up after IPAA (yrs)	9.1±5.3	10.7±6.6	7.6±4.2	5.5±5.6
IPAA (Open/Lap-assisted)	25/3	9/0	32/1	33/4
IPAA # of stages: 1 2 modified 2* 3	 2 18 2 6	 0 7 0 2	 0 24 3 6	 7 23 2 5
Indications for IPAA (Failed Medical Mg/Dysplasia, Cancer/Toxic Colitis)	18/2/8	4/2/3	21/7/5	26/5/6
Average time from IPAA to 1st pouch complication/episode of pouchitis (yrs)	3.23±4.08	3.21±2.44	2.82±2.85	N/A

*Modified 2: patients who underwent IPAA without ileostomy after previous total abdominal colectomy.

Family history, Smoking history, Extraintestinal Manifestations, Follow up, IPAA stage & Indications were all NSD amongst subgroups.

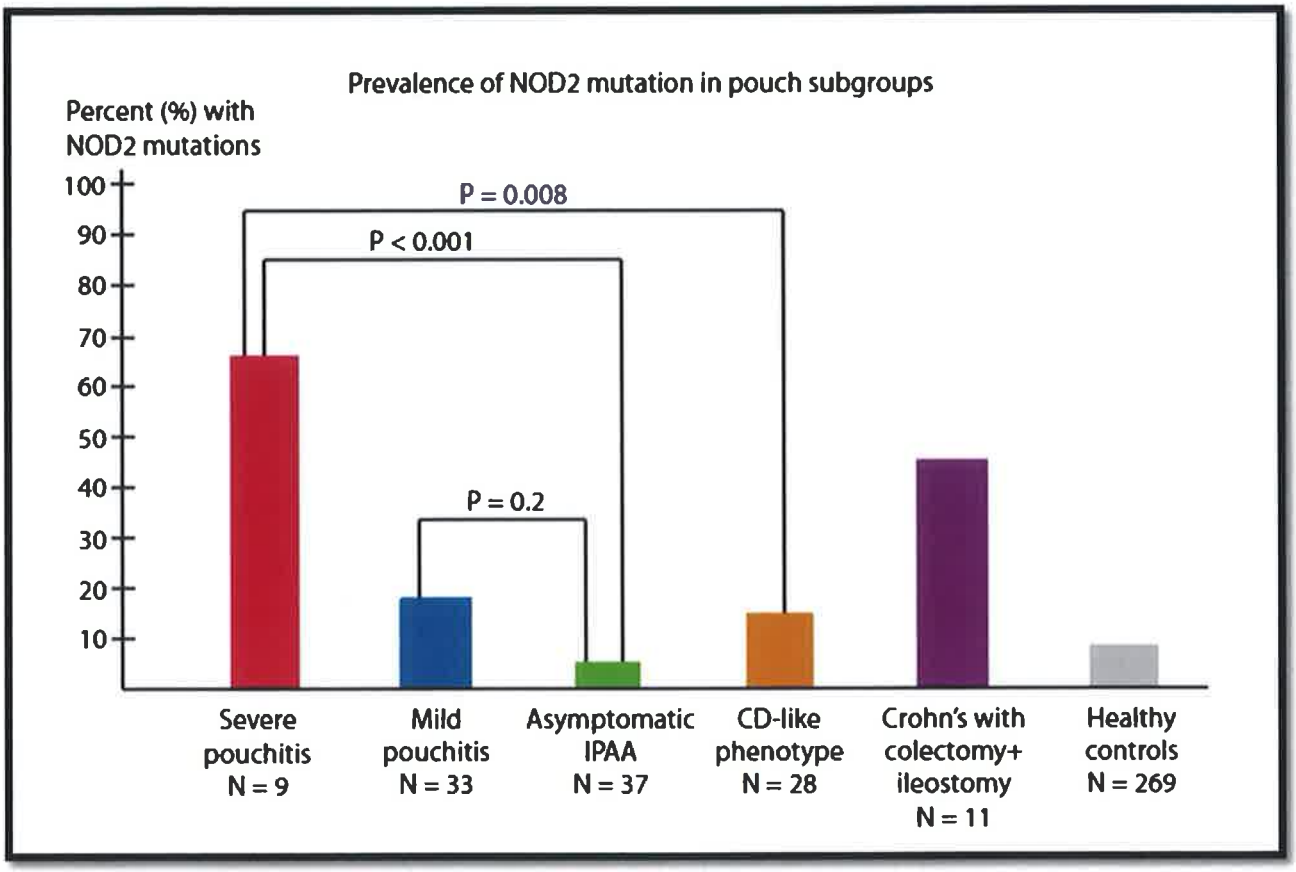

FIGURE 3.2. Prevalence of *NOD2/CARD15* mutations for each subgroup. The incidence of *NOD2/CARD15* mutations was significantly higher in the severe pouchitis group compared with the asymptomatic and Crohn's disease (CD)-like groups. No difference was seen in the mild pouchitisvs asymptomatic groups. Rate of *NOD2/CARD15* mutations was unexpectedly lower in the CD-like complication group compared with patients with Crohn's colitis.

Table 3.4. NOD2/CARD15 Allelic distribution for each subgroup

	Severe Pouchitis	Mild Pouchitis	CD-like Complications	CD treated with TPC+end ileostomy	Healthy Control
1007fsCins (rs2066847)	3/6 (50%)	2/6 (33%)	0/3 (0%)	2/5 (40%)	6/29 (21%)
R702W (rs2066844)	2/6 (33%)	4/6 (67%)	3/4 (75%)	3/5 (60%)	17/29 (59%)
G908R (rs2066845)	1/6 (17%)	0/6 (0%)	1/4 (25%)	0/5 (0%)	6/29 (20%)

No Significant Difference for the distribution of SNP's amongst study groups.

DISCUSSION

Total proctocolectomy with IPAA is the operative gold standard for the surgical treatment of UC. This procedure removes the diseased colon and re-establishes gastrointestinal continuity. It leads to an improved health-related quality of life, decreased risk of side effects from UC-related medications and nearly eliminates the risk of dysplasia and eventual cancer. However, pouchitis and Crohn's like complications confound the benefits of this operation. Whether such complications after surgery are the result of an incorrect preoperative diagnosis or unrelated postoperative events remains unclear. However, if patients prone to such complications could be preoperatively identified, such information could assist in surgical decision making.

Pouchitis is a relatively common complication after the IPAA. Depending on definition, it can affect 24-59% of IPAA patients[Shen2005]. Clinically, pouchitis presents with increased stool frequency, urgency, abdominal cramping, and pelvic discomfort. Endoscopic evaluation together with symptom assessment and histologic evaluation is the key to an accurate diagnosis of pouchitis. Specifically, endoscopy, stool culture and histologic evaluation can distinguish pouchitis from other inflammatory conditions (such as *C. difficile* or cytomegalovirus pouchitis) or functional disorders of the pouch (such as irritable pouch

syndrome). There is no one universally accepted diagnostic or classification criteria for pouchitis but Shen *et al*[Shen B 2003] proposed a pouchitis severity classification based on response to therapy (Figure 3.1). He suggested three distinct groups: (1) antibiotic-responsive pouchitis, a condition in which patients have infrequent episodes (< 4 episodes per year) responding to a 2-week course of a single antibiotic; (2) antibiotic-dependent pouchitis is a condition with frequent episodes (> 4 per year) or with persistent symptoms requiring long-term, continuous antibiotic or probiotic therapy, and (3) antibiotic-refractory pouchitis, where patients fail to respond to a prolonged course of antibiotics and require 5-ASA, corticosteroids or immunomodulator (Imuran, 6MP or Remicade) therapy. The antibiotic refractory group is commonly considered to have a variant of conventional UC more akin to CD, which is clinically reinforced by the observation that immune modulators and/or Tumor Necrosis Factor alpha (TNFα) antagonists can often effectively treat such patients.[Papadakis KA 2003. Shen 2009)

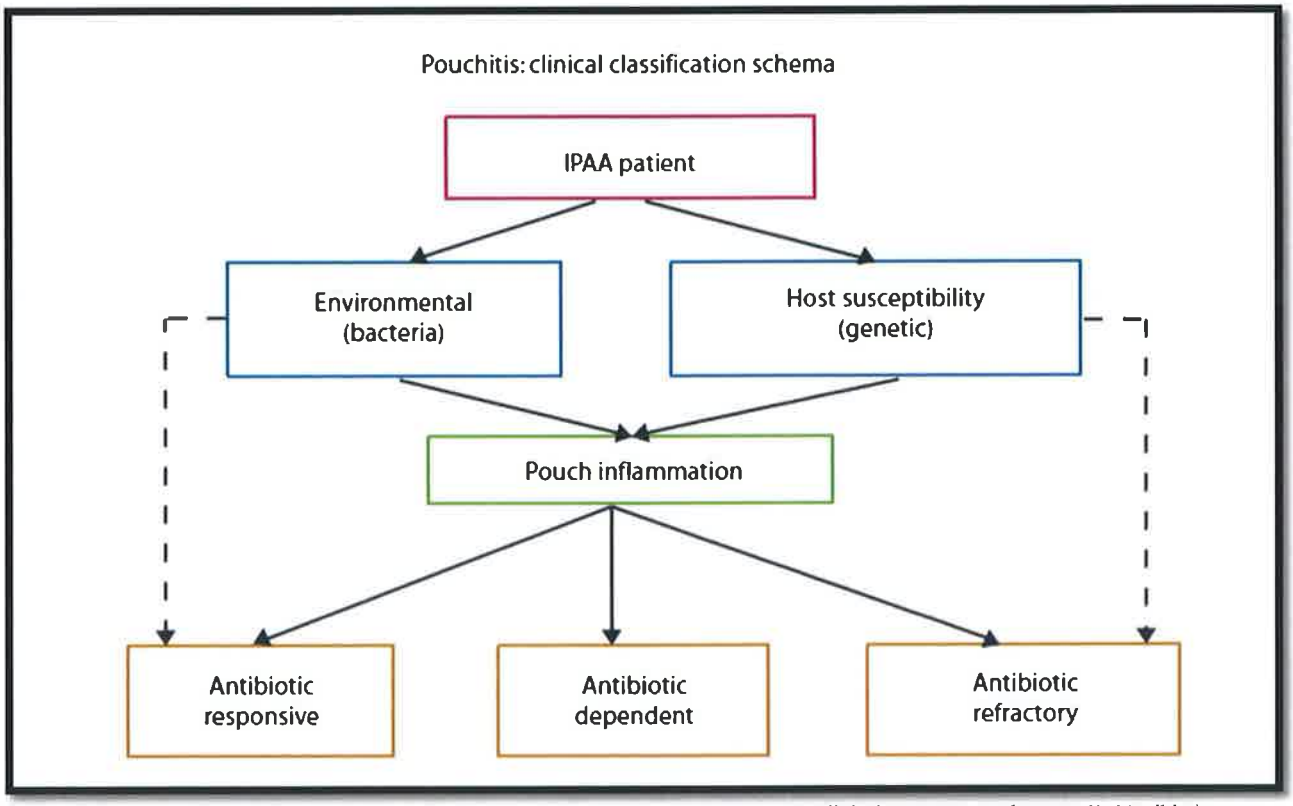

Figure 3.3.Shen *et al* proposed 3 distinct pouchitis severity subgroups based purely on clinical response to therapy: 1) 'Antibiotic-responsive' group, a condition in which patients have infrequent episodes (<4/y) responding to a 2-week course of a single antibiotic; 2) 'antibiotic dependent' pouchitis, a condition with frequent episodes (>4/y) or with persistent symptoms requiring long-term, continuous antibiotic or probiotic therapy; 3) 'antibiotic-refractory' pouchitis, where patients do not respond to a prolonged course of antibiotics and require 5-aminosalicylic acid, corticosteroids, or immunomodulator therapy. Patients with wild-type/functional *NOD2/CARD15* genes probably have the molecular machinery to prevent the development of severe pouchitis, While those patients with *NOD2/CARD15* mutations lack the innate ability to protect themselves and are more prone to antibiotic refractory pouchitis.

In spite of many years of research and clinical experience, the etiology of pouchitis is still largely unknown. However, the fact that it is rarely seen in patients with familial adenomatous polyposis (FAP) and that antibiotics are frequently an effective treatment suggests roles for both a host susceptibility factor and enteric bacteria in disease pathogenesis. Recent studies have also shown an increased number of sulphate-reducing bacteria within pouches created for UC as compared to those given for FAP.[Coffey JC, 2009]Furthermore, the use of oral probiotic therapy has been shown to be often effective in delaying the onset and preventing relapses in patients with pouchitis.[Meier CB 2005. Mimura 2004. Gionchetti 2000] Thus, the bacterial component of this pathophysiologic process seems clear, at least in those that respond to antibiotics. However, those patients with severe pouchitis or antibiotic resistant pouchitis clearly must have other factors that play a significant role, and their lack of responsiveness to antibiotics suggests a different or an additional mechanism in those with severe disease compared to the antibiotic responsive pouchitis patient group.

In the past two decades, genetic variants and their role in the pathogenesis of IBD has been the subject of intensive research. The essence of this exciting work has been to identify the possible etiology of IBD by identifying genetic mutations associated with IBD. Much of this work has focused on identifying specific pathways of immune function and inflammation with the hope of developing new therapies for IBD. However, little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making. Work done in linkage analysis, candidate gene studies and more recently genome wide association studies (GWAS) has identified numerous genetic mutations associated with IBD.[Wellcome Trust Case Control Consortium 2007. Barrett JC 2008. Duerr RH 2006. Fisher SA 2008. Franke A 2008. Hampe J 2007. Libioulle C 2007. Parkes M 2007. Silverberg MS 2009. Mathew CG 2008. Noomen CG 2009. Huebner C 2010. Scherr R 2009. Nakagome S 2010. McGovern DP 2010.] The landmark discovery of mutations in the NOD2/CARD15 gene on chromosome 16 as being associated with CD susceptibility is an example of linkage analysis[Hugot JP 2001]. Mutations within this gene have been found in up to 40% of European and North American CD patients when compared to 10-15% of the healthy population.[Rogler G. 2010] These figures are comparable to our data which shows NOD2/CARD15 mutations in 45% of CD patients versus 8.5% in healthy controls. The NOD2/CARD15 gene encodes for an intracellular protein sensor of MDP, a bacterial cell wall component. Activation of this protein leads to an increase in nuclear factor kappa B (NFκB) and mitogen-activated protein (MAP) kinase signaling pathways, leading to the production of cytokines (TNFα, interleukin-1β) and the

promotion of autophagy of endocytosed bacteria.[Hugot JP 2001. Ogura Y 2001. Brain O 2010] Three single nucleotide polymorphisms (SNPs) (G908R rs2066845, 1007fsCins rs2066847, R702W rs2066844) in this gene account for approximately 82% of the mutated alleles.[Hugot JP 2007] These variants within the NOD2/CARD15 gene have repeatedly been shown to be associated with ileal CD, early age of onset and stricturing and/or penetrating phenotype.[Hampe J 2001. Abreu MT 2002. Ahmad T 2002. Cuthbert AP 2002. Hampe J 2002. Lesage S 2002. Vermeire S 2002. Oostenbrug LE 2006] Both because of its role in innate immunity against enteric bacteria and its association with CD, we investigated whether patients manifesting CD-like complications and/or severe antibiotic resistant or antibiotic dependant pouchitis would have an increased incidence of NOD2/CARD15 mutations. Theoretically, if positive, such a genetic association could lend itself to preoperative testing and possibly playing a role in surgical decision making by avoiding an IPAA in those patients with a high risk of such complications postoperatively.

This study was limited by the small number of recruited patients (107/382). However this did include all of the patients with CD-like complications and severe pouchitis from our practice, since all such patients are repeatedly evaluated in clinic due to their difficulties and readily agreed to inclusion in this research protocol. The mild pouchitis and asymptomatic groups admittedly represented a subset of the much larger total volume of IPAA patients. These two groups nontheless were the largest of the groups studied and represented an unselected subset of the larger group of uncomplicated IPAA patients. Future studies clearly should be prospective and all inclusive to definitively evaluate whether the genetic findings discovered in this smaller trial are borne out.

We found that asymptomatic IPAA patients had an extremelylow incidence (5.4%) of NOD2/CARD15 mutations, not significantly different from healthy controls (8.5%) or mild pouchitis patients (18.2%). However, patients with severe pouchitis had the highest incidence (66.6%) of NOD2/CARD15 mutations suggesting that this group may have a compromised host defense mechanism to enteric bacteria. Similar findings were seen by Meier *et al*[Meier CB 2005] who found an 8% incidence of NOD2/CARD15 mutations in patients without pouchitis, versus 24% in patients with more than 2 episodes of pouchitis per year. Our study found a higher mutation rate in the severe pouchitis group, but our definition of severity was more rigid with 4 or more episodes per year or the continuous need for medical therapy, as compared to only two episodes/year in the Meier study. Nonetheless, both studies suggest that more severe pouchitis patients have an intrinsic defect in innate immunity against

commensal pouch bacteria. In both studies, the frequency of the C-insertion frameshift mutation (rs2066847) was the most prevelant in the severe pouchitis group suggesting that the C-insertion mutation may be the highest risk allotype for pouchitis. Some authors have suggested that NOD2/CARD15 compound heterozygosity may increase the risk of CD.[Heresbach D 2004] In our study only 2 patients carried compound mutations prohibiting statistical analysis. Interestingly, however both of these patients belonged to the severe pouchitis group. Overall, these data suggest a genetic basis for mild versus severe pouchitis (Figure 3.3). Severe pouchitis may be due to a genetic defect in host responsiveness to pouch bacteria, while mild pouchitis could be caused by a transient overgrowth of pathologic bateria that can be treated by antibiotics.

The frequency of Crohn's like complications (fistula, pouch inlet strictures, granuloma formation, proximal small bowel disease) after IPAA ranges from 2.7-13% depending on diagnostic criteria.[Shen B 2010]A diagnosis of CD should be considered in the IPAA patient if 1) a perineal fistula or abscess develops greater than 6 months after IPAA, 2) there are granulomas on pouch or small bowel histology remote from any anastomosis, and 3) there are ulcerated lesions in the afferent limb proximal to the pouch and/or strictures at the pouch inlet in the absence of current NSAID use. Though such complications can often be treated with the reinstitution of immune modulators or other medications, the prognosis for pouch function is worse and specifically, the risk of pouch loss or need for diversion is higher than in those IPAA patients without such complications.[Joyce MR 2009] As mentioned above, mutations within the NOD2/CARD15 gene have repeatedly been associated with CD, thus one might expect the frequency of NOD2/CARD15 mutations in IPAA patients with CD-like complications to be similar to that found in CD patients treated with total proctocolectomy and end ileostomy. Somewhat surprisingly, this was not borne out in this study. Our data showed that the frequency of NOD2/CARD15 in the CD-like IPAA subgroup was only 15% which was significantly different when compared with the severe pouchitis group (66.6% $p=0.008$) but not much different than asymptomatic IPAA patients or healthy controls. This is consistent with clinical and laboratory evidence, however, since NOD2/CARD15 positive CD patients most commonly have ileocolonic stricturing disease and such patients would rarely be confused as having UC and thus would never come to IPAA. It is noteworthy that the CD-like IPAA group was significantly different statistically than the severe pouchitis group, suggesting that the pathophysiologic mechanisms for the respective complications in these two groups were different at least from a genetic standpoint. Thus fistuli, afferent limb

strictures, and pouch granulomata probably have a disease mechanism not related to NOD2/CARD15 dysfunction. In the context of the increasing number of genes now being identified as being associated with CD, further studies are needed to investigate a possible association of these CD-like complications with these more recently discovered gene mutations.

Besides gene haplotypes, serum antibodies (pANCA/ASCA) have also been investigated as prognosticators of IBD severity[Targan SR 1999] and possible predictors for pouch complications. Some investigators have shown pANCA levels to be significantly greater in chronic pouchitis when compared to control groups[Fleshner PR 2001. Sandborn WJ 1995]however, this has been refuted by others[Aisenberg J 2004. Yasuda N 1998]. Such inconsistencies may be due to fluctuations in serum levels of these antibodies associated with disease activity, which would not be an issue with genetic testing.

In summary, we have identified a significant association of mutations in the NOD2/CARD15 gene with severe pouchitis after IPAA but not with mild pouchitis nor CD-like complications. This suggests an intrinsic defect in host immunity to commensal pouch bacteria in severely affected IPAA pouchitis patients that might explain their recalcitrance to conventional antibiotic management. These findings need to be confirmed in larger cohorts of patients from different demographic and geographic locales, as well as in a prospective study. If confirmed, the preoperative measurement of NOD2/CARD 15 status may assist the surgeon and patient being considered for IPAA surgery, especially when the patient is a marginal operative candidate. Future studies looking at other potential genetic associations that could predict CD-like complications are necessary.

Chapter 4

Study #2: Genetic Risk Profiling and Gene Signature Modeling to Predict Risk of Complications after Ileal Pouch Anal Anastomosis

4.1 INTRODUCTION

Ulcerative colitis (UC) is a chronic relapsing and remitting inflammatory disease limited to the mucosa and submucosa of the colon and rectum. Despite advances in medical therapy, approximately 33% of patients with UC eventually require total proctocolectomy with Ileal Pouch-Anal Anastomosis (IPAA).[Shen B 2005]This procedure is now considered the gold standard for the surgical treatment of UC.

However, post operative complications confound the success of the IPAA in UC patients. These include early complications that are usually technically related, or ones occurring late that can suggest recrudescent inflammatory bowel disease (IBD). Two late complications that will be discussed in this study include pouchitis and Crohn's disease (CD) like complications. Pouchitis is an inflammatory condition characterized by increased frequency of bowel movements, diarrhea, tenesmus, malaise and arthralgias that can affect between 15-70% of patients depending on definition and duration of follow-up.[Beliard A 2010] CD-like complications include the development of fistulas, strictures and granulomatous inflammation that can affect up to 13% of patients.[Shen B 2009] Mild pouchitis (MP) a phenomenon that commonly occurs in the IPAA patient, is an acceptable complication since it can be effectively treated with a short course of antibiotics. However severe pouchitis (SP) requires more aggressive therapy sometimes with immunosuppressive medications and like CD-like complications are associated with an increased risk of pouch failure, pouchectomy and permanent stoma. If these more severe post IPAA complications could be predicted, it could assist in pre-operative decision making, possibly avoiding the operation in high risk patients.

In recent years, much work has been done to unravel the possible etiology of IBD by identifying and then correlating genetic mutations found in large populations of IBD patients compared to

healthy controls. We have previously shown that mutations in the *NOD2/CARD15* gene are associated with SP, but not mild pouchitis nor CD-like complications.[Sehgal R 2010]

More recently, genome wide association (GWA) studies have identified approximately 83 single nucleotide polymorphisms (SNPs) within 55 genetic loci to be associated with IBD and were thus investigated in the present study. The aim of this study therefore was to correlate these 83 SNPs with pouchitis (mild vs. severe) and CD-like complications seen after IPAA. Such identified specific SNPs then were used to develop predictive gene signatures which could possibly be utilized to predict post operative complications and thus aid in preoperative surgical decision making.

4.2 METHODS

4.2.1 Patient Recruitment

At The Milton S. Hershey Medical Center from July 1990 to July 2010, a total of 382 patients underwent the IPAA procedure for a pre-operative diagnosis of UC or indeterminate colitis (IC). A subset of 142 patients were recruited into the present study population as they presented to follow up clinic appointments over the study time period September 2008 to September 2010. By the nature of symptomatology, patients with complications, specifically severe pouchitis and CD-like, were seen more frequently, thus recruitment was relatively complete for these patient groups. Patients with asymptomatic pouch function would regularly be seen annually and were thus recruited as they presented for their routine follow up appointments during the study period. These patients were classified into the following categories: 1) IPAA without complications or pouchitis; 2) IPAA with mild pouchitis; 3) IPAA with severe pouchitis; and 4) IPAA with CD-like complications. The exact definitions for each category are shown in Table 4.1.

Table 4.1. Definition for each pouch subgroup		
Group	n	Definitions
Asymptomatic Pouch	63	IPAA without complications or pouchitis for at least 2yr after stoma closure.
Mild Pouchitis	41	<4 episodes/yr for 2 consecutive years, responding to 2-wk course of single Abx.
Severe Pouchitis	12	≥4 episodes/yr for 2 consecutive years or persistent symptoms, requiring long-term continuous Abx.
CD-like	26	The presence of any of the following[1]: • Fistula (n = 12) • Pouch inlet stricture/proximal small-bowel disease (n = 11) • Biopsies showing granulomatous inflammation (n = 3)

CD = Crohn's Disease

1= Any or all of these complications occurred at least 6 months after ileostomy closure.

4.2.2 Patient Classification

Individual episodes of pouchitis were defined by standard clinical, endoscopic and histologic criteria. Symptoms of pouchitis included increased frequency of bowel movements, diarrhea, and tenesmus. Endoscopy showed gross inflammation and biopsy showed acute inflammation superimposed on chronic inflammation. There presently is no one way to classify severity of pouchitis however Shen *et al* (Shen B 2003) proposed a clinical classification schema based on response to therapy. He proposed three main groups: (1) Antibiotic responsive pouchitis, a condition which has <4 episodes per year and responds to a 2 week course of a single antibiotic; (2) Antibiotic dependent pouchitis, defined as ≥4 episodes per year requiring long-term antibiotics; and (3) antibiotic refractory pouchitis, where patients fail to respond to a 2-4 week course of a single antibiotic and require more than >4 weeks with 2 antibiotics or 5-ASA/steroids/immunomodulator therapy. For the purpose of this study, we considered the antibiotic responsive group to be mild pouchitis (MP) and combined the antibiotic dependent and antibiotic refractory groups together to form our severe group. (Table 4.1)

Features that suggest CD in the post IPAA patient include pouch-anal or pouch-intestinal fistulas, afferent limb strictures, and granulomas on pouch or small bowel histology remote from the anastomosis. For the purpose of this study, we considered the diagnosis of CD-like complication only after symptoms developed more than six months post restoration of gastrointestinal continuity.

4.2.3 Patient Demographics

Demographic data and clinical parameters of disease were obtained by reviewing paper and electronic patient charts. These included (1) gender, family history, date of IBD diagnosis, prior number of surgeries, and smoking history; 2) extent of disease, defined by surgical pathology; 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab, and steroid use; 4) frequency of pouchitis attacks and medications used in management; and 5) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. (Table 4.2) The study was approved by The Milton S. Hershey Medical Center Institutional Review Board.

Table 4.2. Patient Demographics					
	CD-like (N=26)	Severe Pouchitis (N=12)	Mild Pouchitis (N=41)	Asymptomatic Pouch (N=63)	p-Value*
Gender:					
Female/Male	10/16	4/8	21/20	20/43	0.25
IBD Family History:					
No/Yes	23/3	7/5	33/8	46/17	0.17
IPAA Stages:					
I	2	0	1	7	0.23
II	16	7	29	42	0 .81
Modified II	3	2	4	4	0.66
III	5	3	7	10	0.89
Mean Pouch Length (cm±SD)	21±5	24±9	21±4	21±3	0.24
Average Time to Surgery after IBD diagnosis(yrs ±SD)	6.2±6.42	6.5±6.42	9.0±9.17	10.3±8.10	0.15
Average Age of IBD at Diagnosis (yrs±SD)	31.0±10.56	36.0±13.69	29.5±11.04	29.0±10.85	0.23
Total Time of Follow-up since the time IPAA (yrs±SD)	10.0±5.55	7.5±4.98	8.0±3.77	6.0±4.77	0.002
Smoking:					
Current	1	0	4	4	0.5954
Ex-smoker	11	6	12	13	0.07696
Never	14	6	25	46	0.2058

*A 4-sample of equal proportions and a one-way analysis of variance was used to test for significant differences in patient demographics over the four clinical subgroups.

4.2.4 DNA/Cell Bank

Patients were recruited into our institutional review board-approved genetic IBD cell/DNA bank originally established in 1998. Informed consent was obtained and patients donated blood samples that were then used to create immortalized B-cell lines using Epstein Barr virus (EBV) that provided for an indefinite source of DNA from each recruited individual. In brief, blood was diluted with sterile phosphate-buffered saline (PBS), and layered onto Ficoll-Paque (Amersham Biosciences). The blood-Ficoll gradient was centrifuged at 1500 rpm for 30 minutes at room temperature. The mononuclear cell interface was extracted and washed with PBS. The washed cell pellet was resuspended in RPMI-1640 (VMR) media containing 12% Fetal bovine serum (Gemini Bioproducts) and 25% EBV supernatant. Inoculated cells were incubated at 37°C in a CO_2 incubator. Once transformed, cells were stored at $1x10^7$ cells/mL in 1-mL aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks till use.

4.2.5 DNA Isolation

DNA was extracted from 1 x 10^6 transformed B cells using a DNA isolation kit (QIAAmp DNA Blood Midi Kit, QIAGEN) following the manufacturer's recommended protocol. DNA was quantified by use of a spectrophotometer at 260 nm. A working solution of DNA was then created by diluting the sample with Tris-EDTA to create stock of 10 ng/μL.

4.2.6 Genotyping

With the help of Illumina® (Illumina, San Diego, CA)[(Gunderson KL 2005)], we developed a customized DNA microarray specific for 83 SNPs previously identified by genome wide association studies (GWAS) to be associated with IBD (Table 4.3). This platform allows for high-throughput genotyping for 96 DNA samples at once interrogating for all 83 SNPs simultaneously. dsDNA concentrations were optimized using an ultrasensitive fluorescent nucleic acid stain Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The samples were then run on Illumina's BeadXpress Reader in Penn State Hershey Medical Center's Functional Genomics Core Facility.

Table 4.3. List of Gene/SNP on Illumina Array		
SNP	Chromosome	GENE
rs2476601	1p13	PTPN22
rs3737240 rs13294	1q21	ECM1
rs3180018	1q22	SCAMP3, MUC1
rs2274910	1q23	ITLN1
rs4656940	1q23	*CD244*, ITLN1
rs1801274	1q23.3	*FCGR2A*
rs9286879	1q24	1q24
rs7517810	1q24	*TNFSF18, TNFSF4, FASLG*
rs1998598	1q31	*DENND1B*
rs1363670	1p31	IL-12B
rs3024505	1q32	IL-10
rs2797685	1p36	*VAMP3*
rs6426833 rs10753575 rs3806308	1p36	RNF186
rs10733113	1q44	*NLRP3*
rs917997	2q11	IL-18R
rs13003464	2p16	*PUS10*

rs10181042	2p16b	*C2orf74, REL*
rs12612347	2q35	ARPC2
rs2241880 rs3828309 rs3792109	2q37	ATG16L1
rs780094	2p23	*GCKR*
rs6738825	2q33	*PLCL1*
rs3197999	3p21	MST1
rs4833103	4p14	TLR1, TLR10, TLR6
rs7720838 rs4613763	5p13	PTGER4
rs17234657 rs9292777	5p13.1	
rs10044354	5q15	ARTS-1
rs1050152	5q31	OCTN1
rs2522057 rs10077785	5q31	IBD5
rs10045431 rs6887695	5q33	IL-12B
rs13361189 rs4958847 rs11747270 rs7714584	5q33	IGRM
rs9268480	6p21.3	BTNL2
rs1059276	6p	MEP1A
rs7746082 rs2844480 rs9271568	6q21	HLA region

rs3794996 rs2395185 rs3763313 rs660895		
rs6908425	6p22	CDKAL1
rs17309827	6p25	*SLC22A23*
rs2301436 rs1456893	7p12	CCR6
rs7849191 rs10758669	9p24	JAK2
rs3810936 rs6478108 rs7848647 rs7869487	9q32	TNFSF15
rs4986790	9q32-q33	TLR 4
rs10870077	9q34.3	CARD9
rs3936503	10p11	CCNY
rs10761659 rs10995271	10q21	ZNF365
rs1248696 rs1248634	10q23-q24	DLG5
rs10883365 rs7081330	10q24	NKX2-3
rs951199	11p15.1	NELL1
rs11175593	12q12	LRRK2
rs2066844 rs2066845 rs2066847	16q12	NOD2

rs916977	15q13.1	HERC2
rs7712957	15q13	S100z
rs744166	17q21	STAT3
rs363617	17q24.3	TNFR2
rs2542151	18p11	PTPN2
rs762421	21q22	ICOSLG
rs12704036		U7
rs6927210		U6

4.2.7 Statistical Analysis

The R statistical software system (version 2.13.0, http://www.r-project.org/) was used to perform statistical analysis. A 4-sample of equal proportions and a one-way analysis of variance was used to test for significant differences in patient demographics over the four clinical subgroups (Table 4.2). Statistical assessment of the genetic associations was evaluated with the Fisher's exact test with power to detect general forms of genetic associations. The SNPs identified as statistically associated with each of CD-like and severe pouchitis groups were used in a multivariate logistic regression model. A backward stepwise selection algorithm was implemented to remove redundant SNPs and yield a parsimonious model with two SNPs in each model. The multivariate logistic regression model yielded probability equations used to predict overall chance of a positive or negative outcome from the SNP-based genetic signature. The final predictive models provide an estimate of the probability a person with a certain genetic signature would display symptoms of the specific modeled complication after undergoing IPAA.

4.3 RESULTS

I. Genetic correlation with Pouchitis and CD-like complications after IPAA

A total of 142 IPAA patients (55 females) were recruited for this study with an overall mean follow up time from the creation of the pouch of 7.4±4.9 years. A detailed account of clinical and demographic data is summarized in Table 2. Patients in the CD-like group developed symptoms within an average of 18 ± 4 months after stoma closure. These patients suffered from pouch-enteric fistulae (n=12), pouch inlet stricture (n=11) and granulomatous inflammation (n=3). (Table 4.1)

Table 4.4 lists the top 6 genes and their respective SNPs with p-values for each sub-group. The genes *ATG16L1*, *U6* and *JAK2* were most significantly associated with MP but their p-values were no better than 0.02. The highest statistical correlation was found in the SP and CD-like complication groups most likely due to the more clearly defined phenotype in these two groups as opposed to the mild pouchitis group. *NOD2*, *TNFSF15* and *S100Z* were associated with SP and the *10q21,CARD9* and *TLR* genes were strongly associated with CD-like complications. Of the top 3 genes for each group, only one has been shown to be associated with only UC (*S100Z*), four are associated with both CD and UC (*JAK2, CARD9, TLR, TNFSF15*); however, the majority are associated with Crohn's disease only (*ATG16L1, U6, NOD2,* and *10q21*).

Table 4.4. Top 6 SNP/Genes For each Sub-group				
Group	# pts N= 142	SNP's	Gene	p- Value
Asymptomatic	63	-	-	-
Mild Pouchitis	41	rs2241880	ATG16L1	0.02
		rs6927210	U6	0.03
		rs7849191	JAK2	0.04
		rs17309827	C6orf85	0.05
		rs10758669	JAK2	0.06
		rs1004819	IL23R	0.08
Severe Pouchitis	12	rs2066844	NOD2	0.0008
		rs7869487	TNFSF15	0.01
		rs7712957	S100Z	0.02
		rs7848647	TNFSF15	0.07
		rs1050152	OCTN1	0.07
		rs6908425	CDKAL1	0.09
CD-like complications	26	rs10761659	10q21	0.006
		rs10870077	CARD9	0.018
		rs4833103	TLR	0.02
		rs3810936	TNFSF15	0.02
		rs17234657	5p13.1	0.04
		rs4613763	PTGER4	0.04

Specific allelic SNP determinants in 9 individual genes/loci appear to correlate with specific complications after IPAA. The majority of these genes have been previously associated with CD and play a role in enteric bacterial recognition and destruction (NOD2, ATG16L1, TLR, CARD 9) and/or NF-kappa B activation (NOD2, TNFSF15). The highest statistical significance was found in the CD-like and severe pouchitis groups. This could be due to the more clearly defined phenotype in these two groups, but may also reflect a more Crohn's-like pathophysiology in these originally diagnosed UC patients.

II. Gene Signature modeling to predict Risk of Crohn's Disease like Complications or Severe Pouchitis after Ileal Pouch Anal Anastomosis.

In order to develop a gene signature to predict adverse outcome after IPAA, we first combined the asymptomatic pouch and MP groups together to form our 'Favorable outcome' group since these two groups are considered to be acceptable outcomes post operatively as well as to increase the total number within the group which would aid in a more robust statistical calculation (N=104, defined as no CD-like complications or SP for at least 2 yrs after IPAA). This combined group was compared to the SP group and the CD-like groups individually to develop the gene signature equations (Table 5).

Table 4.5. Top SNP/Gene's Comparing Severe Pouchitis and CD-like to Favorable group

Group	# pts N= 142	SNP's	Gene	p- Value
Favorable Outcome (Asymptomatic pouch + Mild Pouchitis)	104	-	-	-
Severe Pouchitis	12	rs2066844 rs7869487 rs1050152 rs7848647	NOD2 TNFSF15 OCTN1 TNFSF15	0.003 0.011 0.058 0.064
CD-like complications	26	rs10761659 rs4613763 rs3810936 rs17234657 rs4833103	10q21 PTGER4 TNFSF15 5p13.1 TLR	0.006 0.007 0.011 0.011 0.014

Asymptomatic pouch and Mild Pouchitis groups were combined to form a 'Favorable Outcome'. This combined Favorable Outcome group was then compared to the Severe Pouchitis and the CD-like groups and the top 2 SNPs associated with each of these complications were used to develop gene signature equations.

The top 2 SNPs identified as statistically associated in an independent fashion with each of CD-like and SP in this analysis were then used in a multivariate logistic regression model. These SNPs were used to create probability equations to predict overall chance of a positive or negative outcome for that complication. The top 2 SNPs for CD-like complications were in the 10q21 locus and the gene for PTGER4 (p= 0.006 and 0.007 respectively) while for SP it was NOD2 and TNFSF15 (p= 0.003 and 0.011 respectively). Table 4.6 summarizes the respective predictive formulae and the overall probability of suffering these specific complications based on the various genotype combinations for each gene under each probability equation. These probability equations suggest that the risk of these two complications would markedly increase with increasing number of risk alleles, going as high as 92% for SP and 65% for CD-like complications should both genes in each equation be homozygous for the deleterious or 'at risk' allele.

The mean follow-up time for the combined 'Favourable' group was 6.6± 4.5 years and was not statistically significant when compared to the SP group (7.6±5.0 years, p=0.51). However, the follow up time for the Favorable group was significantly different when compared to the CD-like complication group (10±5.6 years. P=0.005). Since it is unlikely but possible that a patient in the Favourable group may change their phenotype to CD-like with longer follow-up, two additional statistical analyses were performed to evaluate the possible effect of this follow up time difference, by weighting the individual contributions of the patients in the logistic regression model by 1) a linear weighting system proportional to follow up time (ie a patient with ten years of follow up would be weighted twice as much as a patient with 5 years of follow up) and 2) a non-linear log of follow up (where a 10 year follow up patient would get 34% more weight than a 5 year follow up and a 5 year follow up would get 64% more weight than a 2 year follow up). The weighted logistic regression to adjust for follow-up time has been shown to yield informative and reasonable estimates involving patients with variable lengths of follow-up compared to conventional logistic regression and Kaplan-Meier estimation[Hsu CH 2007. Fang MC 2008]. These additional analyses yielded only minor changes in the CD-like gene signature and did not change the order or the identification of the most relevant SNP's, therefore confirming the validity and relative accuracy of the phenotype characterization in spite of the different follow up times.

Table 4.6: Gene Signature Models for Predicting Severe Pouchitis and CD-like complications

Severe Pouchitis Gene Signature
Probability = $\text{logit}^{-1}[-2.9 + 2.3(\text{NOD2}) + 0.3(\text{TNFSF15})]$
Number of 'at risk' alleles (0, 1, or 2) in respective SNP/Gene

# of risk alleles NOD2	# of risk alleles TNFSF15	Probability of Severe Pouchitis (%)	95% Confidence Interval	Patient Distribution (N=12)
0	0	5.8	0.6 – 11.0	****
0	1	7.7	1.5 – 13.9	*
0	2	10.1	0.0 – 24.7	**
1	0	38	8.3 – 67.7	****
1	1	45.3	14.4 – 76.3	
1	2	52.8	6.2 – 99.5	*
2	0	85.9	56.0 – 100	
2	1	89.2	65.2 – 100	
2	2	91.8	70.1 - 100	

Table 4.6: Gene Signature Models for Predicting Severe Pouchitis and CD-like complications (continued)

CD-Like Complication Gene Signature Probability = logit^{-1}[−2.7699 + 0.7746 (10q21) + 0.9131(PTGER4)] Number of 'at risk' alleles (0, 1, or 2) in respective SNP/Gene				
# of risk alleles 10q21	# of risk alleles PTGER4	Probability of CD-like complication (%)	95% Confidence Interval	Patient Distribution (N=26)
0	0	5.9	0.0 - 12.2	
0	1	13.5	0.4 - 26.6	
0	2	28	0.0 - 59.9	
1	0	12	5.1 - 18.9	********
1	1	25.3	12.7 - 37.9	*****
1	2	45.8	13.1 - 78.4	**
2	0	22.8	10.0 – 35.6	*******
2	1	42.4	23.6 - 61.2	***
2	2	64.7	32.9 - 96.5	*
Predictive formulae and overall probability of suffering these specific complications based on the various genotype combinations for each gene are shown. These probability equations suggest that the risk of these two complications would markedly increase with increasing number of risk alleles, going as high as 92% for SP and 65% for CD-like complications. The distributions of the patients in the present study are listed on the right.				

4.4 DISCUSSION

Similar to most other immune mediated diseases such as multiple sclerosis, systemic lupus erythematosus, type 1 diabetes mellitus and rheumatoid arthritis, IBD is a 'complex multigenic' disease that does not follow a 'one gene, one disease' Mendelian pattern of inheritance. These disorders are difficult to classify due to variations in severity of symptoms, clinical features, variable prognosis and age of onset.[Tabor HK 2002] Such complex diseases tend to involve an unknown number of multiple genes that affect various biological pathways and usually interact with a variable number of environmental factors.[Motulsky AG 2006. Moonesinghe R 2010]This wide genetic variation presumably results in the diverse clinical phenotype that is seen in IBD. A single polymorphism is estimated to account for 1-8% of the overall disease risk in such complex diseases.[Moonesinghe R 2010] With the advent of genome wide association studies (GWAS) approximately 83 SNPs within 55 genetic loci have been shown to be associated with IBD (at the time of this study). Genomic profiling is the concept by which multiple genetic loci are tested simultaneously thereby resulting in a more robust prediction of disease outcome.[Moonesinghe R 2010. Yang Q 2003. Evans DM 2009]Therefore, this study investigated 142 UC patients who underwent total proctocolectomy with IPAA and correlated 83 IBD SNPs with specific post operative complications. The identified at risk SNPs were then utilized in a gene signature fashion to predict the likelihood of developing these complications (SP and CD-like) based on genotype in a theoretical attempt to fashion a preoperative decision making aid.

We first sought to determine which SNPs/genes correlated with various pouch associated complications. Our patients were first subcategorized into mild pouchitis, severe pouchitis and CD-like complication groups. This yielded several SNPs that were uniquely associated with each subgroup. In the mild pouchitis group, the most significant SNP was rs2241880 (a threonine-to-alanine substitution at amino acid position 300 of the protein- T300A) within the autophagy-related 16-like 1 (*ATG16L1*) gene found on chromosome 2q37.1.This CD associated gene was first discovered by Hampe *et al*[Hampe J 2007]in 2007 and has since been replicated in several other Caucasian populations.[Rioux JD 2007. Wellcome Trust Case Control Consortium 2007. Baldassano RN 2007]This gene has been shown to be intimately associated with the process of autophagy, a process by which cells remove unwanted cytoplasmic waste materials such as damaged organelles, apoptotic bodies, intracellular viruses, bacteria and parasites by sequestering them into double membrane autophagosomes which are then presented to

lysosomes for degradation.[Fritz T 2011] A deficiency in *ATG16L1* gene is associated with an aberrant formation of the autophagosome and thus in the compromised clearance of unwanted intracellular waste.[Hruz P 2010] Kuballa *et al* studied the role of this T300A variant in human intestinal epithelial cells (Caco-2) and showed the inability of these cells to form autophagosomes around internalized *Salmonella typhimurium.*[Hruz P 2010. Kuballa P 2008] IBD patients carrying the SNP rs2241880 are more prone to ileal CD with no association seen with UC patients.[Prescott NJ 2007] Recent studies have shown that CD patients who are homozygous for the *ATG16L1* risk allele (G) have Paneth cell granule abnormalities that lead to increased expression of acute phase reactants and cytokines such as IL-1β and Il-18 which potentiate the inflammatory response.[Cadwell K 2008. Saitoh T 2008] Even though statistical significance was found within the mild pouchitis group, the lowest p-value was no better than 0.02 most likely owing to the relatively vague definition of this group, that may represent the inclusion of patients with other causes for mild diarrheal states, such as viral infections or dietary indiscretion. Thus this correlation has probably the least pathophysiologic relevance but nonetheless suggests a possible mechanism for mild pouchitis that can direct future research.

The highest statistical correlation was found in the SP and CD-like complication groups most likely due to the more clearly defined phenotype in these two groups as opposed to the mild pouchitis group. The *NOD2* gene highly correlated with SP (p=0.0008) as described in our previously published study.[Sehgal R 2010] This finding has recently been replicated in a much larger retrospective multicenter study.[Hugot J 2001] The *NOD2* gene which is found on chromosome 16 was first discovered to be associated with CD in 2001.[Ogura Y 2001. Hampe J 2001. Rogler G 2010] *NOD2* mutations have been found in up to 40% of European and North American CD patients compared with 10% to 15% of the healthy population.[Spehlmann ME 2009] The protein product of this gene acts as a sensor for the bacterial cell wall component muramyl dipeptide (MDP), which is found on both Gram positive and Gram negative bacteria. Activation of this protein leads to an increase in nuclear factor κB and mitogen activated protein (MAP) kinase signaling pathways, which play key roles in orchestrating the inflammatory and immune response by regulating genes involved in producing several proinflammatory cytokines such as IL-8, IL-6, TNF-α and antimicrobial peptides.[Brain O 2010] Furthermore, *NOD2* has also been shown to promote the process of autophagy mediated endocytosis.[Abreu MT 2002] Increasing number of *NOD2* mutations have repeatedly been shown to be correlated with ileal involvement, early age of onset, and stricturing and/or penetrating CD phenotype.[Cuthbert AP

2002. Hampe J 2002. Lesage S 2002. Haveran LA 2011) How such a genetic defect relates pathophysiologically to SP is unclear, but correlates with the observation that severe pouchitis frequently requires treatment of both enteric bacteria (with antibiotics) and host inflammation in the pouch using ASA derivatives, or even immunosuppressive medications (Yamazaki K 2005) suggesting its pathophysiology is more related to a host immune defect than simply enteric super infection.

Tumor necrosis factor (ligand) superfamily, member 15 (TNFSF15) is unique as it is the only gene to be associated with CD in both Asian and Caucasian populations.(Endo K 2010)TNFSF15's protein product, vascular endothelial cell growth inhibitor (VEGI) is expressed in endothelial cells, lymphocytes, plasma cells, monocytes, and dendritic cells and can bind to the death domain receptor 3 (DR3).(Haritunians T 2011) Microbial stimulation of Toll-like receptors causes an up-regulation of this complex that activates the NF-kappa-B and MAP kinase pathways, thereby leading to the increased production of the anti-apoptotic proteins, interferon gamma (IFN-γ) and IL-8 cytokines. (Haritunians T 2011)

The rs10761659 SNP within the 10q21 gene locus most significantly correlated with CD-like complications (p=0.006). Several European GWA studies have identified SNPs within this locus to be associated with CD in a region closely related to the zinc finger protein 365 (ZNF365) gene.(Rioux JD 2007. Wellcome Trust Case Control Consortium 2007. Vermeire S 2010) Data regarding the function of this gene locus is largely lacking and other causal variants remain to be identified. Recently, Haritunians *et al* provided strong evidence for the ZNF365 gene being responsible for CD susceptibility in the 10q21 locus. They identified the major allele (G) of SNP rs7076156 in ZNF365 isoform D to be strongly associated with CD. This SNP rs7076156 was in linkage equilibrium with other previously identified CD associated SNPs within 10q21 including rs10761659 that was investigated in this study. The SNP rs7076156 in ZNF365 isoform D alters the expression of several genes, including the transcription factor ZNF148 which is known to play a role in gut homeostasis.(Vermeire S 2010)

Even though GWA studies have provided great insights into IBD by identifying specific pathways of immune function and inflammation, little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making. Several studies have attempted to use clinical parameters and serological markers as prognosticators in IBD, however these are largely inconsistent and none are able to accurately predict the disease course pre-

operatively. Using genetics as a marker for predicting outcomes is an attractive prospect as these markers are present long before the onset of disease and remain stable during disease flares.[Weedon MN 2006] The majority of SNPs that have been implicated in IBD so far carry only a low to moderate overall risk for developing disease and predicting outcome with an allelic odds ratio less than 1.5.[Weersma RK 2009. Haritunians T 2010]However an increase in the overall relative risk for developing IBD has been shown when several low-penetrance SNPs are combined together. For example, Weersma *et al* showed a combined increase in the number of risk alleles (*NOD2, IBD5, DLG5, ATG16L1, IL23R*) had a much greater risk for developing CD and with a more severe disease course as seen by the greater need for surgery, a stricturing and/or penetrating behavior and an earlier age of disease onset.[Weersma RK 2009. Haritunians T 2010]In our study, we attempted to combine the top two SNPs identified as statistically associated with each of CD-like and SP group to create probability equations which could theoretically be used to predict overall chance of a positive or negative outcome for that complication based on genotype. The top 2 SNPs used for the gene signature were dissimilar to the SNPs determined in Table 4.5 as the order of significance changed once the Asymptomatic and MP groups were combined together to form the 'Favorable' group. Additionally, the gene signature was constructed from a multivariate model that selected out redundant SNPs due to linkage or correlation of the SNPs remaining in the model thus yielding a parsimonious genetic signature. The SNPs that most strongly correlated with SP compared with the Favorable group were *NOD2* and *TNFSF15* while for CD-like complications the *10q21* locus and *PTGER4* were most significant. These gene-signatures predict that the overall risk for developing these complications increases markedly as the number of risk alleles increases. Individuals who would be homozygous mutant for both genes will have a 65% probability of for developing CD-like complications and be at a 92% risk for of developing SP. Should such predictions be borne out by prospective studies of patients undergoing the IPAA by preoperative genotyping, the utility of such formulae in assisting the surgeon in preoperative decision making could be powerful indeed, especially since genotyping would be free of variability commonly seen in serologic or other clinical measurements.

Phenotype can change over time, however our average follow up for the asymptomatic pouch and mild pouchitis groups was on average 6 and 8 years respectively thus giving us confidence that our clinical classification for the large majority of these patients was robust,

especially since the average time to the development of CD-like complications, severe and mild pouchitis averages 3.2, 3.2 and 2.8 years respectively [(Sehgal R 2010)]. Furthermore, performing additional statistical analyses using a weighting system based on individual patient follow up times in the groups with statistically significant different follow up times, yielded the same identity and priority of SNP's in the gene signatures, confirming that our phenotype characterization with this length of follow up was accurate.

Several other studies have attempted to use data from GWA studies to predict outcome in complex diseases. Haritunians *et al* developed a genetic scoring system for predicting the need for surgery in medically refractory UC patients. A combination of 46 SNPs were associated with medically refractory UC and genetic risk scores were calculated from the total number of risk alleles (0,1 or 2) with greater risk of colectomy associated with an increased number of risk alleles present in their model.[(Haritunians T 2010)]Very interestingly, the gene loci found to be most predictive of lack of response to medical management and thus requiring colectomy were the major histocompatibility complex (MHC) and TNFSF15, as we found in the SP group in our present study. TNFSF15's protein product is a vascular endothelial cell growth inhibitor (VEGI), an anti-angiogenic protein. This suggests that severity of disease whether it be UC, needing colectomy or severe pouchitis, in part relates to the process of healing and angiogenesis. Thus alternative forms of pouchitis therapy that target apparent defects in healing may be proposed. Similarly, Weedon *et al* studied the effects of several known type 2 diabetes SNPs on disease risk in a large case control study. They showed that combining information from several known common risk variants allowed for better identification of patients at higher risk of developing type 2 diabetes when compared to assessment using single variants.[(Weedon MN 2006)] Furthermore, Yang *et al* have showed a substantial improvement in the ability to predict the risk of developing a multifactorial complex disease by using both a panel of genetic variants and environmental factors concurrently, as compared to using only one parameter.[(Yang Q 2003)]

Though our results are exciting and represent essentially the first attempt to use genetics in the surgical prognosis and decision making process of the IBD patient, they should nonetheless be viewed as preliminary. This was a relatively small study done retrospectively in a unique demographic group of patients from the Central Pennsylvania area of the United States. Other genetic associations, specifically the NOD2/CARD15 association with ileal CD, for example, was not replicated in a Japanese population[(Yamazaki K 2002)] exemplifying the need

to carefully control for ethnicity, demographics and possibly even environmental factors that may affect the expression of disease causing genetic determinants.

In conclusion, this study has utilized data from GWA studies to identify genetic variants that correlate with complications after IPAA. The majority of these genes have been previously associated with CD and play a role in enteric bacterial recognition and destruction. The *NOD2* and *TNFSF15* gene had the highest correlation with SP. The 10q21 gene locus and PTGER4 most statistically significantly correlated with post IPAA CD-like complications. Similar to other complex diseases, the risk for developing these complications markedly increased when greater number of risk alleles were present. This small retrospective study needs to be confirmed in a much larger cohort of UC patients and can provide the basis for a prospective multicenter trial to evaluate these SNPs in predicting the development of post IPAA complications. Such risk profiling holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD patients. Genetic profiling pre-operatively could both provide improved counseling to patients regarding their prognosis and assist the clinician with choice of surgery, especially in the marginal operative candidate.

Chapter 5

STUDY # 3: Mutations in IRGM are associated with more frequent need for surgery in ileocolonic Crohn's disease patients.

5.1 INTRODUCTION

Crohn's disease (CD) is an idiopathic inflammatory condition affecting the gastrointestinal tract (GI) that most commonly affects young adults and results in lifelong need for medical and surgical care. Within North America, the prevalence of CD ranges from 26 to 200 cases per 100,000 and most commonly presents between the age of 15 and 30 years. The majority of affected patients have a chronic intermittent disease course, with 13% having an unremitting progressive course, while only 10% live in remission.(Loftus EV 2002)

Although medical management helps to control symptoms, up to two-thirds of patients with CD will require surgical resection of the affected bowel during the course of the disease.(Regueiro M 2011) Surgery for CD is by no means curative and recurrence of symptoms is part of the natural history of the disease. Depending on definition, 70-90% of patients have endoscopic evidence of recurrent CD within one year after intestinal resection.(Regueiro M 2011) However, clinically relevant recurrence is often delayed and up to one-third of patients will not develop clinically significant disease for approximately 15-20 years.(Ritchie JK 1990. Lennard-Jones 1967. Bernell O 2000) Such patients, if they could be identified, could avoid the use of potentially dangerous and expensive anti-TNF therapy. Conversely, some patients develop rapid recurrence that can lead to early and even repetitive surgery that can result in short gut over the long term. Such patients would be candidates for more aggressive medical therapy such as anti-TNF therapy. CD patients are at an increased risk of experiencing postoperative recurrence if they are a current smoker, are positive for granulomas, have undergone previous CD related surgery, and have penetrating disease (e.g. fistula formation, perforation, and abscess).(Swoger JM 2010) Ileocolonoscopy in the early post operative course is the only other diagnostic method by which to identify the patient who will develop early recurrence versus the one who will avoid surgery for many years.

Recently, genome wide association studies (GWAS) have allowed the discovery of single nucleotide polymorphisms (SNPs) and corresponding genes associated with CD. At the time of this study, approximately 80 SNP's within 55 genes (Table 5.1) had been identified to be

associated with inflammatory bowel disease (IBD). This wide variability of disease predisposing genetic haplotypes is presumably one of the reasons for the many variations in disease characteristics or clinical phenotype of IBD patients. Correlation of such genotypes with clinical phenotype promises to allow the sub-classification of IBD patients into categories beyond the simple ulcerative colitis (UC) or CD diagnoses. Therefore, the overall aim of this study was to identify genetic determinants (SNPs) that could be markers of CD severity in surgical patients that may assist in surgical and medical decision making. Though there is no one definition for disease severity in CD, the more frequent need for surgery can be generally accepted as reflecting a more severe disease phenotype. Ileocolonic disease is the most common form of CD and ileocolectomy (IC) is the most commonly performed operation. Therefore this study targeted the most commonly performed intestinal surgery done for CD, namely IC, and attempted to define genetic markers associated with the more frequent need for surgery in this CD patient population.

5.2 METHODS

5.2.1 Patient Recruitment

Operative records were retrospectively reviewed and a total of 275 CD patients who underwent ileocolectomy by the Division of Colon and Rectal Surgery at the Milton S Hershey Medical Center, Penn State College of Medicine were identified over a 10 year period (01/1990- 12/2010). A total of 66 patients were subsequently recruited into our IBD registry started in 1998 and make up the study population presented here without selection bias based on follow-up clinic visits during the study period (September 2008 – September 2010).

Severity of CD was defined as number of ileocolectomy procedures done over the time period between disease diagnosis and patient's last clinic visit, the rationale being that more severe disease would be associated with more frequent operations. Only patients with ileocolonic disease were studied since this is the most common form of CD and also provided for a more uniform study population (Figure 5.1).

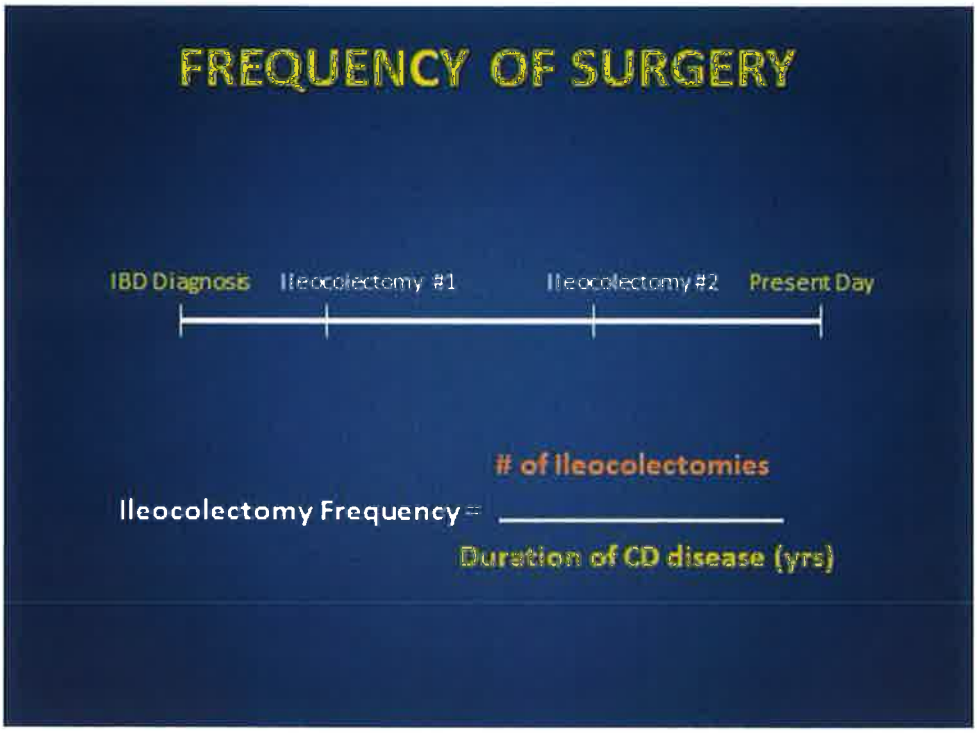

Figure 5.1. The surgical frequency was determined by dividing the total number of IC procedures by the time between IBD diagnosis and the patient's last clinic visit, the rationale being that more severe disease would be associated with more frequent need for surgery.

Patient demographics and documentation of clinical parameters of disease were gathered and included the following: 1) gender, family history, date of IBD diagnosis, prior number of surgeries, and smoking history; 2) Montreal classification (age of diagnosis, disease location, behavior) of disease 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab, and steroid use; and 4) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. The study was approved by The Milton S. Hershey Medical Center Institutional Review Board.

5.2.2 DNA/Cell Bank

The identified CD ileocolectomy patients were recruited into our institutional review board-approved genetic IBD cell/DNA bank originally established in 1998. Informed consent was obtained and patients donated blood samples that were then used to create immortalized B-cell lines using Epstein Barr virus (EBV) that provided for an indefinite source of DNA from each recruited individual. In brief, blood was diluted with sterile phosphate-buffered saline

(PBS), and layered onto Ficoll-Paque (Amersham Biosciences). The blood-Ficoll gradient was centrifuged at 1500 rpm for 30 minutes at room temperature. The mononuclear cell interface was extracted and washed with PBS. The washed cell pellet was resuspended in RPMI-1640 (VMR) media containing 12% fetal bovine serum (Gemini Bioproducts) and 25% EBV supernatant. Infected cells were incubated at 37°C in a CO_2 incubator. Once transformed, cells were stored at $1x10^7$ cells/mL in 1-mL aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks till use.

5.2.3 DNA Isolation

DNA was extracted from 1 x 10^6 transformed B cells using a DNA isolation kit (QIAamp DNA Blood Midi Kit, QIAGEN) following the manufacturer's recommended protocol. DNA was quantified by use of a spectrophotometer. A working solution of DNA was then created by diluting the sample with 10mM Tris-HCl to create stock of 10 ng/ μL.

5.2.4 DNA Genotyping using Illumina® BeadExpress

With the help of Illumina (Illumina, San Diego, CA), we developed a customized DNA microarray specific for 83 SNPs previously identified by GWAS to be associated with IBD (Table 4.3 pg. 106-108) [7-22]. dsDNA concentrations were quantified using an ultrasensitive fluorescent nucleic acid stain Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen™). The samples were then run on Illumina's BeadXpress Reader in Penn State Hershey Medical Center's Functional Genomics Core Facility.

5.2.5 Statistical Analysis

Severity of CD in the individual ileocolonic patients was quantified by dividing the total number of ileocolectomies by the time between IBD diagnosis and the patient's last clinic visit (Figure 5.1). A log transformation of severity was performed in order to yield a more homogenous and more robust measure of severity. Treating log-severity as the primary outcome, three genetic models (generic model, additive model, and dominant model) were considered to identify a possible genetic association with severity (Table 5.2).

Table 5.2. Three Genetic Models for identifying possible genetic association with severity
Generic Model: AA, AB, BB
Additive Model: AA > AB > BB
Dominant Model: AA+AB > BB

Three genetic models were considered to identify a possible genetic association with frequency of surgery. The three models are powered for different types of genetic associations. The generic model is powered to detect an imbalance in any of the three genotypes; the additive model is powered to detect an increasing or decreasing risk with the number of 'A' alleles; the dominant model is powered to detect a change in risk with one or both risk alleles present.

The regression-based genetic models accounted for potentially confounding variables such as age, sex, smoking, location of disease and behavior, and a stepwise model selection procedure was used to retain only the most relevant covariate variables. The Benjamini & Hochberg false discovery method was used to provide adjustments of the p-values for multiple comparisons.[Benjamini Y, Hochberg Y. 1995) Each of the three genetic models involved the testing of 83 SNPs each, therefore a total of 249 hypotheses were considered. Due to the

high level of correlation among the SNPs and among the three different models, this multiple adjustment correction is expected to be highly conservative. The p-value adjustments were implemented by using the p.adjust function within R (www.r-project.org). The R software is a language and environment for statistical computing and graphics. R provides a wide variety of statistical (e.g. linear and nonlinear modeling, classical statistical tests, time-series analysis, classification, clustering etc) and graphical techniques.

5.3 RESULTS

A total of 66 (30 male, 36 female) CD patients who underwent ileocolectomy were recruited for this study. The mean follow up time from the time of disease diagnosis was 14.7 years. The average number of ileocolectomies per patient was 1.7 (range 1-5). The majority of patients had their disease onset within the 17-40 years age group (A2), had predominantly purely ileal involvement (L1) of stricturing type (B2) based on the Montreal classification system. The remaining demographic information is summarized in Table 5.3.

Table 5.3. Patient Demographics	
Gender:	
Male	30
Female	36
Smoker:	
Current	15
Ex-smoker	21
Never	30
FHx of IBD:	
Yes	34
No	32
Medications:	
No IFX/AZA	14
IFX only	7
AZA only	15
Both (IFX/AZA)	30
Montreal Classification:*	
Age: A1/A2/A3	8/54/4
Location: L1/L2/L3	38/0/28
Behavior:B1/B2/B3	5/35/26
Indication for Surgery:	
Fistula	7
Obstruction/Pain	37
Abscess	8
Failed Medical Management	14

*Montreal Classification: Age of IBD diagnosis (A1= below 16yr, A2= between 17-40yrs, A3=above 40yrs); Location (L1= ileal, L2=colonic, L3=ileocolonic); Behavior (B1= non-stricturing, non-penetrating, B2=stricturing, B3=penetrating)

Of the 83 SNPs within 55 IBD associated genes that were interrogated, both SNPs (rs4958847, rs13361189) within the *IRGM* gene, *10q21* (rs10761659), *ATG16L1* (rs2241880), *S100Z* (rs7712957) and *IBD5* (rs10077785) remained highly significant irrespective of the genetic model used. The conventional statistical p-values for these gene/SNPs are listed in Table 5.4. The SNP rs4958847 within the IRGM (immunity-related GTPase family, M) gene was the most significant SNP in all three genetic models ($p = 0.007$, Table 5.4) and remained significant even after applying a Benjamini-Hochberg false discovery method for multiple observations. However there were no patients homozygous for the rs4958847 SNP in our patient cohort making it impossible to assess which genetic model (genetic, additive, or dominant) was responsible for its effect. Patients carrying the 'at risk' allele for this SNP (N=20) had an average of 1 surgery every 6.87±1.33 years as compared to patients carrying the wild type genotype (N=46) who averaged 1 surgery in 11.43±1.21 years (p-value = 0.007, Mann–Whitney *U* test) (Table 5.5).

Patients with ileocolonic disease commonly have a NOD2 mutation. The overall frequency of NOD2 mutations for the entire cohort was 30% (N=20, Table 5.5). There was no correlation between IRGM SNP rs4958847 with NOD2 status as there was 25% NOD2 positivity in the 'at risk' IRGM group compared to 35% in the IRGM wild-type group (p-value = 0.937, Table 5.5).

Table 5.4

Top SNPs: General Genetic Model			
SNP name	Gene Name	p-Value	p-Value after FDR correction
rs4958847	IRGM	0.00009	0.00712
rs13361189	IRGM	0.00738	0.291
rs10761659	10q21	0.0255	0.496
rs2241880	ATG16L1	0.0262	0.496
rs7712957	S100Z	0.032	0.496
Top SNPs: Additive Genetic Model			
rs4958847	IRGM	0.00009	0.00712
rs13361189	IRGM	0.00738	0.291
rs7712957	S100Z	0.032	0.758
rs10758669	STAT3	0.0506	0.758
rs10077785	IBD5	0.0562	0.758
Top SNPs: Dominant Genetic Model			
rs4958847	IRGM	0.00009	0.00712
rs10761659	10q21	0.00705	0.194
rs13361189	IRGM	0.00738	0.194
rs10077785	IBD5	0.0241	0.334
rs2241880	ATG16L1	0.0268	0.334

Several SNP/Genes were shown to be highly significant by using conventional statistics. However, when performing a false discovery rate (FDR) correction for multiple comparisons, only SNP rs4958847 within the IRGM gene remained significant.

Table 5.5

SNP	Genotype*	N	Surgical Frequency (yrs ± SEM)	P-Value	NOD2** mutations
rs4958847	IRGM mutation	20/66	6.87±1.33	0.007	(N=5) 25%
	IRGM wild-type	46/66	11.43±1.21		(N=15) 33%

*'IRGM mutation' represents patients carrying at least one copy of the at-risk allele. 'IRGM wild-type' represents homozygous wild-type.
** Patients with ileocolonic disease commonly have a NOD2 mutation. There was no correlation between IRGM SNP rs4958847 with NOD2 status (p-value = 0.94)

5.4 DISCUSSION

The completion of the International Haplotype Map (HapMap) Project in 2005 and advancements made in microarray-based technologies, promoted interest in identifying the possible etiology of IBD by identifying SNPs and corresponding genes associated with IBD. At the time of this study approximately 80 SNP's within 55 genes had been associated with IBD.[Lees CW 2009. Wellcome Trust Case Control Consortium 2007. Barrett JC 2008. Duerr RH 2006. Fisher SA 2008. Franke A 2008. Hampe J 2007. Libioulle C 2007. Parkes M 2007. Silverberg MS 2009. Mathew CG 2008. Noomen CG 2009. Huebner C 2010. Scherr R 2009. Nakagome S 2010. McGovern DP 2010.] This potential wide variability of genotype is presumably the reason for the many variations in disease characteristics or clinical phenotype of IBD patients. Correlation of such genotypes with clinical phenotype promises to allow for the sub classification of IBD patients into categories beyond the simple ulcerative colitis (UC) or CD categories. In the extreme, genotype may segregate and predict patients' disease characteristics such as anatomic location, behaviour (fistulising vs. stenosing) and even prognosis. Genotypic determinants may even predict responsiveness to specific therapies, or as we suggest in this study, the early recurrence of disease after surgical resection. Genotype may define a 'rapid recurrence' phenotype that would justify early and more aggressive medical management after surgery. To this end, this study investigated 66 CD patients who underwent surgery in order to identify genetic factors that might correlate with high vs. low risk of CD recurrence after ileocolectomy with the goal of identifying patients at high risk for such recurrence. Thus we used frequency of ileocolectomy as a surrogate for disease severity which was determined by calculating the total number of ileocolectomies from the time of initial IBD diagnosis to the last clinic visit, with the rationale being that the more surgeries a patient has to undergo, the more severe the disease course.

After considering three statistical models to identify SNPs that most significantly correlated with severity of CD, the SNP rs4958847 within the *IRGM* gene was the only SNP to maintain its significance even after Benjamini-Hochberg correction in all three genetic models. The Benjamini-Hochberg method is a conservative correction, particularly since many of the SNPs considered in this study were in linkage disequilibrium, which suggests that the *IRGM* gene is strongly statistically associated with severity. CD patients carrying an 'at risk' allele for SNP rs4958847 had an overall more severe disease course, i.e. greater number of surgeries compared to those who were 'wild-type.'

In general, the *IRGM* gene located on the long arm (q) of chromosome 5 at position 33.1 encodes a p47 immunity-related GTPase family member which is involved in the process of autophagy.(Huett A 2008) Autophagy is a teleologically highly conserved process by which cells maintain intracellular homeostasis by degrading potentially dangerous waste materials such as damaged organelles, apoptotic bodies and intracellular pathogens such as viruses, bacteria and parasites by sequestering them within autophagosomes which are then presented to lysosomes for degradation.(Lees CW 2009) siRNA knockdown of *IRGM* in human macrophages has demonstrated an impaired handling of intracellular pathogens such as *Mycobacterium tuberculosis,* leading to prolonged intracellular survival. (Lees CW 2009. Singh SB 2006)

The two non-coding *IRGM* SNPs (rs13361189 and rs4958847) investigated in this study were first identified in 2007 by the Wellcome Trust Case Control Consortium genome wide association study (GWAS).(Wellcome Trust Case Control Consortium 2007) Several studies have since confirmed the association of these IRGM SNPs with CD in various ethnic cohorts, however the exact functional roles of these SNPs are still unclear despite many resequencing attempts.(Huett A 2008. Roberts RL 2008. Latiano A 2009. Weersma RK 2009. Barrett JC 2008. Roberts RL 2008. Fisher SA 2008. Franke A 2008. McCarroll SA 2008) McCarroll *et al* have recently shown SNP rs13361189 to be in perfect linkage disequilibrium with a 20kb deletion polymorphism that affects the expression of *IRGM.*(McCarroll SA 2008) This deletion polymorphism is associated with a SNP (c.313C>T) that has been shown to alter microRNA miR-196 regulation of *IRGM* expression in CD.(Brest P 2001) Our study investigated both these IRGM SNPs, but only rs4958847 maintained significance after Benjamini-Hochberg correction. The other SNP rs13361189 failed to remain significant most probably due to the smaller number of CD patients within this study population. Latiano *et al* have shown the rs4958847 SNP to be associated with fistulizing behavior including perianal fistulas.(Latiano A 2009)

This study also attempted to correlate the frequency of *NOD2/CARD15* variants with *IRGM,* however failed to show any association with severity of disease. Similarly, Latiano *et al* also failed to show any significant interaction between NOD2/CARD15 and IRGM variants in both of their pediatric and adult populations.(Latiano A 2009)

Other investigators have evaluated other modalities to identify early CD recurrence after ileocolectomy. These include changes seen on endoscopy, radiologic studies and serological biomarkers. Rutgeerts *et al* proposed a five grade scoring system in order to predict CD

recurrence based on endoscopic findings.[Rutgeerts P 1990] Patients who showed no ulcers or ≤5 aphthous ulcers in the neoterminal ileum on ileocolonoscopy at 1 year post surgery were at low risk for clinical recurrence. However, patients who showed >5 aphthous ulcers, deep ulcers with more extensive inflammation or severe inflammation with nodularity and stenosis, had a 3 year clinical or surgical recurrence rate of 15, 40 and 90% respectively.[Swoger JM 2010] Calabrese *et al* showed strong correlation of the Rutgeerts scoring system with small intestine contrast ultrasonography[Calabrese E 2009] and similarly Sailer J *et al* correlated the Rutgeerts score with MR enteroclysis.[Sailer J 2008] Furthermore, a recent study conducted by Lamb CA *et al* has demonstrated that the quantitative measurement of faecal calprotectin and lactoferrin levels significantly correlated with disease recurrence in post operative CD patients. These faecal markers were much more sensitive than C-reactive protein, platelet count or endoscopic appearance at predicting clinical disease activity.[Lamb CA 2009] Although using ileocolonoscopy to predict CD recurrence post operatively does show promise, it is an invasive procedure and still carries potential adverse risk. Radiological modalities are less invasive and certainly ultrasonography is advantageous as it carries no risk of radiation exposure, however none of these modalities help to predict the disease course pre-operatively and especially not prior to the actual recurrence of the disease that is visualized by these tests. The potential advantage of our finding is that high risk patients can be identified with a simple blood test and then undergo the initiation of prophylactic medical management well before the onset of disease recurrence.

This study has identified a genetic variant within the *IRGM* gene that could potentially be used as a predictor of early recurrence after ileocolectomy. This small study will need to be confirmed in a much larger cohort of CD patients and can provide the basis for a prospective multicenter trial incorporating populations from different backgrounds to evaluate the *IRGM* SNP rs4958847 in predicting early disease recurrence after ileocolectomy. Genotyping holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD patients.

Chapter 6

General Discussion and Future Prospects

Idiopathic inflammatory bowel disease is a chronic, relapsing inflammatory condition affecting the GI tract that most often affects young adults and results in lifelong need for medical and surgical care. The overall incidence of IBD, especially the rates of CD, has steadily increased since the 1920s with approximately 100-200 per 100,000 of Western Caucasians living with this debilitating disease.

In the past decade, much research has been carried out to aid in understanding the underlying pathophysiology of IBD. The widely accepted basis for the disease is that there is an abnormal immune response to intestinal microflora in a genetically susceptible patient. There has been an ever increasing list of gene loci, mutations or alleles (SNPs) found using genome-wide association studies that are associated with either or both UC and CD. This large number of disease predisposing mutations (SNPs) is presumably the reason for the wide variation in clinical phenotype seen in IBD. To date, most of the research conducted in the realm of IBD genetics has focused on identifying specific pathways of immune function and inflammation, looking to develop newer medicines. However little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making.

The present series of studies performed in this research project sought to bring surgical relevancy to the recently identified genetic mutations associated with IBD. Studies #1 and 2 focused on UC patients who had undergone the IPAA procedure. These patients were retrospectively classified based on the presence or absence of developing an adverse outcome post-operatively. These studies have identified and validated the association of the *NOD2/CARD15* mutation with severe/chronic pouchitis. Several other genetic loci were also identified and used to develop a hypothetical gene signature model in order to predict the overall probability of developing such post-operative adverse outcomes. The results from these small studies need to be confirmed in a much larger cohort of UC patients by conducting a prospective multicenter study to evaluate these SNPs in predicting the development of post IPAA complications. Such risk profiling holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD

patients. Genetic profiling pre-operatively could both provide improved counseling to patients regarding their prognosis and assist the clinician with choice of surgery, especially in the marginal operative candidate.

In addition, we sought to identify gene/SNPs that correlated with rapid recurrence of CD after ileocolectomy. The frequency of surgery was determined by dividing the total number of ileocolectomy procedures performed by the time between IBD diagnosis and the patients' last clinic visit, the rationale being that more severe disease would be associated with more frequent need for surgery. After genotyping our cohort of CD patients, we identified one SNP (rs4958847) within the IRGM gene that significantly correlated with rapid CD recurrence post-operatively. Similar to studies #1-2, this study has identified a genetic variant that could potentially be used as a predictor of early recurrence after ileocolectomy. This small study will need to be confirmed in a much larger cohort of CD patients and can provide the basis for a prospective multicenter study incorporating populations from different backgrounds to evaluate the IRGM SNP rs4958847 SNP in predicting early disease recurrence after ileocolectomy. Similar to studies #1 and 2, genotyping holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD patients. If borne out, this SNP could be a marker used to predict the need for more aggressive prophylactic medical regimens, such as infliximab (Remicade®).

This series of studies has identified several IBD associated genes that are involved in disease pathogenesis and warrant further investigation. For example, NOD2 and IRGM play an important role in the innate immunity against enteric commensal organisms. However, the exact mechanistic relationship between these two genes still needs to be fully elucidated. More functional studies investigating the downstream effects of MDP stimulation and release of cytokines and their effects on the autophagy pathway are warranted. Furthermore, gene-gene interactions should be studied using statistical models and epistasis analysis. The effects of deficient IRGM and ATG16L1 autophagy genes should be investigated in murine models. In particular, interrogating the effects of certain bacteria in mice deficient in these genes and the resultant enhanced and/or deficient immune response should be investigated.

The field of IBD genetics is only in the nascent stages of correlating such genetic variants with the clinical features of IBD, but, as this is carried out, a more accurate picture of the individual patient and their disease will be created. Mutations in the *NOD2/CARD15* gene,

for example, correlate with disease in the terminal ileal region, usually at a younger age. Similarly, other genetic determinants will be associated with clinical features or disease behavior. Increasingly, patients will be sub classified not just on anatomic location and clinical characteristics (as the Montreal classification for example does), but also on genotype. This will provide a third dimension to the classification of the IBD patient, namely one that relates to a pathophysiologic basis of the disease in a particular patient. Such a classification will provide a more uniform patient recruitment into clinical trials and may 'rediscover' treatments that are very effective in certain subgroups of patients that may have been formerly discarded because of the wide genetic variability in the studied patient groups. These genetic data will complement the clinical classification of patients and will also have a direct impact on patient care paradigms.

REFRENCES

Abbott DW, Wilkins A, Asara JM et al. The Crohn's disease protein, NOD2, requires RIP2 in order to induce ubiquitinylation of a novel site on NEMO. *Curr Biol.* 2004; 14:2217-2227.

Abraham C, Cho J. Interleukin-23/Th17 pathways and inflammatory bowel disease. *Inflamm Bowel Dis.* 2009;15(7):1090-100.

Abraham C, Cho JH. IL-23 and autoimmunity: new insights into the pathogenesis of inflammatory bowel disease. *Annu Rev Med.* 2009;60:97-110.

Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med.* 2009;19:361(21):2066-78.

Abreu MT, Taylor KD, Lin YC, et al. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology*. 2002;123:679–688.

Ahmad T, Satsangi J, McGovern D, et al. Review article: the genetics of inflammatory bowel disease. *Aliment Pharmacol Ther*. 2001;15(6):731-48.

Ahmad T, Armuzzi A, Bunce M, et al. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology* 2002; 122:854-866.

Ahmad T, Marshall SE, Jewell D. Genetics of inflammatory bowel disease: the role of the HLA complex. *World J Gastroenterol*. 2006;12(23):3628-35.

Ahmed T, Rieder F, Fiocchi C, et al. Pathogenesis of postoperative recurrence in Crohn's disease. *Gut.* 2011;60(4):553-62.

Aisenberg J, Legnani PE, Nilubol N, et al. Are pANCA, ASCA, or cytokine gene polymorphisms associated with pouchitis? Long term follow-up in 102 ulcerative colitis patients. *Am J Gastroenterol.* 2004;99:432– 441.

Bach SP, Mortensen NJ. Revolution and evolution: 30 years of ileoanal pouch surgery. *Inflamm Bowel Dis.* 2006;12(2):131-45.

Baker PI, Love DR, Ferguson LR. Role of gut microbiota in Crohn's disease. *Expert Rev Gastroenterol Hepatol.* 2009;3(5):535-46.

Bamias G, Martin 3rd C, Marini M, et al. Expression, localization, and functional activity of TL1A, a novel Th1-polarizing cytokine in inflammatory bowel disease. *J Immunol.* 2003;171:4868–74.

Bao XH, Naomoto Y, Hao HF, et al. Autophagy: can it become a potential therapeutic target? *Int. J. Mol. Med.*2010; 25:493–503.

Barmada MM, Brant SR, Nicolae DL, et al. A genome scan in 260 inflammatory bowel disease-affected relative pairs. *Inflamm Bowel Dis*.2004;10(5):513-20.

Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008;40:955–962.

Bartlett JM, Stirling D. A short history of the polymerase chain reaction. *Methods Mol Biol.* 2003;226:3-6.

Baumgart DC, Buning C, Geerdts L, et al. The c.1-260C>T promoter variant of CD14 but not the c.896A>G (p.D299G) variant of toll-like receptor 4 (TLR4) genes is associated with inflammatory bowel disease. *Digestion.* 2007;76(3-4):196-202.

Baumgart DC, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet.* 2007;12;369(9573):1641-57.

Bayless TM, Tokayer AZ, Polito JM, et al. Crohn's disease: concordance for site and clinical type in affected family members--potential hereditary influences. *Gastroenterology* 1996;111: 573±9.

Beliard A, Prudhomme M. Ileal reservoir with ileo-anal anastomosis: long-term complications. *J Visc Surg.* 2010;147:e137-44.

Benevento G, Avellini C, Terrosu G, et al. Diagnosis and assessment of Crohn's disease: the present and the future. *Expert Rev Gastroenterol Hepatol.* 2010;4(6):757-66.

Bernell O, Lapidus A, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann Surg.* 2000;231(1):38-45.

Bernstein C, Rawsthorne P, Cheang M, et al. A population-based case control study of potential risk factors for IBD. *Am J Gastroenterol.* 2006;101:993e1002.

Best WR, Becktel JM, Singleton JW, et al. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology.* 1976;70(3):439–444.

Billmann-Born S, Lipinski S, Böck J, et al. The complex interplay of NOD-like receptors and the autophagy machinery in the pathophysiology of Crohn disease. *Eur J Cell Biol.* 2011; 90:593-602.

Binder V. Genetic epidemiology in inflammatory bowel disease. *Dig. Dis.* 1998;16:351–55.

Binder V. Epidemiology of IBD during the twentieth century: an integrated view. *Best Pract. Res. Clin. Gastroenterol.*2004;18:463–479.

Bonen DK, Ogura Y, Nicolae DL, et al. Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology.* 2003; 124:140–146.

Brain O, Allan P, Simmons A. NOD2-mediated autophagy and Crohn disease. *Autophagy.* 2010;6:412– 414.

Brand S, Staudinger T, Schnitzler F, et al. The role of Toll-like receptor 4 Asp299Gly and Thr399Ile polymorphisms and CARD15/NOD2 mutations in the susceptibility and phenotype of Crohn's disease. *Inflamm Bowel Dis.* 2005;11:645-52.

Brant SR, Fu Y, Fields CT, et al. American families with Crohn's disease have strong evidence for linkage to chromosome 16 but not chromosome 12. *Gastroenterology.* 1998;115:1056–1061.

Braveman JM, Schoetz DJ Jr, Marcello PW, et al. The fate of the ileal pouch in patients developing Crohn's disease. *Dis Colon Rectum.* 2004;47:1613-9.

Browning BL, Huebner C, Petermann I, et al. Has toll-like receptor 4 been prematurely dismissed as an inflammatory bowel disease gene? Association study combined with meta-analysis shows strong evidence for association. *Am J Gastroenterol.* 2007;102:2504-12.

Budarf ML, Labbé C, David G, et al. GWA studies: rewriting the story of IBD. *Trends Genet.* 2009;25(3):137-46.

Burger D, Travis S. Conventional medical management of inflammatory bowel disease. *Gastroenterology.* 2011;140(6):1827-1837.

Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature.* 2008;456:259–263.

Callegaro A, Spinelli R, Beltrame L, et al. Algorithm for automatic genotype calling of single nucleotide polymorphisms using the full course of TaqMan real-time data. *Nucleic Acids Res.* 2006;34:e56.

Caputo JL, Thompson A, McClintock P, et al. An effective method for establishing human B lymphoblastic cell lines using Epstein-Barr virus. *Methods Cell Sci.* 1991;13(1):39-44.

Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. *Infect Immun.* 2000;68(12):7010-7.

Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. *Inflamm Bowel Dis.* 2010;16(9):1583-97.

Carneiro LA, Travassos LH, Girardin SE. Nod-like receptors in innate immunity and inflammatory diseases. *Ann Med.* 2007;39(8):581-93.

Carr I, Mayberry JF. The effects of migration on ulcerative colitis: a three-year prospective study among Europeans and first- and second-generation South Asians in Leicester (1991–1994). *Am J Gastroenterol.*1999;94:2918–2922.

Cavanaugh J. IBD International Genetics Consortium. International collaboration provides convincing linkage replication in complex disease through analysis of a large pooled data set: Crohn disease and chromosome 16. *Am J Hum Genet.* 2001;68(5):1165-71.

Cecconi F, LevineB. The role of autophagy in mammalian development: cell makeover rather than cell death. *Dev. Cell.* 2008;15:344–357.

Cerf-Bensussan N, Gaboriau-Routhiau V. The immune system and the gut microbiota: friends or foes? *Nat Rev Immunol.* 2010;10(10):735-44.

Chamaillard, D. Philpott, S.E. Girardin, et al. Gene–environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* 2003; 3455–3460.

Cheng JF, Ning YJ, Zhang W et al. T300A polymorphism of ATG16L1 and susceptibility to inflammatory bowel diseases: a meta-analysis. *World J Gastroenterol.* 2010;16(10):1258-66.

Cho JH, Abraham C. Inflammatory bowel disease genetics: Nod2. *Annu Rev Med.* 2007;58:401-16.

Cho JH, Nicolae DL, Gold LH, et al. Identification of novel susceptibility loci for inflammatory bowel disease on chromosomes 1p, 3q, and 4q: evidence for epistasis between 1p and IBD1. *Proc Natl Acad Sci U S A.* 1998;95(13):7502-7.

Cho JH. Inflammatory bowel disease: genetic and epidemiologic considerations. *World J Gastroenterol.* 2008;14(3):338-47.

Chow DK, Leong RW, Lai LH et al. Changes in Crohn's disease phenotype over time in the Chinese population: validation of the Montreal classification system. *Inflamm. Bowel Dis.*2008;14(4):536–541.

Coburn LA, Wise PE, Schwartz DA. The successful use of adalimumab to treat active Crohn's disease of an ileoanal pouch during pregnancy. *Dig. Dis. Sci.*2006;51(11):2045–2047.

Coffey JC, McCarthy E, Kavanagh E, et al. Pouchitis: an evolving clinical enigma--a review. *Dis Colon Rectum.* 2009;52(1):140-53.

Coffey JC, Rowan F, Burke J, et al. Pathogenesis of and unifying hypothesis for idiopathic pouchitis. *Am J Gastroenterol.* 2009;104:1013–1023.

Colin Noble, Elaine Nimmo, Daniel Gaya, et al. Novel susceptibility genes in inflammatory bowel disease. *World J Gastroenterol.* 2006;12(13):1991-1999.

Colombel J-F, Grandbastian B, Gower-Rousseau C, et al. Clinical characteristics of Crohn's disease in 72 families. *Gastroenterology.* 1996; 111: 604±7.

Colombel J-F, Richart E, Loftus EV et al. Management of Crohn's disease of the ileoanal pouch with infliximab. *Am. J. Gastroenterol.* 2003;98(10):2239–2244.

Cooney R, Baker J, Brain O, et al. NOD2 stimulation induces autophagy in dendritic cells influencing bacterial handling and antigen presentation. *Nat Med.* 2010;16:90–97.

Corr SC, O'Neill LA. Genetic variation in Toll-like receptor signalling and the risk of inflammatory and immune diseases. *J Innate Immun.* 2009;1(4):350-7.

Cottone M, Rosselli M, Orlando A, et al. Smoking habits and recurrence in Crohn's disease. *Gastroenterology.* 1994;106:643.

Coulombe F, Divangahi M, Veyrier F, et al. Increased NOD2-mediated recognition of N-glycolyl muramyl dipeptide. *J Exp Med.* 2009; 206(8):1709-16.

Creagh EM, and O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol.* 2006; 27(8):352-7.

Crohn BB, Ginzburg L, Oppenheimer GD. Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. *JAMA.* 1984; 251: 73–79.

Cuervo AM. Autophagy and aging: keeping that old broom working. *Trends Genet.* 2008;24:604–612.

Cummings JR, Ahmad T, Geremia A et al. Contribution of the novel inflammatory bowel disease gene IL23R to disease susceptibility and phenotype. *Inflamm. Bowel Dis.* 2007;13(9):1063–1068.

Cuthbert AP, Fisher SA, Mirza MM, et al. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology.* 2002;122:867– 874.

Dawn Teare M, Barrett JH. Genetic linkage studies. Lancet. 2005; 366(9490):1036-44.

de Hertogh G, Aerssens J, Geboes K, et al. Evidence for the involvement of infectious agents in the pathogenesis of Crohn's disease. *World J Gastroenterol.* 2008;14:845e52.

de Silva HJ, Millard PR, Soper N, et al. Effects of the faecal stream and stasis on the ileal pouch mucosa. *Gut.* 1991; 32(10):1166-9.

Dechairo B, Dimon C, van Heel D, et al. Replication and extensionstudies of inflammatory bowel disease susceptibility regions confirm linkage to chromosome 6p (IBD3). *Eur J Hum Genet.* 2001;9:627-633.

Delaney CP, Remzi FH, Gramlich T, et al. Equivalent function, quality of life and pouch survival rates after ileal pouch-anal anastomosis for indeterminate and ulcerative colitis. *Ann Surg.* 2002;236:43–48.

Deretic V, Levine B. Autophagy, immunity, and microbial adaptations. *Cell Host Microbe.* 2009; 18:527-49.

Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes. *Immunol Rev.* 2011; 240(1):92-104.

Duerr RH, Barmada MM, Zhang L, et al. High-density genome scan in Crohn disease shows confirmed linkage to chromosome 14q11-12. *Am J Hum Genet.* 2000; 66(6):1857-62.

Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science.* 2006;314:1461–1463.

Duffy M, O'Mahony L, Coffey JC, et al. Sulfate-reducing bacteria colonize pouches formed for ulcerative colitis but not for familial adenomatous polyposis. *Dis Colon Rectum.* 2002; 45(3):384-8.

Economou M, Trikalinos TA, Loizou KT, et al. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol.*

2004;99:2393-404.

Elson CO, Cong Y, Weaver CT, et al. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology.* 2007;132(7):2359-70.

Endo K, Kinouchi Y, Kakuta Y, et al. Involvement of NF-kappa B pathway in TL1A gene expression induced by lipopolysaccharide. *Cytokine.* 2010;49:215-20.

Espen Melum, Andre Franke, Tom H Karlsen. Genome-wide association studies- A summary for the clinical gastroenterologist. *World J Gastroenterol.* 2009; 15(43): 5377-5396.

Fang L, Adkins B, Deyev V, Podack ER. Essential role of TNF receptor superfamily 25 (TNFRSF25) in the development of allergic lung inflammation. *J Exp Med.* 2008; 205:1037–1048.

Farmer RG, Hawk WA, Turnbull RB Jr. Clinical patterns in Crohn's disease: a statistical study of 615 cases. *Gastroenterology.* 1975. 68: 627–635.

Fazio VW, Tjandra JJ, Lavery IC, et al. Long-term follow-up of strictureplasty in Crohn's disease. *Dis Colon Rectum.* 1993; 36:355-361.

Fernandez L, Mendoza JL, Martinez A, et al. IBD1 and IBD3 determine location of Crohn's disease in the Spanish population. *Inflamm Bowel Dis.* 2004; 10(6):715-22.

Fisher SA, Tremelling M, Anderson CA et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat. Genet.* 2008;40(6),710–712.

Fleshner PR, Vasiliauskas EA, Kam LY, et al. High level perinuclear antineutrophil cytoplasmic antibody (pANCA) in ulcerative colitis patients before colectomy predicts the development of chronic pouchitis after ileal pouch-anal anastomosis. *Gut.* 2001;49:671– 677.

Fowler EV, Doecke J, Simms LA, et al. ATG16L1 T300A shows strong associations with disease subgroups in a large Australian IBD population: further support for significant disease heterogeneity. *Am J Gastroenterol.* 2008;103:2519–2526.

Franchimont D, Vermeire S, El Housni H, et al. Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut.* 2004;53(7):987-92.

Franke A, Balschun T, Karlsen TH, et al. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. *Nat Genet.* 2008;40:713–5.

Franke A, Balschun T, Karlsen TH, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet.* 2008;40:1319 –1323.

Friedman, S. and Blumberg,R.S. (2008) Inflammatory Bowel Disease. In Harrison's Principles of Internal Medicine (17th edn) (Fauci, A.,ed.), pp. 1886–1898, McGraw-Hill.

Friswell M, Campbell B, Rhodes J. The role of bacteria in the pathogenesis of inflammatory

bowel disease. *Gut Liver.* 2010;4(3):295-306.

Fritz T, Niederreiter L, Adolph T, et al. Crohn's disease: NOD2, autophagy and ER stress converge. *Gut.* 2011; 60(11):1580-8.

Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. *Science.* 2002;296:2225–9.

Gasche C, Scholmerich J, Brynskov J et al. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm. Bowel Dis.* 2000; 6(1): 8–15.

Gaya DR, Russell RK, Nimmo ER, Satsangi J. New genes in inflammatory bowel disease: lessons for complex diseases? *Lancet.* 2006; 367:1271–84.

Gertner, D.J., Rampton, D.S., Madden, M.V., et al. Increased leukotriene B4 release from ileal pouch mucosa in ulcerative colitis compared with familial adenomatous polyposis. *Gut.* 1994; 35:1429.

Gionchetti P, Rizzello F, Helwig U, et al. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology.* 2003.124(5):1202-9.

Gionchetti P, Rizzello F, Venturi A, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: adouble-blind, placebo-controlled trial. *Gastroenterology.* 2000; 119:305–309.

Girardin SE, Boneca IG, Viala J, et al. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem.* 2003;278(11):8869-72.

Glick, S. Barth and K.F. Macleod. Autophagy: cellular and molecular mechanisms. *J. Pathol.* 2010; 221: 3–12.

Godet PG, May GR, Sutherland LR. Meta-analysis of the role of oral contraceptive agents in inflammatory bowel disease. *Gut.* 1995;37:668e73.

Goldberg, P.A., Herbst, F., Beckett, et al. Leucocyte typing, cytokine expression and epithelial turnover in the ileal pouch in patients with ulceratice colitis and familial adenomatous polyposis. *Gut.* 1996; 38:549.

Goldstein NS, Sanford WW, Bodzin JH. Crohn's like complications in patients with ulcerative colitis after total proctocolectomy and ileal pouch-anal anastomosis. *Am J Surg Pathol.* 1997;21:1343–1353.

Gordon M, Naidoo K, Thomas AG, et al. Oral 5-aminosalicylic acid for maintenance of surgically-induced remission in Crohn's disease. *Cochrane Database Syst Rev.* 2011; 19;(1):CD008414.

Grant SF, Baldassano RN, Hakonarson H. Classification of genetic profiles of Crohn's disease: a focus on the ATG16L1 gene. *Expert Rev Mol Diagn.* 2008;8(2):199-207.

Grant SF, Hakonarson H. Microarray technology and applications in the arena of genome-wide association. *Clin Chem.* 2008;54(7):1116-24.

Greenstein AJ, Lachman P, Sachar DB et al. Perforating and non-perforating indications for repeated operations in Crohn's disease: evidence of two clinical forms. *Gut.* 1998. 29:588–592.

Gunderson KL, Steemers FJ, Ren H, et al. Whole-genome genotyping. *Methods Enzymol.* 2006;410:359-76.

Gutierrez O, Pipaon C, Inohara N, et al. Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor kappa B activation. *J Biol Chem.* 2002;277:41701e5.

H.J. Buhr and A.J. Kroesen. Surgical therapy of recurrent Crohn's disease. Surgical Treatment: Evidence-Based and Problem-Oriented. Holzheimer RG, Mannick JA, editors. Munich: Zuckschwerdt; 2001.

Halfvarson J, Bodin L, Tysk C, Lindberg E, Jarnerot G. Inflammatory bowel disease in a Swedish twin cohort: a longterm follow-up of concordance and clinical characteristics. *Gastroenterology.* 2003; 124: 1767-1773.

Hampe, A. Franke, P. Rosenstiel, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat. Genet.* 2007; 207–211.

Hampe J, Cuthbert A, Croucher PJ, et al. 2001. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet.* 2001. 357:1925-8.

Hampe J, Frenzel H, Mirza MM, et al. Evidence for a NOD2-independent susceptibility locus for inflammatory bowel disease on chromosome 16p. *Proc Natl Acad Sci U S A.* 2002; 99(1):321-6.

Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet.* 2002;359:1661–1665.

Hampe J, Shaw SH, Saiz R, et al. Linkage of inflammatory bowel disease to human chromosome 6p. Am J Hum Genet. 1999; 65(6):1647-55.

Hancock L, Beckly J, Geremia A, et al. Clinical and molecular characteristics of isolated colonic Crohn's disease. Clinical and molecular characteristics of isolated colonic Crohn's disease. *Inflamm Bowel Dis.* 2008;14(12):1667-77.

Harley JE, Fazio VW, Remzi FH, et al. Analysis of the outcome of ileal pouch-anal anastomosis in patients with Crohn's disease. *Dis Colon Rectum.* 2004;47:1808 –1815.

Haveran LA, Sehgal R, Poritz LS, et al. Infliximab and/or azathioprine in the treatment of Crohn's disease-like complications after IPAA. *Dis Colon Rectum.* 2011; 54(1):15-20.

Heilpern D, Szilagyi A. Manipulation of intestinal microbial flora for therapeutic benefit in inflammatory bowel diseases: review of clinical trials of probiotics, pre-biotics and synbiotics. *Rev Recent Clin Trials.* 2008; 3:167–184.

Heresbach D, Gicquel-Douabin V, Birebent B, et al. NOD2/CARD15 gene polymorphisms in Crohn's disease: a genotypephenotype analysis. *Eur J Gastroenterol Hepatol.* 2004;16:55– 62.

Heuschen UA, Allemeyer EH, Hinz U, et al. Diagnosing pouchitis: comparative validation of two scoring systems in routine follow-up. *Dis Colon Rectum.* 2002;45(6):776-86.

Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. A comprehensive review of genetic association studies. *Genet Med.* 2002; 4(2):45-61.

Hisamatsu, M. Suzuki, H.C. Reinecker,et al. CARD15/NOD2 functions as an antibacterial factor in human intestinal epithelial cells. *Gastroenterology.* 2003; 124:993–1000.

Holly K. Tabor, Neil J. Risch and Richard M. Myers. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nature Reviews Genetics.* 2002; 3:391-397.

Hou JK, El-Serag H, Thirumurthi S. Distribution and manifestations of inflammatory bowel disease in Asians, Hispanics, and African Americans: a systematic review. *Am J Gastroenterol.* 2009;104:2100–9.

Hruz P, Eckmann L. Innate immune defence: NOD2 and autophagy in the pathogenesis of Crohn's disease. *Swiss Med Wkly.* 2010;27:140:w13135.

Hue S, Ahern P, Buonocore S, et al. Interleukin-23 drives innate and T cellmediated intestinal inflammation. *J Exp Med.* 2006;203:2473-83.

Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR. Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. *Inflamm Bowel Dis.* 2010;16:282–295.

Huett A, Xavier R. SNPping away at the pathogenesis of Crohn's disease. *Inflamm Bowel Dis.* 2008;14(1):136-7.

Hugot J, Chamaillard M, Zouali H, et al. Association of NOD2 leucinerich repeat variants with susceptibility to Crohn's disease. *Nature.* 2001;411:599-603.

Hugot JP, Laurent-Puig P, Gower-Rousseau C, et al. Mapping of a susceptibility locus for Crohn's disease on chromosome 16. *Nature.* 1996; 379:821-3.

Hugot JP, Zaccaria I, Cavanaugh J, et al. Prevalence of CARD15/NOD2 mutations in Caucasian healthy people. *Am J Gastroenterol.* 2007;102:1259 –1267.

Hyman NH, FazioVW,Tuckson WB, et al. Consequences of ileal pouch-anal anastomosis for Crohn's colitis. *Dis Colon Rectum.* 1991;34:653–7.

Inohara N, Ogura Y, Fontalba A, et al. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem.* 2003; 278(8):5509-12.

Inohara, Chamaillard, McDonald C, Nunez G. NOD–LRR proteins: role in host–microbial interactions and inflammatory disease. *Annu Rev Biochem.* 2005; 74:355-383.

Inohara, N et al. An induced proximity model for NF-κB activation in the Nod1/RICK and RIP signaling pathways. *J. Biol. Chem.* 2000; 275:27823-27831.

International HapMap Consortium. A haplotype map of the human genome. *Nature*. 2005; 437: 1299–1320.

Ishihara S, Aziz MM, Yuki T, Kazumori H, Kinoshita Y. Inflammatory bowel disease: review from the aspect of genetics. *J Gastroenterol.* 2009;44(11):1097-108.

Ivanov II, Frutos RL, Manel N, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe*. 2008; 4:337–349.

Joyce MR, Fazio VW. Can ileal pouch anal anastomosis be used in Crohn's disease? *Adv Surg.* 2009;43:111–137.

Kakuta Y, Kinouchi Y, Negoro K, Takahashi S, Shimosegawa T. Association study of TNFSF15 polymorphisms in Japanese patients with inflammatory bowel disease. *Gut*. 2006; 55:1527–1528.

Kanneganti TD, Lamkanfi M, Nunez G. Intracellular NOD-like receptors in host defense and disease. *Immunity*. 2007;27:549e59.

Kariv R, Bennett A, Kariv Y, et al. Adenocarcinoma in ileal pouch-anal anastomosis: the Cleveland Clinic experience. *Am J Gastroenterol.* 2007;102(suppl 2):A953.

Kohyama M, Takesue Y, Ohge H, et al. Pouchitis disease activity index (PDAI) does not predict patients with symptoms of pouchitis who will respond to antibiotics. *Surg Today.* 2009;39(11):962-8.

Kruglyak L, Daly MJ, Reeve-Daly MP, et al. Parametric and nonparametric linkage analysis: a unified multipoint approach. *Am J Hum Genet.* 1996; 58:1347–1363.

Kuballa P, Huett A, Rioux JD, et al. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One.* 2008;3(10):e3391.

Lander E, Kruglyak L. Genetic dissection of complex traits: guidelines for interpreting and reporting linkage results. *Nat Genet*. 1995;11:241-247.

Langhorst J, Elsenbruch S, Koelzer J, et al. Noninvasive markers in the assessment of intestinal inflammation in inflammatory bowel diseases: performance of fecal lactoferrin, calprotectin, and PMN-elastase, CRP, and clinical indices. *Am. J. Gastroenterol.* 2008;103(1):162–169.

Lappalainen M, Halme L, Turunen U et al. Association of IL23R, TNFRSF1A, and HLA-DRB1*0103 allele variants with inflammatory bowel disease phenotypes in the Finnish population. *Inflamm. Bowel Dis.* 2008;14(8):1118–1124.

Lecat A, Piette J, Legrand-Poels S. The protein Nod2: an innate receptor more complex than previously assumed. *Biochem Pharmacol.* 2010. 15;80(12):2021-31.

Lee EC, Papaioannou N. Minimal surgery for chronic obstruction in patients with extensive or universal Crohn's disease. *Ann R Coll Surg Engl.* 1982;64(4):229-33.

Lee JH, et al. Lysosomal proteolysis and autophagy require presenilin 1 and are disrupted by Alzheimer-related PS1 mutations. *Cell.* 2010;141:1146–1158.

Lees CW, Satsangi J. Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history. *Expert Rev Gastroenterol Hepatol.* 2009; 3(5):513-34.

Leighton JA, Shen B, Baron TH, et al. Standards of Practice Committee, American Society for Gastrointestinal Endoscopy. Gastrointest Endosc. ASGE guideline: endoscopy in the diagnosis and treatment of inflammatory bowel disease. 2006; 63(4):558-65.

Lennard-Jones JE, Stalder GA. Prognosis after resection of chronic regional ileitis. *Gut.* 1967;8:332-6.

Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl. 1989;170:2-6.

Lesage S, Zouali H, Ce´zard JP, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet.* 2002;70:845–857.

Levin KE, Pemberton JH, Phillips SF, et al. Role of oxygen free radicals in the etiology of pouchitis. *Dis Colon Rectum.* 1992; 35(5):452-6.

Levine B. Cell biology: autophagy and cancer. *Nature*. 2007;446:745–747.

Li X, Conklin L, Alex P. New serological biomarkers of inflammatory bowel disease. *World J Gastroenterol.* 2008;14(33):5115-24.

Libioulle C, Louis E, Hansoul S et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genet.* 2007;3(4),e58.

Loftus EV, Jr, Sandborn WJ. Epidemiology of inflammatory bowel disease. *Gastroenterol Clin North Am.* 2002;31:1-20.

Ma Y, Ohmen JD, Li Z, et al. A genome-wide search identifies potential new susceptibility loci for Crohn's disease. *Inflamm Bowel Dis.* 1999; 5(4):271-8.

Macafee DA, Abercrombie JF, Maxwell-Armstrong C. Pouchitis. *Colorectal Dis.* 2004;6(3):142-52.

Madiba TE, Bartolo DCC. Pouchitis following restorative proctocolectomy for ulcerative colitis: incidence and therapeutic outcome. *J Roy Coll Surg Edinb.* 2001; 46:334–7.

Mahmud N, Weir DG. The urban diet and Crohn's disease: is there a relationship? *Eur J Gastroenterol Hepatol.* 2001;13:93–5.

Mannon PJ, Fuss IJ, Mayer L, et al, Anti-IL-12 Crohn's Disease Study Group. Anti-interleukin-12 antibody for active Crohn's disease. *N Engl J Med.* 2004;351(20):2069-79

Manolio TA, Brooks LD, Collins FS. A HapMap harvest of insights into the genetics of common disease. *J Clin Invest*. 2008;118:1590-1605

Maresso K, Broeckel U. Genotyping platforms for mass-throughput genotyping with SNPs, including human genome-wide scans. *Adv Genet.* 2008;60:107-39.

Márquez A, Núñez C, Martínez A, et al. Role of ATG16L1 Thr300Ala polymorphism in inflammatory bowel disease: a Study in the Spanish population and a meta-analysis. *Inflamm Bowel Dis.* 2009. 15(11):1697-704.

Martinez-Vicente M, et al. Cargo recognition failure is responsible for inefficient autophagy in Huntington's disease. *Nat Neurosci*. 2010;13:567–576.

Martinon F, and Tschopp J. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol.* 2005; 26(8):447-54.)

Massey DC, Parkes M. Genome-wide association scanning highlights two autophagy genes, ATG16L1 and IRGM, as being significantly associated with Crohn's disease. *Autophagy.* 2007;3(6):649-51.

Mathew CG. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. *Nat Rev Genet.*2008;9:9 –14.

Matsui T, Yao T, Sakurai T, et al. Clinical features and pattern of indeterminate colitis: Crohn's disease with ulcerative colitis-like clinical presentation. *J Gastroenterol.* 2003;38(7):647-55.

Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature*. 2008; 453:620–625.

McCarroll SA, Huett A, Kuballa P et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat. Genet.* 2008; 40:1107–1112.

McGeachy MJ, Cua DJ. The link between IL-23 and Th17 cell-mediated immune pathologies. *Semin Immunol*. 2007;19:372-6.

McGovern DP, Gardet A, To¨rkvist L, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet.* 2010;42:332–337.

McLaughlin SD, Clark SK, Tekkis PP, et al. Review article: restorative proctocolectomy, indications, management of complications and follow-up--a guide for gastroenterologists. *Aliment Pharmacol Ther.* 2008;27(10):895-909.

McLeod RS, Wolff BG, Ross S, et al. Investigators of the CAST Trial. Recurrence of Crohn's disease after ileocolic resection is not affected by anastomotic type: results of a multicenter, randomized, controlled trial. *Dis Colon Rectum.* 2009;52(5):919-27.

Meier CB, Hegazi RA, Aisenberg J, et al. Innate immune receptor genetic polymorphisms in

pouchitis: is CARD15 a susceptibilityfactor? *Inflamm Bowel Dis.* 2005;11:965–971.

Meijer AJ, Codogno P. Autophagy: a sweet process in diabetes. *Cell Metab.* 2008;8:275–276.

Melmed GY, Fleshner PR, Bardakcioglu O, et al. Family history and serology predict Crohn's disease after ileal pouch-anal anastomosis for ulcerative colitis. *Dis Colon Rectum.* 2008;51:100–108.

Melton GB, Fazio VW, Kiran RP, et al. Long-term outcomes with ileal pouch-anal anastomosis and Crohn's disease: pouch retention and implications of delayed diagnosis. *Ann Surg.* 2008;248(4):608-16.

Messaris E, Chandolias N, Grand D, Pricolo V. Role of magnetic resonance enterography in the management of Crohn disease. *Arch Surg.* 2010; 145(5):471-5.

Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. *Nature.* 2006;442:39e44.

Meylan F et al. The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases. *Immunity.* 2008;29:79–89.

Michelsen KS, Thomas LS, Taylor KD, et al. IBD-associated TL1A gene (TNFSF15) haplotypes determine increased expression of TL1A protein. *PLoS One.* 2009;4(3):e4719.

Mimura T, Rizzello F, Helwig U, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrentor refractory pouchitis. *Gut.* 2004;53:108 –114.

Miner P, Wedel M, Bane B, Bradley J. An enema formulation of alicaforsen, an antisense inhibitor of intercellular adhesion molecule-1, in the treatment of chronic, unremitting pouchitis. *Aliment Pharmacol Ther.* 2004;19(3):281-6.

Mirza MM, Lee J, Teare D, et al. Evidence of linkage of the inflammatory bowel disease susceptibility locus on chromosome 16 (IBD1) to ulcerative colitis. *J Med Genet.* 1998;35:218–221.

Mizushima N. Autophagy: process and function. *Genes Dev.* 2007;21(22):2861-73.

Montgomery SM, Morris DL, Pounder RE, Wakefield AJ. Asian ethnic origin and the risk of inflammatory bowel disease. *Eur J Gastroenterol Hepatol.* 1999;11:543–546.

Motulsky AG. Genetics of complex diseases. *J Zhejiang Univ Sci B.* 2006;7(2):167-8.

Mowat C, Cole A, Windsor A, Ahmad T, Arnott I, Driscoll R, Mitton S, Orchard T, Rutter M, Younge L, Lees C, Ho GT, Satsangi J, Bloom S; on behalf of the IBD Section of the British Society of Gastroenterology. Guidelines for the management of inflammatory bowel disease in adults. *Gut.* 2011;60(5):571-607.

Nakagome S, Takeyama Y, Mano S, et al. Population-specific susceptibility to Crohn's disease and ulcerative colitis: dominant and recessive relative risks in the Japanese population. *Ann Hum Genet.* 2010;74:126 –136.

National Center for Biotechnology Information, National Library of Medicine. Database of Single Nucleotide Polymorphisms. http://www.ncbi.nlm.nih.gov/SNP/

Netea MG, Joosten LA. A NOD for autophagy. *Nat Med.* 2010;16(1):28-30.

Newman B, Silverberg MS, Gu X, et al. CARD15 and HLA DRB1 alleles influence susceptibility and disease localization in Crohn's disease. *Am J Gastroenterol.* 2004; 99: 306-315.

Nicholls RJ, Banerjee AK. Pouchitis: risk factors, etiology, and treatment. *World J Surg.* 1998;22(4):347-51.

Noomen CG, Hommes DW, Fidder HH. Update on genetics in inflammatory disease. *Best Pract Res Clin Gastroenterol.* 2009;23:233–243.

Nowak D, Hofmann WK, Koeffler HP. Genome-wide Mapping of Copy Number Variations Using SNP Arrays. *Transfus Med Hemother.* 2009;36(4):246-251

Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.* 2001;411:603– 606.

Ohmen JD, Yang HY, Yamamoto KK et al. Susceptibility locus for inflammatory bowel disease on chromosome 16 has a role in Crohns disease, but not in ulcerative colitis. *Hum Mol Genet.* 1996; 5:1679–1683.

Oostenbrug LE, Nolte IM, Oosterom E, et al. CARD15 in inflammatory bowel disease and Crohn's disease phenotypes: an association study and pooled analysis. *Dig Liver Dis.* 2006;38:834–845.

Orchard TR, Chua CN, Ahmad T, et al. Uveitis and erythema nodosum in inflammatory bowel disease: clinical features and the role of HLA genes. *Gastroenterology.* 2002; 123: 714-718.

Orchard TR, Thiyagaraja S, Welsh KI, et al. Clinical phenotype is related to HLA genotype in the peripheral arthropathies of inflammatory bowel disease. *Gastroenterology.* 2000; 118: 274-278.

Orholm M, Binder V, Sorensen TI, et al. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. *Scand J Gastroenterol.* 2000; 35: 1075-1081.

Orholm M, Munkholm P, Langholz E, et al. Familial occurrence of inflammatory bowel disease. *N Engl J Med.* 1991;324(2):84-8.

Ouellette AJ. Paneth cell alpha-defensin synthesis and function. *Curr Top Microbiol Immunol.* 2006 306:1–25

Ozuner G, Fazio VW, Lavery I, Milsom J, Strong S. Reoperative rates for Crohn's disease following strictureplasty. *Dis Colon Rectum.* 1996; 39:1199-1203.

Ozuner G, Fazio VW. Management of gastrointestinal bleeding after strictureplasty for Crohn's disease. *Dis Colon Rectum.* 1995; 38:297-300.

Paavola-Sakki P, Ollikainen V, Helio T, et al. Genome-wide search in Finnish families with inflammatory bowel disease provides evidence for novel susceptibility loci. *Eur J Hum Genet.* 2003; 11(2):112-20.

Papadakis KA, Treyzon L, Abreu MT, et al. Infliximab in the treatment of medically refractory indeterminate colitis. *Aliment Pharmacol Ther.* 2003;18:741–747.

Pappu BP et al. TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease. *J Exp Med.* 2008; 205:1049–1062.

Pardi DS, D'Haens G, Shen B, et al. Clinical guidelines for the management of pouchitis. *Inflamm Bowel Dis.* 2009;15(9):1424-31.

Pardi DS, Shen B. Endoscopy in the management of patients after ileal pouch surgery for ulcerative colitis. *Endoscopy.* 2008;40(6):529-33.

Parkes M, Barmada M, Satsangi J, et al. The IBD2 locus shows linkage heterogeneity between ulcerative colitis and Crohn disease. *Am J Hum Genet.* 2000;67:1605-1610.

Parkes M, Barrett JC, Prescott NJ, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet.* 2007;39:830e2.

Parkes M, Satsangi J, Lathrop GM,et al. Susceptibility loci in inflammatory bowel disease. *Lancet.* 1996; 348:1588.

Parks AG, Nicholls RJ. Proctocolectomy without ileostomy for ulcerative colitis. *Br Med J.* 1978;2(6130):85-8.

Pearson TA, Manolio TA. How to interpret a genome-wide association study. *JAMA.* 2008;299(11):1335-44.

Peeters M, Nevens H, Baert F, et al. Familial aggregation in Crohn's disease: increased age adjusted risk and concordance in clinical characteristics. *Gastroenterology.* 1996; 111:597±603.

Peltekova V, Wintle R, Rubin L, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet.* 2004;36:471-475.

Peltekova VD, Wintle RF, Rubin LA, et al. Functional variants of OCTN cation transporter genes are associated with Crohn disease. *Nat Genet.* 2004;36:471–475.

Penna C, Dozois R, Tremaine W, et al. Pouchitis after ileal pouch-anal anastomosis for ulcerative colitis occurs with increased frequency in patients with associated primary sclerosing cholangitis. *Gut.* 1996;38:234–239.

Peyrègne V, Francois Y, Gilly FN,et al. Outcome of ileal pouch after secondary diagnosis of Crohn's disease. *Int J Colorectal Dis.* 2000;15(1):49-53.

Picornell Y, Mei L, Taylor K, et al. TNFSF15 is an ethnic-specific IBD gene. *Inflamm Bowel Dis.* 2007;13:1333–1338.

Prehn JL, Mehdizadeh S, Landers CJ, et al. Potential role for TL1A, the new TNF-family member and potent costimulator of IFN-gamma, in mucosal inflammation. *Clin Immunol.* 2004;112:66–77.

Prescott NJ, Fisher SA, Franke A, et al. A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5. *Gastroenterology.* 2007;132:1665–1671.

Probert CS, Jayanthi V, Pinder D, et al. Epidemiological study of ulcerative proctocolitis in Indian migrants and the indigenous population of Leicestershire. *Gut.* 1992;33: 687–693.

Radlmayr M, Török HP, Martin K, Folwaczny C. The c-insertion mutation of the NOD2 gene is associated with fistulizing and fibrostenotic phenotypes in Crohn's disease. *Gastroenterology.* 2002;122(7):2091-2.

Raelson JV, Little RD, Ruether A et al. Genome-wide association study for Crohn's disease in the Quebec Founder Population identifies multiple validated disease loci. *Proc. Natl Acad. Sci. USA.* 2007;104(37):14747–14752.

Rallabhandi P, Bell J, Boukhvalova MS, et al. Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. *J Immunol.* 2006;177(1):322-32.

Ravitch MM, Sabiston DC Jr. Anal ileostomy with preservation of the sphincter; a proposed operation in patients requiring total colectomy for benign lesions. *Surg Gynecol Obstet.* 1947;84(6):1095-9.

Reese G, Nanidis T, Borysiewicz C, et al. The effect of smoking after surgery for Crohn's disease: a meta-analysis of observational studies. *Int J Colorectal Dis.* 2008;12:1213e21.

Regueiro M, Kip KE, Schraut W, et al. Crohn's disease activity index does not correlate with endoscopic recurrence one year after ileocolonic resection. *Inflamm Bowel Dis.* 2011;17(1):118-26.

Ricart E, Panaccione R, Loftus EV, Tremaine WJ, Sandborn WJ. Successful management of Crohn's disease of the ileoanal pouch with infliximab. *Gastroenterology.* 1999;117(2):429-32.

Rioux JD, Daly MJ, Green T, et al. Absence of linkage between inflammatory bowel disease and selected loci on chromosomes 3, 7, 12, and 16. *Gastroenterology.* 1998;115:1062–1065.

Rioux JD, Silverberg MS, Daly MJ, et al. Genomewide search in Canadian families with inflammatory bowel disease reveals two novel susceptibility loci. *Am J Hum Genet.* 2000; 66(6):1863-70.

Rioux JD, Xavier RJ, Taylor KD,et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet.* 2007; 39:596–604.

Ritchie JK. The results of surgery for large bowel Crohn's disease. *Ann R Coll Surg Engl.* 1990;72:155-7.

Roberts RL, Hollis-Moffatt JE, Gearry RB, et al. Confirmation of association of IRGM and NCF4 with ileal Crohn's disease in a population-based cohort. *Genes Immun.* 2008;9:561–5.

Rogler G. The importance of gut microbiota in mediating the effect of NOD2 defects in inflammatory bowel disease. *Gut.* 2010;59:153–154.

Rosenstiel P, Fantini M, Brautigam K, et al. TNF-alpha and IFN-gamma regulate the expression of the NOD2 (CARD15) gene in human intestinal epithelial cells. *Gastroenterology.* 2003; 124:1001–9.

Roseth AG, Schmidt PN, Fagerhol MK. Correlation between faecal excretion of indium-111-labelled granulocytes and calprotectin, a granulocyte marker protein, in patients with inflammatory bowel disease. *Scand J Gastroenterol.* 1999;34:50–4.

Roth MP, Petersen GM, McElree C, Feldman E, Rotter JI. Geographic origins of Jewish patients with inflammatory bowel disease. *Gastroenterology.* 1989; 97: 900±4.

Rothfuss KS, Stange EF, Herrlinger KR. Extraintestinal manifestations and complications in inflammatory bowel diseases. *World J Gastroenterol.* 2006;12(30):4819-31.

Rubinsztein DC, DiFiglia M, Heintz N, et al. Autophagy and its possible roles in nervous system diseases, damage and repair. *Autophagy.* 2005; 1:11-22.

Russell RK, Satsangi J. Does IBD run in families? *Inflamm Bowel Dis.* 2008;14 Suppl 2:S20-1.

Rutgeerts P, Goboes K, Peeters M, et al. Effect of faecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet.* 1991;338:771e4.

Ruthruff B. Clinical review of Crohn's disease. *J Am Acad Nurse Pract.* 2007;19(8):392-7.

Sagar PM, Dozois RR, Wolff BG. Long-term results of ileal pouch-anal anastomosis in patients with Crohn's disease. *Dis Colon Rectum.* 1996;39(8):893-8.

Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature.* 2008;456:264–268.

Sandborn WJ, Landers CJ, Tremaine WJ, Targan SR. Antineutrophil cytoplasmic antibody correlates with chronic pouchitis after ileal pouch-anal anastomosis. *Am J Gastroenterol.* 1995;90:740–747.

Sandborn WJ, Tremaine WJ, Batts KP, Pemberton JH, Phillips SF. Pouchitis after ileal pouch-anal anastomosis: a Pouchitis Disease Activity Index. *Mayo Clin Proc.* 1994 May;69(5):409-15.

Sands BE. From symptom to diagnosis: clinical distinctions among various forms of intestinal inflammation. *Gastroenterology.* 2004;126:1518e32.

Satsangi J, Grootscholten C, Holt H, Jewell DP. Clinical patterns of familial inflammatory bowel disease. *Gut.* 1996; 38:738±41.

Satsangi J, Parkes M, Louis E, et al. Two stage genome-wide search in inflammatory bowel disease provides evidence for susceptibility loci on chromosomes 3, 7 and 12. *Nat Genet.* 1996; 14(2):199-202.

Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut. 2006;* 55(6):749–753.

Satsangi J, Welsh KI, Bunce M, et al. Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory bowel disease. *Lancet.* 1996; 347: 1212-1217.

Scherr R, Essers J, Hakonarson H, Kugathasan S. Genetic determinants of pediatric inflammatory bowel disease: is age of onsetgenetically determined? *Dig Dis.* 2009;27:236 –239.

Schork NJ. Genetics of complex disease: approaches, problems, and solutions. *Am J Respir Crit Care Med.* 1997;156(4 Pt 2):S103-9.

Scott NA, Sue-Ling HM, Hughes LE. Anastomotic configuration does not affect recurrence of Crohn's disease after ileocolonic resection. *Int J Colorect Dis.* 1995;10:67–69.

Sehgal R, Koltun WA. Scoring systems in inflammatory bowel disease. *Expert Rev Gastroenterol Hepatol.* 2010;4(4):513-21.

Shen B, Achkar JP, Connor JT, et al. Modified pouchitis disease activity index: a simplified approach to the diagnosis of pouchitis. *Dis Colon Rectum.* 2003;46(6):748-53.

Shen B, Achkar JP, Lashner BA, et al. A randomized clinical trial of ciprofloxacin and metronidazole to treat acute pouchitis. *Inflamm Bowel Dis.* 2001;7(4):301-5.

Shen B, Fazio VF, Remzi FH, et al. Endoscopic balloon dilation of ileal pouch strictures. *Am J Gastroenterol.* 2004;99:2340–7.

Shen B, Fazio VW, Remzi FH, et al. Risk factors for clinical phenotypes of Crohn's disease of the ileal pouch. *Am J Gastroenterol.* 2006;101(12):2760-8.

Shen B, Fazio VW, Remzi FH, et al. Comprehensive evaluation of inflammatory and noninflammatory sequelae of ileal pouch anal anastomoses. *Am J Gastroenterol.* 2005;100:93–101.

Shen B, Fazio VW, Remzi FH, Lashner BA. Clinical approach to diseases of ileal pouch-anal anastomosis. *Am J Gastroenterol.* 2005;100(12):2796-807.

Shen B, Lashner B. Can we immunogenotypically and immunophenotypically profile patients who are at risk for pouchitis? *Am J Gastroenterol.* 2004;99(3):442-4.

Shen B, Patel S, Lian L. Natural history of Crohn's disease in patients who underwent intentional restorative proctocolectomy with ileal pouch-anal anastomosis. *Aliment Pharmacol Ther.* 2010;31:745–753.

Shen B, Remzi FH, Brzezinski A, et al. Risk factors for pouch failure in patients with different

phenotypes of Crohn's disease of the pouch. *Inflamm Bowel Dis.* 2008;14(7):942-8.

Shen B, Remzi FH, Shen L. Efficacy and safety of adalimumab in the treatment of Crohn's disease of the ileal pouch. *Aliment Pharm. Therap.* 2009;29(5):519–526.

Shen B. Crohn's disease of the ileal pouch: reality, diagnosis, and management. *Inflamm Bowel Dis.* 2009;15(2):284-294.

Shen B. Diagnosis and treatment of patients with pouchitis. *Drugs.* 2003;63(5):453-61.

Shih DQ, Michelsen KS, Barrett RJ, et al. Insights into TL1A and IBD pathogenesis. *Adv Exp Med Biol.* 2011;691:279-88.

Shih DQ, Targan SR, McGovern D. Recent advances in IBD pathogenesis: genetics and immunobiology. *Curr Gastroenterol Rep.* 2008;10(6):568-75.

Shih DQ, Targan SR. Insights into IBD Pathogenesis. Curr Gastroenterol Rep. 2009;11:473-80.

Silverberg MS, Cho JH, Rioux JD, et al. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet.* 2009;41:216 –220.

Silverberg MS. OCTNs: will the real IBD5 gene please stand up? *World J Gastroenterol.* 2006 Jun 21;12(23):3678-81.

Simms LA, Doecke JD, Walsh MD, et al. Reduced alpha-defensin expression is associated with inflammation and not NOD2 mutation status in ileal Crohn's disease. *Gut.* 2008; 57:903–910.

Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science.* 2006;313(5792):1438-41.

Singh SB, Ornatowski W, Vergne I, et al. Human IRGM regulates autophagy and cell-autonomous immunity functions through mitochondria. *Nat Cell Biol.* 2010;12(12):1154-65.

Singleton JW. Clinical activity assessment in inflammatory bowel disease. *Dig. Dis. Sci.* 1987;32(Suppl. 12):42S–45S.

Spehlmann ME, Eckmann L. Nuclear factor-kappa B in intestinal protection and destruction. *Curr Opin Gastroenterol.* 2009;25(2):92-9.

Splawski JB, Eisenstein EM, Chualn KS. Isolation of B cell populations. In: Coligan JE, Kruisbeck AM, Margulies DM, Shevach EM, Strober W, eds. Current protocols in immunology. New York, NY: Greene Publishing & Wiley Interscience; 1991:7.1.1-7.1.5.

Stange EF, Travis SP, Vermeire S, et al. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *Gut.* 2006;(55 Suppl 1):i1e15.

Stange EF, Travis SPL, Vermeire S, et al. European evidence-based consensus on the diagnosis and management of ulcerative colitis: definitions and diagnosis.*J Crohns Colitis*. 2008;2:1e23.

Steinhart AH, Girgrah N, McLeod RS. Reliability of a Crohn's disease clinical classification

scheme based on disease behavior. *Inflamm. Bowel Dis.* 1998; 4:228–234.

Strong SA. Surgical management of Crohn's disease. Holzheimer RG, Mannick JA, editors. Surgical Treatment: Evidence-Based and Problem-Oriented. Munich: Zuckschwerdt; 2001.

Sutherland L, Ramcharan S, Bryant H, et al. Effect of cigarette smoking on recurrence of Crohn's disease. *Gastroenterology.* 1990;98:1123e8.

Swoger JM, Regueiro M. Postoperative Crohn's disease: how can we prevent it? *Expert Rev Clin Immunol.* 2010;6:501-4.

Syvanen AC. Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat Rev Genet.* 2001;2:930-42.

Szebeni B, Veres G, Dezsõfi A, et al. Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease. *Clin Exp Immunol.* 2008;151(1):34-41.

Takedatsu H, Michelsen KS, Wei B, et al. TL1A (TNFSF15) regulates the development of chronic colitis by modulating both T-helper 1 and T-helper 17 activation. *Gastroenterology.* 2008;135:552–567.

Targan SR. The utility of ANCA and ASCA in inflammatory bowel disease. *Inflamm Bowel Dis.* 1999;5:61– 63.

Testro AG, Visvanathan K. Toll-like receptors and their role in gastrointestinal disease. *J Gastroenterol Hepatol.* 2009;24(6):943-54.

The Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* 2007; 447:661-78.

Thompson AI, Lees CW. Genetics of ulcerative colitis. *Inflamm Bowel Dis.* 2011;17(3):831-48.

Thompson NP, Driscoll R, Pounder RE, Wakefield AJ. Genetics versus environment in inflammatory bowel disease: results of a British twin study. *BMJ.* 1996; 312: 95-96.

A. Till, P. Rosenstiel, K. Brautigam, et al. A role for membrane-bound CD147 in NOD2-mediated recognition of bacterial cytoinvasion. *J. Cell Sci.* 2008; 121:487–495.

Ting JP, Duncan JA, Lei Y. How the noninflammasome NLRs function in the innate immune system. *Science.* 2010;327(5963):286-90.

Török HP, Glas J, Tonenchi L. Crohn's disease is associated with a toll-like receptor-9 polymorphism. *Gastroenterology.* 2004;127(1):365-6.

Tracy L. Hull. Surgical Management Of Crohn's Disease. American Society of Colon and Rectal Surgeons. 1999.
http://www.fascrs.org/physicians/education/core_subjects/1999/chrohns_surgical_management/

Travassos LH, Carneiro LA, Ramjeet M, et al. Nod1 and Nod2 direct autophagy by recruiting

ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol.* 2010;11:55–62.

Tremelling M, Berzuini C, Massey D, et al. Contribution of TNFSF15 gene variants to Crohn's disease susceptibility confirmed in UK population. *Inflamm Bowel Dis.* 2008;14:733–737.

Tremelling M, Cummings F, Fisher SA et al. IL23R variation determines susceptibility but not disease phenotype in inflammatory bowel disease. *Gastroenterology.* 2007;132(5):1657–1664.

Truelove SC, Witts LF. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br. Med. J.* 1955; 2(4947):1041–1048.

Tysk C, Lindberg E, Järnerot G, Flodérus-Myrhed B. Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut.* 1988;29(7):990-6.

van Heel DA, Fisher SA, Kirby A, et al. Inflammatory bowel disease susceptibility loci defined by genome scan meta-analysis of 1952 affected relative pairs. *Hum. Mol. Genet.* 2004; 13(7):763-770.

Van LJ, Russell RK, Nimmo ER et al. IL23R Arg381Gln is associated with childhood onset inflammatory bowel disease in Scotland. *Gut.* 2007;56(8),1173–1174.

Vermeire S, Peeters M, Vlietinck R, et al. Exclusion of linkage of Crohn's disease to previously reported regions on chromosomes 12, 7, and 3 in the Belgian population indicates genetic heterogeneity. *Inflamm Bowel Dis.* 2000; 6:165–170.

Vermeire S, Rutgeerts P, Van Steen K, et al. Genome wide scan in a Flemish inflammatory bowel disease population: support for the IBD4 locus, population heterogeneity, and epistasis. *Gut.* 2004; 53(7):980-6.

Vermeire S, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? *Gut.* 2006; 55(3):426-31.

Vermeire S, Wild G, Kocher K, et al. CARD15 genetic variation in a Quebec population: prevalence, genotype-phenotype relationship, and haplotype structure. *Am J Hum Genet.* 2002;71:74–83.

Wang J, Wang X, Yang H, Wu D, Wang L, Qian J. Contribution of the IBD5 locus to inflammatory bowel disease: a meta-analysis. *Hum Genet.* 2011;129(6):597-609.

Watanabe T, Kitani A, Murray PJ, et al. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol.* 2004;5:800e8.

Watanabe T, Kitani A, Strober W. NOD2 regulation of Toll-like receptor responses and the pathogenesis of Crohn's disease. *Gut.* 2005;54:1515e18.

Weersma RK, Stokkers PC, Cleynen I, et al. Confirmation of multiple Crohn's disease susceptibility loci in a large Dutch–Belgian cohort. *Am J Gastroenterol.* 2009;104:630–638.

Weersma RK, Stokkers PC, van Bodegraven AA et al. Molecular prediction of disease risk and

severity in a large Dutch Crohn's disease cohort. *Gut*. 2008;58(3):388–395.

Weersma RK, Zhernakova A, Nolte IM et al. ATG16L1 and IL23R are associated with inflammatory bowel diseases but not with celiac disease in the Netherlands. *Am. J. Gastroenterol.* 2008;103(3):621–627.

Wehkamp J, Salzman NH, Porter E, et al. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A*. 2005;102:18129e34.

Wiekowski MT, Leach MW, Evans EW,et al. Ubiquitous transgenic expression of the IL-23 subunit p19 induces multiorgan inflammation, runting, infertility, and premature death. *J Immunol.* 2001;166(12):7563-70.

Williams CN, Kocher K, Lander ES, et al. Using a genome-wide scan and meta-analysis to identify a novel IBD locus and confirm previously identified IBD loci. *Inflamm Bowel Dis*. 2002; 8(6):375-81.

Witte JS. Genome-wide association studies and beyond. *Annu Rev Public Health*. 2010;31:9-20.

Wolf JM, Achkar J-P, Lashner BA, et al. Afferent limb ulcers predict Crohn's disease in patients with ileal pouch-anal anastomosis. *Gastroenterology.* 2004;126:1686–91.

Wu H, Shen B. Crohn's disease of the pouch: diagnosis and management. *Expert Rev Gastroenterol Hepatol*. 2009;3(2):155-65.

Xavier RJ, Rioux JD. Genome-wide association studies: a new window into immune-mediated diseases. *Nat Rev Immunol*. 2008;8(8):631-43.

Yamazaki K, McGovern D, Ragoussis J et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. *Hum. Mol. Genet.* 2005;14(22):3499–3506.

Yang H, Plevy SE, Taylor K, et al. Linkage of Crohn's disease to the major histocompatibility complex region is detected by multiple non-parametric analyses. *Gut*. 1999; 44: 519-526.

Yasuda N, Thomas P, Ellis H, et al. Perinuclear anti-neutrophil cytoplasmic antibodies in ulcerative colitis after restorative proctocolectomy do not correlate with the presence of pouchitis. *Scand J Gastroenterol.* 1998;33:509–513.

Yen D, Cheung J, Scheerens H, et al. IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest.* 2006;116:1310-6.

Yoshida EM. The Crohn's Disease Activity Index, its derivatives and the Inflammatory Bowel Disease Questionnaire: a review of instruments to assess Crohn's disease. *Can. J. Gastroenterol.* 1999;13(1):65–73.

Yu ED, Shao Z, Shen B. Pouchitis. *World J Gastroenterol.* 2007;13(42):5598-604.

Yuan Q, Walker WA. Innate immunity of the gut: mucosal defense in health and disease. *J Pediatr Gastroenterol Nutr*. 2004;38(5):463-73.

Zhang WJ, Thompson JL, Chorney MJ, Koltun WA. Phenotyping of IL4-induced nuclear Stat6 activity in humans: quantitation after gel shift assay using immortalized cell lines. *Oncol Rep.*2003;10:1281–1288.

APPENDIX #1

CONSENT FOR RESEARCH
Penn State College of Medicine
The Milton S. Hershey Medical Center

Title of Project: A Proposal for the Creation of an Inflammatory Bowel Disease Registry

Principal Investigator: Walter Koltun, M.D.

Other Investigators: Lisa Poritz, M.D., Marjorie Lebo, C.R.N.P., Danielle Pastor, M.D., Gaylene Webber, Zhenwu Lin, Ph.D., Amy Sheranko, M.A., Ashley Kelly, B.A. , Gail Matters, Ph.D., Robin Wilson, Ph.D., Wei Yu, M.D., Ph.D. John Hegary, M.S., Rishabh Sehgal, M.D., Sue Deiling, B.A. Kevin McKenna, M.D., David Stewart, M.D., Nathan Hansen, M.D., David Brinton, C.P.N.P.

Consent: Adult with IBD

Participant's Printed Name: _____

This is a research study. Research studies include only people who voluntarily choose to take part. This consent form gives you information about this research, which will be discussed with you. This consent form may contain words or procedures that you do not understand. You are urged to ask questions about anything that is unclear to you. Discuss it with your family and friends and take your time to make your decision. You will receive a copy of the signed and dated consent form to keep.

1. **Purpose of the Study**: You have been given the opportunity to participate in this study because:
   - You have inflammatory bowel disease (IBD).
   - You **may or may not** have family members with inflammatory bowel disease (IBD).

   There are two main forms of IBD: Crohn's disease and ulcerative colitis. IBD is a chronic inflammatory condition affecting the intestines and resulting in abdominal pain, diarrhea, anemia, and malnutrition. There is no cure for IBD, and its cause remains unknown. Some studies have suggested that this disease may be, in part, hereditary. This study will investigate the role of genes in IBD. Approximately 2000 people will be enrolled in this study at Hershey Medical Center.

2. **Procedures to be Followed**: If you agree to participate in this study, you will complete a questionnaire about your health and family history. You are free to skip any questions you would

prefer not to answer. If a question is unclear and you would prefer to try to answer it, you are encouraged to call us for clarification (see Contact Information, Section 12).
A sample of your blood (4 to 5 teaspoons) will be taken to examine the possible genes that are associated with IBD.

We will create a "cell line" from your blood by treating the white blood cells with something which causes them to repeatedly divide under specific laboratory conditions. This enables us to grow the cells over time which we cannot do with untreated blood cells. We then prepare and store frozen stocks of each cell line so that we have "fresh cells" as we need them. It is from this supply of cells that we extract the DNA (genetic or hereditary material) which we use to conduct our genetic research on IBD.

Additionally a portion of your blood will be used to isolate serum, which we will look at to study protein expression. This information will be considered in light of the genetic findings to better study IBD. Unlike the cells we cannot "grow" the serum in the lab, so we will isolate a given amount of it from your blood and divide it up into small quantities. We will then freeze these samples and use them as needed until they are depleted.

The cell and serum samples we collect from you will be entered into our "IBD Registry," a bank of samples we catalogue and store as possible for the duration of this study.

In addition, if you are undergoing surgery to remove a part of your intestine, you give permission for some of your removed intestine, which would be otherwise discarded, to be used in our research. We will process small segments of the tissue for genetic and metabolic analysis. Agreeing to this does not alter your surgery or your care in any way, and no additional tissue will be removed, only that tissue which would be removed due to your disease will be studied in such a fashion, and only after all other test necessary for your care are done first. We may also take and store a digital photograph of your removed intestine for future reference.

If and only if you agree, your samples will be stored beyond the duration of this study and used for other research studies. If you refuse to have your samples stored for these other research studies, you will still have the opportunity to participate in this IBD research study. This option and related details are addressed in the last section of this document.

In order to determine the extent of your disease, your medical records will be obtained from your primary physician, gastroenterologist(s), and/or surgeon(s) for review.

Additionally, it is possible that over time it will be necessary for an investigator to call you to update your questionnaire information. Typically this type of communication takes 5 to 15 minutes. Additionally it is possible that at some point your cells and/or serum will need to be replenished. If that is the case you may be contacted by an investigator to arrange for another blood draw. This could occur as soon as a month after your first draw or as late as years later, and in each case your participation is, as always, entirely voluntary. You are not agreeing to any future participation at this time. You will be asked to sign an addendum to this consent form for any additional blood samples requested.

3. **Discomforts and Risks**: The discomfort associated with removing blood by insertion of a needle into a vein (venipuncture) in your arm, using standard procedure, is a slight pinch or pin prick when the sterile needle enters the skin. The risks from removing blood by needle include mild discomfort and/or black and blue mark at the site of puncture. Less common

risks include a small blood clot, infection, or bleeding at the puncture site, and on rare occasions fainting during the procedure.

If you are undergoing surgery, and agree to allow us to study your removed intestine, there will be no difference in risk or discomfort beyond that which your planned surgery would be expected to incur.

You may be uncomfortable discussing your health. You may refuse to answer any questions that make you feel uncomfortable. There is also a risk of loss of confidentiality if your medical information is obtained by someone other than the researchers but precautions will be taken to prevent this from happening.

4. **(a) Benefits to You**: There is no promise or guarantee of any medical benefits to you resulting from participation in this study. Neither you nor your doctor will receive results of these research tests.

   (b) Potential Benefits to Society: This study may provide the medical community and society with information about the genetic factors that contribute to the development of IBD, potentially resulting in better treatment of IBD.

5. **Other Options That Could Be Used Instead Of This Research**: You may choose not to participate in the study.

6. **Time Duration of the Procedures and Study**: The initial visit for the consenting and blood-draw typically takes one half hour. The questionnaire which is typically sent home with the participant takes about 1 to 2 hours to complete. Since this is an ongoing study with stored samples, we do often contact participants months to years after the initial consent. Future participation is likely to be in the form of a 5 to 15 minute phone call to update the questionnaire information. As described in the procedure section (2), it is also possible that you will be called at a future date and asked to donate a blood sample again. If you agree that usually takes about one half hour.

7. **Statement of Confidentiality**:
   (a) **Privacy and confidentiality measures:** The research records that we generate from your participation will be reviewed, stored, and analyzed at The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU). Research results generated in the laboratory in this study will be labeled with a code, and stored at-large in the secured laboratories of Dr. Koltun. Likewise, the samples we collect from you for research purposes will be labeled with a code, and stored at the requisite temperature in the secured laboratories of Dr. Koltun.
   The research information we collect from you that has identifying information, such as the questionnaire you will be asked to complete, will be stored in a locked filing cabinet in the secured laboratories of Dr. Koltun.
   A list linking codes to identifiers will be kept in a separate locked filing cabinet in the secured laboratories of Dr. Koltun.

   In the event that research data and/or samples are sent to an institution outside of HMC/COM you will not be identified by name, social security number, address or phone number. The data and/or samples will be labeled with a code and will not be identifiable to you in any way. The list

that matches your name with the code number will be kept in a locked file at this institution and will not be shared with another institution.

In the event of any publication resulting from the research, no personally identifiable information will be shared.

(b) **The use of private health information:** Health information about you will be collected if you choose to be part of this research study. Health information is protected by law as explained in the HMC Privacy Notice. If you have not received this notice, please request a copy from the researcher. At The Milton S. Hershey Medical Center (HMC) and Penn State College of Medicine (PSU) your information will only be used or shared as explained and authorized in this consent form or when required by law. It is possible that some of the other people/groups who receive your health information may not be required by Federal privacy laws to protect your information and may share it without your permission.

To participate in this research you must allow the research team to use your health information. If you do not want us to use your protected health information you may not participate in this research.

Your permission for the use, retention, and sharing of your identifiable health information will continue indefinitely. Any research information in your medical record will be kept indefinitely. If you consent to the storage of your samples for future research, the period for the use of the samples is unknown.

If you choose to participate, you are free to withdraw your permission for the use and sharing of your health information and your samples at any time. You must do this in writing. Write to Dr. Koltun and let him know that you are withdrawing from the research study. His mailing address is 500 University Drive, Department of Colon and Rectal Surgery, H137, Hershey, PA 17033.

If you withdraw your permission, we will no longer use or share medical information about you or your samples for this research study, except when the law allows us to do so. We are unable to take back anything we have already done or any information we have already shared with your permission, and we may continue using and sharing the information obtained prior to your withdrawal if it is necessary for the soundness of the overall research. Also we will keep our records of the care that we provided to you as long as the law requires.

The research team may use the following sources of health information from you, your primary physician, your gastroenterologist(s), and/or your surgeon(s):

- Blood drawn for this research
- Progress Notes, as they relate to this research
- Endoscopy Reports, Radiology Reports, Operative Reports, and Pathology Reports, as they relate to this research
- Questionnaire about your health and family history.

In most cases records are pulled from the date of IBD diagnosis and onward.

Representatives of the following people/groups within HMC/PSU may use your health information and share it with other specific groups in connection with this research study.

- The principal investigator, Dr. Koltun
- The HMC/PSU Institutional Review Board
- The HMC/PSU Human Subjects Protection Office

- The Research Team

The above people/groups may share your health information with the following people/groups outside HMC/PSU for their use in connection with this research study. These groups, while monitoring the research study, may also review and/or copy your original PSU/HMC records.

- The Office of Human Research Protections in the U.S. Department of Health and Human Services
- The Philadelphia Health Care Trust Fund
- American Society of Colon and Rectal Surgeons
- Pennsylvania Department of Health's Health Research Formula Funding Program – Tobacco Settlement Grant

8. **Costs for Participation:** There are no costs to you for participating in this research study.

9. **Compensation for Participation:** There is no financial compensation for participation in this study. In the event of the development of commercial products from the findings of this research, there are no plans to share any of the profits with you.

10. **Research Funding:** The institution and investigators are receiving a grant from the Philadelphia Health Care Trust Fund, the Carlino Gift Fund for IBD Research and the Tobacco Settlement Grant to support this study.

11. **Voluntary Participation**: Taking part in this research study is voluntary. If you choose to take part in this research, your major responsibilities will include: providing a venipuncture blood sample and the completion of questionnaires. You do not have to participate in this research. If you choose to take part, you have the right to stop at any time. If you decide not to or if you decide to stop taking part in the research at a later date, there will be no penalty or loss of benefits to which you are entitled. In other words, your decision to not participate in this research or to stop taking part in the research will not affect your medical services.

12. **Contact Information for Questions or Concerns**: You have the right to ask any questions you may have about this research. If you have questions later or concerns related to this study, or if you believe you may have developed an injury that is related to this research, you should contact Dr. Walter Koltun at (717) 531-5164 or Ashley Kelly (717) 531-5325.

    If you have questions regarding your rights as a research participant or you have concerns or general questions about the research or about your privacy and the use of your personal health information, contact the research protection advocate in the HMC Human Subjects Protection Office at 717-531-5687. You may also call this number if you cannot reach the research team or wish to talk to someone else.

    For more information about participation in a research study and about the Institutional Review Board (IRB), a group of people who review the research to protect your rights, please visit the HMC IRB's Web site at http://www.hmc.psu.edu/irb. Included on this web site, under the heading "Participant Info", you can access federal regulations and information about the protection of human research participants. If you do not have access to the internet, copies of these federal regulations are available by calling the HSPO at (717) 531-5687.

Signature and Consent/Permission to be in the Research

Before making the decision regarding enrollment in this research you should have:

- Discussed this study with an investigator,
- Reviewed the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Participant: By signing this consent form, you indicate that you are voluntarily choosing to take part in this research.

Signature of Participant	Date	Printed Name

Person Explaining the Research: Your signature below means that you have explained the research to the participant/participant representative and have answered any questions he/she has about the research.

Signature of person who explained this research	Date	Printed Name

Optional Storage of Samples and Questionnaires for Future Research Studies: By definition this study is establishing a collection of stored samples (cells, serum, and DNA) and questionnaires to be used for ongoing research for the duration of this study. However, these samples and questionnaires may not be stored beyond the duration of this study or used for other research without your explicit permission.

Agreeing to the storage of your samples and questionnaire beyond the duration of this study and use for other research is optional and will not affect your participation in this research study. You can participate in the main part of the research without agreeing to allow your samples or questionnaire to be used for this optional part.

These future studies may provide additional information that will be helpful in understanding IBD, but it is unlikely that these studies will have a direct benefit to you. Neither you nor your doctor will receive results of these future research tests. The results of these tests will not have an effect on your care, nor will the results be put in your health record. Sometimes a sample and questionnaire is used for genetic research about diseases that are passed on in families. Even if your samples and questionnaire are used for this kind of research, the results will not be put in your health records. If you have any questions, you should contact Dr. Walter Koltun at (717) 531-5164.

Your samples will be labeled with a code number and stored. Specifically, your cells will be labeled with a code, the date they were frozen down, and stored in a tank of liquid nitrogen in the secured laboratories of Dr. Koltun. Your serum and DNA will be labeled with a code, the date extracted, and stored in a -80C freezer in the secured laboratories of Dr. Koltun. Your questionnaire will remain in your file in a locked filing cabinet. If you agree to allow your samples and questionnaire to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Walter Koltun at (717) 531-5164 and let

him know you wish to withdraw your permission for your samples and questionnaire to be used for future research. In that case your unused samples and your questionnaire would be destroyed at the termination of this study and not used for future studies.

Please **INITIAL** *<u>one</u> of the following:*

_____ initial	Your samples and questionnaire **may** be stored beyond the duration of this study and used for **any** future research studies involving this or any other project without your further permission.

OR

_____ initial	Your samples and questionnaire **may not** be used for future research studies. Your samples and questionnaire may be stored for the duration of this study only. When this study is over your samples and questionnaire will be destroyed.

<u>Participant</u>: By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part of the research study.

_____	_____	_____
Signature of Participant	Date	Printed Name

<u>Person Explaining the Research</u>: Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

_____	_____	_____
Signature of person who explained this research	Date	Printed Name

APPENDIX #2

PENNSTATE

Human Subjects Protection Office
Institutional Review Board
Room - H6509

Penn State College of Medicine
The Milton S. Hershey Medical Center
Human Subjects Protection Office, H138
500 University Drive
Hershey, PA 17033-2390

(717) 531-5687
Fax: (717) 531-3937
E-mail: hspo@psu.edu

DATE: July 23, 1998

TO: Walter A. Koltun, M.D., Surgery/General Surgery

FROM: Kevin Gleeson, M.D., Chairman
Institutional Review Board

K Gleeson MD

RE: IRB Protocol No. 98-057 - A Proposal for the Creation of an Inflammatory Bowel Disease Registry

Thank you for your application for the above research. The Institutional Review Board (IRB) granted approval for this investigation for a one year period, effective July 20, 1998. This approval includes the consent form (dated 7/15/98). A total of five hundred (500) subjects may be enrolled. You may proceed with the research, as outlined.

Please include the IRB protocol number on all future correspondence and documents related to this investigation.

All original, signed consent forms for this research must be forwarded to the IRB for the central files. Please ensure that both an approved investigator and the participant sign and date the consent form. Provide a copy of the form to the participant. If the participant is a patient, include a copy of the signed consent form and the protocol abstract in the medical record.

Federal regulations require prompt reporting to the IRB of any proposed changes in a research activity and prior approval before changes are initiated, except where necessary to eliminate apparent immediate hazards to the subject. Any serious, life-threatening or unexpected adverse events related to this project should be reported immediately to the IRB using the Adverse Event and Safety Report form. All other adverse events (such as mild or expected reactions) should be reported on the Progress Report for continuing review.

The Institutional Review Board appreciates your efforts to conduct research in compliance with the federal regulations that have been established to ensure the protection of human subjects.

KG\dme

APPENDIX #3

PENNSTATE HERSHEY
Milton S. Hershey Medical Center

PENNSTATE HERSHEY
Colon and Rectal Surgery

Walter A. Koltun, MD
Division Chief
Peter & Marshia Carlino Chair in Inflammatory Bowel Disease

Lisa Poritz, MD
Associate Professor of Surgery and Cellular & Molecular Physiology
Director of Colon & Rectal Research

Kevin J. McKenna, MD
Associate Professor of Surgery

David B. Stewart, MD
Assistant Professor of Surgery

Evangelos Messaris, MD, PhD
Assistant Professor of Surgery

Marjorie Lebo, MSN, CRNP
Nurse Practitioner

David Brinton, MSN, CRNP
Nurse Practitioner

Amy Sheranko, MA
Medical Assistant

IRB# 98-057 Inflammatory Bowel Disease Registry

STUDY PARTICIPANT QUESTIONNAIRE

This packet contains a questionnaire that we ask all study participants to fill out in addition to giving a blood sample. It can take anywhere from 30 minutes to 2 hours to fill out. Please mail this packet back to us in the pre-stamped self-addressed envelope provided by us.

The Complete Family History section is quite extensive, but can only be filled out according to the size of your family and your knowledge of your family's health history. Please fill it out to the best of your ability. Please take care to note if someone is a "half" or "step" relation, if there are twins, or any other details that could be important to our genetic studies.

While you should not answer any question that makes you uncomfortable as we explained in the consent, please do not hesitate to contact us if it is just a matter of a question being unclear.

If you have questions about the questionnaire or need further information, please contact me:
Sue at 717-531-0003 x 285223 or sdeiling@psu.edu

We sincerely thank you for your participation in this study.

Sue Deiling, B.A.
Walter A. Koltun, M.D

Personal Information

1. Subject Name: _____
Last First Middle Initial

2. Address: _____
Street Address

_____
City State Zip Code

3. Phone Number: (____) ____-____(H) (____) ____-____(W)
Area code Area code

4. E-mail Address: _____
(If applicable)

5. Family Physician: _____
Last First Middle Initial

6. Physician's Address: _____
Street Address

_____
City State Zip Code

7. Physician's Phone Number: (____) ____-____
Area code

8. Gastroenterologist: _____
(If applicable)
Last First Middle Initial

9. Gastroenterologist's Address: _____
Street Address

_____
City State Zip Code

10. Gastroenterologist's Phone Number: (____) ____-____
Area code

Complete Family History

Please indicate whether the following members of your immediate (biologic) family have ever been diagnosed with IBD, Diverticulitis, or colorectal cancer. Enter Yes, No, or Unknown.

Relationship			IBD	Diverticulitis	Cancer	Comments (twin?, step?, deceased?, other disease, type cancer, etc)
Paternal	Grandfather	009				
	Grandmother	010				
Maternal	Grandfather	011				
	Grandmother	012				
Father		013				
Mother		014				
Spouse		019				
Brother		015				
Brother		015				
Brother		015				
Brother		015				
Sister		017				
Sister		017				
Sister		017				
Sister		017				
Son		020				
Son		020				
Son		020				
Son		020				
Daughter		021				
Daughter		021				
Daughter		021				
Daughter		021				

Relationship		IBD	Diverticulitis	Cancer	Comments (twin?, step?, deceased?, other disease, type cancer, etc)
Son's son	022				
Son's son	022				
Daughter's son	024				
Daughter's son	024				
Son's Daughter	023				
Son's Daughter	023				
Daughter's Daughter	025				
Daughter's Daughter	025				

Please indicate whether members of the your extended family, such as cousins, uncles, aunts, etc, have ever been diagnosed with IBD, Diverticulitis, or colorectal cancer. Enter Yes, No, or Unknown.

Relationship	IBD	Diverticulitis	Cancer	Comments (Mother's side?, Father's side?, twin?, deceased?, other disease, type cancer?, etc)
1.				
2.				
3.				
4.				
5.				
6.				
7.				
8.				
9.				
10.				
11.				
12.				
13.				
14.				
15.				

Demographic Information

13. What is your date of birth?

_ _ /_ _ /_ _ _ _
month day year

14. What is your race/ethnic?
- ☐ Aleut, Eskimo, or American Indian
- ☐ Asian
- ☐ Black, Hispanic / Latino
- ☐ Black, Not Hispanic / Latino
- ☐ Pacific Islander
- ☐ White, Hispanic / Latino
- ☐ White, Not Hispanic / Latino
- ☐ Other _____

15. Where were you born?

_____
City State

_ _ _ _ _
Zip (if known)

_ _ _ _____
(Admin use) Country

16. Where did you spend the majority of your childhood?

_____
City State

_ _ _ _ _
Zip (if known)

_ _ _ _____
(Admin use) Country

17. Where do you currently live?

_____
City State

_ _ _ _ _
Zip (if known)

_ _ _ _____
(Admin use) Country

18. Has a doctor ever told you that you have Inflammatory Bowel Disease (Crohn's Disease, Ulcerative Colitis, Indeterminate Colitis)?
    - ☐ Yes
    - ☐ No *(☞ **Please go to question #19.**)*

    18a. If Yes, please specify what type:
    - ☐ Crohn's Disease
    - ☐ Ulcerative Colitis
    - ☐ Indeterminate Colitis
    - ☐ Unknown

19. What is your biologic father's ethnic background? ***(Use Ancestry Codes below)***

    __ __ _____
    __ __ _____

20. What is your biologic mother's ethnic background? ***(Use Ancestry Codes below)***

    __ __ _____
    __ __ _____

ANCESTRY CODES

Most people in the United States have ancestors who came from other parts of the world. This lists some ethnic backgrounds. Please choose the one or two that most closely describe your ancestry.

African 11
American Indian 17
Canadian 24
Central American 21
Chinese 14
Croatia 07
Cuban 23
Czech Republic 07
Danish 08
English 01
Finnish 08
French 04
German 03
Greek 06
Hungarian 07
Indian 13
Irish 02
Italian 05
Japanese 15
Jewish / Ashkenazi 28
Mexican 18
Middle Eastern 12
Norwegian 08
Pacific Islander 16
Pakistani 13
Pennsylvania Dutch 26
Polish 07
Portugese 09
Puerto Rican 19
Russian 07
Scottish 01
Serbia 07
Slovakia 07
South American 22
Spanish 09
Swedish 08
Ukraine 07
Welsh 01
West Indian 20
Yugoslavia 07
Other Asian (Please specify) .16
Other European (Please specify) 10
Other (Please specify) 25
Unknown 27

21. In which religion were you raised (pick one)?
- ❑ Amish
- ❑ Baptist
- ❑ Catholic
- ❑ Jewish
- ❑ Lutheran
- ❑ Mennonite / Brethren
- ❑ Methodist
- ❑ Other Protestant _____ (Please specify)
- ❑ Other _____ (Please specify)
- ❑ None

22. Smoking History *(Cigarettes only)*

22a. Are you.....
- ❑ Current Smoker
- ❑ Ex-Smoker
- ❑ Never Smoked (Skip to Question 23)

22b. How many years have you, or did you, smoke?
_ _ years

22c. What is the average number of packs per day that you smoke(d)?
_ _ • _ packs per day

22d. If you did quit smoking, how long ago did you quit?

_ _ years _ _ months

23. Have you ever spent at least 2 consecutive years living in an active agricultural area with cows?
- ❑ Yes
- ❑ No
- ❑ Unknown

24. Were you breast fed as a baby?
- ❑ Yes
- ❑ No
- ❑ Unknown

Medical History

Do you now, or have you ever been diagnosed with... (*Please select only **one** answer for **each** question)*

		No	Yes	Unknown
25a.	Measles?			
25b.	Chicken Pox?			
25c.	Mumps?			
25d.	Lupus?			
25e.	Rheumatoid Arthritis?			
25f.	Diabetes?			
25g.	☞If yes, how controlled? *(Circle only one)* Insulin, oral meds, diet only			
25h.	Multiple Sclerosis (MS)?			
25i	Celiac Disease			

		No	Currently	Previously	Unknown
25j.	Pyoderma Gangrenosum?				
25k.	Anal Abscesses or Fistulae?				
25l.	Recurrent Mouth Sores?				
25m.	Uveitis, Iritis, or Chronic Irritation of the Eyes?				
24n.	Erythema Nodosum or other persistent skin disorders? (Please specify) _____				
25o.	Primary Sclerosing Cholangitis (PSC) or other liver disease, excluding gallstones? (Please specify) _____				

Have you ever been diagnosed, by a physician, with any of the following disease or conditions: *(Please select only **one** answer for **each** question)*			
	No	Yes →	If Yes, Year Diagnosed
26a. Anal Cancer?			
26b. Anemia?			
26c. Appendicitis?			
26d. Collagenous Colitis?			
26e. Colon Cancer?			
26f. Diverticulitis?			
26g. Infectious Colitis?			
26h. Intestinal Cancer?			
26i. Irritable Bowel Syndrome?			
26j. Proctitis?			
26k. Prostrate Cancer			
26l. Rectal Cancer?			
26m. Reflux?			
26n. Stomach Cancer?			
26o. Ulcer Disease?			
26p. Whipple's Disease?			
26q. Other (Please specify) _____			

27. Have you ever had a stoma?
- ❑ Yes
- ❑ No *(☞ **Please go to question #28.**)*

27a. If Yes, do you currently have a stoma?
- ❑ Yes *(☞ **Please go to question #33, if applicable.**)*
- ❑ No *(☞ **Please go to question #28.**)*

28. How many Bowel Movements (BMs) do you have in a typical day?
- ❑ Less than 1 per day
- ❑ 1 per day
- ❑ 1-3 per day
- ❑ More than 3 per day

29. Are your BMs typically:
- ❑ Diarrhea without form?
- ❑ Loose?
- ❑ Soft with form?
- ❑ Formed?
- ❑ Constipated?

30. How often do you have blood in your stools?
- ❑ Never
- ❑ Rarely
- ❑ Sometimes
- ❑ Often
- ❑ Always

31. Do you suffer from diarrhea (watery, loose stools)?
- ❑ Rarely
- ❑ Occasionally (2-3 times per year)
- ❑ Regularly (every month)
- ❑ Frequently (every week)

32. When you suffer from diarrhea, it typically:
- ❑ Resolves in a few days, requiring no medication.
- ❑ Lasts longer than a week, but resolves with no medication required.
- ❑ Requires medical treatment (Kaopectate, Imodium, or a visit to the doctor) to resolve.

If you have **never** been diagnosed with IBD, you can stop here! Thank you for taking the time to complete this form. Don't forget to mail it back to us!

If you have **EVER** been diagnosed with IBD, please continue to complete the next section.

IBD Characterization

(Please complete if you have ever been diagnosed with IBD)

33. When were you diagnosed with Inflammatory Bowel Disease (IBD)?

_ _ / _ _ _ _
month year

34. When did your initial treatment start after your diagnosis?

_ _ / _ _ _ _
month year

35. Are you currently being treated by a Gastroenterologist for your Inflammatory Bowel Disease?
- ☐ Yes
- ☐ No

36. Have you had any hospitalization or surgeries related to your Inflammatory Bowel Disease?
- ☐ Yes ***(☞ Please complete the table below.)***
- ☐ No ***(☞ Please go to question #35.)***

If Yes, please complete.	Total	In the past 2 years
How many hospitalizations?		
How many surgeries?		

37. Have you ever had emergency abdominal surgery because of your Inflammatory Bowel Disease?
- ☐ Yes
- ☐ No

38. How many Bowel Movements (BMs) do you have in a typical day? (When your disease is in its usual state of activity (i.e., not during a flare) and when you are taking your medicine.)

_ _ BMs

39. How often do you experience belly pain with your Inflammatory Bowel Disease?
- ☐ Every day
- ☐ Every week
- ☐ Occasionally
- ☐ Only when I eat wrong or don't use my medicine
- ☐ Never

40. Have you ever received a blood transfusion?
- ❑ Yes
- ❑ No

41. How many total days in the past 12 months have you spent in the hospital or visited your physician (1 office visit = 1 day) because of your Inflammatory Bowel Disease?

_ _ _

42. Please characterize your disease:
- ❑ Rarely gives me problems; minimal need for medicine.
- ❑ Rarely gives me problems as long as I take my medicine.
- ❑ Occasionally gives me problems, but generally is under control.
- ❑ Frequently gives me problems.
- ❑ A constant battle; severely affects my life and ability to function.

43. Please rate on a scale from 0 (None) to 9 (Most Severe) the following Inflammatory Bowel Disease symptoms for when your disease is in its usual state of activity (i.e., not during a flare) and when you are taking your medicine(s).

(☞ Circle the number that is most clearly associated with the severity of each symptom.)

	None		Mild			Moderate				Severe
a. Abdominal pain	0	1	2	3	4	5	6	7	8	9
b. Abdominal bloating	0	1	2	3	4	5	6	7	8	9
c. Nausea/Vomiting	0	1	2	3	4	5	6	7	8	9
d. Diarrhea	0	1	2	3	4	5	6	7	8	9
e. Rectal Bleeding	0	1	2	3	4	5	6	7	8	9
f. Anal Fistuli/Abscesses	0	1	2	3	4	5	6	7	8	9

IBD

Inflammatory Bowel Disease Registry

Subject ID: ___-___-__

Date Completed: __ __ / __ __ / __ __ __ __
Month day year

Please provide us with a complete list of all medications you have ever taken for IBD. Remember if the medication was taken in the past to indicate how long ago the medication was last taken.

Answer for ***each*** of the following medications.	Never Taken	Currently Taking	Taken in the past	How long ago did you stop?
Imuran® or 6-MP (azathioprine) 001	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Flagyl® (metronidazole) 002	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Asacol® (mesalamine) 003	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Pentasa® (mesalamine) 004	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Cipro® (ciprofloxacin hydrochloride) 005	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Azulfidine® (sulfasalazine) 006	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Folic Acid 007	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
B_{12} Shots 008	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Prednisone 009	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Remicade 016	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Humira® 067	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Lialda® 069	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
Cimzia® 075	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
List any other medications you are currently using, or used in the past, for IBD:				
	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago

Answer for ***each*** of the following medications.	Never Taken	Currently Taking	Taken in the past	How long ago did you stop?
	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago
	❑	❑	❑ →	○ Less than 3 months ago ○ 3 - 12 months ago ○ More than 12 months ago

44. What ***one*** medication do you feel best controls, or controlled, your Inflammatory Bowel Disease?

_ _ _ _____

Thank you for taking the time to complete this form. Don't forget to mail it back to us!

APPENDIX #4

ORIGINAL CONTRIBUTION

NOD2/CARD15 Mutations Correlate With Severe Pouchitis After Ileal Pouch-Anal Anastomosis

Rishabh Sehgal, M.B., B.Ch.[1] • Arthur Berg, Ph.D.[2] • John P. Hegarty, Ph.D.[1]
Ashley A. Kelly, B.A.[1] • Zhenwu Lin, Ph.D.[1] • Lisa S. Poritz, M.D.[1]
Walter A. Koltun, M.D.[1]

1 Division of Colon and Rectal Surgery, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania
2 Department of Public Health Sciences, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania

PURPOSE: Pouchitis and Crohn's-like complications can plague patients after IPAA. NOD2 is an intracellular sensor for bacterial cell wall peptidoglycan. NOD2 mutations compromise host response to enteric bacteria and are increased in Crohn's disease. We hypothesize that IPAA patients with complications (Crohn's disease-like/pouchitis) have a higher rate of NOD2 mutations compared with asymptomatic IPAA patients.

METHODS: Patients were retrospectively subclassified into the following groups: 1) IPAA with Crohn's-like complications (n = 28, perianal fistula, pouch inlet stricture/upstream small-bowel disease, or biopsies showing granulomata) occurring at least 6 months after ileostomy closure; 2) IPAA with mild pouchitis (n = 33, ≤3 episodes/y for 2 consecutive years); 3) IPAA with severe pouchitis (n = 9, ≥4 episodes/y for 2 consecutive years or need for continuous antibiotics); 4) IPAA without complications or pouchitis (n = 37); 5) patients with Crohn's disease with colitis undergoing total proctocolectomy/ileostomy (n = 11); and 6) healthy controls (n = 269). The 3 NOD2 single-nucleotide polymorphism mutations (rs2066844, rs2066845, and rs2066847) previously identified as associated with Crohn's disease were genotyped using polymerase chain reaction. Groups were compared by use of χ^2 with Yates continuity correction.

RESULTS: NOD2 mutations were found in 8.5% of healthy controls. NOD2 mutations were significantly higher in the severe pouchitis group (67%) compared with both asymptomatic IPAA (5.4%, $P < .001$) and IPAA with Crohn's disease-like complications (14.3%, $P = .008$) groups.

CONCLUSIONS: 1) Asymptomatic IPAA patients have a low incidence of NOD2 mutations not significantly different from patients with mild pouchitis or healthy controls. 2) Patients with severe pouchitis had the highest incidence of NOD2 mutations, suggesting that this group may have a compromised host defense mechanism to enteric bacteria. 3) Patients with Crohn's-like complications after IPAA have a significantly lower incidence of NOD2 mutations than patients with severe pouchitis, suggesting a different genetic makeup in these 2 patient groups. Preoperative assessment of NOD2 in the equivocal IPAA candidate may predict severe pouchitis and might assist in preoperative surgical decision making.

KEY WORDS: Classification; Crohn's disease; Genetics; Inflammatory bowel disease; *NOD2/CARD15*; Pouchitis; Ulcerative colitis.

Ulcerative colitis (UC) is an inflammatory condition of the colon that has no clear etiology or medical cure. Although medical therapy aids in controlling the inflammation, approximately 25% to 33%[1] of patients will eventually require surgery to alleviate their symptoms and improve quality of life. Total proctocolectomy with IPAA is now considered to be the operative standard for the treatment of UC. This procedure avoids a permanent stoma, decreases the risk of dysplasia and cancer, and allows patients to discontinue steroids and other immune therapies that can have significant side effects.

Financial Disclosure: None reported.

Presented at meeting of The American Society of Colon and Rectal Surgeons, Minneapolis, MN, May 15 to 19, 2010.

Correspondence: Rishabh Sehgal, M.B., B.Ch., or Walter Koltun, M.D., Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Division of Colon and Rectal Surgery, H137, 500 University Dr, P.O. Box 850, Hershey, PA 17033-0850. E-mail: rsehgal@hmc.psu.edu or wkoltun@hmc.psu.edu

Dis Colon Rectum 2010; 53: 1487–1494
DOI: 10.1007/DCR.0b013e3181f22635

Postoperative complications related to the IPAA can occur, however. These include early complications that are usually technically related, or ones occurring later that can suggest recrudescent IBD, such as perianal fistulae, afferent limb stricture, or severe pouchitis recalcitrant to conventional management with antibiotics. The severity of such complications can sometimes suggest a diagnosis of Crohn's disease (CD), which is usually considered a preoperative contraindication for IPAA, because of historically high failure rates.[2]

In the past 2 decades, genetic variants and their role in the pathogenesis of IBD (both UC and CD) have been the subject of intensive research. The goal of this exciting work is to discover the possible etiology of IBD by identifying genetic mutations associated with IBD through studying large populations of IBD patients. One of the earliest genes in which mutations have been shown to be associated with Crohn's disease was the *NOD2/CARD15* gene on chromosome 16.[3,4] This gene encodes a protein that functions as an intracellular sensor of muramyl-dipeptide, a component of bacterial cell walls. Three common mutations within this gene lead to a compromised host immune response to enteric bacteria. Pouchitis is due, in part, to an overgrowth of bacteria as evidenced by its responsiveness to antibiotics. Mutations in the *NOD2/CARD15* gene could possibly contribute to pouchitis and/or predict Crohn's-like complications such as fistulae or strictures in patients thought to have UC. The present study therefore was undertaken to test the hypothesis that patients experiencing complications after IPAA (CD-like fistulae, strictures, granulomata, and/or pouchitis) have a higher-than-expected rate of NOD2 mutations compared with IPAA patients without such complications.

METHODS

Patients

A retrospective patient chart review was conducted by reviewing all patients who underwent a total proctocolectomy with IPAA reconstruction at The Milton S. Hershey Medical Center from July 1990 to July 2009. From a total of 382 IPAA patients, 107 patients were subsequently recruited into the study population presented here. All patients with CD-like complications and severe pouchitis were fully recruited. Patients with mild pouchitis and an asymptomatic pouch were recruited without selection bias based on availability defined by follow-up clinic visits during the study period (September 2008 to September 2009). Patients were classified into the following groups: 1) IPAA without complications or pouchitis; 2) IPAA with mild pouchitis; 3) IPAA with severe pouchitis; and 4) IPAA with CD-like complications. Control populations of patients were CD patients with colitis undergoing total proctocolectomy/ileostomy and otherwise healthy controls. The criteria defining these patient groups are presented in Table 1. Medical records, including progress notes, hospital admission and discharge documents, surgical operative notes, endoscopy reports, and pathology reports were reviewed.

Individual episodes of pouchitis were defined by conventional clinical criteria including symptoms (increased frequency of bowel movements, diarrhea, tenesmus), pouchoscopy (gross inflammation), and biopsy showing inflammation superimposed on chronic inflammation. Patients were defined as having severe vs mild pouchitis according to criteria established by Shen et al.[5]

Patient demographics and documentation of clinical parameters of disease were gathered and included the following: 1) gender, family history, date of IBD diagnosis, prior number of surgeries, and smoking history; 2) extent of disease, defined by surgical pathology; 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab, and steroid use; 4) frequency of pouchitis attacks and medications used in management; and 5) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. The study was approved by The Milton S. Hershey Medical Center Institutional Review Board.

DNA/Cell Bank

The identified IPAA patients were contacted and recruited into our institutional review board-approved genetic IBD cell/DNA bank originally established in 1998. Informed consent was obtained and patients donated blood samples that were used to create immortalized B-cell lines using Epstein Barr virus to provide an indefinite source of DNA from each recruited individual.[6] In brief, blood was placed into sterile phosphate-buffered saline, mixed, and slowly layered onto sterile Ficoll-Paque (Amersham Biosciences) solution. The blood-Ficoll mixture was centrifuged in a Sorvall RT centrifuge at 1000 rpm for 30 minutes at room temperature until cellular gradient achieved. The lymphocyte interface was carefully extracted. These cells were then washed with phosphate-buffered saline, mixed, and centrifuged at 1000 rpm for 10 minutes. The cellular pellet was resuspended in media containing Epstein Barr virus for transformation and incubated. Once transformed, cells were stored by placing 1×10^7 cells/mL in 1-mL aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks till use.

DNA Isolation

DNA was extracted from 1×10^6 transformed B cells using a DNA isolation kit (QIAAmp DNA Blood Midi Kit, QIAGEN) following the manufacturer's recommended protocol. DNA was quantified by use of a spectrophotometer at 260 nm. A working solution of DNA was then created by diluting the sample with Tris-EDTA to create stock of 10 ng/μL.

TABLE 1. Definition for each pouch subgroup

Pouch subgroups	*n*	*Definition*
IPAA without complications or pouchitis	37	IPAA without complications or pouchitis for at least 2 y after stoma closure
IPAA with mild pouchitis	33	≤3 episodes/y for 2 consecutive years, effectively treated with 7–10 d of ciprofloxacin or metronidazole
IPAA with severe pouchitis	9	≥4 episodes/y for 2 consecutive years OR the need for continuous antibiotics
IPAA with CD-like complications[a]	28	The presence of any of the following[b]: • Perianal fistula (n = 12) • Pouch inlet stricture/proximal small-bowel disease (n = 11) • Biopsies showing granulomata (n = 3) • Antibiotic-resistant pouchitis (n = 5)
Patients with CD colitis (controls)	11	Treated with total proctocolectomy with end ileostomy
Healthy patients (controls)	269	No immunologically based disease, no gastrointestinal surgery

CD = Crohn's disease.
[a]Patients in IPAA with CD-like complications may have had more than 1 defining complication.
[b]Any or all of these complications occurred at least 6 months after ileostomy closure.

Polymerase Chain Reaction Genotyping

Once patients were classified into their respective clinical subgroups, polymerase chain reaction (PCR) for the 3 *NOD2/CARD15* mutations was performed after appropriate annealing temperatures and reaction conditions were optimized (Table 2). PCR products were separated by electrophoresis on a 2% Tris-acetate-EDTA-ethidium bromide agarose gel at 100 V for 30 minutes and visualized on an ultraviolet transilluminator. PCR data were further verified with a customized Illumina BeadArray using VeraCode technology programmed for 2 of the 3 *NOD2/CARD15* polymorphisms (rs2066844 p.Arg702Trp and rs2066845 p.Gly908Arg) with 100% agreement between PCR data and BeadArray on all patients.

Statistical Analysis

The R statistical software system (version 2.9.2, http://www.r-project.org/) was used to perform statistical analysis. Data (reported as mean ± SD) was analyzed using χ^2 with Yates correction with significant as $P < .05$.

RESULTS

One hundred seven IPAA patients were recruited into the various groups and their associated clinical data are summarized in Table 3. The overall mean follow-up for each group was more than 5 years. The average time to the development of complications was over 3 years in the CD-like and severe pouchitis groups, and 2.8 years in the mild pouchitis group.

The incidence of *NOD2/CARD15* mutations among the various study groups is shown in Figure 1. The mutation incidence among the healthy population cohort was 8.5% (23/269), whereas mutation incidence in the known CD group was 45.5% (5/11). The severe pouchitis group had the highest *NOD2/CARD15* frequency, which was significantly elevated when compared with the asymptomatic IPAA group and healthy controls (66.6% vs 5.4% vs 8.5% respectively, $P < .001$). There was no significant increase in *NOD 2/CARD15* mutations observed in the mild pouchitis or CD-like IPAA groups compared with healthy controls

TABLE 2. NOD2 PCR reaction summary

SNPs	*Mutation*	*Primer*	*Primer sequence*	*PCR reaction*	*Fragment length (bp)*
rs2066844	R702W	Wild-type forward: Mutant forward: Reverse:	5′-ATC TGA GAA GGC CCT GTT CC-3′ 5′-ATC TGA GAA GGC CCT GTT CT-3′ 5′-CCC ACA CTT AGC CTT GAT G-3′	Denature 95°C 5 min. 94°C 30 s, 60°C 30 s, 72°C 30 s 30 cycles Extension 72°C 2 min.	438
rs2066845	G908R	Forward: Reverse:	5′-CCC AGC TCC TCC CTC TTC-3′ 5′-AAG TCT GTA ATG TAA AGC CAC-3′	Denature 95°C 5 min 94°C 30 s, 56°C 30 s, 72°C 30 s 30 cycles Extension 72°C 2 min	380
rs2066847	3020insC	Forward: Wild-type reverse: Mutant reverse:	5′ CTT CAA CCA CAT CCC CAT TCC-3′ 5′-AAG CCC TCC TGC AGG CCC T-3′ 5′-AGC CCT CCT GCA GGC CCC-3′	Denature 95°C 5 min 94°C 30 s, 67.0°C 60 s, 15 cycles 94°C 15 s, 63.0°C 30 s, 72°C 30 s 20 cycles Extension 72°C 2 min	330

SNPs = single-nucleotide polymorphisms; PCR = polymerase chain reaction.

TABLE 3. Patient demographic/operative data

	Crohn's like (n = 28)	*Severe pouchitis (n = 9)*	*Mild pouchitis (n = 33)*	*Asymptomatic (n = 37)*
Sex M/F	16/12	5/4	17/16	26/11
Family history for IBD (yes/no)	4/24	4/5	9/24	10/27
Smoking (current/exsmoker/never)	1/6/21	0/3/6	0/10/23	0/6/31
Extraintestinal manifestations				
PSC	1	1	3	3
Dermatologic	1	0	0	0
Ocular	1	0	0	0
Arthritis	3	0	0	1
Average time to IPAA after diagnosis (y)	5.7 ± 5.4	5.8 ± 7.0	7.9 ± 8.4	11.2 ± 9.0
Average follow-up after IPAA (y)	9.1 ± 5.3	10.7 ± 6.6	7.6 ± 4.2	5.5 ± 5.6
IPAA (open/lap-assisted)	25/3	9/0	32/1	33/4
IPAA stage				
1	2	0	0	7
2	18	7	24	23
Modified 2[a]	2	0	3	2
3	6	2	6	5
Indications for IPAA (failed medical mg/dysplasia, cancer/toxic colitis)	18/2/8	4/2/3	21/7/5	26/5/6
Average time from IPAA to 1st pouch complication/episode of pouchitis (y)	3.23 ± 4.08	3.21 ± 2.44	2.82 ± 2.85	N/A

Family history, smoking history, extraintestinal manifestations, follow-up, IPAA stage and indications were all not significantly different among subgroups.
PSC = primary sclerosing cholangitis; lap = laparoscopically; mg = management; N/A = not applicable.
[a]Modified 2: patients who underwent IPAA without ileostomy after previous total abdominal colectomy.

or asymptomatic IPAA patients (18.2% vs 14.8% vs 8.5% vs 5.4%, respectively). The allelic distribution of *NOD2/CARD15* mutational variants for each patient subgroup is summarized in Table 4. Although not statistically significant, all groups had the R702W (rs2066844) as the most common, with the exception of the severe pouchitis group, where the C-insertion mutation (rs2066847) had the greatest incidence.

DISCUSSION

Total proctocolectomy with IPAA is the operative standard for the surgical treatment of UC. This procedure removes the diseased colon and reestablishes gastrointestinal continuity. It leads to an improved health-related quality of life, results in a decreased risk of side effects from UC-related medications, and nearly eliminates the risk of dysplasia

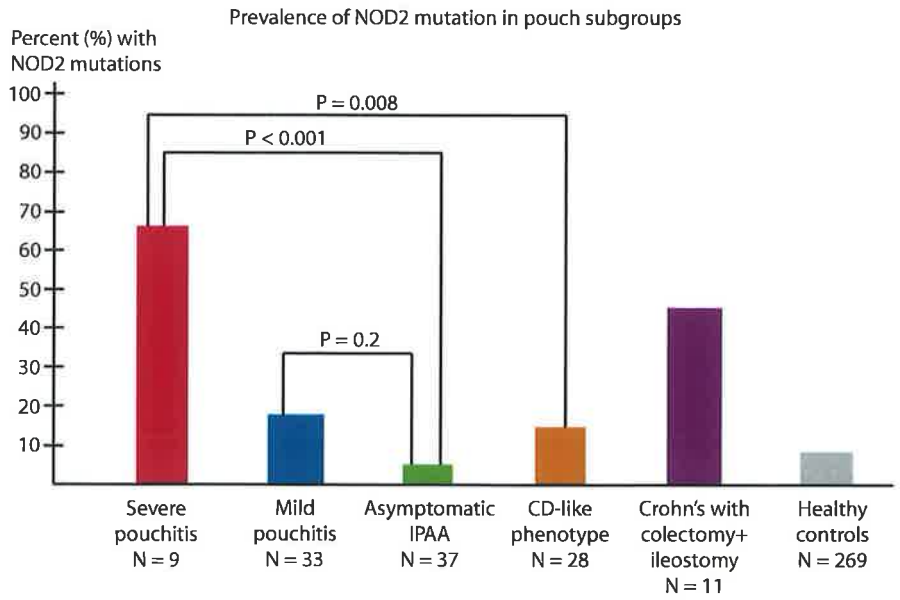

FIGURE 1. Prevalence of *NOD2/CARD15* mutations for each subgroup. The incidence of *NOD2/CARD15* mutations was significantly higher in the severe pouchitis group compared with the asymptomatic and Crohn's disease (CD)-like groups. No difference was seen in the mild pouchitis vs asymptomatic groups. Rate of *NOD2/CARD15* mutations was unexpectedly lower in the CD-like complication group compared with patients with Crohn's colitis.

TABLE 4. *NOD2/CARD15* allelic distribution for each subgroup

	Severe pouchitis	*Mild pouchitis*	*CD-like complications*	*CD treated with TPC + end ileostomy*	*Healthy control*
1007fsCins (rs2066847)	3/6 (50%)	2/6 (33%)	0/3 (0%)	2/5 (40%)	6/29 (21%)
R702W (rs2066844)	2/6 (33%)	4/6 (67%)	3/4 (75%)	3/5 (60%)	17/29 (59%)
G908R (rs2066845)	1/6 (17%)	0/6 (0%)	1/4 (25%)	0/5 (0%)	6/29 (20%)

Not significantly different for distribution of single-nucleotide polymorphisms among study groups.
CD = Crohn's disease; TPC = total proctocolectomy.

and eventual cancer. However, pouchitis and Crohn's-like complications confound the benefits of this operation. Whether such complications after surgery are the result of an incorrect preoperative diagnosis or unrelated postoperative events remains unclear. However, if patients prone to such complications could be preoperatively identified, such information could assist in surgical decision making.

Pouchitis is a relatively common complication after IPAA. Depending on its definition, it can affect 24% to 59% of IPAA patients.[7] Clinically, pouchitis presents with increased stool frequency, urgency, abdominal cramping, and pelvic discomfort. Endoscopic evaluation together with symptom assessment and histologic evaluation is the key to an accurate diagnosis of pouchitis. Specifically, endoscopy, stool culture, and histologic evaluation can distinguish pouchitis from other inflammatory conditions (such as *Clostridium difficile* or cytomegalovirus pouchitis) or functional disorders of the pouch (such as irritable pouch syndrome). There are no universally accepted diagnostic or classification criteria for pouchitis, but Shen et al[5] proposed a pouchitis severity classification based on response to therapy (Fig. 2). He suggested 3 distinct groups: 1) antibiotic-responsive pouchitis, a condition in which patients have infrequent episodes (<4 episodes per year) responding to a 2-week course of a single antibiotic; 2) antibiotic-dependent pouchitis, a condition with frequent episodes (>4 per year) or with persistent symptoms requiring long-term, continuous antibiotic or probiotic therapy; and 3) antibiotic-refractory pouchitis, where patients do not respond to a prolonged course of antibiotics and require 5-aminosalicylic acid, corticosteroids, or immunomodulator (Imuran, 6-mercaptopurine, or Remicade) therapy. The antibiotic-refractory group is commonly considered to have a variant of conventional UC more akin to CD, which is clinically reinforced by the observation that immune modulators and/or tumor necrosis factor α antagonists can often effectively treat such patients.[8,9]

Despite many years of research and clinical experience, the etiology of pouchitis is still largely unknown. However, the fact that it is rarely seen in patients with familial adenomatous polyposis, and that antibiotics are frequently an effective treatment, suggests roles for both a host susceptibility factor and enteric bacteria in disease pathogenesis. Recent studies have also shown an increased number of sulfate-reducing bacteria within pouches created for UC compared with those given for familial adenomatous polyposis.[10] Furthermore, the use of oral probiotic therapy has been shown to often be effective in delaying the onset and preventing relapses in patients with pouchitis.[11–13] Thus, the bacterial component of this pathophysiologic process seems clear, at least in the patients that respond to antibiotics. However, patients with severe pouchitis or antibiotic-resistant pouchitis clearly must have other factors that play a significant role, and their lack of responsiveness to antibiotics suggests a different or an additional mechanism acting in those with severe disease compared with patients with antibiotic-responsive pouchitis.

In the past 2 decades, genetic variants and their role in the pathogenesis of IBD have been the subject of intensive research. The essence of this exciting work has been to identify the possible etiology of IBD by identifying genetic mutations associated with IBD. Much of this work has focused on identifying specific pathways of immune function and inflammation with the hope of developing new therapies for IBD. However, little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making. Work done in linkage analysis, candidate gene studies, and, more recently, genome-wide association studies has identified numerous genetic mutations associated with IBD.[14–28] The landmark discovery of mutations in the *NOD2/CARD15* gene on chromosome 16 as being associated with CD susceptibility is an example of linkage analysis.[3] Mutations within this gene have been found in up to 40% of European and North American CD patients when compared with 10% to 15% of the healthy population.[29] These figures are comparable to our data that show *NOD2/CARD15* mutations in 45% of CD patients vs 8.5% in healthy controls. The *NOD2/CARD15* gene encodes for an intracellular protein sensor of muramyl-dipeptide, a bacterial cell wall component. Activation of this protein leads to an increase in nuclear factor κB and mitogen-activated protein kinase signaling pathways, leading to the production of cytokines (tumor necrosis factor α, interleukin-1β) and the promotion of autophagy of endocytosed bacteria.[3,4,30] Three single-nucleotide polymorphisms (G908R rs2066845, 1007fsCins rs2066847, and R702W rs2066844) in this gene account for approximately 82% of the mutated alleles.[31] These variants within the *NOD2/*

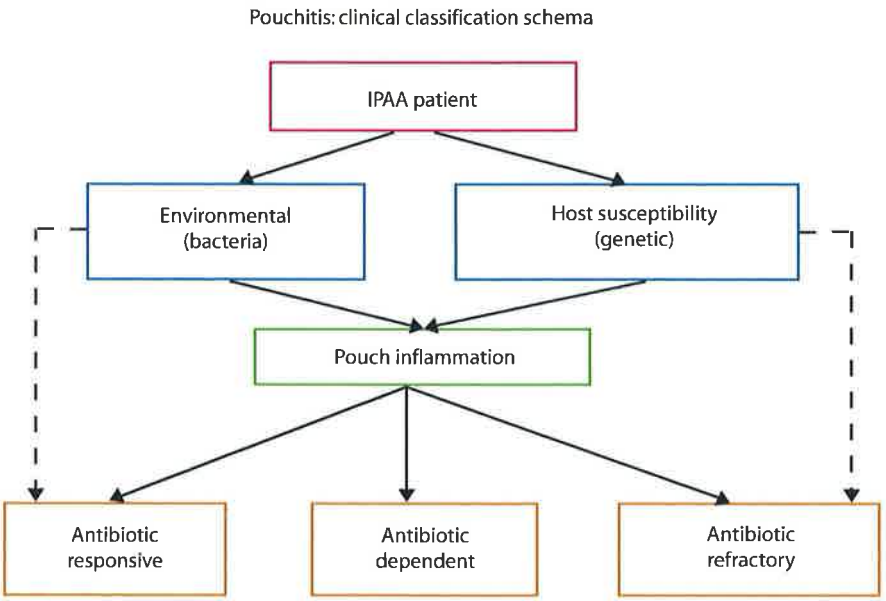

FIGURE 2. Shen et al[5] proposed 3 distinct pouchitis severity subgroups based purely on clinical response to therapy: 1) Antibiotic-responsive group, a condition in which patients have infrequent episodes (<4/y) responding to a 2-week course of a single antibiotic; 2) antibiotic-dependent pouchitis, a condition with frequent episodes (>4/y) or with persistent symptoms requiring long-term, continuous antibiotic or probiotic therapy; 3) antibiotic-refractory pouchitis, where patients do not respond to a prolonged course of antibiotics and require 5-aminosalicylic acid, corticosteroids, or immunomodulator therapy. Patients with wild-type/functional *NOD2/CARD15* genes probably have the molecular machinery to prevent the development of severe pouchitis, Although those patients with *NOD2/CARD15* mutations lack the innate ability to protect themselves and are more prone to antibiotic refractory pouchitis.

CARD15 gene have repeatedly been shown to be associated with ileal CD, early age of onset, and a stricturing and/or penetrating phenotype.[32–39] Both because of its role in innate immunity against enteric bacteria and its association with CD, we investigated whether patients manifesting CD-like complications and/or severe antibiotic-resistant or antibiotic-dependant pouchitis would have an increased incidence of *NOD2/CARD15* mutations. Theoretically, if positive, such a genetic association could lend itself to preoperative testing and possibly playing a role in surgical decision making by avoiding an IPAA in those patients with a high risk of such complications postoperatively.

The present study was limited by the small number of recruited patients (107/382). However, it did include all of the patients with CD-like complications and severe pouchitis from our practice, because all such patients are repeatedly evaluated in clinic owing to their difficulties and they readily agreed to inclusion in this research protocol. The mild pouchitis and asymptomatic groups admittedly represented a subset of the much larger total volume of IPAA patients. These 2 groups nonetheless were the largest of the groups studied and represented an unselected subset of the larger group of patients with uncomplicated IPAA. Future studies clearly should be prospective and all-inclusive to definitively evaluate whether the genetic findings discovered in this smaller trail are borne out.

We found that asymptomatic IPAA patients had an extremely low incidence (5.4%) of *NOD2/CARD15* mutations, not significantly different from healthy controls (8.5%) or patients with mild pouchitis (18.2%). However, patients with severe pouchitis had the highest incidence (66.6%) of *NOD2/CARD15* mutations, suggesting that this group may have a compromised host defense mechanism to enteric bacteria. Similar findings were seen by Meier et al,[11] who found an 8% incidence of *NOD2/CARD15* mutations in patients without pouchitis vs 24% in patients with more than 2 episodes of pouchitis per year. Our study found a higher mutation rate in the severe pouchitis group, but our definition of severity was more rigid with 4 or more episodes per year or the continuous need for medical therapy, compared with only 2 episodes per year in the Meier study. Nonetheless, both studies suggest that patients with more severe pouchitis have an intrinsic defect in innate immunity against commensal pouch bacteria. In both studies, the frequency of the C-insertion frameshift mutation (rs2066847) was the most prevalent in the severe pouchitis group suggesting that the C-insertion mutation may be the allotype with the highest risk for pouchitis. Some authors have suggested that *NOD2/CARD15* compound heterozygosity may increase the risk of CD.[40] In our study, only 2 patients carried compound mutations prohibiting statistical analysis. It is interesting, however, that both of these patients belonged to the severe pouchitis group. Overall, these data suggest a genetic basis for mild vs severe pouchitis (Fig. 2). Severe pouchitis may be due to a genetic defect in host responsiveness to pouch bacteria, whereas mild pouchitis could be caused by a transient

overgrowth of pathologic bacteria that can be treated by antibiotics.

The frequency of Crohn's-like complications (fistula, pouch inlet strictures, granuloma formation, proximal small-bowel disease) after IPAA ranges from 2.7% to 13% depending on diagnostic criteria.[2] A diagnosis of CD should be considered in the IPAA patient if 1) a perineal fistula or abscess develops more than 6 months after IPAA, 2) there are granulomas on pouch or small-bowel histology remote from any anastomosis, and 3) there are ulcerated lesions in the afferent limb proximal to the pouch and/or strictures at the pouch inlet in the absence of current nonsteroidal anti-inflammatory drug use. Although such complications can often be treated with the reinstitution of immune modulators or other medications, the prognosis for pouch function is worse and, specifically, the risk of pouch loss or need for diversion is higher than in those IPAA patients without such complications.[41] As mentioned above, mutations within the *NOD2/CARD15* gene have repeatedly been associated with CD; thus, one might expect the frequency of *NOD2/CARD15* mutations in IPAA patients with CD-like complications to be similar to that found in CD patients treated with total proctocolectomy and end ileostomy. Somewhat surprisingly, this was not borne out in this study. Our data showed that the frequency of *NOD2/CARD15* in the CD-like IPAA subgroup was only 15%, which was significantly different when compared with the severe pouchitis group (66.6%, $P = .008$), but not much different than asymptomatic IPAA patients or healthy controls. This is consistent with clinical and laboratory evidence, however, because *NOD2/CARD15*-positive CD patients most commonly have ileocolonic stricturing disease and such patients would rarely be confused as having UC and thus would never come to IPAA. It is noteworthy that the CD-like IPAA group was significantly different statistically than the severe pouchitis group, suggesting that the pathophysiologic mechanisms for the respective complications in these 2 groups were different at least from a genetic standpoint. Thus, fistulae, afferent limb strictures, and pouch granulomata probably have a disease mechanism not related to *NOD2/CARD15* dysfunction. In the context of the increasing number of genes now being identified as being associated with CD, further studies are needed to investigate a possible association of these CD-like complications and these more recently discovered gene mutations.

Besides gene haplotypes, serum antibodies (pANCA/ASCA) have also been investigated as prognosticators of IBD severity[42] and possible predictors for pouch complications. Some investigators have shown pANCA levels to be significantly greater in chronic pouchitis compared with control groups,[43,44] but this has been refuted by others.[45,46] Such inconsistencies may be due to fluctuations in serum levels of these antibodies associated with disease activity, which would not be an issue with genetic testing.

CONCLUSIONS

We have identified a significant association of mutations in the *NOD2/CARD15* gene with severe pouchitis after IPAA, but not with mild pouchitis or CD-like complications. This suggests an intrinsic defect in host immunity to commensal pouch bacteria in severely affected IPAA pouchitis patients that might explain their recalcitrance to conventional antibiotic management. These findings need to be confirmed in larger cohorts of patients from different demographic and geographic locales and in a prospective study. If confirmed, the preoperative measurement of *NOD2/CARD15* status may assist the surgeon and patient being considered for IPAA surgery, especially when the patient is a marginal operative candidate. Future studies looking at other potential genetic associations that could predict CD-like complications are necessary.

REFERENCES

1. Shen B, Fazio VW, Remzi FH, Lashner BA. Clinical approach to diseases of ileal pouch-anal anastomosis. *Am J Gastroenterol.* 2005;100:2796–2807.
2. Shen B, Patel S, Lian L. Natural history of Crohn's disease in patients who underwent intentional restorative proctocolectomy with ileal pouch-anal anastomosis. *Aliment Pharmacol Ther.* 2010;31:745–753.
3. Hugot JP, Chamaillard M, Zouali H, et al. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature.* 2001;411:599–603.
4. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature.* 2001;411:603–606.
5. Shen B. Diagnosis and treatment of patients with pouchitis. *Drugs.* 2003;63:453–461.
6. Zhang WJ, Thompson JL, Chorney MJ, Koltun WA. Phenotyping of IL4-induced nuclear Stat6 activity in humans: quantitation after gel shift assay using immortalized cell lines. *Oncol Rep.* 2003;10:1281–1288.
7. Shen B, Fazio VW, Remzi FH, et al. Comprehensive evaluation of inflammatory and noninflammatory sequelae of ileal pouch-anal anastomoses. *Am J Gastroenterol.* 2005;100:93–101.
8. Papadakis KA, Treyzon L, Abreu MT, Fleshner PR, Targan SR, Vasiliauskas EA. Infliximab in the treatment of medically refractory indeterminate colitis. *Aliment Pharmacol Ther.* 2003;18:741–747.
9. Shen B, Remzi FH, Lavery IC, et al. Administration of adalimumab in the treatment of Crohn's disease of the ileal pouch. *Aliment Pharmacol Ther.* 2009;29:519–526.
10. Coffey JC, Rowan F, Burke J, Dochery NG, Kirwan WO, O'Connell PR. Pathogenesis of and unifying hypothesis for idiopathic pouchitis. *Am J Gastroenterol.* 2009;104:1013–1023.
11. Meier CB, Hegazi RA, Aisenberg J, et al. Innate immune receptor genetic polymorphisms in pouchitis: is CARD15 a susceptibility factor? *Inflamm Bowel Dis.* 2005;11:965–971.
12. Mimura T, Rizzello F, Helwig U, et al. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut.* 2004;53:108–114.
13. Gionchetti P, Rizzello F, Venturi A, et al. Oral bacteriotherapy as maintenance treatment in patients with chronic pouchitis: a

double-blind, placebo-controlled trial. *Gastroenterology.* 2000; 119:305–309.
14. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* 2007;447:661–678.
15. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008;40:955–962.
16. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science.* 2006;314:1461–1463.
17. Fisher SA, Tremelling M, Anderson CA, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat Genet.* 2008;40:710–712.
18. Franke A, Balschun T, Karlsen TH, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet.* 2008;40:1319–1323.
19. Hampe J, Franke A, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet.* 2007;39:207–211.
20. Libioulle C, Louis E, Hansoul S, et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genet.* 2007; 3:e58.
21. Parkes M, Barrett JC, Prescott NJ, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet.* 2007;39: 830–832.
22. Silverberg MS, Cho JH, Rioux JD, et al. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet.* 2009;41:216–220.
23. Mathew CG. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. *Nat Rev Genet.* 2008;9:9–14.
24. Noomen CG, Hommes DW, Fidder HH. Update on genetics in inflammatory disease. *Best Pract Res Clin Gastroenterol.* 2009;23: 233–243.
25. Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR. Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. *Inflamm Bowel Dis.* 2010;16:282–295.
26. Scherr R, Essers J, Hakonarson H, Kugathasan S. Genetic determinants of pediatric inflammatory bowel disease: is age of onset genetically determined? *Dig Dis.* 2009;27:236–239.
27. Nakagome S, Takeyama Y, Mano S, et al. Population-specific susceptibility to Crohn's disease and ulcerative colitis: dominant and recessive relative risks in the Japanese population. *Ann Hum Genet.* 2010;74:126–136.
28. McGovern DP, Gardet A, Törkvist L, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet.* 2010;42:332–337.
29. Rogler G. The importance of gut microbiota in mediating the effect of NOD2 defects in inflammatory bowel disease. *Gut.* 2010;59:153–154.
30. Brain O, Allan P, Simmons A. NOD2-mediated autophagy and Crohn disease. *Autophagy.* 2010;6:412–414.
31. Hugot JP, Zaccaria I, Cavanaugh J, et al. Prevalence of CARD15/ NOD2 mutations in Caucasian healthy people. *Am J Gastroenterol.* 2007;102:1259–1267.
32. Hampe J, Cuthbert A, Croucher PJ, et al. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet.* 2001;357:1925–1928.
33. Abreu MT, Taylor KD, Lin YC, et al. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology.* 2002;123:679–688.
34. Ahmad T, Armuzzi A, Bunce M, et al. The molecular classification of the clinical manifestations of Crohn's disease. *Gastroenterology.* 2002;122:854–866.
35. Cuthbert AP, Fisher SA, Mirza MM, et al. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology.* 2002;122:867–874.
36. Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet.* 2002;359:1661–1665.
37. Lesage S, Zouali H, Cézard JP, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet.* 2002;70: 845–857.
38. Vermeire S, Wild G, Kocher K, et al. CARD15 genetic variation in a Quebec population: prevalence, genotype-phenotype relationship, and haplotype structure. *Am J Hum Genet.* 2002;71: 74–83.
39. Oostenbrug LE, Nolte IM, Oosterom E, et al. CARD15 in inflammatory bowel disease and Crohn's disease phenotypes: an association study and pooled analysis. *Dig Liver Dis.* 2006;38: 834–845.
40. Heresbach D, Gicquel-Douabin V, Birebent B, et al. NOD2/ CARD15 gene polymorphisms in Crohn's disease: a genotype-phenotype analysis. *Eur J Gastroenterol Hepatol.* 2004;16:55–62.
41. Joyce MR, Fazio VW. Can ileal pouch anal anastomosis be used in Crohn's disease? *Adv Surg.* 2009;43:111–137.
42. Targan SR. The utility of ANCA and ASCA in inflammatory bowel disease. *Inflamm Bowel Dis.* 1999;5:61–63.
43. Fleshner PR, Vasiliauskas EA, Kam LY, et al. High level perinuclear antineutrophil cytoplasmic antibody (pANCA) in ulcerative colitis patients before colectomy predicts the development of chronic pouchitis after ileal pouch-anal anastomosis. *Gut.* 2001;49:671–677.
44. Sandborn WJ, Landers CJ, Tremaine WJ, Targan SR. Antineutrophil cytoplasmic antibody correlates with chronic pouchitis after ileal pouch-anal anastomosis. *Am J Gastroenterol.* 1995;90: 740–747.
45. Aisenberg J, Legnani PE, Nilubol N, et al. Are pANCA, ASCA, or cytokine gene polymorphisms associated with pouchitis? Long-term follow-up in 102 ulcerative colitis patients. *Am J Gastroenterol.* 2004;99:432–441.
46. Yasuda N, Thomas P, Ellis H, Herbst F, Nicholls J, Ciclitira P. Perinuclear anti-neutrophil cytoplasmic antibodies in ulcerative colitis after restorative proctocolectomy do not correlate with the presence of pouchitis. *Scand J Gastroenterol.* 1998;33:509–513.

ORIGINAL CONTRIBUTION

Genetic Risk Profiling and Gene Signature Modeling to Predict Risk of Complications After IPAA

Rishabh Sehgal, M.B., B.Ch., M.R.C.S.[1] • Arthur Berg, Ph.D.[1,2]
Joseph I. Polinski, B.S.[1] • John P. Hegarty, Ph.D.[1] • Zhenwu Lin, Ph.D.[1]
Kevin J. McKenna, M.D.[1] • David B. Stewart, M.D.[1] • Lisa S. Poritz, M.D.[1,3]
Walter A. Koltun, M.D.[1]

1 Division of Colon and Rectal Surgery, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania
2 Center for Statistical Genetics, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania
3 Department of Cellular and Molecular Physiology, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania

BACKGROUND: Severe pouchitis and Crohn's disease-like complications are 2 adverse postoperative complications that confound the success of the IPAA in patients with ulcerative colitis. To date, approximately 83 single nucleotide polymorphisms within 55 genes have been associated with IBD.

OBJECTIVE: The aim of this study was to identify single-nucleotide polymorphisms that correlate with complications after IPAA that could be utilized in a gene signature fashion to predict postoperative complications and aid in preoperative surgical decision making.

DESIGN: One hundred forty-two IPAA patients were retrospectively classified as "asymptomatic" (n = 104, defined as no Crohn's disease-like complications or severe pouchitis for at least 2 years after IPAA) and compared with a "severe pouchitis" group (n = 12, ≥4 episodes pouchitis per year for 2 years including the need for long-term therapy to maintain remission) and a "Crohn's disease-like" group (n = 26, presence of fistulae, pouch inlet stricture, proximal small-bowel disease, or pouch granulomata, occurring at least 6 months after surgery). Genotyping for 83 single-nucleotide polymorphisms previously associated with Crohn's disease and/or ulcerative colitis was performed on a customized Illumina genotyping platform. The top 2 single-nucleotide polymorphisms statistically identified as being independently associated with each of Crohn's disease-like and severe pouchitis were used in a multivariate logistic regression model. These single-nucleotide polymorphisms were then used to create probability equations to predict overall chance of a positive or negative outcome for that complication.

RESULTS: The top 2 single-nucleotide polymorphisms for Crohn's disease-like complications were in the *10q21* locus and the gene for *PTGER4* ($p = 0.006$ and 0.007), whereas for severe pouchitis it was *NOD2* and *TNFSF15* ($p = 0.003$ and 0.011). Probability equations suggested that the risk of these 2 complications greatly increased with increasing number of risk alleles, going as high as 92% for severe pouchitis and 65% for Crohn's disease-like complications.

CONCLUSION: In this IPAA patient cohort, mutations in the *10q21* locus and the *PTGER4* gene were associated with Crohn's disease-like complications, whereas mutations in *NOD2* and *TNFSF15* correlated with severe pouchitis. Preoperative genetic analysis and use of such gene signatures hold promise for improved preoperative surgical patient selection to minimize these IPAA complications.

Funding/Support: This work was funded by the Research Foundation of The American Society of Colon and Rectal Surgeons. Dr Sehgal is the International Fellowship Award Recipient for year 2010.

Financial Disclosure: None reported.

Presented at the meeting of The American Society of Colon and Rectal Surgeons, Vancouver, BC, Canada, May 14 to 18, 2011.

Correspondence: Rishabh Sehgal, M.B., B.Ch., or Walter Koltun, M.D., Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Division of Colon & Rectal Surgery, H137, 500 University Dr, PO Box 850, Hershey, PA 17033-0850. E-mail: rishsehgal@gmail.com or wkoltun@hmc.psu.edu.

Dis Colon Rectum 2012; 55: 239–248
DOI: 10.1097/DCR.0b013e31823e2d18

KEY WORDS: Inflammatory bowel disease; Crohn's disease; Genetics; Classification; Pouchitis; Crohn's disease-like complications.

Ulcerative colitis (UC) is a chronic relapsing and remitting inflammatory disease limited to the mucosa and submucosa of the colon and rectum. Despite advances in medical therapy, approximately 33% of patients with UC eventually require total proctocolectomy with IPAA.[1] This procedure is now considered the standard for the surgical treatment of UC.

However, postoperative complications confound the success of the IPAA in UC patients. These include early complications that are usually technically related, or complications occurring late that can suggest *recrudescent* IBD. Two late complications that will be discussed in this study include pouchitis and Crohn's disease (CD)-like complications. Pouchitis is an inflammatory condition characterized by increased frequency of bowel movements, diarrhea, tenesmus, malaise, and arthralgias that can affect between 15% to 70% of patients depending on definition and duration of follow-up.[2] CD-like complications include the development of fistulas, strictures, and granulomatous inflammation that can affect up to 13% of patients.[3] Mild pouchitis (MP), a phenomenon that commonly occurs in the IPAA patient, is an acceptable complication because it can be effectively treated with a short course of antibiotics. However, severe pouchitis (SP) requires more aggressive therapy, sometimes with immunosuppressive medications, and, similar to CD-like complications, SP is associated with an increased risk of pouch failure, pouchectomy, and permanent stoma. The ability to predict these more severe post-IPAA complications could assist in preoperative decision making, possibly avoiding the operation in high-risk patients.

In recent years, much work has been done to unravel the possible origin of IBD by identifying and then correlating genetic mutations found in large populations of IBD patients in comparison with healthy controls. We have previously shown that mutations in the *NOD2/CARD15* gene are associated with SP, but not MP or CD-like complications.[4]

More recently, genome-wide association studies (GWAS) have identified approximately 83 single-nucleotide polymorphisms (SNPs) within 55 genetic loci to be associated with IBD, and these SNPs were thus investigated in the present study. The aim of this study therefore was to correlate these 83 SNPs with pouchitis (mild vs severe) and CD-like complications seen after IPAA. Such identified specific SNPs were then used to develop predictive gene signatures that could possibly be used to predict postoperative complications and thus aid in preoperative surgical decision making.

METHODS

Patient Recruitment

At the Milton S. Hershey Medical Center from July 1990 to July 2010, a total of 382 patients underwent the IPAA procedure for a preoperative diagnosis of UC or indeterminate colitis (IC). A subset of 142 patients were recruited into the present study population as they presented to follow-up clinic appointments over the study period from September 2008 to September 2010. By the nature of symptomatology, patients with complications, specifically SP and CD-like complications, were seen more frequently; thus, recruitment was relatively complete for these patient groups. Patients with asymptomatic pouch function would regularly be seen annually and were thus recruited as they presented for their routine follow-up appointments during the study period. These patients were classified into the following categories: 1) IPAA without complications or pouchitis; 2) IPAA with MP; 3) IPAA with SP; and 4) IPAA with CD-like complications. The exact definitions for each category are shown in Table 1.

Patient Classification

Individual episodes of pouchitis were defined by standard clinical, endoscopic, and histologic criteria. Symptoms of pouchitis included increased frequency of bowel movements, diarrhea, and tenesmus. Endoscopy showed gross

TABLE 1. Definition for each pouch subgroup

Group	*n*	*Definitions*
Asymptomatic pouch	63	IPAA without complications or pouchitis for at least 2 y after stoma closure.
Mild pouchitis	41	<4 episodes/y for 2 consecutive years, responding to 2-wk course of single antibiotic.
Severe pouchitis	12	≥4 episodes/y for 2 consecutive years or persistent symptoms, requiring long-term continuous antibiotics.
CD-like	26	The presence of any of the following[a]: Fistula (n = 12) Pouch inlet stricture/proximal small-bowel disease (n = 11) Biopsies showing granulomatous inflammation (n = 3)

CD = Crohn's disease.
[a]Any or all of these complications occurred at least 6 months after ileostomy closure.

inflammation, and biopsy showed acute inflammation superimposed on chronic inflammation. There presently is no one way to classify severity of pouchitis, but Shen[5] proposed a clinical classification schema based on response to therapy. He proposed 3 main groups: 1) antibiotic-responsive pouchitis, a condition that has <4 episodes per year and responds to a 2-week course of a single antibiotic; 2) antibiotic-dependent pouchitis, defined as ≥4 episodes per year requiring long-term antibiotics; and 3) antibiotic-refractory pouchitis, where patients do not respond to a 2- to 4-week course of a single antibiotic and require >4 weeks with 2 antibiotics or 5-aminosalicylic acid/steroids/immunomodulator therapy. For the purpose of this study, we considered the antibiotic-responsive group to be MP and combined the antibiotic-dependent and antibiotic-refractory groups together to form our severe group (Table 1).

Features that suggest CD in the post-IPAA patient include pouch-anal or pouch-intestinal fistulas, afferent limb strictures, and granulomas on pouch or small-bowel histology remote from the anastomosis. For the purpose of this study, we considered the diagnosis of CD-like complication only after symptoms developed more than 6 months postrestoration of gastrointestinal continuity.

Patient Demographics

Demographic data and clinical parameters of disease were obtained by reviewing paper and electronic patient charts. These included 1) sex, family history, date of IBD diagnosis, previous number of surgeries, and smoking history; 2) extent of disease, defined by surgical pathology; 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab, and steroid use; 4) frequency of pouchitis attacks and medications used in management; and 5) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations (Table 2). The study was approved by The Milton S. Hershey Medical Center Institutional Review Board.

DNA/Cell Bank

Patients were recruited into our institutional review board-approved genetic IBD cell/DNA bank originally established in 1998. Informed consent was obtained, and patients donated blood samples that were then used to create immortalized B-cell lines using Epstein Barr virus that provided for an indefinite source of DNA from each recruited individual. In brief, blood was diluted with sterile phosphate-buffered saline, and layered onto Ficoll-Paque (Amersham Biosciences). The blood-Ficoll gradient was centrifuged at 1500 rpm for 30 minutes at room temperature. The mononuclear cell interface was extracted and washed with phosphate-buffered saline. The washed cell pellet was resuspended in RPMI-1640 (VMR) media containing 12% fetal bovine serum (Gemini Bioproducts) and 25% Epstein Barr virus supernatant. Inoculated cells were incubated at 37°C in a CO_2 incubator. Once transformed, cells were stored at 1×10^7 cells/mL in 1-mL aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks until use.

DNA Isolation

DNA was extracted from 1×10^6 transformed B cells using a DNA isolation kit (QIAAmp DNA Blood Midi Kit, QIAGEN) according to the manufacturer's recommended protocol. DNA was quantified by use of a spectrophotometer at 260 nm. A working solution of DNA was

TABLE 2. Patient demographics

	CD-like (n = 26)	*Severe pouchitis (n = 12)*	*Mild pouchitis (n = 41)*	*Asymptomatic pouch (n = 63)*	*p*[a]
Sex					
Female/male	10/16	4/8	21/20	20/43	0.25
IBD family history					
No/yes	23/3	7/5	33/8	46/17	0.17
IPAA stages:					
I	2	0	1	7	0.23
II	16	7	29	42	0.81
Modified II	3	2	4	4	0.66
III	5	3	7	10	0.89
Mean pouch length, cm± SD	21 ± 5	24 ± 9	21 ± 4	21 ± 3	0.24
Average time to surgery after IBD diagnosis, y ± SD	6.2 ± 6.42	6.5 ± 6.42	9.0 ± 9.17	10.3 ± 8.10	0.15
Average age of IBD at diagnosis, y± SD	31.0 ± 10.56	36.0 ± 13.69	29.5 ± 11.04	29.0 ± 10.85	0.23
Total time of follow-up since the time IPAA, y ± SD	10.0 ± 5.55	7.5 ± 4.98	8.0 ± 3.77	6.0 ± 4.77	0.002
Smoking:					
Current	1	0	4	4	0.5954
Ex-smoker	11	6	12	13	0.07696
Never	14	6	25	46	0.2058

[a]A 4-sample of equal proportions and a 1-way analysis of variance was used to test for significant differences in patient demographics over the 4 clinical subgroups.

TABLE 3. List of genes/SNPs on Illumina array

SNP	*Chromosome*	*Gene*
rs2476601	1p13	PTPN22
rs3737240	1q21	ECM1
rs13294		
rs3180018	1q22	SCAMP3, MUC1
rs2274910	1q23	ITLN1
rs4656940	1q23	*CD244*, ITLN1
rs1801274	1q23.3	*FCGR2A*
rs9286879	1q24	1q24
rs7517810	1q24	*TNFSF18, TNFSF4, FASLG*
rs1998598	1q31	*DENND1B*
rs1363670	1p31	IL-12B
rs3024505	1q32	IL-10
rs2797685	1p36	*VAMP3*
rs6426833	1p36	RNF186
rs10753575		
rs3806308		
rs10733113	1q44	*NLRP3*
rs917997	2q11	IL-18R
rs13003464	2p16	*PUS10*
rs10181042	2p16b	*C2orf74, REL*
rs12612347	2q35	ARPC2
rs2241880	2q37	ATG16L1
rs3828309		
rs3792109		
rs780094	2p23	*GCKR*
rs6738825	2q33	*PLCL1*
rs3197999	3p21	MST1
rs4833103	4p14	TLR1, TLR10, TLR6
rs7720838	5p13	PTGER4
rs4613763		
rs17234657	5p13.1	
rs9292777		
rs10044354	5q15	ARTS-1
rs1050152	5q31	OCTN1
rs2522057	5q31	IBD5
rs10077785		
rs10045431	5q33	IL-12B
rs6887695		
rs13361189	5q33	IGRM
rs4958847		
rs11747270		
rs7714584		
rs9268480	6p21.3	BTNL2
rs1059276	6p	MEP1A
rs7746082	6q21	HLA region
rs2844480		
rs9271568		
rs3794996		
rs2395185		
rs3763313		
rs660895		
rs6908425	6p22	CDKAL1
rs17309827	6p25	*SLC22A23*
rs2301436	7p12	CCR6
rs1456893		
rs7849191	9p24	JAK2
rs10758669		
rs3810936	9q32	TNFSF15
rs6478108		
rs7848647		
rs7869487		
rs4986790	9q32-q33	TLR 4
rs10870077	9q34.3	CARD9
rs3936503	10p11	CCNY
rs10761659	10q21	ZNF365
rs10995271		
rs1248696	10q23-q24	DLG5
rs1248634		
rs10883365	10q24	NKX2-3
rs7081330		
rs951199	11p15.1	NELL1
rs11175593	12q12	LRRK2
rs2066844	16q12	NOD2
rs2066845		
rs2066847		
rs916977	15q13.1	HERC2
rs7712957	15q13	S100z
rs744166	17q21	STAT3
rs363617	17q24.3	TNFR2
rs2542151	18p11	PTPN2
rs762421	21q22	ICOSLG
rs12704036		U7
rs6927210		U6

SNP = single-nucleotide polymorphism.

then created by diluting the sample with Tris-EDTA to create stock of 10 ng/L.

Genotyping

With the help of Illumina (Illumina, San Diego, CA),[6] we developed a customized DNA microarray specific for 83 SNPs previously identified by GWAS to be associated with IBD (Table 3). This platform allows for high-throughput genotyping for 96 DNA samples interrogating for all 83 SNPs simultaneously. dsDNA concentrations were optimized by the use of an ultrasensitive fluorescent nucleic acid stain Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). The samples were then run on Illumina's BeadXpress Reader in Penn State Milton S. Hershey Medical Center's Functional Genomics Core Facility.

Statistical Analysis

The R statistical software system (version 2.13.0, http://www.r-project.org/) was used to perform statistical analysis. A 4-sample of equal proportions and a 1-way analysis of variance was used to test for significant differences in patient demographics over the 4 clinical subgroups (Table 2). Statistical assessment of the genetic associations was evaluated with the Fisher exact test with power to detect general forms of genetic associations. The SNPs identified as statistically associated with each of CD-like and SP groups were used in a multivariate logistic regression model. A backward stepwise selection algorithm was implemented to remove redundant SNPs and yield a parsimonious model with 2 SNPs in each model. The

multivariate logistic regression model yielded probability equations used to predict overall chance of a positive or negative outcome from the SNP-based genetic signature. The final predictive models provide an estimate of the probability that a person with a certain genetic signature would display symptoms of the specific modeled complication after undergoing IPAA.

RESULTS

Genetic Correlation with Pouchitis and CD-Like Complications After IPAA

A total of 142 IPAA patients (55 females) were recruited for this study with an overall mean follow-up time from the creation of the pouch of 7.4 ± 4.9 years. A detailed account of clinical and demographic data is summarized in Table 2. Patients in the CD-like group developed symptoms within an average of 18 ± 4 months after stoma closure. These patients had pouch-enteric fistulas (n = 12), pouch inlet stricture (n = 11), and granulomatous inflammation (n = 3) (Table 1).

Table 4 lists the top 6 genes and their respective SNPs with *p* values for each subgroup. The genes *ATG16L1*, *U6*, and *JAK2* were most significantly associated with MP but their *p* values were no better than 0.02. The highest statistical correlation was found in the SP and CD-like complication groups, most likely because of the more clearly defined phenotype in these 2 groups as opposed to the MP group. *NOD2*, *TNFSF15*, and *S100Z* were associated with SP, and the *10q21*,*CARD9*, and *TLR* genes were strongly associated with CD-like complications. Of the top 3 genes for each group, only one has been shown to be associated with only UC (*S100Z*), 4 are associated with both CD and UC (*JAK2, CARD9, TLR, TNFSF15*); however, the majority are associated with CD only (*ATG16L1, U6, NOD2,* and *10q21*).

TABLE 4. Top 6 SNP/genes for each subgroup

Group	No. of patients (N = 142)	SNP	Gene	p
Asymptomatic	63	—	—	—
Mild pouchitis	41	rs2241880	ATG16L1	0.02
		rs6927210	U6	0.03
		rs7849191	JAK2	0.04
		rs17309827	C6orf85	0.05
		rs10758669	JAK2	0.06
		rs1004819	IL23R	0.08
Severe pouchitis	12	rs2066844	NOD2	0.0008
		rs7869487	TNFSF15	0.01
		rs7712957	S100Z	0.02
		rs7848647	TNFSF15	0.07
		rs1050152	OCTN1	0.07
		rs6908425	CDKAL1	0.09
CD-like complications	26	rs10761659	10q21	0.006
		rs10870077	CARD9	0.018
		rs4833103	TLR	0.02
		rs3810936	TNFSF15	0.02
		rs17234657	5p13.1	0.04
		rs4613763	PTGER4	0.04

Specific allelic SNP determinants in 9 individual genes/loci appear to correlate with specific complications after IPAA. The majority of these genes have been previously associated with CD and play a role in enteric bacterial recognition and destruction (NOD2, ATG16L1, TLR, CARD 9) and/or nuclear factor κB activation (NOD2, TNFSF15). The highest statistical significance was found in the CD-like and severe pouchitis groups. This could be due to the more clearly defined phenotype in these 2 groups, but may also reflect a more Crohn's-like pathophysiology in these originally diagnosed UC patients.
CD = Crohn's disease; UC = ulcerative colitis; SNP = single-nucleotide polymorphism.

TABLE 5. Top SNP/Genes comparing severe pouchitis and CD-like groups with the favorable group

Group	No. of patients (N = 142)	SNP	Gene	p
Favorable outcome (asymptomatic pouch + mild pouchitis)	104	—	—	—
Severe pouchitis	12	rs2066844	NOD2	0.003
		rs7869487	TNFSF15	0.011
		rs1050152	OCTN1	0.058
		rs7848647	TNFSF15	0.064
CD-like complications	26	rs10761659	10q21	0.006
		rs4613763	PTGER4	0.007
		rs3810936	TNFSF15	0.011
		rs17234657	5p13.1	0.011
		rs4833103	TLR	0.014

Asymptomatic pouch and mild pouchitis groups were combined to form a "favorable outcome." This combined favorable outcome group was then compared with the severe pouchitis and the CD-like groups, and the top 2 SNPs associated with each of these complications were used to develop gene signature equations.
CD = Crohn's disease; SNP = single-nucleotide polymorphism.

Gene Signature Modeling to Predict Risk of CD-Like Complications or SP After IPAA

To develop a gene signature to predict adverse outcomes after IPAA, we first combined the asymptomatic pouch and MP groups together to form our "favorable outcome" group, because these 2 groups are considered to have acceptable outcomes postoperatively and to increase the total number within the group, which would aid in a more robust statistical calculation (N = 104, defined as no CD-like complications or SP for at least 2 years after IPAA). This combined group was compared with the SP group and the CD-like groups individually to develop the gene signature equations (Table 5).

The top 2 SNPs identified as statistically associated in an independent fashion with each of CD-like and SP in this analysis were then used in a multivariate logistic regression model. These SNPs were used to create probability equations to predict the overall chance of a positive or negative outcome for that complication. The top 2 SNPs for CD-like complications were in the *10q21* locus and the gene for *PTGER4* ($p = 0.006$ and 0.007), whereas for SP it was *NOD2* and *TNFSF15* ($p = 0.003$ and 0.011). Table 6 summarizes the respective predictive formulas and the overall

TABLE 6. Gene signature models for predicting SP and CD-like complications

SP gene signature[a]				
No. of risk alleles		*Probability of SP, %*	*95% CI*	*Patient distribution (n = 12)*
NOD2	*TNFSF15*			
0	0	5.8	0.6–11.0	4
0	1	7.7	1.5–13.9	1
0	2	10.1	0.0–24.7	2
1	0	38	8.3–67.7	4
1	1	45.3	14.4–76.3	
1	2	52.8	6.2–99.5	1
2	0	85.9	56.0–100	
2	1	89.2	65.2–100	
2	2	91.8	70.1–100	

CD-like complication gene signature[b]				
No. of risk alleles		*Probability of CD-like complication, %*	*95% CI*	*Patient distribution (n = 26)*
10q21	*PTGER4*			
0	0	5.9	0.0–12.2	
0	1	13.5	0.4–26.6	
0	2	28	0.0–59.9	
1	0	12	5.1–18.9	8
1	1	25.3	12.7–37.9	5
1	2	45.8	13.1–78.4	2
2	0	22.8	10.0–35.6	7
2	1	42.4	23.6–61.2	3
2	2	64.7	32.9–96.5	1

Predictive formulae and overall probability of experiencing these specific complications based on the various genotype combinations for each gene are shown. These probability equations suggest that the risk of these 2 complications would markedly increase with increasing number of risk alleles, going as high as 92% for SP and 65% for CD-like complications. The distributions of the patients in the present study are listed on the right.

SP = severe pouchitis; CD = Crohn's disease; SNP = single-nucleotide polymorphism.

[a]Probability = logit^{-1} (−2.9 + 2.3(NOD2) + 0.3(TNFSF15)). Number of "at- risk" alleles (0, 1, or 2) in respective SNP/gene.

[b]Probability = logit^{-1} (−2.7699 + 0.7746 (10q21) + 0.9131(PTGER4)). Number of "at- risk" alleles (0, 1, or 2) in respective SNP/gene.

probability of experiencing these specific complications based on the various genotype combinations for each gene under each probability equation. These probability equations suggest that the risk of these 2 complications would greatly increase with increasing number of risk alleles, going as high as 92% for SP and 65% for CD-like complications should both genes in each equation be homozygous for the deleterious or "at risk" allele.

The mean follow-up time for the combined "favorable" group was 6.6 ± 4.5 years and was not statistically significant in comparison with the SP group (7.6 ± 5.0 years, $p = 0.51$). However, the follow-up time for the favorable group was significantly different in comparison with the CD-like complication group (10 ± 5.6 years; $p = 0.005$). Because it is unlikely, but possible, that a patient in the favorable group may change their phenotype to CD-like with longer follow-up, 2 additional statistical analyses were performed to evaluate the possible effect of this follow-up time difference, by weighting the individual contributions of the patients in the logistic regression model by 1) a linear weighting system proportional to follow-up time (ie, a patient with 10 years of follow-up would be weighted twice as much as a patient with 5 years of follow-up) and 2) a nonlinear log of follow-up (where a 10-year follow-up patient would get 34% more weight than a 5-year follow-up and a 5-year follow-up would get 64% more weight than a 2-year follow-up). The weighted logistic regression to adjust for follow-up time has been shown to yield informative and reasonable estimates involving patients with variable lengths of follow-up in comparison with conventional logistic regression and Kaplan-Meier estimation.[7,8] These additional analyses yielded only minor changes in the CD-like gene signature and did not change the order or the identification of the most relevant SNPs, therefore confirming the validity and relative accuracy of the phenotype characterization despite the different follow-up times.

DISCUSSION

Similar to most other immune-mediated diseases, such as multiple sclerosis, systemic lupus erythematosus, type 1 diabetes mellitus, and rheumatoid arthritis, IBD is a "complex multigenic" disease that does not follow a "one gene, one disease" Mendelian pattern of inheritance. These disorders are difficult to classify because of variations in severity of symptoms, clinical features, variable prognosis, and age of onset.[9] Such complex diseases tend to involve an unknown number of multiple genes that affect various biological pathways and usually interact with a variable number of environmental factors.[9,10] This wide genetic variation presumably results in the diverse clinical phenotype that is seen in IBD. A single polymorphism is estimated to account for 1% to 8% of the overall disease risk in such complex diseases.[11] With the advent of GWAS, approximately 83 SNPs within 55 genetic loci have been shown to be associated with IBD (at the time of this study). Genomic profiling is the concept by which multiple genetic loci are tested simultaneously, thereby resulting in a more robust prediction of disease outcome.[11–13] Therefore, this study investigated 142 UC patients who underwent total proctocolectomy with IPAA and correlated 83 IBD SNPs with specific postoperative complications. The identified at-risk SNPs were then utilized in a gene signature fashion to predict the likelihood of developing these complications (SP and CD-like) based on genotype in a theoretical attempt to fashion a preoperative decision-making aid.

We first sought to determine which SNPs/genes correlated with various pouch-associated complications. Our patients were first subcategorized into MP, SP, and CD-like complication groups. This yielded several SNPs that were uniquely associated with each subgroup. In the MP

group, the most significant SNP was rs2241880 (a threonine-to-alanine substitution at amino acid position 300 of the protein-T300A) within the autophagy-related 16-like 1 (*ATG16L1*) gene found on chromosome 2q37.1. This CD associated gene was first discovered by Hampe et al[14] in 2007 and has since been replicated in several other white populations.[15–17] This gene has been shown to be intimately associated with the process of autophagy, a process by which cells remove unwanted cytoplasmic waste materials such as damaged organelles, apoptotic bodies, intracellular viruses, bacteria, and parasites by sequestering them into double-membrane autophagosomes, which are then presented to lysosomes for degradation.[18] A deficiency in *ATG16L1* gene is associated with an aberrant formation of the autophagosome and thus with the compromised clearance of unwanted intracellular waste.[19] Hruz and Eckmann[19] and Kuballa et al[20] studied the role of this T300A variant in human intestinal epithelial cells (Caco-2) and showed the inability of these cells to form autophagosomes around internalized *Salmonella typhimurium*. IBD patients carrying the SNP rs2241880 are more prone to ileal CD with no association seen with UC patients.[21] Recent studies have shown that CD patients who are homozygous for the *ATG16L1* risk allele (G) have Paneth cell granule abnormalities that lead to increased expression of acute-phase reactants and cytokines, such as interleukin (IL)-1β and IL-18 that potentiate the inflammatory response.[22,23] Even though statistical significance was found within the MP group, the lowest p value was no better than 0.02, most likely owing to the relatively vague definition of this group that may represent the inclusion of patients with other causes for mild diarrheal states, such as viral infections or dietary indiscretion. Thus, this correlation probably has the least pathophysiologic relevance, but nonetheless suggests a possible mechanism for MP that can direct future research.

The highest statistical correlation was found in the SP and CD-like complication groups most likely because of the more clearly defined phenotype in these 2 groups as opposed to the MP group. The *NOD2* gene highly correlates with SP ($p = 0.0008$), as described in our previously published study.[4] This finding has recently been replicated in a much larger retrospective multicenter study (A. D. Tyler, Ph.D., unpublished data, 2011). The *NOD2* gene that is found on chromosome 16 was first discovered to be associated with CD in 2001.[24–26] *NOD2* mutations have been found in up to 40% of European and North American CD patients compared with 10% to 15% of the healthy population.[27] The protein product of this gene acts as a sensor for bacterial cell wall component muramyl dipeptide, which is found on both gram-positive and gram-negative bacteria. Activation of this protein leads to an increase in nuclear factor κB and mitogen-activated protein kinase signaling pathways, which play key roles in orchestrating the inflammatory and immune response by regulating genes involved in producing several proinflammatory cytokines such as IL-8, IL-6, tumor necrosis factor-α, and antimicrobial peptides.[28] Furthermore, *NOD2* has also been shown to promote the process of autophagy-mediated endocytosis.[29] Increasing number of *NOD2* mutations have repeatedly been shown to be correlated with ileal involvement, early age of onset, and stricturing and/or penetrating CD phenotype.[30–33] How such a genetic defect relates pathophysiologically to SP is unclear, but it correlates with the observation that SP frequently requires treatment of both enteric bacteria (with antibiotics) and host inflammation in the pouch by the use of 5-aminosalicylic acid derivatives, or even immunosuppressive medications,[34] suggesting that its pathophysiology is more related to a host-immune defect than simply enteric superinfection.

Tumor necrosis factor (ligand) superfamily, member 15 (*TNFSF15*) is unique because it is the only gene to be associated with CD in both Asian and white populations.[35] *TNFSF15*'s protein product, vascular endothelial cell growth inhibitor is expressed in endothelial cells, lymphocytes, plasma cells, monocytes, and dendritic cells and can bind to the death domain receptor 3.[36] Microbial stimulation of Toll-like receptors causes an upregulation of this complex that activates the nuclear factor κB and mitogen-activated protein kinase pathways, thereby leading to the increased production of the antiapoptotic proteins, interferon γ, and IL-8 cytokines.[36]

The rs10761659 SNP within the *10q21* gene locus most significantly correlated with CD-like complications ($p = 0.006$). Several European GWAS have identified SNPs within this locus to be associated with CD in a region closely related to the zinc finger protein 365 (*ZNF365*) gene.[15,16,37] Data regarding the function of this gene locus are largely lacking, and other causal variants remain to be identified. Recently, Haritunians et al[37] provided strong evidence for the *ZNF365* gene being responsible for CD susceptibility in the *10q21* locus. They identified the major allele (G) of SNP rs7076156 in *ZNF365* isoform D to be strongly associated with CD. This SNP rs7076156 was in linkage equilibrium with other previously identified CD-associated SNPs within *10q21*, including rs10761659 that was investigated in this study. The SNP rs7076156 in *ZNF365* isoform D alters the expression of several genes, including the transcription factor *ZNF148* that is known to play a role in gut homoeostasis.

Even though GWAS have provided great insights into IBD by identifying specific pathways of immune function and inflammation, little work has focused on how such genetic discoveries can assist the surgeon in surgical decision making. Several studies have attempted to use clinical parameters and serological markers as prognosticators in IBD, but these are largely inconsistent and none are able to accurately predict the disease course preoperatively. Using

genetics as a marker for predicting outcomes is an attractive prospect, because these markers are present long before the onset of disease and remain stable during disease flares.[38] The majority of SNPs that have been implicated in IBD so far carry only a low to moderate overall risk for developing disease and predicting outcome with an allelic odds ratio less than 1.5.[39,40] However, an increase in the overall relative risk for developing IBD has been shown when several low-penetrance SNPs are combined together. For example, Weersma et al[40] showed that a combined increase in the number of risk alleles (*NOD2, IBD5, DLG5, ATG16L1, IL23R*) had a much greater risk for developing CD and, with a more severe disease course as seen by the greater need for surgery, a stricturing and/or penetrating behavior and an earlier age of disease onset. In our study, we attempted to combine the top 2 SNPs identified as statistically associated with each of CD-like and SP group to create probability equations that could theoretically be used to predict overall chance of a positive or negative outcome for that complication based on genotype. The top 2 SNPs used for the gene signature were dissimilar to the SNPs determined in Table 4, because the order of significance changed once the asymptomatic and MP groups were combined together to form the "favorable" group. In addition, the gene signature was constructed from a multivariate model that selected out redundant SNPs because of linkage or correlation of the SNPs remaining in the model, thus yielding a parsimonious genetic signature. The SNPs that most strongly correlated with SP compared with the favorable group were *NOD2* and *TNFSF15*, whereas, for CD-like complications, the *10q21* locus and *PTGER4* were most significant. These gene signatures predict that the overall risk for developing these complications increase greatly as the number of risk alleles increases. Individuals who would be homozygous mutant for both genes will have a 65% probability for developing CD-like complications and at 92% risk for developing SP. Should such predictions be borne out by prospective studies of patients undergoing the IPAA by preoperative genotyping, the utility of such formulas in assisting the surgeon in preoperative decision making could be powerful indeed, especially because genotyping would be free of the variability commonly seen in serologic or other clinical measurements.

Phenotype can change over time, but our average follow-up for the asymptomatic pouch and MP groups was on average 6 and 8 years, thus giving us confidence that our clinical classification for the large majority of these patients was robust, especially because the average time to the development of CD-like complications, SP, and MP averages 3.2, 3.2, and 2.8 years.[4] Furthermore, performing additional statistical analyses using a weighting system based on individual patient follow-up times in the groups with statistically significant different follow-up times, yielded the same identity and priority of SNPs in the gene signatures, confirming that our phenotype characterization with this length of follow-up was accurate.

Several other studies have attempted to use data from GWAS to predict outcome in complex diseases. Haritunians et al developed a genetic scoring system for predicting the need for surgery in patients with medically refractory UC. A combination of 46 SNPs were associated with medically refractory UC, and genetic risk scores were calculated from the total number of risk alleles (0, 1, or 2), with the greater risk of colectomy associated with an increased number of risk alleles present in their model.[41] Very interestingly, the gene loci found to be most predictive of lack of response to medical management and thus requiring colectomy were the major histocompatibility complex and *TNFSF15*, as we found in the SP group in our present study. *TNFSF15*'s protein product is a vascular endothelial cell growth inhibitor, an antiangiogenic protein. This suggests that severity of disease, whether it be UC needing colectomy or SP, in part relates to the process of healing and angiogenesis. Thus, alternative forms of pouchitis therapy that target apparent defects in healing may be proposed. Similarly, Weedon et al[39] studied the effects of several known type 2 diabetes SNPs on disease risk in a large case control study. They showed that combining information from several known common risk variants allowed for better identification of patients at higher risk of developing type 2 diabetes in comparison with assessment using single variants. Furthermore, Yang et al have shown a substantial improvement in the ability to predict the risk of developing a multifactorial complex disease by use of both a panel of genetic variants and environmental factors concurrently, compared with using only one parameter.[12]

Although our results are exciting and represent essentially the first attempt to use genetics in the surgical prognosis and decision-making process of the IBD patient, they should nonetheless be viewed as preliminary. This was a relatively small study done retrospectively in a unique demographic group of patients from the central Pennsylvania area of the United States. Other genetic associations, specifically the *NOD2*/CARD15 association with ileal CD, for example, were not replicated in a Japanese population[42] exemplifying the need to carefully control for ethnicity, demographics, and possibly even environmental factors that may affect the expression of disease-causing genetic determinants.

CONCLUSION

This study has utilized data from GWAS to identify genetic variants that correlate with complications after IPAA. The majority of these genes have previously been associated with CD and play a role in enteric bacterial recognition and destruction. The *NOD2* and *TNFSF15* gene had the highest correlation with SP. The *10q21* gene locus and *PTGER4* most significantly correlated with post-IPAA CD-like

complications. Similar to other complex diseases, the risk for developing these complications greatly increased when a greater number of risk alleles were present. This small retrospective study needs to be confirmed in a much larger cohort of UC patients and can provide the basis for a prospective multicenter trial to evaluate these SNPs in predicting the development of post-IPAA complications. Such risk profiling holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD patients. Genetic profiling preoperatively could both provide improved counseling to patients regarding their prognosis and assist the clinician with choice of surgery, especially in the marginal operative candidate.

REFERENCES

1. Shen B, Fazio VW, Remzi FH, Lashner BA. Clinical approach to diseases of ileal pouch-anal anastomosis. *Am J Gastroenterol.* 2005;100:2796–2807.
2. Beliard A, Prudhomme M. Ileal reservoir with ileo-anal anastomosis: long-term complications. *J Visc Surg.* 2010;147:e137–e144.
3. Shen B. Crohn's disease of the ileal pouch: reality, diagnosis, and management. *Inflamm Bowel Dis.* 2009;15:284–294.
4. Sehgal R, Berg A, Hegarty JP, et al. NOD2/CARD15 mutations correlate with severe pouchitis after ileal pouch-anal anastomosis. *Dis Colon Rectum.* 2010;53:1487–1494.
5. Shen B. Diagnosis and treatment of patients with pouchitis. *Drugs.* 2003;63:453–461.
6. Gunderson KL, Steemers FJ, Lee G, Mendoza LG, Chee MS. A genome-wide scalable SNP genotyping assay using microarray technology. *Nat Genet.* 2005;37:549–554.
7. Hsu CH, Green SB, He Y. A weighted logistic regression model for estimation of recurrence of adenomas. *Stat Med.* 2007;26:1567–1578.
8. Fang MC, Go AS, Chang Y, et al. Comparison of risk stratification schemes to predict thromboembolism in people with nonvalvular atrial fibrillation. *J Am Coll Cardiol.* 2008;51:810–815.
9. Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet.* 2002;3:391–397.
10. Motulsky AG. Genetics of complex diseases. *J Zhejiang Univ Sci B.* 2006;7:167–168.
11. Moonesinghe R, Liu T, Khoury MJ. Evaluation of the discriminative accuracy of genomic profiling in the prediction of common complex diseases. *Eur J Hum Genet.* 2010;18:485–489.
12. Yang Q, Khoury MJ, Botto L, Friedman JM, Flanders WD. Improving the prediction of complex diseases by testing for multiple disease-susceptibility genes. *Am J Hum Genet.* 2003;72:636–649.
13. Evans DM, Visscher PM, Wray NR. Harnessing the information contained within genome-wide association studies to improve individual prediction of complex disease risk. *Hum Mol Genet.* 2009;18:3525–3531.
14. Hampe J, Franke A, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet.* 2007;39:207–211.
15. Rioux JD, Xavier RJ, Taylor KD, et al. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet.* 2007;39:596–604.
16. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* 2007;447:661–678.
17. Baldassano RN, Bradfield JP, Monos DS, et al. Association of the T300A non-synonymous variant of the *ATG16L1* gene with susceptibility to paediatric Crohn's disease. *Gut.* 2007;56:1171–1173.
18. Fritz T, Niederreiter L, Adolph T, Blumberg RS, Kaser A. Crohn's disease: NOD2, autophagy and ER stress converge [published online ahead of print January 19, 2011]. *Gut.* doi: 10.1136/gut.2009.206466.
19. Hruz P, Eckmann L. Innate immune defense: NOD2 and autophagy in the pathogenesis of Crohn's disease. *Swiss Med Wkly.* 2010;27:140:w13135.
20. Kuballa P, Huett A, Rioux JD, Daly MJ, Xavier RJ. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. *PLoS One.* 2008;3:e3391.
21. Prescott NJ, Fisher SA, Franke A, et al. A nonsynonymous SNP in ATG16L1 predisposes to ileal Crohn's disease and is independent of CARD15 and IBD5. *Gastroenterology.* 2007;132:1665–1671.
22. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. *Nature.* 2008;456:259–263.
23. Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. *Nature.* 2008;456:264–268.
24. Hugot J, Chamaillard M, Zouali H, et al. Association of NOD2 leucinerich repeat variants with susceptibility to Crohn's disease. *Nature.* 2001;411:599–603.
25. Ogura Y, Bonen DK, Inohara N, et al. A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. *Nature.* 2001;411:603–606.
26. Hampe J, Cuthbert A, Croucher PJ, et al. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet.* 2001;357:1925–1928.
27. Rogler G. The importance of gut microbiota in mediating the effect of NOD2 defects in inflammatory bowel disease. *Gut.* 2010;59:153–154.
28. Spehlmann ME, Eckmann L. Nuclear factor-kappa B in intestinal protection and destruction. *Curr Opin Gastroenterol.* 2009;25:92–99.
29. Brain O, Allan P, Simmons A. NOD2-mediated autophagy and Crohn disease. *Autophagy.* 2010;6:412–414.
30. Abreu MT, Taylor KD, Lin YC, et al. Mutations in NOD2 are associated with fibrostenosing disease in patients with Crohn's disease. *Gastroenterology.* 2002;123:679–688.
31. Cuthbert AP, Fisher SA, Mirza MM, et al. The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology.* 2002;122:867–874.
32. Hampe J, Grebe J, Nikolaus S, et al. Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet.* 2002;359:1661–1665.
33. Lesage S, Zouali H, Cézard JP, et al. CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease. *Am J Hum Genet.* 2002;70:845–857.

34. Haveran LA, Sehgal R, Poritz LS, McKenna KJ, Stewart DB, Koltun WA. Infliximab and/or azathioprine in the treatment of Crohn's disease-like complications after IPAA. *Dis Colon Rectum.* 2011;54:15–20.
35. Yamazaki K, McGovern D, Ragoussis J, et al. Single nucleotide polymorphisms in TNFSF15 confer susceptibility to Crohn's disease. *Hum Mol Genet.* 2005;14:3499–3506.
36. Endo K, Kinouchi Y, Kakuta Y, Ueki N, Takahashi S, Shimosegawa T. Involvement of NF-kappa B pathway in TL1A gene expression induced by lipopolysaccharide. *Cytokine.* 2010;49:215–220.
37. Haritunians T, Jones MR, McGovern DP, et al. Variants in ZNF365 isoform D are associated with Crohn's disease [published online ahead of print January 21, 2011]. *Gut.* doi: gut.2010.227256v1.
38. Vermeire S, Van Assche G, Rutgeerts P. Role of genetics in prediction of disease course and response to therapy. *World J Gastroenterol.* 2010;16:2609–2615.
39. Weedon MN, McCarthy MI, Hitman G, et al. Combining information from common type 2 diabetes risk polymorphisms improves disease prediction. *PLoS Med.* 2006;3:e374.
40. Weersma RK, Stokkers PC, van Bodegraven AA, et al. Dutch Initiative on Crohn and Colitis (ICC). Molecular prediction of disease risk and severity in a large Dutch Crohn's disease cohort. *Gut.* 2009;58:388–395.
41. Haritunians T, Taylor KD, Targan SR, et al. Genetic predictors of medically refractory ulcerative colitis. *Inflamm Bowel Dis.* 2010;16:1830–1840.
42. Yamazaki K, Takazoe M, Tanaka T, Kazumori T, Nakamura Y. Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease. *J Hum Genet.* 2002;47:469–472.

ORIGINAL CONTRIBUTION

Mutations in *IRGM* Are Associated With More Frequent Need for Surgery in Patients With Ileocolonic Crohn's Disease

Rishabh Sehgal, M.B., B.Ch., M.R.C.S.[1] • Arthur Berg, Ph.D.[1,2] • Joseph I. Polinski, B.S.[1]
John P. Hegarty, Ph.D.[1] • Zhenwu Lin, Ph.D.[1] • Kevin J. McKenna, M.D.[1]
David B. Stewart, M.D.[1] • Lisa S. Poritz, M.D.[1,3] • Walter A. Koltun, M.D.[1]

1 Division of Colon and Rectal Surgery, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania
2 Center for Statistical Genetics, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania
3 Department of Cellular and Molecular Physiology, Penn State Milton S. Hershey Medical Center, Hershey, Pennsylvania

BACKGROUND: There are no clear criteria for judging the severity of disease in patients with Crohn's disease. Yet classification of patients into low- and high-risk severity groups would benefit both medical and surgical management. At the time of this study, approximately 80 single-nucleotide polymorphisms within 55 genes had been associated with IBD.

OBJECTIVE: The aim of this study was to identify genetic determinants (single-nucleotide polymorphisms) that could be markers of Crohn's disease severity by the use of frequency of ileocolic surgery as a surrogate for disease severity.

DESIGN: Sixty-six patients (30 male) with ileocolonic Crohn's disease who previously underwent ileocolectomy were retrospectively studied. The severity of Crohn's disease was quantified by dividing the total number of ileocolectomy procedures by the time between IBD diagnosis and the patient's last clinic visit, the rationale being that more severe disease would be associated with a more frequent need for surgery. Genotyping for the 83 single-nucleotide polymorphisms associated with IBD was done on a customized Illumina Veracode genotyping platform. Three genetic models (general, additive, and dominant) were used to statistically quantify the genetic association of the studied single-nucleotide polymorphisms to the frequency of surgery after adjusting for covariates (age, smoking, family history, disease location, and disease behavior).

RESULTS: For the entire group the average number of ileocolectomies per patient was 1.7 (range, 1–5) with an average duration of disease of 14.7 years. Single-nucleotide polymorphism rs4958847 in the *IRGM* gene (immunity-related GTPase family, M) was the most significant single-nucleotide polymorphism in all 3 models tested ($p = 0.007$) as being associated with ileocolectomy, and it remained significant even after a Benjamini-Hochberg false-discovery correction for multiple observations. Patients carrying the "at-risk" allele for this single-nucleotide polymorphism ($n = 20$) had an average of 1 surgery every 6.87 ± 1.33 years in comparison with patients carrying the wild-type genotype ($n = 46$) who averaged 1 surgery in 11.43 ± 1.21 years ($p = 0.007$, Mann-Whitney *U* test).

CONCLUSIONS: Single-nucleotide polymorphism rs4958847 in the *IRGM* gene correlated very significantly with frequency of surgery in patients with ileocolonic Crohn's disease. *IRGM* is a mediator of innate immune responses and is involved in autophagy. The presence of this *IRGM* SNP may be a marker for disease severity and/or early recurrence after ileocolectomy and may assist in surgical and medical decision making.

KEY WORDS: Classification; Crohn's disease; Genetics; Genome-wide association studies; Inflammatory bowel disease; Infliximab; Surgical recurrence.

Financial Disclosures: None reported.

Podium presentation at the meeting of The American Society of Colon and Rectal Surgeons, Vancouver, BC, Canada, May 14 to 18, 2011.

Recipient the 2011 ASCRS Harry E. Bacon Foundation Award for Best Basic Science Podium Presentation.

Correspondence: Rishabh Sehgal, M.B., B.Ch., or Walter Koltun, M.D., Penn State Milton S. Hershey Medical Center, Penn State College of Medicine, Division of Colon and Rectal Surgery, H137, 500 University Dr, PO Box 850, Hershey, PA 17033-0850. E-mail: rishsehgal@gmail.com or wkoltun@hmc.psu.edu

Dis Colon Rectum 2012; 55: 115–121
DOI: 10.1097/DCR.0b013e31823ccea8

Crohn's disease (CD) is an idiopathic inflammatory condition affecting the GI tract that most commonly affects young adults and results in lifelong need for medical and surgical care. Within North America, the prevalence of CD ranges from 26 to 200 cases per 100,000 and most commonly presents between the ages of 15 and 30 years. The majority of affected patients have a chronic intermittent disease course, with 13% having an unremitting progressive course, whereas only 10% live in remission.[1]

Although medical management helps to control symptoms, up to two-thirds of patients with CD will require surgical resection of the affected bowel during the course of the disease.[2] Surgery for CD is by no means curative, and recurrence of symptoms is part of the natural history of the disease. Depending on definition, 70% to 90% of patients have endoscopic evidence of recurrent CD within 1 year after intestinal resection.[2] However, clinically relevant recurrence is often delayed, and up to one-third of patients will not develop clinically significant disease for approximately 15 to 20 years.[3–5] Such patients, if they could be identified, could avoid the use of potentially dangerous and expensive anti-tumor necrosis factor (anti-TNF) therapy. Conversely, some patients develop rapid recurrence that can lead to early and even repetitive surgery that can result in a short gut over the long term. Such patients would be candidates for more aggressive medical therapy such as anti-TNF therapy. CD patients are at an increased risk of experiencing postoperative recurrence if they are a current smoker, are positive for granulomas, have undergone previous CD-related surgery, and have penetrating disease (eg, fistula formation, perforation, and abscess).[6] Ileocolonoscopy in the early postoperative course is the only other diagnostic method by which to identify the patient who will develop early recurrence versus the one who will avoid surgery for many years.

Recently, genome-wide association studies (GWAS) have allowed the discovery of single-nucleotide polymorphisms (SNPs) and corresponding genes associated with CD. At the time of this study, approximately 80 SNPs within 55 genes (Table 1) had been identified to be associated with IBD. This wide variability of disease-predisposing genetic haplotypes is presumably one of the reasons for the many variations in disease characteristics or clinical phenotype of IBD patients. Correlation of such genotypes with clinical phenotype promises to allow the subclassification of IBD patients into categories beyond the simple ulcerative colitis or CD diagnoses. Therefore, the overall aim of this study was to identify genetic determinants (SNPs) that could be markers of CD severity in surgical patients that may assist in surgical and medical decision making. Although there is no one definition for disease severity in CD, the more frequent need for surgery can be generally accepted as reflecting a more severe disease phenotype. Ileocolonic disease is the most common form of CD and ileocolectomy is the most commonly performed operation. Therefore, this study targeted the most commonly performed intestinal surgery done for CD, namely ileocolectomy, and attempted to define genetic markers associated with the more frequent need for surgery in this CD patient population.

METHODS

Patient Recruitment

Operative records were retrospectively reviewed and a total of 275 CD patients who underwent ileocolectomy by the Division of Colon and Rectal Surgery at the Milton S. Hershey Medical Center, Penn State College of Medicine were identified over a 10-year period (January 1990 to December 2010). A total of 66 patients were subsequently recruited into our IBD registry started in 1998, and these patients make up the study population presented here without selection bias based on follow-up clinic visits during the study period (September 2008 to September 2010).

Severity of CD was defined as number of ileocolectomy procedures done over the period between disease diagnosis and the patient's last clinic visit, the rationale being that more severe disease would be associated with more frequent operations. Only patients with ileocolonic disease were studied because this is the most common form of CD and also provides for a more uniform study population (Fig. 1).

Patient demographics and documentation of clinical parameters of disease were gathered and included the following: 1) sex, family history, date of IBD diagnosis, previous number of surgeries, and smoking history; 2) Montreal classification (age of diagnosis, disease location, behavior) of disease; 3) medication/dose history including 6-mercaptopurine/azathioprine, infliximab, and steroid use; and 4) presence or absence of extraintestinal manifestations including primary sclerosing cholangitis, arthropathy, uveitis, aphthous stomatitis, or dermatological manifestations. The study was approved by The Milton S. Hershey Medical Center Institutional Review Board.

DNA/Cell Bank

The identified CD ileocolectomy patients were recruited into our institutional review board-approved genetic IBD cell/DNA bank that was originally established in 1998. Informed consent was obtained and patients donated blood samples that were then used to create immortalized B-cell lines by the use of Epstein-Barr virus that provided for an indefinite source of DNA from each recruited individual. In brief, blood was diluted with sterile phosphate-buffered saline, and layered onto Ficoll-Paque (Amersham Biosciences). The blood-Ficoll gradient was centrifuged at 1500 rpm for 30 minutes at room temperature. The mononuclear cell interface was extracted and washed with phosphate-buffered saline. The washed cell pellet was resuspended in RPMI

TABLE 1. List of gene/SNP on Illumina array

SNP	*Chromosome*	*Gene*
rs2476601	1p13	PTPN22
rs3737240	1q21	ECM1
rs13294		
rs3180018	1q22	SCAMP3, MUC1
rs2274910	1q23	ITLN1
rs4656940	1q23	*CD244*, ITLN1
rs1801274	1q23.3	*FCGR2A*
rs9286879	1q24	1q24
rs7517810	1q24	*TNFSF18, TNFSF4, FASLG*
rs1998598	1q31	*DENND1B*
rs1363670	1p31	IL-12B
rs3024505	1q32	IL-10
rs2797685	1p36	*VAMP3*
rs6426833	1p36	RNF186
rs10753575		
rs3806308		
rs10733113	1q44	*NLRP3*
rs917997	2q11	IL-18R
rs13003464	2p16	*PUS10*
rs10181042	2p16b	*C2orf74, REL*
rs12612347	2q35	ARPC2
rs2241880	2q37	ATG16L1
rs3828309		
rs3792109		
rs780094	2p23	*GCKR*
rs6738825	2q33	*PLCL1*
rs3197999	3p21	MST1
rs4833103	4p14	TLR1, TLR10, TLR6
rs7720838	5p13	PTGER4
rs4613763		
rs17234657	5p13.1	
rs9292777		
rs10044354	5q15	ARTS-1
rs1050152	5q31	OCTN1
rs2522057	5q31	IBD5
rs10077785		
rs10045431	5q33	IL-12B
rs6887695		
rs13361189	5q33	IGRM
rs4958847		
rs11747270		
rs7714584		
rs9268480	6p21.3	BTNL2
rs1059276	6p	MEP1A
rs7746082	6q21	HLA region
rs2844480		
rs9271568		
rs3794996		
rs2395185		
rs3763313		
rs660895		
rs6908425	6p22	CDKAL1
rs17309827	6p25	*SLC22A23*
rs2301436	7p12	CCR6
rs1456893		
rs7849191	9p24	JAK2
rs10758669		
rs3810936	9q32	TNFSF15
rs6478108		
rs7848647		
rs7869487		
rs4986790	9q32-q33	TLR 4
rs10870077	9q34.3	CARD9
rs3936503	10p11	CCNY
rs10761659	10q21	ZNF365
rs10995271		
rs1248696	10q23-q24	DLG5
rs1248634		
rs10883365	10q24	NKX2–3
rs7081330		
rs951199	11p15.1	NELL1
rs11175593	12q12	LRRK2
rs2066844	16q12	NOD2
rs2066845		
rs2066847		
rs916977	15q13.1	HERC2
rs7712957	15q13	S100z
rs744166	17q21	STAT3
rs363617	17q24.3	TNFR2
rs2542151	18p11	PTPN2
rs762421	21q22	ICOSLG
rs12704036		U7
rs6927210		U6

SNP = single-nucleotide polymorphism.

1640 media containing 12% fetal bovine serum (Gemini Bioproducts) and 25% Epstein-Barr virus supernatant. Infected cells were incubated at 37°C in a CO_2 incubator. Once transformed, cells were stored at 1×10^7 cells/mL in 1-mL aliquots of fetal bovine serum with 10% dimethyl sulfoxide in secure liquid nitrogen tanks until use.

DNA Isolation

DNA was extracted from 1×10^6 transformed B cells by the use of a DNA isolation kit (QIAamp DNA Blood Midi Kit, QIAGEN) according to the manufacturer's recommended protocol. DNA was quantified by the use of a spectrophotometer. A working solution of DNA was then created by diluting the sample with 10 mM Tris-HCl to create stock of 10 ng/μL.

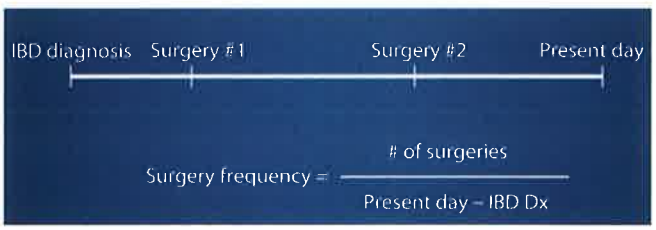

FIGURE 1. Assessing the severity of Crohn's disease. The surgical frequency was determined by dividing the total number of ileocolectomy procedures by the time between IBD diagnosis and the patient's last clinic visit, the rationale being that more severe disease would be associated with a more frequent need for surgery. Dx = diagnosis.

TABLE 2. Three genetic models

Generic model: AA, AB, BB
Additive model: AA > AB > BB
Dominant model: AA + AB > BB

Three genetic models were considered to identify a possible genetic association with frequency of surgery. The 3 models are powered for different types of genetic associations. The generic model is powered to detect an imbalance in any of the 3 genotypes; the additive model is powered to detect an increasing or decreasing risk with the number of "A" alleles; and the dominant model is powered to detect a change in risk with one or both risk alleles present.

DNA Genotyping With the Use of Illumina BeadExpress

With the help of Illumina (Illumina, San Diego, CA), we developed a customized DNA microarray specific for 83 SNPs previously identified by GWAS to be associated with IBD (Table 1).[7–22] dsDNA concentrations were quantified using an ultrasensitive fluorescent nucleic acid stain Quant-IT PicoGreen dsDNA Assay Kit (Invitrogen). The samples were then run on Illumina's BeadXpress Reader in Penn State Milton S. Hershey Medical Center's Functional Genomics Core Facility.

Statistical Analysis

Severity of CD in the individual ileocolonic patients was quantified by dividing the total number of ileocolectomies by the time between IBD diagnosis and the patient's last clinic visit (Fig. 1). A log transformation of severity was performed to yield a more homogenous and more robust measure of severity. Treating log severity as the primary outcome, 3 genetic models (generic model, additive model, and dominant model) were considered to identify a possible genetic association with severity (Table 2).

The regression-based genetic models accounted for potentially confounding variables such as age, sex, smoking, location of disease and behavior, and a stepwise model selection procedure was used to retain only the most relevant covariate variables. The Benjamini-Hochberg false-discovery method was used to provide adjustments of the p values for multiple comparisons.[23] Each of the 3 genetic models involved the testing of 83 SNPs each; therefore, a total of 249 hypotheses were considered. Because of the high level of correlation among the SNPs and among the 3 different models, this multiple adjustment correction is expected to be highly conservative. The p value adjustments were implemented by the use of the p.adjust function within R (www.r-project.org).

RESULTS

A total of 66 (30 male, 36 female) CD patients who underwent ileocolectomy were recruited for this study. The mean follow-up time from the time of disease diagnosis was 14.7 years. The average number of ileocolectomies per patient was 1.7 (range, 1–5). The majority of patients had their disease onset within the 17 to 40 years age group (A2), and had predominantly purely ileal involvement (L1) of stricturing type (B2) based on the Montreal classification system. The remaining demographic information is summarized in Table 3.

TABLE 3. Patient demographics

Sex	
Male	30
Female	36
Smoker	
Current	15
Ex-smoker	21
Never	30
FHx of IBD	
Yes	34
No	32
Medications	
No IFX/AZA	14
IFX only	7
AZA only	15
Both (IFX/AZA)	30
Montreal classification[a]	
Age: A1/A2/A3	8/54/4
Location: L1/L2/L3	38/0/28
Behavior:B1/B2/B3	5/35/26
Indication for surgery	
Fistula	7
Obstruction/pain	37
Abscess	8
Failed medical management	14

FHx = family history; IFX = infliximab; AZA = azathioprine.
[a]Montreal classification: age of IBD diagnosis (A1 = below 16 y; A2 = between 17 and 40 y; and A3 = above 40 y); location (L1 = ileal; L2 = colonic; L3 = ileocolonic); and behavior (B1 = nonstricturing, nonpenetrating; B2 = stricturing; B3 = penetrating).

Of the 83 SNPs within 55 IBD-associated genes that were interrogated, both SNPs (rs4958847, rs13361189) within the *IRGM* gene, *10q21* (rs10761659), *ATG16L1* (rs2241880), *S100Z* (rs7712957), and *IBD5* (rs10077785) remained highly significant irrespective of the genetic model used. The conventional statistical p values for these gene/SNPs are listed in Table 4. The SNP rs4958847 within the *IRGM* (immunity-related GTPase family, M) gene was the most significant SNP in all 3 genetic models ($p = 0.007$; Table 4) and remained significant even after applying a Benjamini-Hochberg false-discovery method for multiple observations. However there were no patients homozygous for the rs4958847 SNP in our patient cohort, making it impossible to assess which genetic model (genetic, additive, or dominant) was responsible for its effect. Patients carrying the "at risk" allele for this SNP (n = 20) had an average of 1 surgery every 6.87 ± 1.33 years compared with patients carrying the wild-type genotype (n = 46) who averaged 1 surgery in 11.43 ± 1.21 years ($p = 0.007$, Mann-Whitney U test; Table 5).

Patients with ileocolonic disease commonly have a *NOD2* mutation. The overall frequency of *NOD2* mutations for the entire cohort was 30% (n = 20; Table 5). There was no correlation between *IRGM* SNP rs4958847

TABLE 4. Top single-nucleotide polymorphisms

SNP name	Gene name	p	p after FDR correction
General genetic model			
rs4958847	*IRGM*	0.00009	0.00712
rs13361189	*IRGM*	0.00738	0.291
rs10761659	*10q21*	0.0255	0.496
rs2241880	*ATG16L1*	0.0262	0.496
rs7712957	*S100Z*	0.032	0.496
Additive genetic model			
rs4958847	*IRGM*	0.00009	0.00712
rs13361189	*IRGM*	0.00738	0.291
rs7712957	*S100Z*	0.032	0.758
rs10758669	*STAT3*	0.0506	0.758
rs10077785	*IBD5*	0.0562	0.758
Dominant genetic model			
rs4958847	*IRGM*	0.00009	0.00712
rs10761659	*10q21*	0.00705	0.194
rs13361189	*IRGM*	0.00738	0.194
rs10077785	*IBD5*	0.0241	0.334
rs2241880	*ATG16L1*	0.0268	0.334

Several SNP/genes were shown to be highly significant by the use of conventional statistics. However, when performing an FDR correction for multiple comparisons,[39] only SNP rs4958847 within the *IRGM* gene remained significant.
SNP = single-nucleotide polymorphism; FDR = false-discovery rate.

with *NOD2* status, because there was 25% *NOD2* positivity in the "at risk" *IRGM* group compared with 35% in the *IRGM* wild-type group ($p = 0.937$; Table 5).

DISCUSSION

The completion of the International Haplotype Map (HapMap) Project in 2005 and the advancements made in microarray-based technologies promoted interest in identifying the possible etiology of IBD by identifying SNPs and corresponding genes associated with IBD. At the time of this study, approximately 80 SNPs within 55 genes had been associated with IBD.[7–22] This potential wide variability of genotype is presumably the reason for the many variations in disease characteristics or clinical phenotype of IBD patients. Correlation of such genotypes with clinical phenotype promises to allow for the subclassification of IBD patients into categories beyond the simple ulcerative colitis or CD categories. In the extreme, genotype may segregate and predict patients' disease characteristics such as anatomic location, behavior (fistulizing vs stenosing), and even prognosis. Genotypic determinants may even predict responsiveness to specific therapies, or, as we suggest in this study, the early recurrence of disease after surgical resection. Genotype may define a "rapid recurrence" phenotype that would justify early and more aggressive medical management after surgery. To this end, this study investigated 66 CD patients who underwent surgery to identify genetic factors that might correlate with the high versus low risk of CD recurrence after ileocolectomy with the goal of identifying patients at high risk for such recurrence. Thus, we used frequency of ileocolectomy as a surrogate for disease severity, which was determined by calculating the total number of ileocolectomies from the time of initial IBD diagnosis to the last clinic visit, with the rationale being that the more surgeries a patient has to undergo, the more severe the disease course.

After considering 3 statistical models to identify SNPs that most significantly correlated with severity of CD, the SNP rs4958847 within the *IRGM* gene was the only SNP to maintain its significance even after Benjamini-Hochberg correction in all 3 genetic models. The Benjamini-Hochberg method is a conservative correction, in particular, because many of the SNPs considered in this study were in linkage disequilibrium, which suggests that the *IRGM* gene is strongly statistically associated with severity. CD patients carrying an "at risk" allele for SNP rs4958847 had an overall more severe disease course, ie, greater number of surgeries in comparison with those who were "wild-type."

In general, the *IRGM* gene located on the long arm (q) of chromosome 5 at position 33.1 encodes a p47 immunity-related GTPase family member that is involved in the process of autophagy.[24] Autophagy is a *teleologically* highly conserved process by which cells maintain intracellular homeostasis by degrading potentially dangerous waste materials, such as damaged organelles, apoptotic bodies, and intracellular pathogens, such as viruses, bacteria, and parasites by sequestering them within autophagosomes, which are then presented to lysosomes for degradation.[7] siRNA knockdown of *IRGM* in human macrophages has demonstrated an impaired handling of intracellular pathogens such as *Mycobacterium tuberculosis*, leading to prolonged intracellular survival.[7,25]

The 2 noncoding *IRGM* SNPs (rs13361189 and rs4958847) investigated in this study were first identified in 2007 by the Wellcome Trust Case Control Consortium

TABLE 5. Summary of IRGM rs4958847

SNP	Genotype[a]	N	Surgical frequency (y ± SEM)	p	NOD2[b] mutations
rs4958847	*IRGM* mutation	20/66	6.87 ± 1.33	0.007	(n = 5) 25%
	IRGM wild type	46/66	11.43 ± 1.21		(n = 15) 33%

SNP = single-nucleotide polymorphism.
[a]"*IRGM* mutation" represents patients carrying at least 1 copy of the at-risk allele. "IRGM wild type" represents the homozygous wild type.
[b]Patients with ileocolonic disease commonly have a NOD2 mutation. There was no correlation between *IRGM* SNP rs4958847 with NOD2 status ($p = 0.94$).

GWAS.[8] Several studies have since confirmed the association of these *IRGM* SNPs with CD in various ethnic cohorts, but the exact functional roles of these SNPs are still unclear despite many resequencing attempts.[24,26–33] McCarroll et al[33] have recently shown that SNP rs13361189 is in perfect linkage disequilibrium with a 20-kb deletion polymorphism that affects the expression of *IRGM*. This deletion polymorphism is associated with a SNP (c.313C>T) that has been shown to alter microRNA miR-196 regulation of *IRGM* expression in CD.[34] Our study investigated both these *IRGM* SNPs, but only rs4958847 maintained significance after Benjamini-Hochberg correction. The other SNP rs13361189 failed to remain significant most probably because of the smaller number of CD patients within this study population. Latiano et al[35] have shown that the rs4958847 SNP is associated with fistulizing behavior, including perianal fistulas.

This study also attempted to correlate the frequency of *NOD2/CARD15* variants with *IRGM*; however, it failed to show any association with the severity of disease. Similarly, Latiano et al[35] also failed to show any significant interaction between *NOD2/CARD15* and *IRGM* variants in both of their pediatric and adult populations.

Other investigators have evaluated other modalities to identify early CD recurrence after ileocolectomy. These include changes seen on endoscopy, radiological studies, and serological biomarkers. Rutgeerts et al[36] proposed a 5-grade scoring system to predict CD recurrence based on endoscopic findings. Patients who showed no ulcers or ≤5 aphthous ulcers in the neoterminal ileum on ileocolonoscopy at 1 year postsurgery were at low risk for clinical recurrence. However, patients who showed >5 aphthous ulcers, deep ulcers with more extensive inflammation, or severe inflammation with nodularity and stenosis, had a 3-year clinical or surgical recurrence rate of 15%, 40%, and 90%.[6] Calabrese et al[37] showed strong correlation of the Rutgeerts scoring system with small-intestine contrast ultrasonography and similarly Sailer et al[38] correlated the Rutgeerts score with MR enteroclysis. Furthermore, a recent study conducted by Lamb et al[39] demonstrated that the quantitative measurement of fecal calprotectin and lactoferrin levels significantly correlated with disease recurrence in postoperative CD patients. These fecal markers were much more sensitive than C-reactive protein, platelet count, or endoscopic appearance at predicting clinical disease activity. Although using ileocolonoscopy to predict CD recurrence postoperatively does show promise, it is an invasive procedure and still carries potential adverse risk. Radiological modalities are less invasive and certainly ultrasonography is advantageous because it carries no risk of radiation exposure, but none of these modalities help to predict the disease course preoperatively, and especially not before the actual recurrence of the disease that is visualized by these tests. The potential advantage of our finding is that high-risk patients can be identified with a simple blood test and then undergo the initiation of prophylactic medical management well before the onset of disease recurrence.

This study has identified a genetic variant within the *IRGM* gene that could potentially be used as a predictor of early recurrence after ileocolectomy. This small study will need to be confirmed in a much larger cohort of CD patients and can provide the basis for a prospective multicenter trial incorporating populations from different backgrounds to evaluate the *IRGM* SNP rs4958847 in predicting early disease recurrence after ileocolectomy. Genotyping holds promise for playing a role in both surgical and medical decision making in the clinical management of IBD patients.

REFERENCES

1. Loftus EV Jr, Schoenfeld P, Sandborn WJ. The epidemiology and natural history of Crohn's disease in population-based patient cohorts from North America: a systematic review. *Aliment Pharmacol Ther.* 2002;16:51–60.
2. Regueiro M, Kip KE, Schraut W, et al. Crohn's disease activity index does not correlate with endoscopic recurrence one year after ileocolonic resection. *Inflamm Bowel Dis.* 2011;17:118–126.
3. Ritchie JK. The results of surgery for large bowel Crohn's disease. *Ann R Coll Surg Engl.* 1990;72:155–157.
4. Lennard-Jones JE, Stalder GA. Prognosis after resection of chronic regional ileitis. *Gut.* 1967;8:332–336.
5. Bernell O, Lapidus A, Hellers G. Risk factors for surgery and recurrence in 907 patients with primary ileocaecal Crohn's disease. *Br J Surg.* 2000;87:1697–1701.
6. Swoger JM, Regueiro M. Postoperative Crohn's disease: how can we prevent it? *Expert Rev Clin Immunol.* 2010;6:501–504.
7. Lees CW, Satsangi J. Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history. *Expert Rev Gastroenterol Hepatol.* 2009;3:513–534.
8. Wellcome Trust Case Control Consortium. Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature.* 2007;447:661–678.
9. Barrett JC, Hansoul S, Nicolae DL, et al. Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008;40:955–962.
10. Duerr RH, Taylor KD, Brant SR, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science.* 2006;314:1461–1463.
11. Fisher SA, Tremelling M, Anderson CA, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat Genet.* 2008;40:710–712.
12. Franke A, Balschun T, Karlsen TH, et al. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat Genet.* 2008;40:1319–1323.
13. Hampe J, Franke A, Rosenstiel P, et al. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet.* 2007;39:207–211.
14. Libioulle C, Louis E, Hansoul S, et al. Novel Crohn disease locus identified by genome-wide association maps to a gene desert on 5p13.1 and modulates expression of PTGER4. *PLoS Genet.* 2007;3:e58.
15. Parkes M, Barrett JC, Prescott NJ, et al. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet.* 2007;39:830–832.

16. Silverberg MS, Cho JH, Rioux JD, et al. Ulcerative colitis-risk loci on chromosomes 1p36 and 12q15 found by genome-wide association study. *Nat Genet.* 2009;41:216–220.
17. Mathew CG. New links to the pathogenesis of Crohn disease provided by genome-wide association scans. *Nat Rev Genet.* 2008;9:9–14.
18. Noomen CG, Hommes DW, Fidder HH. Update on genetics in inflammatory disease. *Best Pract Res Clin Gastroenterol.* 2009;23: 233–243.
19. Huebner C, Petermann I, Lam WJ, Shelling AN, Ferguson LR. Characterization of single-nucleotide polymorphisms relevant to inflammatory bowel disease in commonly used gastrointestinal cell lines. *Inflamm Bowel Dis.* 2010;16:282–295.
20. Scherr R, Essers J, Hakonarson H, Kugathasan S. Genetic determinants of pediatric inflammatory bowel disease: is age of onset genetically determined? *Dig Dis.* 2009;27:236–239.
21. Nakagome S, Takeyama Y, Mano S, et al. Population-specific susceptibility to Crohn's disease and ulcerative colitis: dominant and recessive relative risks in the Japanese population. *Ann Hum Genet.* 2010;74:126–136.
22. McGovern DP, Gardet A, Törkvist L, et al. Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat Genet.* 2010;42:332–337.
23. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser B.* 1995;57:289–300.
24. Huett A, Xavier R. SNPping away at the pathogenesis of Crohn's disease. *Inflamm Bowel Dis.* 2008;14:136–137.
25. Singh SB, Davis AS, Taylor GA, Deretic V. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science.* 2006 8;313:1438–1441.
26. Roberts RL, Hollis-Moffatt JE, Gearry RB, Kennedy MA, Barclay ML, Merriman TR. Confirmation of association of IRGM and NCF4 with ileal Crohn's disease in a population-based cohort. *Genes Immun.* 2008;9:561–565.
27. Latiano A, Palmieri O, Cucchiara S, et al. Polymorphism of the IRGM gene might predispose to fistulizing behavior in Crohn's disease. *Am J Gastroenterol.* 2009;104:110–116.
28. Weersma RK, Stokkers PC, Cleynen I, et al. Confirmation of multiple Crohn's disease susceptibility loci in a large Dutch–Belgian cohort. *Am J Gastroenterol.* 2009;104:630–638.
29. Barrett JC, Hansoul S, Nicolae DL, et al. Genomewide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet.* 2008;40:955–962.
30. Roberts RL, Hollis-Moffatt JE, Gearry RB, et al. Confirmation of association of IRGM and NCF4 with ileal Crohn's disease in a population-based cohort. *Genes Immun.* 2008;9:561–565.
31. Fisher SA, Tremelling M, Anderson CA, et al. Genetic determinants of ulcerative colitis include the ECM1 locus and five loci implicated in Crohn's disease. *Nat Genet.* 2008;40:710–712.
32. Franke A, Balschun T, Karlsen TH, et al. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. *Nat Genet.* 2008;40: 713–715.
33. McCarroll SA, Huett A, Kuballa P, et al. Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn's disease. *Nat Genet.* 2008;40:1107–1112.
34. Brest P, Lapaquette P, Souidi M, et al. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. *Nat Genet.* 201;43:242–245.
35. Latiano A, Palmieri O, Cucchiara S, et al. Polymorphism of the IRGM gene might predispose to fistulizing behavior in Crohn's disease. *Am J Gastroenterol.* 2009;104:110–116.
36. Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology.* 1990;99:956–963.
37. Calabrese E, Petruzziello C, Inali S, et al. Severity of postoperative recurrence in Crohn's disease: correlation between endoscopic and sonographic findings. *Inflamm Bowel Dis.* 2009;15: 1635–1642.
38. Sailer J, Peloschek P, Reinisch W, Vogelsang H, Turetschek K, Schima W. Anastomotic recurrence of Crohn's disease after ileocolic resection: comparison of MR enteroclysis with endoscopy. *Eur Radiol.* 2008;18:2512–2521.
39. Lamb CA, Mohiuddin MK, Gicquel J, et al. Faecal calprotectin or lactoferrin can identify postoperative recurrence in Crohn's disease. *Br J Surg.* 2009;96:663–674.

EXPERT REVIEWS

Scoring systems in inflammatory bowel disease

Expert Rev. Gastroenterol. Hepatol. 4(4), 513–521 (2010)

Rishabh Sehgal[1] and Walter A Koltun[†1]

[1]Section of Colon and Rectal Surgery, Penn State Milton S Hershey Medical Center, PO Box 850, Hershey, PA 17033, USA
[†]Author for correspondence:
Tel.: +1 717 531 5164
Fax: +1 717 531 0646
wkoltun@psu.edu

Inflammatory bowel disease (IBD) is a chronic, relapsing condition affecting the GI tract that can affect individuals of any age and results in lifelong treatment, frequently including the need for surgery. Historically, the lack of a single effective and sensitive test for IBD has posed a great challenge in assessing disease severity, effectiveness of medication and predicting outcomes for this complex condition. Several IBD scoring and classification systems have been developed over many years to classify and characterize IBD patients, with the goal of helping to better define the disease status and effectiveness of therapy. Recent genetic investigations have revealed the complexity of IBD at the pathophysiologic level, revealing numerous genetic mutations associated with the disease. Thus, these clinically based IBD classification systems can provide the basis for the eventual correlation between the underlying genotype with clinical expression of disease and lead to better characterization of disease subtypes and, hopefully, customized treatment regimens.

Keywords: classification • Crohn's disease • genetics • inflammatory bowel disease • scoring • ulcerative colitis

Inflammatory bowel disease (IBD) is a chronic, relapsing inflammatory condition affecting the GI tract that most often affects young adults and results in lifelong need for medical and surgical care. There are two main subtypes: Crohn's disease (CD), where inflammation can affect any part of the gut, but most commonly the terminal ileum and colon; and ulcerative colitis (UC), where the disease is limited to the colon and rectum. The location of bowel affected in CD and UC produce the much varied clinical symptoms among patients (Table 1).

There is still no known exact etiology for these conditions; however, both environmental and genetic factors have been implicated in the pathogenesis of IBD. The generally agreed mechanism is that there is an exaggerated or poorly regulated immune response to an environmental agent in a genetically susceptible individual [1]. The discovery in CD patients of mutations in the *NOD2/CARD15* gene, which produces a protein that identifies a cell wall component of certain bacteria, reinforces this concept of a host–environment interaction as the basis of the disease. Apart from smoking, which has repeatedly been shown to worsen CD and conversely be protective in UC [2–4], other environmental factors, such as the use of oral contraceptives, high sugar and fat diet, breastfeeding, appendectomy and vaccinations, have shown conflicting results [5]. More recent discoveries suggesting defects in autophagy that play a role in innate intestinal immunity again point to commensal bacteria as being the environmental trigger for disease in a genetically susceptible patient [6].

The lack of a single effective and sensitive test for diagnosing IBD or assessing its severity has posed a great challenge in managing this complex condition. Several IBD classification systems and scoring schema have thus been developed to characterize disease severity and clinical phenotype, with the goal of helping to better define therapy and prognosis. These classification systems are generally of three broad types: anatomically based systems that define areas of the gut affected by disease; severity-based systems that generally use clinical symptoms or simple tests to assess the severity of disease; and quality of life systems that generally use patient questionnaires to assess overall well-being and social functioning. The first two types of classification schema will be discussed here, since intuitively the measurement of disease severity and extent can potentially lend insight into disease pathogenesis.

Using such classification systems theoretically promotes reproducibility between different care centers, as well as providing improved prognostic

 10.1586/EGH.10.40 ISSN 1747-4124

Table 1. Comparisons of various clinical factors in Crohn's disease and ulcerative colitis.

Clinical factors	Crohn's disease	Ulcerative colitis
Terminal ileum involvement	Common	Reflux ileitis
Colon involvement	Common	Usually
Rectum involvement	Possible	Always
Anal disease	Common	Rare
Bile duct involvement	Low	Higher rate
Distribution of disease	Entire gut possible, skip lesions	Continuous inflammation from rectum proximal
Endoscopy	Geographic and serpiginous ulcers	Continuous mucosal inflammation
Depth of inflammation	May be transmural, deep into tissues	Shallow, mucosal
Fistulae	Common	Rare
Stenosis	Common	Rare
Granulomas on biopsy	Can have granulomas	Granulomas not seen
Surgical cure	Often returns following removal of affected part	Usually cured by removal of colon and rectum
Smoking	Higher risk for smokers	Lower risk for smokers

information by better defining patient disease and objective response to therapy. The chronic relapsing and remitting nature of IBD also adds to the dynamic clinical picture that physicians face; for example, the clinical picture that a patient initially presents with may not necessarily be the same after a 10-year period of disease. Another key advantage of using such classification systems is, therefore, their ability to define and follow disease progression over time. With the significant genetic research that is being carried out in IBD, it is becoming increasingly important to correlate such genetic findings with a clinically accurate picture of diseased patients. Thus, these clinical classification systems should now provide the basis for evolving genetic definitions of disease that will become increasingly relevant in the future, both for clinical investigation and the administering of newer therapies that may target specific disease subtypes determined by both clinical and genetic criteria.

Crohn's disease (anatomic) classification systems

Rome/Vienna classification system

Since the publication of Dr Burrill B Crohn's 1932 landmark article 'Regional ileitis: a pathologic and clinical entity' [7], much work has gone into trying to classify the disease. Farmer *et al.* based the earliest classification on the anatomical location of disease in 1975. In his study entitled 'Clinical patterns in Crohn's disease: a statistical study of 615 cases' [8], Farmer hypothesized that disease location may directly determine the clinical course and prognosis of the disease. Disease locations were segregated into ileocolic, small intestine, colon and anorectal. Farmer noted that patients who had colonic involvement required operations less frequently than those with ileocolic disease. However, it was the colonic group that most often presented with rectal bleeding, perianal fistulae, toxic megacolon and arthritis. Those with small bowel disease presented earlier with obstructive symptoms and, thus, had a more frequent need for surgery. With the ileocolic subgroup of patients, the main presenting symptoms were perianal and rectal fistulae, abscesses and intestinal obstruction. Building on this, in 1988, Greenstein *et al.* observed that surgical indications remained the same for repeated operations and influenced the speed with which reoperation occurred [9]. The authors noted two different clinical patterns, independent of anatomic distribution: the perforating disease type, which was shown to be more aggressive; and the nonperforating type, which had a more 'indolent course'. This behavioral classification, in conjunction with Farmer's anatomic classification, allowed the Working Party of Gastroenterologists to introduce the 1991 Rome Classification, where further phenotypic characterization was carried out by looking at: location (i.e., stomach/duodenum, jejunum, ileum, colon, rectum or anal/perianal); behavior, defined as primarily inflammatory, primarily fistulizing or primarily fibrostenotic; extent of disease (i.e., localized or diffuse); and operative history (i.e., primary or recurrent). By using this classification, it was recognized that there could be as many as 756 subgroups of CD. Steinhard *et al.* found that there were insufficient definitions of disease behavior, which resulted in only a 'fair interobserver agreement' when used by multiple different caregivers [10]. The aim of the International Working Party became, therefore, to develop a simpler classification of CD based on objective and reproducible clinical variables.

The Working Party made many revisions and modifications to the Rome Classification and came up with their final draft in 1998, which is now known as the Vienna classification (Table 2) [11]. This classification was created in order to standardize the description of study populations in clinical trials and to aid in correlating putative etiologic factors with particular clinical phenotypes. The final version incorporated three main disease descriptors: age of diagnosis (A1–2), location of disease along the GI tract (L1–3) and clinical behavior, such as stricturing, inflammatory or fistulizing disease (B1–3). Validation of this classification using population clusters from Europe, Scandinavia and the USA showed a high degree of interobserver agreement in classifying patients [11].

Table 2. Vienna and Montreal classifications for Crohn's Disease.

Clinical factors	Vienna	Montreal
Age at onset	A1: <40 years A2: ≥40 years	A1: below 16 years A2: between 17 and 40 years A3: above 40 years
Disease location	L1: terminal ileum L2: colon L3: ileocolon	L1: ileal L2: colonic L3: ileocolonic L4: isolated upper disease†
Disease behavior	B1: inflammatory B2: stricturing B3: penetrating	B1: nonstricturing, nonpenetrating B2: stricturing B3: penetrating 'p': perianal disease modifier

†L4 is a modifier that can be added to L1–3 when concomitant upper GI disease is present.
'p' is added to B1–3 when concomitant perianal disease is present.
Adapted with permission from [12].

Montreal classification system

The Montreal classification was introduced in 2005 (Table 2) [12]. This was a further refinement of the Vienna classification, in that there was no change in the three major categories, but modifications were made within each of the categories. An additional age subgroup allowed pediatric CD to be defined (below 16 years of age). In the location category, upper GI disease (L4) could now be added to each of the L1–3 subclasses, as it had been noted that upper GI involvement was relatively common, and could coexist with ileal and colonic disease. The behavior category was amended in that perianal disease ('p') was added to the B1–3 classes as it was shown that perianal fistulizing disease can be variably associated with intestinal fistulizing disease. Patients with intestinal fistulizing disease, however, had a higher frequency of surgery compared with those with perianal fistulizing disease.

The Montreal classification system is still in its relative infancy and studies are underway to validate and assess this new scoring system among different populations. Chow *et al.* recently showed that the Montreal classification was more sensitive to behavioral phenotypic changes compared with the Vienna classification, after excluding perianal disease from the fistulising disease category [13]. Furthermore, it was useful in predicting the clinical course and the need for surgery. Their retrospective longitudinal study looked at a total of 109 patients with CD who were followed-up for an average of 4 years. Using the Montreal classification, CD behavior changed 3 years after diagnosis with an increase in stricturing and penetrating phenotypes, but this was only detected by the Vienna classification after 5 years, suggesting a better sensitivity to disease behavior characteristics. Disease location remained stable on follow-up in both classification systems. In total, 31% of patients with stricturing and penetrating phenotype underwent major surgery during the follow-up period, as determined by the Montreal classification.

Crohn's disease severity systems

Crohn's Disease Activity Index

To assess disease severity, CD activity indexes have been developed. In 1976, Best *et al.* published their proposed Crohn's Disease Activity Index (CDAI) [14–16], which was ultimately adopted by the National Cooperative Crohn's Disease Study Group. To create the index, 18 proposed predictor variables were initially gathered from 112 patients with CD at each clinic visit. Clinic scores were compared with scores from previous visits and critiqued by an attending physician. By using multiple regression analysis, the 18-predictor variables were eventually consolidated down to eight clinical variables. Three of the eight variables were derived from a 1-week patient diary (Table 3). Each independent variable was coded so that 0 corresponded to good health and increasing positive values corresponded to greater degrees of illness. A formula assigning weighting factors to multiply each disease value by was devised. A CDAI value of 150 and below was defined as quiescent disease. Values above that indicated active disease, while those above 450 reflected extremely severe disease. Changes in CDAI values from each visit correlated well with

Table 3. Crohn's Disease Activity Index items and weighting factors.

Item (cumulative 7 day score)	Weighting factors (multiply item)
Number of liquid or very soft stools	2
Abdominal pain score in 1 week (rating, 0–3)	5
General well-being (rating, 1–4)	7
Sum of physical findings per week: – Arthritis/arthralgia – Mucocutaneous lesions (e.g., erythema nodosum, aphthous ulcers) – Iritis/uveitis – Anal disease (e.g., fissure, fistula) – External fistula (e.g., enterocutaneous, vesicle, vaginal) – Fever over 37.8°C	20
Antidiarrheal use (e.g., diphenoxylate)	30
Abdominal mass (no = 0, equivocal = 2, yes = 5)	10
47 minus hematocrit (males) or 42 minus hematocrit (females)	6
100 × (1- [bodyweight divided by a standard weight])	1

Remission of Crohn's disease is defined as a Crohn's Disease Activity Index of less than 150. Severe disease was defined as a value of greater than 450. Most major research studies on medications in Crohn's disease define response as a fall of the Crohn's Disease Activity Index of greater than 70 points.
Adapted with permission from [14].

Box 1. Harvey–Bradshaw Index (1980).

General well-being
0 = Very well
1 = Slightly below par
2 = Poor
3 = Very poor
4= Terrible
Abdominal pain
0 = None
1 = Mild
2 = Moderate
3 = Severe
Number of liquid stools per day
Abdominal mass
0 = None
1 = Dubious
2 = Definite
3 = Definite and tender
Complications (score 1 per item)
- Arthralgia
- Uveitis
- Erythema nodosum
- Aphthous ulcers
- Pyoderma gangrenosum
- Anal fissure
- New fistula
- Abscess

Adapted with permission from [17,19].

physicians' assessments of change in patient status. The CDAI has become the most commonly used clinical severity-scoring index, especially in therapeutic research protocols. However, the need to keep a 7-day patient diary has been a major criticism of the CDAI. It is also highly dependent on subjective variables (e.g., abdominal pain and general well-being) and the score can potentially become skewed secondary to the use of medications, such as antidiarrheals, that influence the number of bowel movements. Furthermore, CD patients who have undergone a colectomy may have a higher defecation rate and produce looser stools in the absence of active disease, which again can affect the score.

Harvey–Bradshaw Index

In order to overcome the drawbacks associated with the subjective aspect of the CDAI, a number of other different scoring systems have been devised. One such system is the Harvey–Bradshaw Index, also known as the Simple Index or the Modified CDAI (Box 1). This system uses a 24-h diary entry and excludes body weight, hematocrit and the use of antidiarrheals or narcotics. It reduces the original eight variables in the CDAI down to five and the values are simply added together rather than summing the products of weighted values. The Harvey–Bradshaw Index showed a 93% correlation with the CDAI [17]. Its easier practical application and amenability to immediate calculation at a clinic visit makes this scoring system advantageous. However, using only a single day's symptom calculation raises the possibility of less reliability compared with the CDAI's 7-day patient diary and some studies have shown less consistent intraclass correlation [18].

van Hees Index

Shortly after the publication of the CDAI, van Hees *et al.* published their retrospective study of 63 patients who underwent 83 clinical examinations [19]. An initial list of 18 independent variables (known as the 'measurement set') was used in the development of this activity index. The measurement set was analyzed by three gastroenterologists from outside institutions to assess for correlation between evaluation scores and disease activity. The measurement set was then further reduced to nine variables by using a multiple regression model, which achieved a correlation coefficient of 95% with the corresponding physician's rating. The final variables selected in the van Hees Index were: sex, serum albumin, erythrocyte sedimentation rate and clinical features, which included the presence of an abdominal mass, temperature, stool consistency, bowel resection for CD and extraintestinal manifestations (e.g., arthritis, stomatitis, erythema nodosum, episcleritis, iritis and iridocyclitis). Scores were determined by inserting values into the multiple regression equation (Table 4). A van Hees Index score of less than 100 correlated with indolent disease, less than 150 indicated mild disease, less than 210 implied moderate disease and a score greater than 210 indicated severe disease activity. Although this scoring system is a great deal more objective than the CDAI, relying less on subjective

Table 4. van Hees Index (1980)†.

Variable (Xi)	Regression coefficient (b_i)
Albumin (g/l)	-5.48
Erythrocyte sedimentation (mm after 1 h)	0.29
Quetelet Index: weight (kg)/H²(m)	-0.22
Abdominal mass: 1 = No 2 = Dubious 3 = Diameter <6 cm 4 = Diameter 6–12 cm 5 = Diameter >12 cm	7.83
Sex: 1 = Male 2 = Female	-12.3
Temperature (°C)	16.4
Stool consistency 1 = Well-formed 2 = Soft, variable 3 = Watery	8.46
Resection: 1 = No 2 = Yes	-9.17

†Activity Index = b0 + ∑i bixi
b0: Constant (-209); bi: Regression coefficient; xi: Variable.
Adapted with permission from [17,19].

Table 5. Truelove–Witts Severity Index for ulcerative colitis.

Parameters	Mild	Severe
Bowel movements	≤4	≥6
Fever	Absent	>99.5°F (37.5°C) × 2 days
Heart rate (beats/min)	<100	>100
Hemoglobin	Normal	≤75% of normal for sex
Blood in stools	Streaks	Grossly bloody
Erythrocyte sedimentation (mm)	<30	>30

Adapted with permission from [20].

clinical criteria, determining the final score using the somewhat complicated equation is more cumbersome. van Hees reported a marginal correlation of only 0.57 with the CDAI making this scoring system less popular overall.

Ulcerative colitis classification systems

Truelove–Witts Severity Index

In 1955, Truelove and Witts published their landmark paper 'Cortisone in ulcerative colitis: final report on a therapeutic trial' in the *British Medical Journal* [20]. In order to study the effects of cortisone versus placebo in their randomized controlled trial, the authors devised a method to segregate patients according to their disease severity. Six parameters were studied that included: number of bowel movements in 24 h, blood in stool, core body temperature, heart rate, hemoglobin and erythrocyte sedimentation rate.

A total of 210 UC patients were studied, out of which 109 were treated with 100 mg of cortisone for 6 weeks and 101 patients received placebo. For every stage of disease severity, patients treated with cortisone did much better compared with the corresponding control patients.

The Truelove–Witts Severity Index was the first attempt to classify disease severity of UC patients into mild, moderate and severe disease (Table 5). This scoring system is popular in the clinical arena owing to its objectivity and ease of use. It has stood the test of time amongst other similar scoring systems that incorporate added clinical and biochemical parameters in their criteria. It continues to be used today in assessing response to therapy in the acutely flaring patient, because of its simplicity and clinical relevancy.

Powell-Tuck Index (St Mark's Index)

In 1964, Baron *et al.* had observed certain sigmoidoscopic features of colitic mucosa that were relatively characteristic with minimal interobserver variation [21]. In 1982, Powell-Tuck took these endoscopic findings and correlated them with other clinical and laboratory parameters [22]. The appearance of the mucosa was: grade 0 or nonhemorrhagic when there was no spontaneous bleeding upon light touch; grade 1 or hemorrhagic when bleeding was seen on light touch but no spontaneous bleeding was seen ahead of the instrument; and grade 2 or hemorrhagic when spontaneous bleeding was seen ahead of the instrument upon initial inspection. Clinical factors in the Powell-Tuck Index included bowel frequency, bloody stools, sense of well-being, abdominal pain, stool consistency, nausea, weight loss, extraintestinal signs and fever. Powell-Tuck noted a significant correlation between the severity of diseased mucosa on endoscopic evaluation and the overall clinical picture of disease severity as defined by the studied clinical criteria; for example, patients whose mucosa only bled upon touch experienced less rectal bleeding and bowel frequency than those with a spontaneously hemorrhagic mucosa. In spite of this endoscopic correlation with clinical picture, this scoring system has not been broadly accepted owing to its ambitious use of numerous clinical variables, which does not seem to add much to the more simpler indexes.

Mayo Clinical Score/Disease Activity Index

In 1987, Schroeder *et al.* published their double-blind, placebo-controlled study of an oral 5-aminosalicylic acid preparation using a pH-sensitive polymer coating in 87 patients with mild-to-moderately active UC [23]. In their study, disease severity was judged by both clinical and endoscopic criteria that included: the frequency of bowel movements in 24 h, presence of visible rectal bleeding, flexible sigmoidsocopic findings and the physician's global assessment of disease status (Box 2). Each category

Box 2. Mayo Score/Ulcerative Colitis Disease Activity Index.

Stool frequency
0 = Normal
1 = 1–2 stools/day more than normal
2 = 3–4 stools/day more than normal
3 = 4 stools/day more than normal

Rectal bleeding
0 = None
1 = Visible blood with stool less than half the time
2 = Visible blood with stool half of the time or more
3 = Passing blood alone

Mucosal appearance at endoscopy
0 = Normal or inactive disease
1 = Mild disease (erythema, decreased vascular pattern, mild friability)
2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions)
3 = Severe disease (spontaneous bleeding, ulceration)

Physician rating of disease activity
0 = Normal
1 = Mild
2 = Moderate
3 = Severe

Adapted with permission from [23].

Box 3. Montreal classification of ulcerative colitis.

Extent

- E1: ulcerative proctitis (involvement limited to the rectum)
- E2: left-sided ulcerative colitis (UC; involvement limited to colorectum distal to the splenic flexure)
- E3: extensive UC (involvement extends proximal to the splenic flexure)

Severity

- S0: clinical remission (asymptomatic)
- S1: mild UC
  - Passage of four or fewer stools/day with or without blood, absence of any systemic illness and normal erythrocyte sedimentation
- S2: moderate UC
  - Passage of more than four stools per day but with minimal signs of systemic toxicity
- S3: severe UC
  - Passage of at least six bloody stools daily
  - Pulse rate of at least 90 beats/min
  - Temperature of at least 37.5°C
  - Hemoglobin of less than 10.5 g/100 ml
  - Erythrocyte sedimentation of at least 30 mm/h

Adapted with permission from [12].

ranged from 0 to 3, with a maximum score of 12. A high score was associated with an increase in disease severity. Owing to its simplicity and the fact that this scoring system takes into account both clinical and endoscopic parameters, the Mayo Clinical Score has become one of the more popular scoring system used for assessing severity in UC.

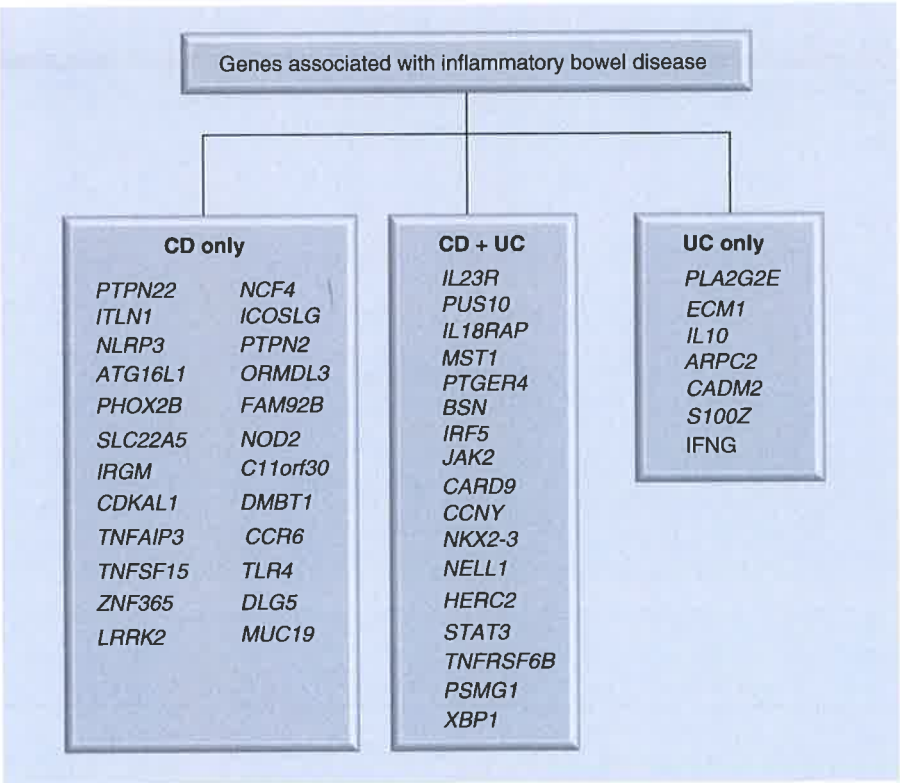

Figure 1. Genes associated with inflammatory bowel disease.
CD: Crohn's disease; UC: Ulcerative colitis.

Montreal classification for ulcerative colitis

The Montreal Working Party decided to not only make improvements to the initial Vienna CD classification, but to also introduce a classification system for UC [12]. The Working Party devised a classification system by first defining anatomical disease extent and then including disease severity characteristics (Box 3). The extent of disease categories are defined as proctitis (E1), left-sided colitis (E2) and pancolitis (E3). The severity of disease category definitions took into account clinical parameters very akin to the Truelove–Witts criteria, where S0 defined clinical remission, while S3 had at least six bloody bowel movements a day, tachycardia, fever, anemia and elevated erythrocyte sedimentation. Therefore, this system utilized criteria that were very clinically relevant from both an anatomic and symptomatic perspective and which were previously accepted features of disease severity found that are in other grading schema.

Towards a genetic classification of disease

In the past decade, much research has been carried out to aid in understanding the underlying pathophysiology of IBD. The widely accepted basis for the disease is that there is an abnormal immune response to intestinal microflora in a genetically susceptible patient. There has been an ever increasing list of gene loci, mutations or alleles (now approaching 100) found using genome-wide association studies that are

associated with either or both UC and CD (Figure 1) [24–27]. The wide variation in clinical phenotype of IBD patients, which historically dictated the development of the aforementioned classification schemas, in fact predicted this complexity of genetic variability. The field is only in the nascent stages of correlating these genetic findings with the clinical features of IBD, but, as this is carried out, a more accurate picture of the individual patient and his/her disease will be created. Mutations in the *NOD2/CARD15* gene, for example, correlate with disease in the terminal ileal region, usually at a younger age [28]. Similarly, other genetic determinants will be associated with clinical features or disease behavior. Increasingly, patients will be subclassified not just on anatomic location and clinical characteristics (as the Montreal classification presently does), but also on genotype. This will provide a third dimension to the classification of the IBD patient, namely one that relates to a pathophysiologic basis of the disease in a particular patient. Such a classification will provide more uniform patient recruitment into clinical trials and may 'rediscover' treatments that are very effective in certain subgroups of patients that may have been formerly discarded because of the wide genetic variability in the studied patient groups. These genetic data will complement the clinical classification of patients and will also have a direct impact on patient care paradigms.

Conclusion

The lack of a single effective sensitive test for IBD has historically posed a great challenge in classifying patients and assessing disease status in this complex condition. Disease classification and severity systems have, thus, been developed to not only classify a varied patient population, but also provide criteria by which to assess the success of therapy and help in predicting disease behavior (Table 6).

New evidence from genome-wide association studies have contributed great insight into the understanding of the patient's genetic predisposition to IBD. The aforementioned clinical classification

Table 6. Scoring systems summary.

Scoring system	Type	Advantages	Disadvantages	Best use
Crohn's disease				
Rome	C	Detailed classification: – Location – Behavior – Extent of disease – Operative history	Numerous patient groups	Identifying uniform patient groups for clinical trails
Vienna	C	Simpler than Rome: – Age – Location – Behavior	Not as detailed as Rome	Identifying uniform patient groups for clinical trails
Montreal	C	More detailed: – Age – Location and upper GI – Behavior and perianal involvement	Limited number of validation studies	Identifying uniform patient groups for clinical trails
Crohn's Disease Activity Index	S	Assesses disease activity over 1 week	Need to keep 7-day diary Subjective variables (pain, diarrhea)	To assess response to therapy
Harvey–Bradshaw Index	S	24-h diary Easier to calculate Objective variables	Variability due to calculation based on a single day	To assess response to therapy
van Hees Index	S	More objective than Crohn's Disease Activity Index	Cumbersome mathematical equation	To assess response to therapy
Ulcerative colitis				
Truelove–Witts Index	S	Objective variables, easy to use	No anatomic variables	Clinic setting/response to therapy
Powell-Tuck Index	S	Endoscopic, clinical and laboratory indices	Numerous clinical and endoscopic variables	Clinic setting/response to therapy
Mayo Score	S	Simplified endoscopic and clinical assessment	Does not assess extent of disease	Clinic setting/response to therapy
Montreal	C/S	Assesses extent of disease and clinical indices	Limited number of validation studies	Clinic setting/response to therapy

C: Classification system; S: Severity scoring system.

and scoring systems can help in the correlation of this new found genetic information with the clinical phenotype. Disease subtypes and behavior will be more objectively defined and therapeutic management can be more effectively tailored when it is based on both the clinical phenotype and underlying genotype.

Five-year view

In recent years, great technological advancements have led to exciting new insights into the pathogenesis of IBD. Data from genome-wide association studies are providing a better understanding of the molecular pathways involved in the inflammatory process called IBD. In earlier years, IBD was classified by clinical criteria that related to disease extent and severity of inflammation, as suggested by patient subjective complaints, physical exam findings and some basic laboratory testing. Now, the trend is towards subclassifying IBD patients by correlating the relevant clinical features that were defined in earlier classification schema with contemporay genetic discoveries that relate to disease pathogenesis. The landmark discovery of mutations in *NOD2/CARD15* and its association with the early onset of ileal disease is just one example of the way genetic factors are redefining our understanding of this complex disease. Categorizing patients as having either UC or CD may soon be replaced by many subgroups based on both phenotypic and genotypic determinants. With our better understanding of the underlying genetic and immunologic pathophysiology of IBD, it is hoped that patients will receive specific treatment strategies tailored for each individual based on specific clinical and genetic correlates.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- Currently, there is no single effective marker that can be used to grade disease severity or outcome in inflammatory bowel disease.
- Scoring systems help in subclassifying patient disease and provide a common basis for the scientific and clinical community to compare results.
- Clinically subclassifying patients provides a more uniform phenotype that can be used to more effectively assess therapeutic outcomes and disease progression.
- Clinical classification and scoring systems are the foundations upon which to integrate new genetic data.
- Clinical classification schema help to identify relatively uniform groups of patients that allow accurate correlation of genetic mutations relevant to disease pathogenesis.

References

Papers of special note have been highlighted as:
• of interest
•• of considerable interest

1 Podolsky DK. Inflammatory bowel disease. *N. Engl. J. Med.* 347, 417–429 (2002).

2 Tobin MV, Logan RF, Langman MJ, McConnell RB, Gilmore IT. Cigarette smoking and inflammatory bowel disease. *Gastroenterology* 93(2), 316–321 (1987).

3 Logan RF, Kay CR. Oral contraception, smoking and inflammatory bowel disease – findings in the Royal College of General Practitioners Oral Contraception Study. *Int. J. Epidemiol.* 18(1), 105–107 (1989).

•• **Landmark paper describing the pathologic and clinical details of Crohn's disease.**

4 Halfvarson J, Jess T, Magnuson A *et al.* Environmental factors in inflammatory bowel disease: a co-twin control study of a Swedish-Danish twin population. *Inflamm. Bowel Dis.* 12(10), 925–933 (2006).

5 Loftus EV Jr. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 126(6), 1504–1517 (2004).

6 Galluzzi L, Kepp O, Zitvogel L, Kroemer G. Bacterial invasion: linking autophagy and innate immunity. *Curr Biol.* 20(3), R106–R108 (2010).

7 Crohn BB, Ginzburg L, Oppenheimer GD. Landmark article Oct 15, 1932. Regional ileitis. A pathological and clinical entity. *JAMA* 251, 73–79 (1984).

8 Farmer RG, Hawk WA, Turnbull RB Jr. Clinical patterns in Crohn's disease: a statistical study of 615 cases. *Gastroenterology* 68, 627–635 (1975).

9 Greenstein AJ, Lachman P, Sachar DB *et al.* Perforating and non-perforating indications for repeated operations in Crohn's disease: evidence of two clinical forms. *Gut* 29, 588–592 (1988).

10 Steinhart AH, Girgrah N, McLeod RS. Reliability of a Crohn's disease clinical classification scheme based on disease behavior. *Inflamm. Bowel Dis.* 4, 228–234 (1998).

11 Gasche C, Scholmerich J, Brynskov J *et al.* A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm. Bowel Dis.* 6(1), 8–15 (2000).

•• **Development of the Vienna Classification system.**

12 Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 55(6), 749–753 (2006).

• **Summarizes the differences between the Vienna and Montreal classifications.**

13 Chow DK, Leong RW, Lai LH *et al.* Changes in Crohn's disease phenotype over time in the Chinese population: validation of the Montreal classification system. *Inflamm. Bowel Dis.* 14(4), 536–541 (2008).

14 Best WR, Becktel JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 70(3), 439–444 (1976).

• **Landmark paper about how the Crohn's Disease Activity Index was developed.**

15 Singleton JW. Clinical activity assessment in inflammatory bowel disease. *Dig. Dis. Sci.* 32(Suppl. 12), 42S–45S (1987).

16 Yoshida EM. The Crohn's Disease Activity Index, its derivatives and the Inflammatory Bowel Disease Questionnaire: a review of instruments to assess Crohn's disease. *Can. J. Gastroenterol.* 13(1), 65–73 (1999).

17 Harvey RF, Bradshaw JM. A simple index of Crohn's-disease activity. *Lancet* 1(8167), 514 (1980).

- **Landmark paper of the development of Harvey–Bradshaw Index.**

18 Best WR. Predicting the Crohn's disease activity index from the Harvey–Bradshaw Index. *Inflamm. Bowel Dis.* 12(4), 304–310 (2006).

19 van Hees PA, van Elteren PH, van Lier HJ, van Tongeren JH. An index of inflammatory activity in patients with Crohn's disease. *Gut* 21(4), 279–286 (1980).

20 Truelove SC, Witts LF. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br. Med. J.* 2(4947), 1041–1048 (1955).

- **Landmark study that led to the development of Truelove–Witts Severity Index.**

21 Baron JH, Connell AM, Lennard-Jones JE. Variation between observers in describing mucosal appearances in proctocolitis. *BMJ* 1(5375), 89–92 (1964).

22 Powell-Tuck J, Day DW, Buckell NA, Wadsworth J, Lennard-Jones JE. Correlations between defined sigmoidoscopic appearances and other measures of disease activity in ulcerative colitis. *Dig. Dis. Sci.* 27(6), 533–537 (1982).

23 Schroeder KW, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N. Engl. J. Med.* 317(26), 1625–1629 (1987).

24 Lees CW, Satsangi J. Genetics of inflammatory bowel disease: implications for disease pathogenesis and natural history. *Expert Rev. Gastroenterol. Hepatol.* 3(5), 513–534 (2009).

25 Abraham C, Cho JH. Inflammatory bowel disease. *N. Engl. J. Med.* 361(21), 2066–2078 (2009).

- **Comprehensive review of the current genes associated in inflammatory bowel disease.**

26 Barrett JC, Hansoul S, Nicolae DL *et al.* Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat. Genet.* 40(8), 955–962 (2008).

27 McGovern DP, Gardet A, Törkvist L *et al.* Genome-wide association identifies multiple ulcerative colitis susceptibility loci. *Nat. Genet.* 42(4), 332–337 (2010).

28 Hampe J, Grebe J, Nikolaus S *et al.* Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study. *Lancet* 359(9318), 1661–1665 (2002).

- **Landmark study that linked the *NOD2/CARD15* gene to Crohn's disease.**

The *NOD2*insC polymorphism is associated with worse outcome following ileal pouch-anal anastomosis for ulcerative colitis

Andrea D Tyler, Raquel Milgrom, Joanne M Stempak, et al.

Gut 2013 62: 1433-1439 originally published online August 9, 2012
doi: 10.1136/gutjnl-2011-301957

ORIGINAL ARTICLE

The *NOD2*insC polymorphism is associated with worse outcome following ileal pouch-anal anastomosis for ulcerative colitis

Andrea D Tyler,[1,2] Raquel Milgrom,[2] Joanne M Stempak,[2] Wei Xu,[3] John Hunter Brumell,[1,4,5] Aleixo M Muise,[1,4,6] Rishabh Sehgal,[7] Zane Cohen,[1,2] Walter Koltun,[7,8] Bo Shen,[9] Mark S Silverberg[1,2]

▶ Additional supplementary files are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2011-301957).

For numbered affiliations see end of article.

Correspondence to
Dr Mark S Silverberg, Mount Sinai Hospital, 600 University Ave, Room 441, Toronto, ON M5G 1X5, Canada; msilverberg@mtsinai.on.ca

Revised 21 May 2012
Accepted 22 June 2012
Published Online First 8 September 2012

ABSTRACT

Background Inflammatory complications after ileal pouch-anal anastomosis (IPAA) for ulcerative colitis (UC) are common.

Objective To investigate whether genetic factors are associated with adverse pouch outcomes such as chronic pouchitis (CP) and a Crohn's disease-like (CDL) phenotype.

Design 866 patients were recruited from three centres in North America: Mount Sinai Hospital (Toronto, Ontario, Canada), the Cleveland Clinic (Cleveland, Ohio, USA) and Penn State Milton S Hershey Medical Center (Hershey, Pennsylvania, USA). DNA and clinical and demographic information were collected. Subjects were classified into post-surgical outcome groups: no chronic pouchitis (NCP), CP and CDL phenotype.

Results Clinical and genetic data were available on 714 individuals. 487 (68.2%) were classified as NCP, 118 (16.5%) CP and 109 (15.3%) CDL. The presence of arthritis or arthropathy (p=0.02), primary sclerosing cholangitis (p=0.009) and duration of time from ileostomy closure to recruitment (p=0.001) were significantly associated with outcome. The *NOD2*insC (rs2066847) risk variant was the single nucleotide polymorphism (SNP) most significantly associated with pouch outcome ($p=7.4\times10^{-5}$). Specifically, it was associated with both CP and CDL in comparison with NCP (OR=3.2 and 4.3, respectively). Additionally, SNPs in *NOX3* (rs6557421, rs12661812), *DAGLB* (rs836518) and *NCF4* (rs8137602) were shown to be associated with pouch outcome with slightly weaker effects. A multivariable risk model combining previously identified clinical (smoking status, family history of inflammatory bowel disease), serological (anti-*Saccharomyces cerevisiae* antibody IgG, perinuclear antineutrophil cytoplasmic antibody and anti-CBir1) and genetic markers was constructed and resulted in an OR of 2.72 ($p=8.89\times10^{-7}$) for NCP versus CP/CDL and 3.22 ($p=4.11\times10^{-8}$) for NCP versus CDL, respectively.

Conclusion Genetic polymorphisms, in particular, the *NOD2*insC risk allele, are associated with chronic inflammatory pouch outcomes among patients with UC and IPAA.

Significance of this study

What is already known on this subject?

- Several genetic factors have previously been associated with inflammatory pouch outcomes in small cohorts.
- Patients with familial adenomatous polyposis and a pelvic pouch do not typically develop inflammatory pouch complications, suggesting that genetic factors are important in pouchitis and a Crohn's disease-like phenotype among individuals with ulcerative colitis.

What are the new findings?

- The *NOD2*insC polymorphism, which is important in innate immune recognition of microbial muramyl dipeptide, is associated with inflammatory pouch outcomes.
- Additional genes which are mediators in the generation of reactive oxygen species may also have a role in pouch inflammation.

How might it impact on clinical practice in the foreseeable future?

- These polymorphisms may be useful for predicting which patients are at an increased risk of developing pouch inflammatory complications after surgery for ulcerative colitis.
- A combination of clinical, serological and genetic factors may be a useful clinical tool for assisting in decision making for patients considering a colectomy and pelvic pouch for ulcerative colitis.

Linked

▶ http://dx.doi.org/10.1136/gutjnl-2012-303188

To cite: Tyler AD, Milgrom R, Stempak JM, *et al*. *Gut* 2013;**62**: 1433–1439.

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disorder of unknown aetiology. About 20% of affected individuals will require colectomy at some point in their disease.[1–4] Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the preferred surgical procedure in fulminant or chronic, treatment-refractory UC and UC-associated colonic neoplasia.[5 6] However, de novo inflammation of the ileal reservoir (pouchitis) is a common post-surgical complication with a prevalence ranging from 12% to >50%.[5 7–9] An additional subset of patients (up to 17%) will develop a Crohn's disease-like (CDL) phenotype characterised by abdominal or perianal fistulae and/or inflammation of the small bowel proximal to the pouch (afferent limb).[10]

The causes of ileal inflammation in the pouch in patients with a preoperative diagnosis of UC are unknown. Clearly, evolution of the microbiome of the pouch after closure of a diverting ileostomy plays a role, as patients rarely develop pouchitis before restoration of continuity of the fecal stream. The decreased risk of inflammation among those with familial adenomatous polyposis and reports by some groups that a family history of CD may increase the risk for the CDL phenotype in the pouch, suggests that heritable susceptibility may be a critical factor in disease pathogenesis.[11 12] However, previous genetic studies have been small in scale and have not yielded consistently reproducible results.[13 14] Among others, variants in *IL1RN*[15 16] and *TLR1*[17] have been associated with pouch inflammation. Additional studies have tentatively linked *NOD2* variants with chronic pouchitis.[18–20] The aim of this study was to evaluate whether inflammatory bowel disease (IBD) susceptibility polymorphisms which have been previously implicated in CD, UC or pouchitis are associated with chronic pouch inflammatory outcomes after surgery for UC in a large IPAA cohort.

METHODS

Study population

All study protocols were carried out in accordance with the research ethics boards at each centre and informed consent was obtained from all patients before their enrolment. Patients who had undergone colectomy with IPAA at Mount Sinai Hospital (MSH), Cleveland Clinic and the Penn State Milton S Hershey Medical Center (HMC) were contacted by research staff between 2007 and 2010. The demographic and clinical features of subjects from MSH have been previously reported and described in detail,[21] and the *NOD2* genotype status has been previously reported for the HMC cohort.[20] Any patients with a confirmed precolectomy diagnosis of UC and who had had their ileostomy closed a minimum of 1 year before study enrolment were included in the study. UC diagnosis was confirmed based on clinical, endoscopic and pathological evidence—particularly from the colectomy specimen. Those who had undergone their procedure outside any of these institutions were included only if the medical documentation was available. Patients with CD, IBD of the colon-type unclassified or indeterminate colitis based on precolectomy medical chart review or colectomy surgical pathology were excluded.

Clinical data collection

Study data were obtained through a detailed retrospective chart review and patient questionnaire with investigators blinded to patients' genetic results. To ensure uniformity between sites, rigorous definitions were agreed upon and applied to classify patients into outcome groups. Data collected directly by patient interview included gender, age, family history of IBD, smoking status and clinical symptoms of pouch function after surgery (daily number of bowel movements, incontinence, presence or absence of blood in stool). UC diagnosis date, surgical history and dates, presence of any extraintestinal manifestations of IBD such as arthritis or arthropathy, osteoporosis/osteopenia, erythema nodosum, pyoderma gangrenosum, primary sclerosing cholangitis (PSC) or ocular inflammation, as well as the post-surgical outcome were confirmed through medical chart review. The extent of precolectomy disease was classified based on the Montreal classification.[22] The presence of backwash ileitis (inflammation of the terminal ileum in individuals with pancolitis) precolectomy was documented and patients were included only if all other findings were consistent with UC.

Post-surgical classification

Patients were classified into one of three outcome groups based on clinical, endoscopic and histological factors. The 'no chronic pouchitis' (NCP) group included individuals without an episode of pouchitis, and those who had had fewer than four clinical acute pouchitis episodes per year, each responding to 2 weeks or less of antibiotics (ciprofloxacin, metronidazole, or combination). This clinical definition of acute pouchitis has been previously described.[23] Typically, these subjects may not have had a full clinical assessment but clearly represent a distinct category as demonstrated by their immediate response to antibiotics. The 'chronic pouchitis' (CP) group included antibiotic-dependent and antibiotic-refractory patients who required prolonged (>1 month) antibiotic treatment, or medical intervention for pouchitis more than three times a year, or the use of second- or third-line drugs (5-acetylsalicylic acid, steroids, immunomodulators, biological agents). All such subjects had endoscopic assessment at some point during their disease.[23] The final group was the CDL phenotype. Individuals classified into this group met at least one of the following criteria: (a) development of a perianal fistula more than 1 year after ileostomy closure documented through physical examination, examination under anaesthesia or imaging; (b) development of a stricture proximal to the pouch which was not related to a surgical complication and was confirmed by endoscopy or small-bowel imaging; (c) evidence of inflammation (ulceration, erythema, friability) extending above the pouch inlet and into the afferent limb/pre-pouch ileum or more proximal small intestine detected on pouchoscopy or upper endoscopy. Anastomotic ulceration or ulceration around the pouch inlet alone was not sufficient to classify patients into the CDL outcome group. Additional post-surgical outcomes, including mechanical or surgical complications or cuffitis, were also documented.[23] Time to diagnosis of pouchitis was defined as the time from ileostomy closure to the time of onset of symptoms and diagnosis.

DNA collection and genotyping

Whole blood (3–6 ml) was obtained by venepuncture using standard EDTA collection tubes at both MSH and HMC. DNA was extracted using the QIAGEN Gentra Puregene blood kit (Qiagen, CA, USA). DNA samples from the Cleveland Clinic were extracted from clotted blood using the Maxwell 16 Tissue DNA purification kit (Promega, WI, USA), according to manufacturer's protocol. DNA from all study sites was then stored in sealed Matrix screw top tubes at 4°C before a spectrophotometric quality and quantity check using the NanoDrop 1000 (Thermo Scientific, IL, USA). Most DNA samples had concentrations ranging from 100 to 300 ng/μl with all concentrations >20 ng/μl. Those falling below 18 ng/μl were whole-genome amplified using the Illustra Genomiphi HY DNA Amplification Kit (GE Healthcare, NJ, USA). Single nucleotide polymorphism (SNP) genotyping was performed using the Illumina Goldengate custom SNP assay on the Illumina BeadStation500G (San Diego, California, USA) at The Center for Applied Genomics (TCAG, Toronto, Canada) and the Sequenom iPLEX platform (Génome Québec, Montreal, Canada). *NOD2*insC genotyping was performed using the TaqMan SNP genotyping platform (TCAG).

Statistical analysis and quality control

Descriptive statistics were reported as mean and range for continuous variables and frequencies and proportions for categorical variables. For phenotypic results, Fisher's exact test and Pearson's χ^2 test were used to compare proportions; non-

parametric tests were used to compare the continuous variables. PLINK version 1.06[24] was used to obtain descriptive statistics of the SNPs such as the allele frequency, genotype distribution and to test for Hardy–Weinberg equilibrium (HWE) for each marker based on Pearson's χ^2 test. All SNPs which were successfully genotyped in at least 95% of the study cohort, satisfied HWE criteria (p HWE >0.001) and had a minor allele frequency >0.01, were included in the analysis. To ensure that results were not due to population stratification, only Caucasian individuals were included in the genetic analysis. Logistic regression models were applied for the association analysis. Although an additive genetic model was used for the primary analysis,[25] we also explored dominant and recessive genetic models. Throughout this report the p values are those obtained from the additive genetic model unless otherwise stated. ORs and 95% CIs were calculated. Two-sided statistical tests were applied and all analyses were performed with SAS V.9.2 (SAS Institute).

A list of 646 SNPs was generated and chosen for the analysis in the initial cohort based on their previous known associations with CD, UC or pouchitis. To accommodate the Sequenom platform, the top 46 hits from the initial analysis as well as 20 previously described important CD/UC/pouchitis-associated SNPs were selected for genotyping in the replication cohort (online supplementary table 1). Owing to the large number of tests performed, nominal significance was defined in both the preliminary analysis and the combined cohort as $p<0.01$. Stringent Bonferroni correction was applied to the final p value to adjust for multiple comparisons with $p<7.5\times10^{-4}$ required to declare significance.

Finally, multivariate analysis was performed with factors previously shown by our group and others to be associated with pouch outcome. These included the serological markers anti-*Saccharomyces cerevisiae* antibody (ASCA) IgG, perinuclear antineutrophil cytoplasmic antibody (pANCA) and anti-CBir1[21]; clinical factors included smoking and family history of IBD; and the SNPs which were significantly associated with outcome in our Caucasian cohort. This analysis was performed in a subset of 341 patients in whom all of the preceding data was available. A stepwise procedure was performed to generate a risk score based on the significant risk factors from the multivariate analysis. The risk score was based on the weighted combination of the risk factors with the standardised logistic regression coefficient as the weight.[26] Receiver operating characteristic curves were generated to calculate the area under the curve and the sensitivity and specificity of the risk score.[26]

RESULTS

Study cohort

The initial cohort consisted of 399 patients recruited from MSH and the second included 467 patients recruited from MSH, Cleveland Clinic and HMC, for a total cohort of 866. Within both the first and second cohorts, 339 and 401, respectively had both genotype and phenotype information available for a total genetic cohort of 740. Among these individuals 714 (96.5%) were Caucasian and only these subjects were subsequently analysed.

Clinical variables associated with pouch outcome

Clinical and phenotypic characteristics of the study population are shown in table 1. A description of the pouch outcome groups among the combined Caucasian cohort is shown in figure 1. Of the 714 individuals, 487 (68.2%) were classified as NCP, 118 (16.5%) CP and 109 (15.3%) CDL. The mean age at UC diagnosis (29, range 2–59 years) and at IPAA (37, range 7–61 years) was the same between groups. Within the CDL group, 56.6% were diagnosed based on the presence of a fistula or abscess developing more than 1 year after surgery, 36.4% based on inflammation extending into the afferent limb and 7.0% met both criteria. Factors previously related to pouch outcome such as smoking and family history of IBD[10 12] were not found to be significantly associated in this cohort. However, the presence of large-joint arthritis (p=0.02) and PSC (p=0.009) were significantly associated with both the CP and CDL outcomes. A longer duration of time from ileostomy closure to study enrolment was associated with a worse pouch outcome (p=0.001). The mean time to diagnosis of CP and CDL was 2.4 and 4.4 years, respectively, well below the mean post-surgical follow-up time for any of the outcome groups.

Association of SNPs with pouch outcome

Of the 646 SNPs selected for analysis in the initial cohort of subjects (n=369), 66 SNPs which met initial criteria for significance or were SNPs of importance in prior IBD genetic studies were genotyped in the additional cohort (n=345). Of these, 12 had a low call rate and were not included in the analysis. Of the remaining 54 SNPs, five were significantly associated with outcome at a p value threshold of $p<0.01$ in a three-way comparison. However, only the *NOD2* insertion variant (*NOD2*insC, rs2066847) remained significantly associated with pouch outcome after stringent Bonferroni correction ($p=7.4\times10^{-5}$; $p_{corr}=4.9\times10^{-3}$) (table 2). Specifically, the *NOD2*insC variant was detected significantly more frequently

Table 1 Clinical and phenotypic characteristics of the study population

Feature	No chronic pouchitis (n=487)	Chronic pouchitis (n=118)	Crohn's disease-like (n=109)	p Value
Gender (% female)	47.0	43.2	50.5	0.55
Mean age at UC diagnosis (years, range)	30 (7–59)	30 (11–55)	28 (2–55)	0.85
Mean age at surgery (years, range)	37 (7–61)	37 (12–59)	36 (8–56)	0.84
Ashkenazi Jewish (% Jewish)	6.0	10.2	8.3	0.23
Smoking (n=704)				
Never	59.5	58.5	54.1	0.72
Ex-smoker	33.3	34.7	33.9	
Current	5.7	6.8	9.2	
Family history of IBD (%)	9.8	4.2	11.0	0.16
Duration of pouch (years), mean (SD) (n=665)	8.6 (6.4)	9.1 (6.3)	11.0 (6.3)	0.001
Arthritis or arthropathy (%) (n=447)	10.6	19.4	22.2	0.02
Primary sclerosing cholangitis (%) (n=617)	1.4	6.7	4.2	0.009

IBD, inflammatory bowel disease; UC, ulcerative colitis.

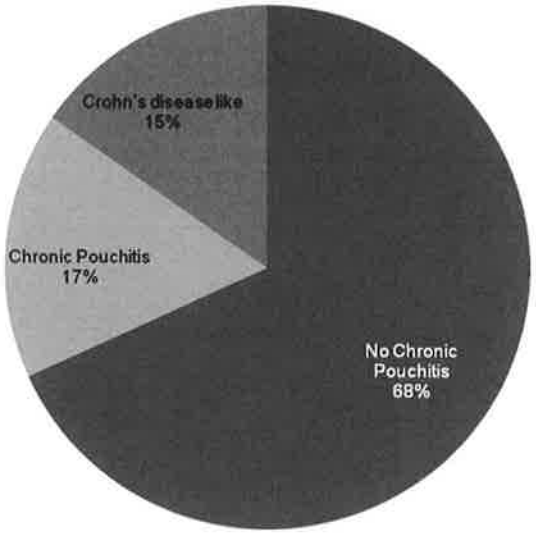

Figure 1 Proportion of individuals in each specific outcome group.

among individuals with CP and CDL than among those with NCP, with an OR of 3.21 (CI=1.38 to 7.47) and 4.30 (CI=1.90 to 9.77), respectively (table 3). This association remained significant even when the previously reported samples obtained from HMC were excluded.

Given the low minor allele frequency of the *NOD2*insC allele in our cohort, to fully assess the risk associated with variants in *NOD2* and pouch-related inflammatory complications, we examined the risk associated with compound heterozygosity or homozygosity for combinations of markers (rs2066847, rs2066845 and rs2066844). We found that increased numbers of variants at this locus were associated with increased risk of CDL in comparison with NCP, (p=0.002; OR=2.08; CI=1.31 to 3.50); however, no increased risk was seen when NCP was compared with CP.

Additional SNPs associated with outcome in the three-way analysis (p<0.01) were variants in *NOX3* (rs6557421) and *DAGLB* (rs836518); however, these did not remain significant after correction for multiple testing (table 2). In the pairwise analysis, rs6557421 showed a trend towards being protective for both of the inflammatory outcomes, and rs836518 was protective against only the CDL phenotype (table 3). Additional variants in *NOX3* (rs12661812) and *NCF4* (rs8137602) were found to be associated with outcome (p<0.01) using the dominant genetic model, with variants at both loci more common in individuals with inflammatory pouch phenotypes. However, these polymorphisms did not remain significantly associated with outcome after correction for multiple testing.

We next conducted analyses in which we compared the NCP group with the combined CP/CDL cohort (n_{comb}=227). We found that both rs2066847 (*NOD2*insC) and rs6557421 (*NOX3*) were associated with outcome with the minor allele of rs2066847 ($p=3.3\times10^{-4}$) increasing disease risk and that of rs6557421 ($p=2.8\times10^{-4}$) protective against inflammatory phenotypes (table 3).

Variants which had been previously associated with pouch complications, including those in *TLR1*,[17] *CD14*, *TLR9*[14] and other *NOD2* variants, were not found to be associated with outcome among our cohort (table 4). *IL1RN*[16] showed a trend towards significance in our initial analysis, but did not remain significant after correction for multiple testing. Additionally, many of the other well known IBD-associated genetic polymorphisms including those specific to CD (ie, *ATG16L1*) and those associated with both CD and UC (ie, *IL23R*), were not associated with any of the outcome groups.

Multivariate analysis and risk score

In the preliminary multivariate analysis, five factors were found to be independently associated with outcome and were included in the subsequent multivariate analysis, including rs2066847 (*NOD2*insC), rs6557421 (*NOX3*), rs836518 (*DAGLB*), anti-CBir1 and smoking (table 5). These factors were used to generate a weighted risk score, with the comparison of both NCP with CDL ($p=4.11\times10^{-8}$; OR=3.22, CI=2.12 to 4.89) and NCP with combined CP and CDL ($p=8.89\times10^{-7}$; OR=2.72; CI=1.89 to 3.91) resulting in highly significant associations. The sensitivity and specificity were assessed by generating receiver operating characteristic curves. The best model was that comparing CDL with NCP and containing all three genetic markers, as well as the serological and clinical factors (sensitivity = 80.0%; specificity = 70.3%). This model performed significantly better than those generated using smoking alone, or smoking and anti-CBir1 (figure 2).

DISCUSSION

Our results demonstrate that phenotypic characteristics, including PSC and arthritis, as well as several SNPs are associated with pouch outcome after IPAA in individuals with UC. PSC has previously been associated with chronic pouchitis,[27] as have general autoimmune disorders including arthritis.[28] The co-occurrence of additional inflammatory disorders previously documented in CD and UC, such as those described above, with pouch complications suggest overlapping disease mechanisms may be important in the onset and propagation of inflammatory outcomes. Interestingly, we did not observe any association between a family history of IBD or smoking and pouch outcome.[11 21] The sample size of this

Table 2 Single nucleotide polymorphisms (SNPs) associated with outcome at a nominal significance threshold of p<0.01

		Cohort 1 (n=369)	Cohort 2 (n=345)	Combined cohort (n=714)				
				MAF				
Gene	SNP	p Value	p Value	NCP	CP	CDL	p Value	p Value corr
*NOD2*insC	rs2066847	0.09	5.0×10^{-3}	0.01	0.05	0.06	7.4×10^{-5}	4.9×10^{-3}
NOX3	rs6557421	0.03	0.04	0.26	0.16	0.17	8.2×10^{-4}	0.05
*NOX3**	rs12661812	>0.1	0.05	0.08	0.11	0.16	7.5×10^{-3}	0.50
DAGLB	rs836518	0.02	3.3×10^{-3}	0.22	0.21	0.22	3.4×10^{-3}	0.22
*NCF4**	rs8137602	9.3×10^{-4}	>0.1	0.09	0.12	0.10	7.2×10^{-4}	0.05

Bonferroni corrected p values are also listed. The additive genetic model was applied except where indicated by * (dominant genetic model).
CDL, Crohn's disease-like; CP, chronic pouchitis; MAF, minor allele frequency; NCP, no chronic pouchitis.

Table 3 ORs and CIs for the five single nucleotide polymorphisms (SNPs) which are associated with outcome

		CP vs NCP		CDL vs NCP		CDL vs CP		CP/CDL vs NCP	
Gene	SNP	OR	CI	OR	CI	OR	CI	OR	CI
*NOD2*insC	rs2066847	3.21	1.38 to 7.47	4.30	1.90 to 9.77	1.28	0.57 to 2.88	3.67	1.81 to 7.45
NOX3	rs6557421	0.55	0.36 to 0.82	0.56	0.37 to 0.87	1.05	0.62 to 1.79	0.56	0.40 to 0.76
*NOX3**	rs12661812	1.25	0.71 to 2.19	1.09	0.59 to 2.00	0.87	0.41 to 1.83	1.17	0.74 to 1.84
DAGLB	rs836518	0.93	0.63 to 1.38	0.45	0.27 to 0.74	0.49	0.27 to 0.88	0.69	0.50 to 0.96
*NCF4**	rs8137602	1.01	0.58 to 1.74	1.10	0.63 to 1.93	1.09	0.54 to 2.21	1.05	0.69 to 1.61

*Dominant genetic model.
CDL, Crohn's disease-like; CP, chronic pouchitis; NCP, no chronic pouchitis.

study, which was approximately double that of previous studies, provided us with increased power to detect true associations. Our data describing no correlation between IBD family history or smoking and outcome suggest that these previously described associations may have been the result of a type I error.

We have shown that several genetic polymorphisms are associated with pouch inflammation, confirming that host genetic factors are critical in the aetiology of both pouchitis and the CDL phenotype. The well established *NOD2*insC polymorphism, which has previously been implicated in ileal CD[29 30] and which results in a truncation of the leucine-rich repeat region of the NOD2 protein,[19] is associated with CP and a CDL outcome in our large cohort. While the precise effect of this polymorphism is unknown, gene knockout studies have demonstrated that loss of *NOD2* is associated with reduced ability to detect microbial pathogens.[31 32] Further, *NOD2* variants have been implicated in intestinal allograft rejection and graft-versus-host disease after allogeneic stem cell transplantation.[33 34] Our findings demonstrating the association between *NOD2* and pouch complications, confirm results seen in several smaller cohorts,[20 35] and suggest that studies failing to detect significant associations were likely underpowered.[17] This growing body of evidence demonstrates that pathways which are important in ileal CD may also be critical in inflammation of the ileal pouch after surgery.

It is interesting to note that the *NOD2*insC variant is typically only rarely found in UC. In the MSH UC patient population, for example, the *NOD2*insC allele frequency is 0.014 which is comparable to the reported allele frequency in this study for those without inflammatory pouch complications.[36] However, in the 227 subjects with CP or CDL, the *NOD2*insC allele frequency is >0.05. These patients were all carefully chart reviewed to exclude any clinical or pathological evidence of CD or IBD of the colon-type unclassified before colectomy. Additionally, the colectomy pathology was all carefully reviewed and no features of CD were seen in these subjects. These data therefore suggest that the utility of traditional clinical and histological diagnostic classification and phenotyping is not sufficient to categorise subjects for prognosis after pelvic pouch surgery. Thus, a diagnosis of clinical UC may not imply that pouch complications are less likely to arise but rather that the subject's genotype may be a more important determinant of outcome.

Other variants which demonstrated modest association with outcome include those which are located in the non-coding regions of *NOX3* and *DAGLB* or adjacent to *NCF4*. *NCF4*, encoding p40phox which is a component of the NADPH oxidase complex, was recently identified in a genome-wide association study as an ileal CD susceptibility gene.[37] Mechanistic studies have demonstrated that patients with CD who are carriers of risk alleles at this locus have significantly reduced amounts of reactive oxygen species—important for host innate immunity and defence from microbial pathogens—generated from granulocyte-macrophage colony-stimulating factor-primed neutrophils compared with patients not carrying these mutations.[38] NOX3, another component of this complex in various tissue types, is also important in the production of reactive oxygen species.[39 40] Together, these findings suggest an important role for this pathway in the pathogenesis of pouch inflammatory outcomes and IBD, in general. The function of *DAGLB* and a potential role for it in IBD pathogenesis remains unclear; however, the SNP in this gene is in a region of high linkage disequilibrium with *RAC1*—a gene involved in host immune defence and which has been previously associated with UC.[41]

Other variants which have been previously associated with pouch outcome among smaller cohorts were not associated with outcome in our group. A possible explanation for the difference between our results and others is that this study has a much larger sample size, which would help to reduce type I error. Additionally, population stratification may account for different results as some previous studies did not control for ethnicity.[17 42] To limit these effects, only Caucasian individuals were analysed

Table 4 Single nucleotide polymorphisms (SNPs) previously associated with pouch outcome. *IL1RN* did not meet specified quality criteria for combined analysis (successfully genotyped in <95% of the cohort)

		MAF				
Gene	SNP	NCP	CP	CDL	p Value	p Value corr
TLR1[17]	rs4833103	0.47	0.43	0.51	4.0×10^{-2}	NS
CD14[14]	rs2569190	0.49	0.52	0.40	NS	NS
NOD2 (SNP8)[20]	rs2066844	0.04	0.05	0.06	NS	NS
NOD2 (SNP12)[20]	rs2066845	0.02	0.01	0.02	NS	NS
IL1RN[42]*	rs419598	0.25	0.20	0.35	3.0×10^{-3}	NS
TLR9[14]*	rs352140	0.46	0.47	0.51	NS	NS

*Replication genotyping did not pass quality control measures.
CDL, Crohn's disease-like; CP, chronic pouchitis; MAF, minor allele frequency; NCP, no chronic pouchitis.

Table 5 Factors significantly associated with outcome by multivariate analysis

	CP vs NCP		CDL vs NCP		CDL vs CP	
Factor	**OR (CI)**	**p Value**	**OR (CI)**	**p Value**	**OR (CI)**	**p Value**
*NOD2*insC* rs2066847	2.62 (0.39 to 17.64)	0.32	13.51 (2.00 to 90.9)	0.008	2.99 (0.45 to 20.0)	0.26
*NOX3** rs6557421	0.54 (0.31 to 0.94)	0.03	0.35 (0.15 to 0.78)	0.01	0.62 (0.24 to 1.59)	0.32
*DAGLB** rs836518	1.10 (0.69 to 1.74)	0.70	0.29 (0.12 to 0.67)	0.004	0.25 (0.10 to 0.66)	0.005
Anti-CBir1 (±)	2.30 (0.84 to 3.21)	0.15	6.38 (2.81 to 14.43)	<0.0001	3.48 (1.36 to 8.90)	0.009
Smoking						
Current smoking vs never smoked	2.30 (0.55 to 9.71)	0.38	11.96 (3.07 to 46.53)	0.0002	5.68 (1.10 to 29.25)	0.04
Ex-smoker vs never smoked	1.52 (0.83 to 2.77)	0.99	2.20 (0.95 to 5.10)	0.30	1.16 (0.44 to 3.05)	0.18

*For the genetic markers an additive genetic model to compare homozygote recessive versus heterozygote vs. homozygote dominant genotypes was applied.
CDL, Crohn's disease-like; CP, chronic pouchitis; NCP, no chronic pouchitis.

in our study. We also applied very rigorous statistical correction to our data in order to reduce the likelihood of reporting false positive results, which may have been too stringent to allow detection of weaker associations. The four variants discussed in this paper which did not remain significant after multiple testing correction, may therefore warrant further investigation in an additional cohort to definitively assess their importance in pouchitis pathogenesis. This would ideally be attempted in the setting of the large International IBD Genetics Consortium Cohort but would require detailed phenotyping of precolectomy data as well as of pouch outcomes, both of which may not be readily available in existing databases.

We hypothesise that patients with *NOD2*insC may be more likely to develop inflammatory pouch complications owing to alteration in the microbial composition of the pouch mucosa. This hypothesis is supported by our data showing that patients with UC with a CDL phenotype after pelvic pouch surgery are more likely to be positive for ASCA and anti-CBir1,[21] serological markers more typically associated with CD, and by the data from other groups demonstrating a strong association between the common UC-associated serum marker, pANCA and pouch outcome.[43 44] Additional evidence that *NOD2* variants affect the composition of the ileal flora suggests that there may be a relationship between IBD-associated genetic polymorphisms and the presence of serum antibodies.[32 45] Our findings of the presence of both serological markers of CD and genetic polymorphisms which have been previously associated with ileal CD showing association with pouch outcome, suggest that the mechanism of ileal inflammation which leads to a CP or CDL outcome in the pouch may be similar to those which lead to the development of inflammation within the ileum. Additionally, the inclusion of individuals with non-chronic pouchitis in the NCP group and the lack of evidence for any associations between genotype and this outcome, suggests that the chronic inflammatory phenotypes proceed via diverging mechanistic pathways from those which lead to antibiotic-responsive pouchitis. Furthermore, the overlap in genetic susceptibility between CP and CDL suggests that distinction between these outcomes may be of limited consequence compared with the more clinically relevant phenotype of chronic, medication-refractory inflammation which is characteristic of both disorders and results from a common aetiology.

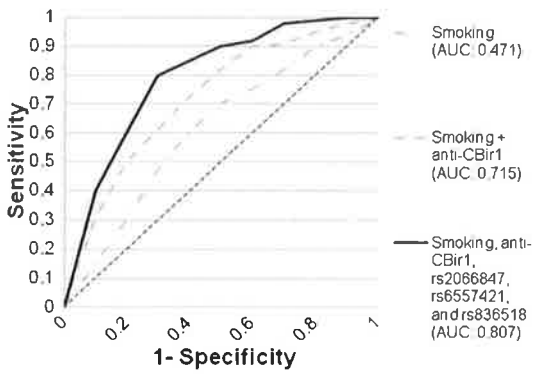

Figure 2 Receceiver operating characteristic curves generated for the risk score analysis. *NOD2*insC—rs2066847; *NOX3*—rs6557421; *DAGLB*—rs836518. AUC, area under the curve.

While it is conceivable that these patients with CDL phenotype were misclassified before colectomy, the stringent phenotypic classification which was used to determine which patients were included in this analysis would suggest that this is unlikely to be the case. Rather, our results combined with recent data published by Waterman *et al* showing few differences in the prevalence of several IBD-associated SNPs between those with UC and those with CD,[36] support the concept of IBD as a mosaic of inflammatory disorders. The varying phenotypes associated with overlapping genetic susceptibility loci suggest that while genetic predisposition may be a key inflammatory mediator, other factors such as bacteria or as yet unknown environmental stimuli are necessary for immune dysregulation and inflammation to occur.

The aetiology of pouch complications remains unknown. However, the data presented in this paper emphasise the similarities between pouch inflammation and IBD in general and suggest that the pelvic pouch model may be useful for evaluating factors which contribute to de novo inflammation. It will be important to evaluate the impact of the pouch microbiome and the impact of host genotype on the composition of the microbiome to fully understand the mechanisms of pouch inflammation. Our data also demonstrate that a model for assessing risk of pouch complications which encompasses genetic and serological factors may be a more useful tool than current clinical assessment.

Author affiliations
[1]Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada
[2]Zane Cohen Centre for Digestive Diseases, Mount Sinai Hospital IBD Group, Toronto, Ontario, Canada
[3]Department of Public Health Sciences, University of Toronto, Toronto, Ontario, Canada
[4]Program in Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada
[5]Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada
[6]Division of Gastroenterology, Hepatology and Nutrition, Department of Paediatrics, The Hospital for Sick Children, Toronto, Canada
[7]Division of Colon and Rectal Surgery, Department of Surgery, Penn State Milton S Hershey Medical Center, Hershey, Pennsylvania, USA
[8]Department of Cellular and Molecular Physiology, Penn State Milton S Hershey Medical Center, Hershey, Pennsylvania, USA
[9]Center for Inflammatory Bowel Disease, Digestive Disease Institute, Cleveland Clinic, Cleveland, Ohio, USA

Acknowledgements The authors thank Diane Verbeeten and Lucy T Zhang for their assistance with the study and Harden Huang and Brenda O'Connor for maintaining and providing access to the Mount Sinai Hospital Pelvic Pouch Database.

Contributors ADT implemented the trial in Toronto and was responsible for data acquisition, analysis and interpretation and drafting and revising the manuscript. RM data analysis and critical revision of the manuscript. JMS implemented the trial in Toronto and carried out critical revision of the manuscript. WX statistical analysis and revision of the manuscript. JHB, AM critical revision of the manuscript. ZC critical revision of the manuscript and assisted with patient recruitment. RS implemented the trial for the Hershey site and monitored data collection for that site. WK implemented the trial in Hershey and carried out critical revision of the manuscript. BS implemented the trial in Cleveland and carried out critical revision of the manuscript. MSS initiated the collaborative project, designed data collection tools, implemented the trial in Toronto and carried out critical revision of the manuscript. ADT and MSS are guarantors.

Funding This study was supported by a grant from the Crohn's and Colitis Foundation of Canada. MSS is partially supported by the Gale and Graham Wright Research Chair in Digestive Disease. Partial funding for genotyping was supported by a CIHR operating grant (MOP97756) to AMM and JHB.

Competing interests MSS receives speaker fees, consulting fees and research support from Prometheus Laboratories.

Ethics approval Research ethics boards of Mount Sinai Hospital, Cleveland Clinic and Milton S Hershey Medical Center.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Selby W. The natural history of ulcerative colitis. *Baillieres Clin Gastroenterol* 1997;11:53–64.
2 Etchevers MJ, Aceituno M, Garcia-Bosch O, *et al*. Risk factors and characteristics of extent progression in ulcerative colitis. *Inflamm Bowel Dis* 2009;15:1320–5.
3 Farmer RG, Easley KA, Rankin GB. Clinical patterns, natural history and progression of ulcerative colitis. A long-term follow-up of 1116 patients. *Dig Dis Sci* 1993;38:1137–46.
4 Allison J, Herrinton LJ, Liu L, *et al*. Natural history of severe ulcerative colitis in a community-based health plan. *Clin Gastroenterol Hepatol* 2008;6:999–1003.
5 Marcello PW, Roberts PL, Schoetz DJ Jr, *et al*. Long-term results of the ileoanal pouch procedure. *Arch Surg* 1993;128:500–3; discussion 3–4.
6 Cohen Z, McLeod RS, Stern H, *et al*. The pelvic pouch and ileoanal anastomosis procedure. Surgical technique and initial results. *Am J Surg* 1985;150:601–7.
7 Simchuk EJ, Thirlby RC. Risk factors and true incidence of pouchitis in patients after ileal pouch-anal anastomoses. *World J Surg* 2000;24:851–6.
8 Kuisma J, Jarvinen H, Kahri A, *et al*. Factors associated with disease activity of pouchitis after surgery for ulcerative colitis. *Scand J Gastroenterol* 2004;39:544–8.
9 Dayton MT, Larsen KR, Christiansen DD. Similar functional results and complications after ileal pouch-anal anastomosis in patients with indeterminate vs ulcerative colitis. *Arch Surg* 2002;137:690–4; discussion 4–5.
10 Shen B, Remzi FH, Brzezinski A, *et al*. Risk factors for pouch failure in patients with different phenotypes of Crohn's disease of the pouch. *Inflamm Bowel Dis* 2008;14:942–8.
11 Shen B, Remzi FH, Hammel JP, *et al*. Family history of Crohn's disease is associated with an increased risk for Crohn's disease of the pouch. *Inflamm Bowel Dis* 2009;15:163–70.
12 Melmed GY, Fleshner PR, Bardakcioglu O, *et al*. Family history and serology predict Crohn's disease after ileal pouch-anal anastomosis for ulcerative colitis. *Dis Colon Rectum* 2008;51:100–8.
13 Facklis K, Plevy SE, Vasiliauskas EA, *et al*. Crohn's disease-associated genetic marker is seen in medically unresponsive ulcerative colitis patients and may be associated with pouch-specific complications. *Dis Colon Rectum* 1999;42:601–5; discussion 5–6.
14 Lammers KM, Ouburg S, Morre SA, *et al*. Combined carriership of TLR9-1237C and CD14-260T alleles enhances the risk of developing chronic relapsing pouchitis. *World J Gastroenterol* 2005;11:7323–9.
15 Aisenberg J, Legnani PE, Nilubol N, *et al*. Are pANCA, ASCA, or cytokine gene polymorphisms associated with pouchitis? Long-term follow-up in 102 ulcerative colitis patients. *Am J Gastroenterol* 2004;99:432–41.
16 Carter MJ, Di Giovine FS, Cox A, *et al*. The interleukin 1 receptor antagonist gene allele 2 as a predictor of pouchitis following colectomy and IPAA in ulcerative colitis. *Gastroenterology* 2001;121:805–11.
17 Ferrante M. Development of pouchitis following ileal-pouch anal anastomosis (IPAA) for ulcerative colitis: a role for serological markers and microbial pattern recognition receptor genes. *J of Crohn's and Colitis* 2008;2:142–51.
18 Hugot JP, Chamaillard M, Zouali H, *et al*. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* 2001;411:599–603.
19 Ogura Y, Bonen DK, Inohara N, *et al*. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* 2001;411:603–6.
20 Sehgal R, Berg A, Hegarty JP, *et al*. NOD2/CARD15 mutations correlate with severe pouchitis after ileal pouch-anal anastomosis. *Dis Colon Rectum* 2010;53:1487–94.
21 Tyler AD, Milgrom R, Xu W, *et al*. Anti-microbial antibodies are associated with a Crohn's disease-like phenotype following ileal pouch-anal anastomosis. *Clin Gastroenterol Hepatol* 2012;10:507–12.e1.
22 Silverberg MS, Satsangi J, Ahmad T, *et al*. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005;19(Suppl A):5–36.
23 Shen B, Remzi FH, Lavery IC, *et al*. A proposed classification of ileal pouch disorders and associated complications after restorative proctocolectomy. *Clin Gastroenterol Hepatol* 2008;6:145–58; quiz 24.
24 Purcell S, Neale B, Todd-Brown K, *et al*. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–75.
25 Freidlin B, Zheng G, Li Z, *et al*. Trend tests for case-control studies of genetic markers: power, sample size and robustness. *Hum Hered* 2002;53:146–52.
26 Wang Y, Jatkoe T, Zhang Y, *et al*. Gene expression profiles and molecular markers to predict recurrence of Dukes' B colon cancer. *J Clin Oncol* 2004;22:1564–71.
27 Abdelrazeq AS, Kandiyil N, Botterill ID, *et al*. Predictors for acute and chronic pouchitis following restorative proctocolectomy for ulcerative colitis. *Colorectal Dis* 2008;10:805–13.
28 Shen B, Remzi FH, Nutter B, *et al*. Association between immune-associated disorders and adverse outcomes of ileal pouch-anal anastomosis. *Am J Gastroenterol* 2009;104:655–64.
29 Economou M, Trikalinos TA, Loizou KT, *et al*. Differential effects of NOD2 variants on Crohn's disease risk and phenotype in diverse populations: a metaanalysis. *Am J Gastroenterol* 2004;99:2393–404.
30 Hampe J, Cuthbert A, Croucher PJ, *et al*. Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations. *Lancet* 2001;357:1925–8.
31 Kim YG, Kamada N, Shaw MH, *et al*. The Nod2 sensor promotes intestinal pathogen eradication via the chemokine CCL2-dependent recruitment of inflammatory monocytes. *Immunity* 2011;34:769–80.
32 Frank DN, Robertson CE, Hamm CM, *et al*. Disease phenotype and genotype are associated with shifts in intestinal-associated microbiota in inflammatory bowel diseases. *Inflamm Bowel Dis* 2011;17:179–84.
33 Fishbein TM, Zasloff M. Association of Crohn's disease-associated NOD2 variants with intestinal failure requiring small bowel transplantation and clinical outcomes. *Gut* 2011;60:877–8.
34 Holler E, Rogler G, Brenmoehl J, *et al*. The role of genetic variants of NOD2/CARD15, a receptor of the innate immune system, in GvHD and complications following related and unrelated donor haematopoietic stem cell transplantation. *Int J Immunogenet* 2008;35:381–4.
35 Meier CB, Hegazi RA, Aisenberg J, *et al*. Innate immune receptor genetic polymorphisms in pouchitis: is CARD15 a susceptibility factor? *Inflamm Bowel Dis* 2005;11:965–71.
36 Waterman M, Xu W, Stempak JM, *et al*. Distinct and overlapping genetic loci in crohn's disease and ulcerative colitis: correlations with pathogenesis. *Inflamm Bowel Dis* 2011;17:1936–42.
37 Muise AM, Xu W, Guo CH, *et al*. NADPH oxidase complex and IBD candidate gene studies: identification of a rare variant in NCF2 that results in reduced binding to RAC2. *Gut* 2011;17:179–84.
38 Somasundaram R, Deuring JJ, van der Woude CJ, *et al*. Linking risk conferring mutations in NCF4 to functional consequences in Crohn's disease. *Gut* 2012;61:1097.
39 Wientjes FB, Hsuan JJ, Totty NF, *et al*. p40phox, a third cytosolic component of the activation complex of the NADPH oxidase to contain src homology 3 domains. *Biochem J* 1993;296:557–61.
40 Ueno N, Takeya R, Miyano K, *et al*. The NADPH oxidase Nox3 constitutively produces superoxide in a p22phox-dependent manner: its regulation by oxidase organizers and activators. *J Biol Chem* 2005;280:23328–39.
41 Muise AM, Walters T, Xu W, *et al*. Single nucleotide polymorphisms that increase expression of the guanosine triphosphatase RAC1 are associated with ulcerative colitis. *Gastroenterology* 2011;141:633–41.
42 Brett PM, Yasuda N, Yiannakou JY, *et al*. Genetic and immunological markers in pouchitis. *Eur J Gastroenterol Hepatol* 1996;8:951–5.
43 Fleshner P, Ippoliti A, Dubinsky M, *et al*. Both preoperative perinuclear antineutrophil cytoplasmic antibody and anti-CBir1 expression in ulcerative colitis patients influence pouchitis development after ileal pouch-anal anastomosis. *Clin Gastroenterol Hepatol* 2008;6:561–8.
44 Fleshner PR, Vasiliauskas EA, Kam LY, *et al*. High level perinuclear antineutrophil cytoplasmic antibody (pANCA) in ulcerative colitis patients before colectomy predicts the development of chronic pouchitis after ileal pouch-anal anastomosis. *Gut* 2001;49:671–7.
45 Devlin SM, Yang H, Ippoliti A, *et al*. NOD2 variants and antibody response to microbial antigens in Crohn's disease patients and their unaffected relatives. *Gastroenterology* 2007;132:576–86.