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MULTIFOCAL DIVERTICULITIS: A SEVERE SUBSET OF DISEASE WITH DISTINCT CLINICAL AND BIOLOGIC FEATURES

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ABSTRACT

Diverticular disease is characterized by diverticula, or outpouchings in the wall of the colon. Although diverticular disease is common, its pathophysiology is poorly understood. The majority of patients with diverticula remain asymptomatic, but some patients develop inflammation of these diverticula, known as diverticulitis. Diverticulitis is often managed medically and without hospitalization, but it can sometimes result in severe complications and require surgical resection. Due to the poor understanding of the factors contributing to disease progression, it can be difficult to identify patients who require surgery to prevent severe disease. To further evaluate the disease, we identified a subset of patients with multifocal diverticulitis (MFD), or multiple episodes of diverticulitis occurring at different locations within the colon. We hypothesized that these patients would display different clinical and transcriptomic characteristics in comparison to patients with conventional unifocal diverticulitis (UFD).

We performed a retrospective study of 404 patients with diverticulitis. Of these patients, 28 had diverticulitis in at least two different colonic locations, and thus were classified as MFD patients. A comparison with the UFD patients found that MFD patients had more episodes, more family history of diverticulitis, more right sided disease, were more likely to require surgery, and were more likely to have recurrence after surgery. Transcriptomic analysis was then performed using RNA-seq on full-thickness colonic tissues of 10 MFD and 11 UFD patients matched for age, sex, BMI, and smoking history. We identified 69 differentially expressed genes and MFD patients displayed down-regulation of immune-associated gene sets.

Our study found clinical and biologic features that differentiates MFD from UFD. MFD appears to be a more severe disease with a possible genetic component. RNA-seq demonstrates immune dysregulation in MFD. The identification of this subtype adds information about the disease as well as its pathophysiology and may lead to improved management decisions.

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DD	Diverticular disease
GI	Gastrointestinal
SUDD	Symtpomatic uncomplicated diverticular disease
CT	Computed tomography
BMI	Body mass index
SNP	Single nucleotide polymorphism
TNFSF15	Tumor necrosis factor superfamily 15 gene
LAMB4	laminin β 4
COL3A1	Collagen III gene
GWAS	Genome wide association studies
ARHGAP15	Rho-GTPase activating protein 15
COLQ	Collagen like tail protein of acetylcholinesterase
FAM155A	Family with sequence similarity 155 member A
UK	United Kingdom
MGI	Michigan genomics initiative
IBD	Inflammatory bowel disease
TNF-α	Tumor necrosis factor alpha
IL-6	Interleukin 6
MFD	Multifocal diverticulitis
UFD	Unifocal diverticulitis
ICD-9	International classification of diseases, ninth revision
VST	Variance-stabilizing transformation
CCL18	C-C motif chemokine ligand 18
CCL20	C-C motif chemokine ligand 20

CCL23	C-C motif chemokine ligand 23
MIP-3α	Macrophage inhibitory protein 3α
CCR6	C-C chemokine receptor 6

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Chapter 1

Introduction

1.1 Epidemiology and Classification of Diverticular Disease

Diverticular disease (DD) is a common condition of the gastrointestinal (GI) tract, especially in Western countries. The disease is characterized by colonic diverticula, or outpouchings in the wall of the colon. The colon receives partially digested food from the small intestine, and its primary function is to absorb water and move the remaining stool to the rectum where it is stored before evacuation. The colon is made up of an ascending, transverse, descending, and sigmoid portion. These sections and their anatomic location are shown in figure 1-1.

In the United States alone, DD contributes to over 1.9 million annual ambulatory visits and over 200,000 annual hospitalizations, with an estimated cost of over 5.4 billion dollars in 2015 [1]. Diverticula are most commonly found in the sigmoid colon in Western countries, while right sided diverticula more common in Asia [2, 3]. DD encompasses a spectrum of changes from asymptomatic diverticulosis to complicated, and at times life threatening, episodes of diverticulitis. Figure 1-2 represents the changes in the colon associated with DD.

Diverticulosis refers to the presence of diverticula without inflammation. The majority of patients with diverticula are asymptomatic and diverticulosis is often discovered incidentally on colonoscopy [4]. Approximately 50% of individuals at age 60 have diverticulosis, and the prevalence increases to almost 80% by age 85 [4, 5]. Although diverticulosis was once thought to be rare in adults under 40, the prevalence may actually be as high as 20% [4]. Patients may develop symptomatic uncomplicated diverticular disease (SUDD) which can involve chronic

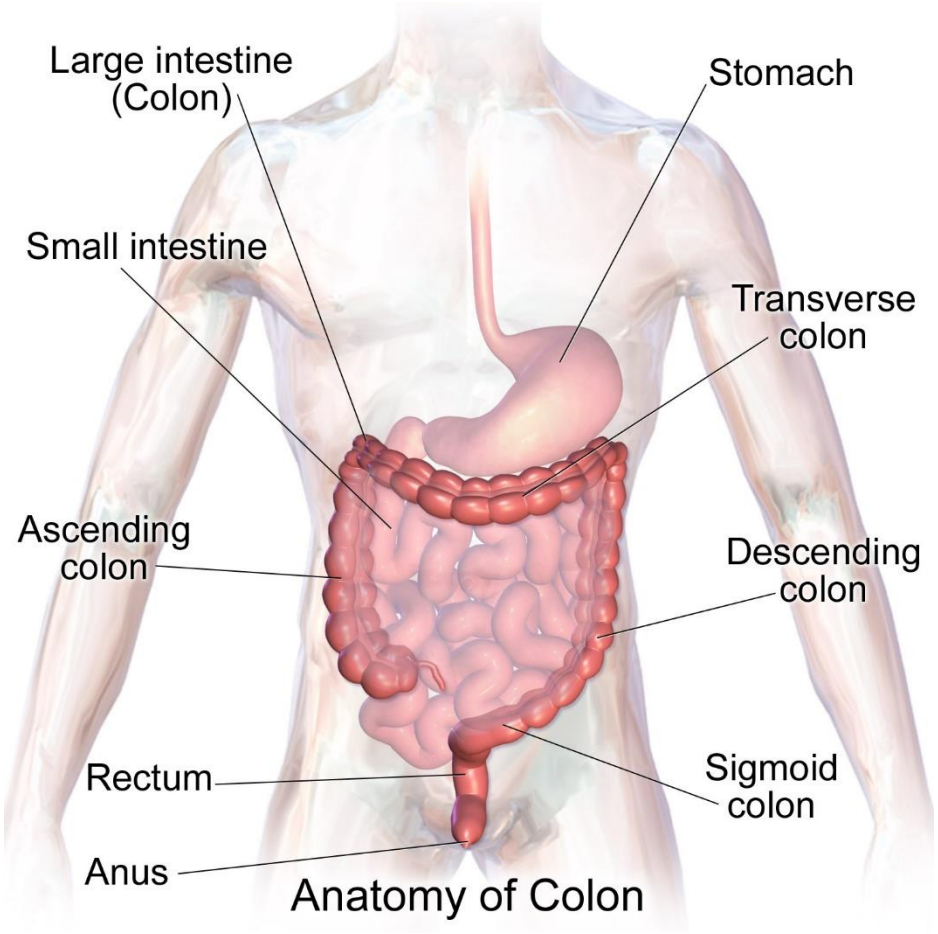

Figure 1-1: Anatomy of the Colon

Anatomic location of the ascending, transverse, descending, and sigmoid colon. The relationship between the colon and other GI organs such as the small bowel and rectum are also shown.
Source:
https://en.wikiversity.org/wiki/WikiJournal_of_Medicine/Medical_gallery_of_Blausen_Medical_2014

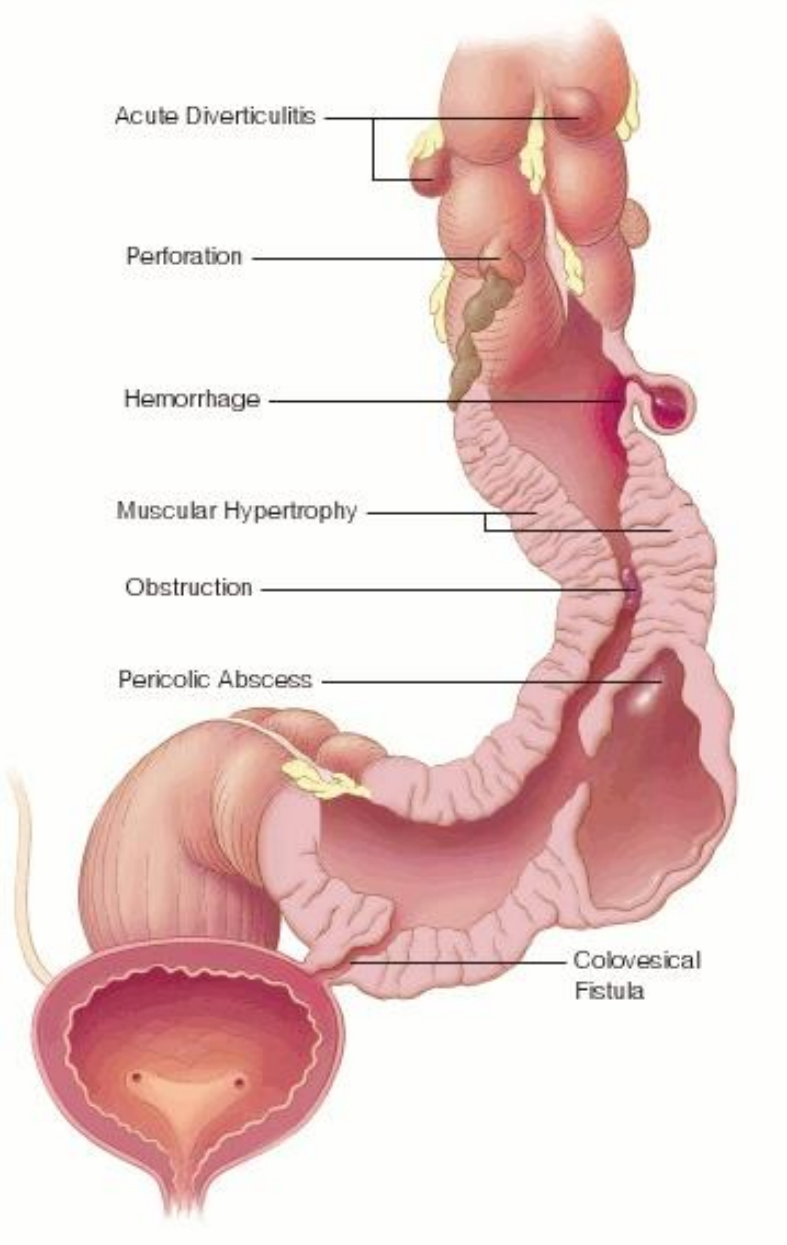

Figure 1-2: Diverticular changes in the colon

Illustration of the sigmoid colon with inflamed diverticula (acute diverticulitis), perforation, obstruction due to muscular hypertrophy or stricture, pericolic abscess, and fistula between the colon and bladder (colovesical fistula). Source: https://diverticulitismedication.wordpress.com/

abdominal pain, bloating and/or irregular bowel habits without radiographic or endoscopic evidence of diverticular inflammation [6]. If diverticular inflammation is present, this is termed diverticulitis.

The incidence rate of diverticulitis in patients with diverticulosis is currently under debate. While early literature suggested that approximately 20% of patients with diverticulosis will develop diverticulitis [4, 5], a more recent study found an incidence of 4% based on clinical symptoms [7]. Even fewer patients, around one percent, have episodes of diverticulitis that are confirmed by imaging or surgery [7]. The clinical symptoms associated with an acute episode of diverticulitis are left lower quadrant abdominal pain, alteration in bowel habits, leukocytosis, and fever [8]. Although acute diverticulitis can often be diagnosed by history and physical examination, computed tomography (CT) imaging is frequently used to aid in diagnosis and assess severity [9].

Diverticulitis is usually uncomplicated and can be managed without hospitalization [10]. However, complications occur in approximately 10-25% of patients with diverticulitis [10-12]. Acute complications of diverticulitis are often classified according to the Hinchey grading system, developed in the 1970s [13]. This system increases in severity from localized abscess (Hinchey grade I) to free perforation with feces in the abdominal cavity (Hinchey grade IV) [13]. The most common complication of acute diverticulitis is a diverticular abscess (Hinchey grades I and II [13]). An abscess is found in approximately 20-40% of complicated cases [14, 15]. Hinchey grades III and IV are more severe and involve perforation leading to generalized peritonitis [13]. Acute complications are associated with significant morbidity, and one study found a 4.5-fold increase in one year mortality in patients with a perforation or abscess compared to the general population [16]. Complications from chronic DD include stricture and fistula [5]. Strictures can develop due to fibrosis of the colon wall, often due to recurrent episodes [17]. A

fistula is an abnormal connection between the diseased colon and another organ, most commonly the bladder or the vagina [18]. Figure 1-3 represents the subdivisions of diverticular disease.

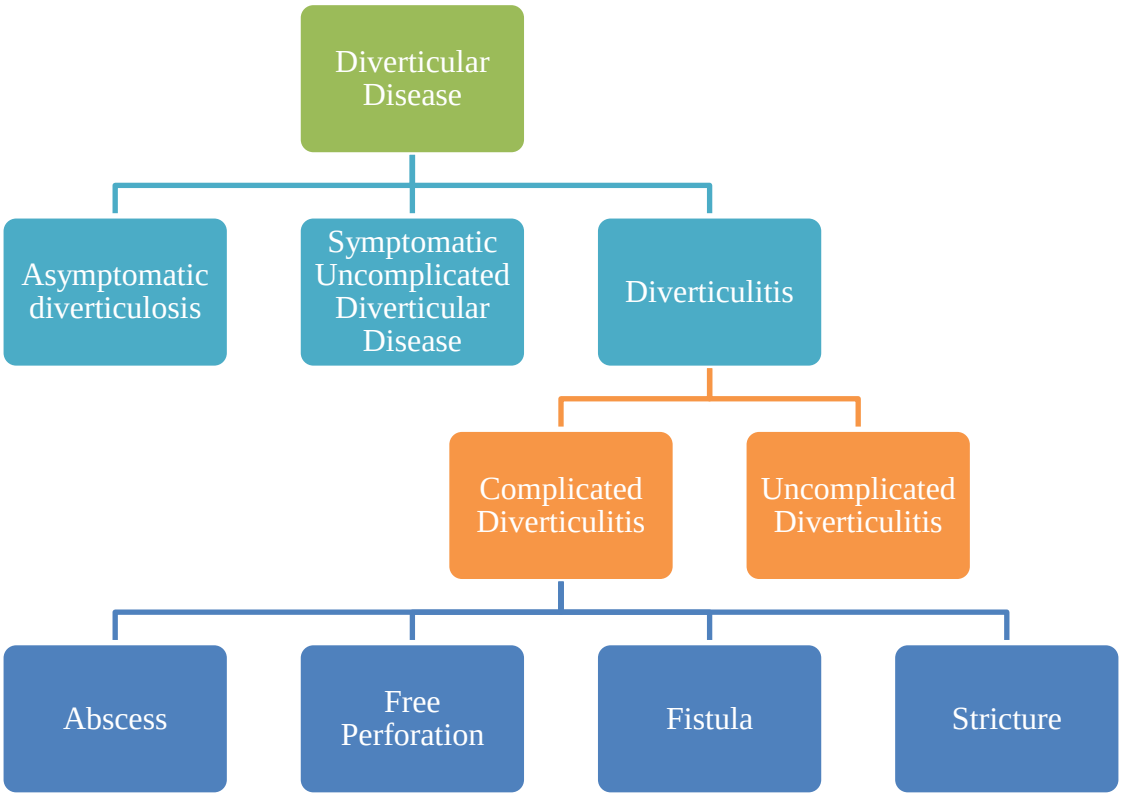

Figure 1-3: Subdivisions of diverticular disease

A schematic depicting the different categories of diverticular disease, how diverticulitis can be stratified as complicated or uncomplicated, and the major phenotypic presentations of complicated diverticulitis.

1.2 Management of Diverticulitis

When discussing the management of diverticulitis, care strategies can refer to both patients suffering from acute episodes and those with more chronic forms of the disease. In those with acute diverticulitis, outpatient management with oral antibiotics is generally recommended for clinically stable and reliable patients with an uncomplicated episode [9]. However, opinions are currently shifting as recent randomized studies have failed to demonstrate significantly better outcomes in patients treated with antibiotics compared to those treated without [19-21]. Patients with an abscess (Hinchey grades I and II) are managed acutely with drainage in addition to antibiotics [9]. Surgical resection in acute diverticulitis is reserved for patients who do not respond to nonoperative management or for patients with free perforation (Hinchey grades III and IV) [9].

Elective colectomy was previously recommended after two episodes of uncomplicated or one episode of complicated diverticulitis, but now a more individualized approach is recommended [9]. Originally, there was concern that without surgical resection, a future episode could be more severe. However, recent studies found that the first episode is frequently the most severe [22, 23]. In terms of the overall risk of recurrence, a study of over 200,000 patients who presented to hospitals in California with diverticulitis, 85% of patients were managed medically, and 16.3% of these patients had a second diverticulitis episode [23]. Other studies have shown a recurrence rate of approximately 28-36% in patients who do not undergo elective resection [24-26]. In these reports, only 4% of patients presented with a complicated recurrence such as abscess, fistula, or free perforation [24]. Predictors of recurrence include number of previous episodes, age at first episode, family history of diverticulitis, smoking history, or complications at their initial presentation [23, 24, 27]. Although these predictors are taken into account when determining management, the pathophysiology of DD must be better understood to more

accurately identify patients who will suffer from recurrent episodes and therefore may benefit from earlier surgery.

1.3 Pathophysiology and Risk Factors

The pathogenesis of DD is still poorly understood. Diverticulosis is thought to be related to defects in colonic wall structure, colonic motility, diet, obesity, and physical activity [6]. Diverticulitis is believed to develop when a diverticulum becomes obstructed by a fecalith, leading to bacterial overgrowth and subsequent immune response [6, 28]. Factors such as colonic wall structure, motility, and the immune response may have genetic predispositions [29-31], and these will be discussed more in depth subsequently. There are still significant gaps in our knowledge of how all of these factors interact and contribute to the development of DD. Factors associated with DD and their proposed role in disease progression are shown in Figure 1-4.

Diverticula form at an area of weakness in the colon wall where the vasa recta perforate through the muscularis propria to supply the mucosa and submucosa [32]. Although this anatomy is common to all individuals, several changes in the bowel wall have been noticed in patients with DD. Studies have found increased elastin deposition in the colon wall of patients with diverticulosis [33, 34]. Increased collagen crosslinking has also been identified in the colon wall of individuals with asymptomatic diverticulosis and patients undergoing surgery for diverticulitis [35, 36]. Patients with DD have increased thickness of the muscular layers of the colon with smooth muscle cell alterations and increased connective tissue [37].

In addition to changes within the wall itself, herniation through the weak areas of the colon wall could also be due to increased intraluminal pressure in the colon and dysmotility [28]. Colonic pressures have been shown to be higher in DD than in control individuals [38]. Longitudinal muscle from patients with diverticulosis shows abnormalities in relaxation and

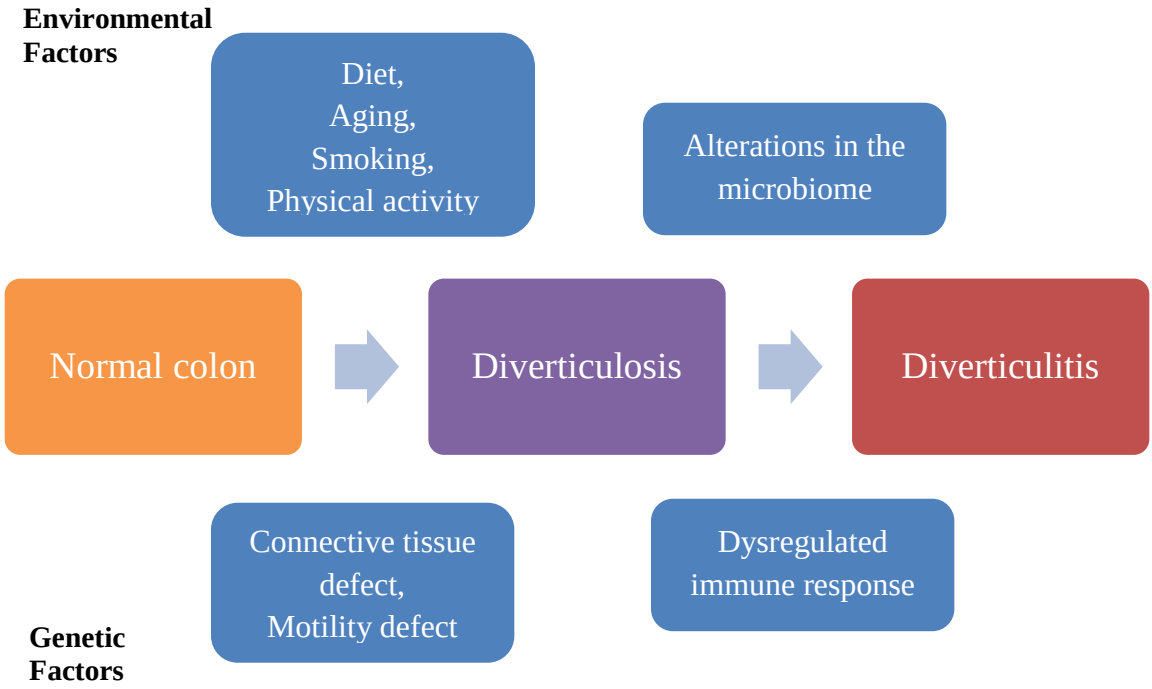

Figure 1-4: Factors associated with diverticular disease

The progression of diverticular disease has been suggested to be associated with both environmental and genetic factors. Diet, aging, smoking, physical activity, and defects in connective tissue and motility are often found to associate with development of diverticulosis. Alterations in the microbiome and dysregulation in the immune response may increase the risk of developing diverticulitis. Adapted from Connelly et al. [31]

lower nitric oxide synthase expression [34]. The number of interstitial cells of Cajal (the pacemaker cells of the GI tract) are decreased in DD, and this potentially contributes to dysmotility [39].

Smoking, diet, physical activity, and body weight are often discussed as modifiable environmental risk factors for DD [40, 41]. Although these risk factors have been suggested to impact the pathophysiology of the disease in various ways, their role is unclear. Additionally, there is still debate over whether some of these factors are actually associated with increased risk.

In two Swedish studies, smoking was associated with a 24% increase in hospitalization for DD in women and a 60% increase in men [42, 43]. A systematic review and meta-analysis performed on five prospective studies with a total of 385,291 participants, of which 6,076 developed DD [44]. This study found a 36% increased risk of DD in current smokers and 17% increased risk in ex-smokers [44]. Smoking has also been associated with an increased risk of diverticulitis requiring hospitalization or surgery in retrospective studies [45, 46].

Dietary modification, specifically increasing fiber intake, is often recommended to decrease the risk of diverticulitis episodes, but the actual significance of fiber intake in diverticulitis has been debated [47, 48]. Dietary habits are often discussed as the explanation for differences in DD prevalence between Asia and the West. In comparison to native Westerners, non-Western immigrants to Western countries have a lower risk for hospital admission from DD [49, 50], but the risk increases with time of residence in the country [49]. A study of 47,033 individuals in England and Scotland found a 31% lower risk of DD in vegetarians compared to meat eaters [51]. This study also found that individuals with a high dietary fiber intake (>26 g/day) had a 41% lower risk of DD than those with low fiber intake (<14 g/day) [51]. A systematic review of three randomized controlled trials and one case-control study did find evidence of improved pain scores in patients with SUDD who followed a high-fiber diet, but the quality of these studies was questioned as there was a significant placebo effect present [48]. The

American Gastroenterological Association notes that high quality evidence is lacking to support the argument that a high fiber diet reduces the risk of recurrent diverticulitis [47].

A study of 47,230 males in the United States found that vigorous physical activity was associated with a 34% reduction in the risk of diverticulitis [52]. A prospective cohort study of 7,494 men in Sweden found that obesity (defined as a body mass index or BMI greater than 30) was associated with a fourfold higher risk of hospitalization from DD compared to men with a normal BMI [43]. A similar Swedish study performed in 36,592 women found BMI greater than 30 was associated with a 33% higher risk of DD compared to normal BMI [53]. A recent meta-analysis of 11 cohort studies found increasing risk of DD with increasing BMI and decreasing risk with physical activity[54].

Finally, there are several connective tissue diseases found to be associated with DD. These diseases may give insight into the pathogenesis. Early case reports noted an association between Marfan's syndrome and DD [55]. Diverticulosis has frequently been found in patients with Ehlers-Danlos syndrome [56, 57]. Additionally, intra-abdominal cysts were found in 71.4% of 238 patients with DD compared to 22.5% of 369 controls [58]. The association of these diseases reinforce the argument that DD is at least in part due to defects in the connective tissue.

1.4 Genetics

Although DD was previously attributed to factors such as diet or advancing age, there has been a more recent focus on the role of genetics. Right sided DD (diverticula in the ascending colon) may be associated with a genetic predisposition, as right sided DD is higher in non-Western populations and remains high even as overall diverticulitis rates increase with Westernization [59]. Two large twin studies in Scandinavian countries found the heritability of DD to be 40-50% [60, 61]. The first study was performed on 104,452 twins in the Swedish Twin

registry, and the authors found 2296 individuals who were diagnosed with DD. The odds ratio of a twin being diagnosed with DD was 7.15 in monozygotic and 3.2 in dizygotic twins, and the heritability was estimated at 40% [60]. A second study was performed using the Danish twin registry of 30,322 twins, in which 923 were found to have DD. The relative risk of a twin being diagnosed with DD was 14.5 in monozygotic twins and 5.5 in dizygotic twins, and the heritability was estimated at 53% [62].

DD has now been linked to several single nucleotide polymorphisms (SNPs) in various genes. A list of these SNPs and potential genes associated with them are presented in table 1-1. The first SNPs were identified through small cohort studies [29-31]. In 2014, the SNP rs7848647 in the tumor necrosis factor superfamily 15 gene (*TNFSF15*) was associated with the need for surgery in a 21-patient discovery group and then validated in a test group of 34 patients [31]. *TNFSF15* is an immunoregulatory gene that encodes TL1A and has previously been associated with intestinal inflammation [63]. Another study identified a rare variant in the laminin β 4 gene (*LAMB4*) from whole exome sequencing performed on 148 patients with diverticulitis. Targeted re-sequencing of *LAMB4* identified other variants in the gene, and patients with *LAMB4* variants had decreased LAMB4 protein expression in the colonic myenteric plexus [29]. LAMB4 is a subunit of laminin, a major subunit of the extracellular matrix, which contributes to intestinal differentiation [64]. Finally, a recent study identified an association between the SNP rs3134646 in the collagen III gene (*COL3A1*) in 422 Caucasian patients with diverticulosis compared to 285 Caucasian controls [30]. However, when adjusted for age and BMI, the association was only present in men. *COL3A1* is associated with Ehlers Danlos Syndrome and gastroesophageal reflux disease, and rs3134646 is associated with hiatal hernia in men [65].

Other SNPs have been identified by genome wide association studies (GWAS). The first GWAS evaluated 5,426 Icelandic individuals with DD and compared them to 245,951 controls. The top 16 variants were then tested in an independent cohort of 5,970 Danish individuals and

3,020 controls. Ultimately, three loci were identified as significantly associated with DD or diverticulitis: rs4662344 in rho-GTPase activating protein 15 (*ARHGAP15*), rs7609897 in collagen like tail protein of acetylcholinesterase (*COLQ*), and rs67153654 in family with sequence similarity 155 member A (*FAM155A*) [66]. *ARHGAP15* has many intracellular functions, but may play a role in the immune system as it has been shown to help regulate neutrophil function including chemotaxis, phagocytosis, and bactericidal activity [67]. *COLQ* encodes a protein that anchors acetylcholinesterase in the neuromuscular junction [68], and therefore may influence gastrointesintal motility. *FAM155A* is poorly understood due to minimal study, but it was recently identified as a regulator of the proliferation and migration of stem cells derived from supernumerary teeth [69]. Of note, the 5,426 Icelandic individuals with DD were split up into 2,764 individuals with diverticulitis and 2,662 with diverticulosis, but the diagnosis of diverticulitis was not available for the Danish sample. Therefore, although rs67153654 in *FAM155A* is stated to be associated with diverticulitis, a significant association was only found when combining the 2,764 Icelanders with diverticulitis and 5,970 Danish with DD [66].

A second GWAS was performed on 27,444 individuals with DD and 382,284 controls in a United Kingdom (UK) Biobank. The results were then tested for replication in a cohort of 1,854 individuals with diverticulosis, 718 individuals with diverticulitis, and 28,649 controls in a biobank held by the Michigan Genomics Initiative (MGI) [70]. Overall, 40 significant loci were identified in the UK cohort (including the three SNPs from the first GWAS), and eight of these were replicated in the MGI sample. Of the original three SNPs, only rs4662344 in *ARHGAP15* and rs67153654 in *FAM155A* were replicated, and only in individuals with diverticulosis, not diverticulitis. The MGI sample identified two additional loci that did not meet genome wide significance in the UK sample, leading the authors to conclude that this study identified 39 novel loci that may contribute to the pathophysiology of DD [70]. These SNPs and possible target genes are listed in table 1-1.

The most recent GWAS was also performed using the UK biobank, comparing 31,964 individuals with DD to 419,135 controls [71]. The top 51 associated loci were validated in a European sample of 3893 individuals with DD and 2829 controls. After validation, the study identified 48 risk loci for DD (listed in table 1-1), including 12 novel loci [71]. The original three SNPs from the initial GWAS (rs4662344 in *ARHGAP15*, rs7609897 in *COLQ*, and rs67153654 in *FAM155A* [66]) were also confirmed by this study. The identification of genes associated with DD will hopefully allow focused study into the pathophysiology of the disease.

1.5 Microbiome

The human gut microbiome is comprised of bacteria, viruses, fungi, and other microbes that contribute to metabolic functions and interact with the immune system [72]. The role of the microbiome in DD is unclear, but the recommendation for management of diverticulitis with antibiotics is based on a long held belief that diverticulitis is caused by bacterial infection [9]. The human GI tract harbors over 1000 species of bacteria, most belonging to the Bacteroidete and Firmicute phyla [72]. The majority of the Firmicutes are classified as *Clostridia* [73]. The physical barrier between the lumen and mucosal tissue is made up of a single layer of epithelial cells and mucus which is produced by goblet cells [74]. The mucus is organized into two layers and mucin 2 (MUC2) is the major constituent in both layers. The inner layer is tightly packed to prevent bacterial infiltration, while the outer layer is nonattached and provides a habitat for the GI flora [75]. When these protective layers break down, bacteria can translocate across the epithelium and lead to an inflammatory response [72]. Intestinal permeability can be effected by diet, intrinsic defects in the GI tract, or gut dysbiosis [74, 76].

Table 1-1: SNPs associated with diverticular disease

SNP	Nearest Gene	Associated Subtype	Study
rs7848647	*TNFSF15*	Surgical diverticulitis	Connelly et al. 2014 [31]
N/A[1]	*LAMB4*	Diverticulitis	Coble et al. 2017 [29]
rs3134646	*COL3A1*	Diverticulosis in white males	Reichert et al. 2018 [30]
rs4662344	*ARHGAP15*	Diverticular disease	Sigurdsson et al. 2017 [66] Maguire et al. 2018 [70] Schafmayer et al. 2019 [71]
rs7609897	*COLQ*	Diverticular disease	Sigurdsson et al. 2017 [66] Maguire et al. 2018 (UK Biobank only) [70] Schafmayer et al. 2019 [71]
rs67153654	*FAM155A*	Diverticular disease[2]	Sigurdsson et al. 2017 [66] Maguire et al. 2018 [70] Schafmayer et al. 2019 [71]
rs4333882 rs3113037 rs875107 rs3823878	*SLC35F3* *SHFM1* *FADD* *ELN*	Diverticulosis[3]	Maguire et al. 2018 [70] Schafmayer et al. 2019 [71]
rs10519134 rs138699	*ISL2* *GTPBP1*	Diverticulosis[3]	Maguire et al. 2018 [70]
rs70862491 rs582094 rs1544387	*GPR158* *ABO* *BMPR1B*	Diverticulitis[4]	Maguire et al. 2018 [70] Schafmayer et al. 2019 [71]
rs962369 rs10472291 rs2131755 rs4871180 rs2049865 rs10120333 rs62126581 rs1802575 rs2280028 rs714724331 rs61823192 rs1888693 rs2784255 rs9856118 rs11667256 rs69493912 rs61814883 rs7098322	*BDNF* *WDR70* *CRISPLD2* *HAS2* *TRPS1* *PCSK5* *NT5C1B* *EFEMP1* *LINC01082* *DISP21* *LYPLAL1* *CACNB2* *HLX* *P2RY122* *PPP1R14A* *FAM185A* *S100A10* *SLC25A28*	Diverticular disease	Maguire et al. 2018 (UK Biobank only) [70] Schafmayer et al. 2019 [71]

SNP	Nearest Gene(s)	Associated Subtype	Study
rs75434097	*COL6A1*	Diverticular disease	Maguire et al. 2018 (UK Biobank only) [70] Schafmayer et al. 2019 [71]
rs4839715	*ENSG00000224849*		
rs1381335	*NOV*		
rs8074740	*UBTF*		
rs12293535	*CALCA*		
rs10471645	*CWC27*		
rs2470653	*EDEM1*		
rs148376933	*UNC50*	Diverticular disease	Maguire et al. 2018 (UK Biobank only) [70]
rs11934833	*ENSG00000251283*		
rs12942267	*ZBTB4*		
rs115490395	*UBL4B*		
rs10173528	*RBKS*		
rs2056544	*SCAPER*	Diverticular disease	Schafmayer et al. 2019 [71]
rs9960286	*CTAGE1*		
rs6001870	*TNRC6B*		
rs4132788	*C1QTNF7*		
rs387505	*PIAS1*		
rs34126945	*SNX24*		
rs1973232	*TIMP2*		
rs208814	*PPP1R16B*		
rs7990	*HLA-DQA1*		
rs139760870	*PLEKHA1*		
rs6714546	*LTBP1*		
rs1473813	*STARD13*		

[1]The variant in *LAMB4* has no Reference SNP cluster ID number but is a cytosine to thymine mutation at position 107738905. [2]rs67153654 in *FAM155A* was labeled as associated with diverticulitis in the Sigurdsson study but only found to be associated with diverticulosis in the Maguire study. [3]The MGI sample showed association with diverticulosis. [4]The MGI sample showed association with diverticulitis.

Microbial dysbiosis has been associated with several diseases, including inflammatory bowel disease (IBD) [77], necrotizing enterocolitis [78], and GI cancers [79] as well as non-GI diseases such as asthma [80]. SUDD shares many symptoms with irritable bowel syndrome, which is associated with decreased levels of *Coliformis*, *Lactobacilli*, and *Bifidobacteria* [81]. A decrease in Bacteroidetes has been seen in obesity, a disease which is associated with DD [82] This deficit in Bacteroidetes can be reversed with weight loss on a low calorie diet [82].

Few studies have directly evaluated the relationship between the microbiome and DD. In one, fecal samples were taken from 31 patients with acute diverticulitis and compared to samples from 25 controls [83]. Although the diversity of the two most common phyla Bacteroidetes and Firmicutes did not differ, the diversity of the Proteobacteria phylum was higher in patients with diverticulitis [83]. The most discriminative family was found to be *Enterobacteriaceae*, which includes species such as *E. coli, K. pneumoniae, and Enterobacter aerogenes*. This study suggested that the microbiome could be used to diagnose diverticulitis [83]. Other tests have evaluated mucosal samples as opposed to fecal samples to identify changes in the microbiome. One study used qualitative and quantitative PCR to identify and quantify, respectively, the most common bacteria within the genus of *Bifidobacterium* from colon mucosal samples from 21 patients with colorectal cancer, nine with diverticulitis, and four with IBD. *B. longum* was the most common species found. Compared to patients with IBD and colorectal cancer, patients with diverticulitis had higher total levels of *Bifidobacterium*, higher levels of *B. longum*, and a higher proportion of *B. longum* compared to all other *Bifidobacterium* species [84]. Studies requiring tissue suffer from a lack of appropriate controls, so one study addressed this by comparing chronically diseased diverticular tissue to non-affected adjacent tissue to identify differences in the microbiome within the same patient [85]. This study found that *Micobacteriaceae* and *Ascomycota* were more associated with diseased tissue while *Pseudomonas* and *Actinobacteria* were more associated with adjacent tissue [85]. Although the specific differences in microbiome

between normal and diseased digestive tracts are still being determined, current evidence suggests that microbial dysregulation may play a role in the development of DD.

1.6 Role of the immune system

The immune system has both innate and adaptive components. The innate immune system is the first line of response against microbial pathogens [86]. In the gut as in the rest of the body, phagocytic cells such as macrophages and dendritic cells recognize pathogenic antigens and carry out an early immune response [87]. Innate immune dysregulation, specifically altered toll-like receptor signaling, has been associated with intestinal inflammation [88]. The adaptive immune system is mediated by T and B lymphocytes, which carry out a delayed but enhanced immune response and then develop memory to pathogens that can improve the response on subsequent encounters [87]. Many of the studies on intestinal immune dysregulation have examined IBD, while only a few studies focus on the immune systems role in DD.

An unbiased study comparing sigmoid colon transcriptomes between 20 patients with a history (but not grossly active) diverticulitis and five control patients identified an upregulation of immune response pathways in diverticulitis [89]. Both the innate and adaptive immune systems were represented. This study identified four hub genes with high interactivity within the immune-associated group: *RASAL3*, *SASH3*, *PTPRC*, and *INPP5D*. These four genes were more highly expressed in the colon of diverticulitis patients compared to controls, suggesting a deregulation of the immune system even when patients' acute episode has symptomatically resolved [89].

Pro-inflammatory cytokine expression and macrophage infiltration have been shown to be associated with diverticulitis. Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) are increased in the colon of patients with history of diverticulitis compared to asymptomatic diverticulosis [90]. A study of 101 patients with diverticulitis found an increase in

CD68+CD163+ macrophages in patients with complicated sigmoid diverticulitis [91]. Additionally, a recent study of 16 patients with asymptomatic diverticulosis, eight patients with SUDD, and 14 control patients evaluated changes in mucosal immune cells from colonoscopic biopsy specimens [92]. This study found an over 70% increase in colonic macrophages in patients with diverticula, regardless of symptoms. Interestingly, tissue distant from the diverticular region was also biopsied, and found to have increased macrophages compared to controls, suggesting an overall dysregulation in the gastrointestinal immune system of patients with any DD, not just diverticulitis [92].

Although there have been various proposed mechanisms that contribute to the pathophysiology of DD, the role of each portion and the interaction between them remains unclear. This limited understanding of the pathophysiology leads to difficulty in identifying patients who will have severe disease and therefore require more aggressive management. The research presented in this thesis identifies clinical and physiologic differences between two distinct groups of patients with diverticular disease. This provides more information on the underlying basis of the disease and could lead to determinants that could help guide management.

Chapter 2

Multifocal Versus Conventional Unifocal Diverticulitis: A Comparison of Clinical and Transcriptomic Characteristics

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2.1 Abstract

Background The management of diverticulitis is compromised by difficulty in identifying patients who require surgery for recurrent or persistent disease. Here we introduce the concept of multifocal diverticulitis (MFD), characterized by multiple episodes of diverticulitis occurring at different locations within the colon.

Aims To compare clinical characteristics, success of surgical management, and colonic transcriptomes of MFD patients to patients with conventional unifocal diverticulitis (UFD).

Methods This retrospective study included 404 patients with CT confirmed diverticulitis episodes. Patients with diverticulitis seen in at least two different colonic locations were classified as the MFD group and compared to the UFD group based on number of episodes, sites of disease, family history, surgeries performed, and postoperative recurrence. RNA-seq was conducted on full-thickness colonic tissues of 10 MFD and 11 UFD patients.

Results Twenty-eight patients (6.9%) with MFD were identified. MFD patients had more diverticulitis episodes and were more likely to have positive family history, have right sided disease, require surgery, and have recurrence after surgery. All MFD patients treated with segmental resection had recurrence, while recurrence was less common in patients undergoing more extensive surgery ($P<0.001$). Using RNA-seq, we identified 69 genes that were differentially expressed between MFD and UFD patients. Significantly down-regulated genes were associated with immune response pathways.

Conclusions MFD appears to be a more severe subset of diverticulitis with a possible genetic component. Transcriptomic data suggests that MFD may be associated with alteration of the immune response.

2.2 Introduction

Diverticulosis is a common condition characterized by outpouchings in the wall of the colon. Although colonic diverticula alone are asymptomatic, they can become inflamed in diverticulitis leading to pain, abscess, and even peritonitis from free perforation. The prevalence of diverticulosis appears to have increased since the early 20th century, correlating with the increase of urbanization, especially in Western countries [17]. Currently, diverticulosis is estimated to be present in 10% of the population over 45 years old and can be found in 50-80% of people over 85 years old [5, 17]. It is estimated that less than 20% of patients with diverticulosis develop diverticulitis [5]. However, diverticulitis is the third most common gastrointestinal disorder requiring hospitalization and accounting for an estimated $2.1 billion in healthcare costs in the United States in 2009 [93].

The pathogenesis of diverticular disease appears to be based on a combination of underlying colonic anatomy, motility, diet, and inflammatory response [94, 95]. A recent meta-analysis has shown BMI is associated with increased risk of diverticular disease and increased physical activity may be a protective factor [54]. Despite the previous belief that dietary fiber is protective, current research has shown that it may actually increase risk of disease [96]. Genetic factors have long been proposed due to familial inheritance patterns [97] but now genes of interest have been identified through GWAS and candidate allele analysis [31, 66]. Ultimately, disease progression is probably mediated by both genetic and environmental factors [31].

When considering elective management of diverticular disease, individualized treatment is recommended, weighing the persistence of symptoms, frequency or severity of attacks, or presence of complications of the disease, such as obstruction or fistula [9, 98]. However, these characteristics are often only apparent after an extended period of evaluation, leading at times to a delay in definitive care for those who eventually undergo resection. The individualized

management of diverticulitis would be aided by being able to identify subsets of patients who are at higher risk of having recurrent episodes and therefore candidates for earlier and more definitive surgical management.

Our group observed that some patients who develop multiple episodes of diverticulitis have such occurrences in different locations within the colon. Some of these episodes were identified in patients after previous surgical resection, but such recurrences were remote from the vicinity of the anastomosis and thus not related to insufficient surgical resection at the initial surgery. These patients were frequently young, often had a family history, and appeared to have an overall more aggressive clinical course of disease. Such multifocal patients appeared to be a distinct subset of the broader population of diverticulitis patients. The present study therefore sought to identify the incidence and clinical characteristics of patients with evidence of MFD. Additionally, using RNA-seq, a genome-wide approach to evaluate the intestinal transcriptome, we aimed to identify pathways that differentiate patients with MFD from those with UFD. We hypothesized that MFD is a more severe form of diverticulitis with distinct transcriptomic features in the colon compared to UFD. Therefore, these patients may require a more aggressive approach to management involving a more extensive resection to prevent future episodes.

2.3 Methods

2.3.1 Clinical Comparison of MFD and UFD Patients

A retrospective cohort study was performed using patient information gathered at the Pennsylvania State University College of Medicine in Hershey, Pennsylvania. Using the diagnosis of diverticulitis by International Classification of Diseases, Ninth Revision (ICD-9) diagnosis code (562.00), patients were identified from a CT database spanning a period from

1/2/2004 to 12/23/2014. In total, data were gathered from 404 consecutive diverticulitis patients undergoing CT scanning over this ten-year period. The research was approved by the Institutional Review Board of the Penn State College of Medicine.

The electronic medical record was reviewed to examine specific characteristics of the patients identified. The following clinical characteristics were evaluated: age of disease onset, gender, race, BMI, smoking history, pandiverticulosis (diverticula proximal and distal to the splenic flexure on CT or contrast enema), anatomic sites of diverticulitis, family history of diverticulitis, number of episodes, any surgical procedures performed, and postoperative recurrence of diverticulitis. Right sided diverticulitis was defined as inflammation proximal to the splenic flexure and left sided diverticulitis was localized at or distal to the splenic flexure. Every episode of diverticulitis required CT confirmation to be counted in this study.

MFD was defined and identified as diverticulitis seen in at least two different sites of the colon, separated by at least 10 cm when observed on separate CT scans. CT scans were read initially by board certified radiologists, then by the study authors to define anatomic location of each episode. A subset of the MFD patients identified had undergone surgery between episodes of diverticulitis. For these patients, any diverticulitis recurrence seen on CT scan within 10 cm of the anastomosis (after previous resection) was considered an anastomotic recurrence (possibly due to inadequate initial resection) and were not included as MFD but were included in the UFD group instead.

Statistical analysis was performed using two-tailed Fisher's exact test and student's *t*-tests using R software with $P < 0.05$ considered significant.

2.3.2 RNA Sequencing

In total, 10 Caucasian MFD patients were previously consented to have tissue collected into the Penn State Hershey Colorectal Diseases Biobank. Eleven Caucasian UFD patients were identified as a control group, matched by age, sex, BMI, and smoking history. Tissue from all 21 patients was obtained from elective surgery performed ≥ 6 weeks after an acute episode and thus was not grossly inflamed. One MFD patient underwent two surgical resections, and in this patient only tissue from the first resection was evaluated. At the time of surgery, resected colonic tissue was immediately brought to the surgical pathology laboratory where a full-thickness section of sigmoid colon tissue was obtained and stored in RNAlater (Invitrogen, Carlsbad, CA). RNA was isolated using a trizol-chloroform and RNeasy (Qiagen, Germantown, MD) hybrid protocol as previously described [89]. RNA-seq was performed as previously reported [89]. Read quality was assessed using FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects.fastqc). FASTQ files were uploaded to the Galaxy web platform and the public server at usegalaxy.org was used to for pre-processing steps [99]. Reads were aligned using RNA STAR (Galaxy version 2.5.2b-0) [100] to GRCh37.p13 (GENCODE release 19) [101] using default settings. Aligned reads were counted using HTSeq-Count (Galaxy version 0.6.1galaxy3) [102] set to union mode with a minimum alignment quality of 10.

Reads were evaluated for differential expression using *DESeq2* [103] in R version 3.4.3 (R Software for Statistical Computing, Vienna, Austria). Genes were excluded if expression was not seen in at least 10% of the entire study cohort. Differentially expressed genes were visualized by volcano plot using the R package *plotly* [104]. Variance-stabilizing transformation (VST) of read counts was performed by *DESeq2* for hierarchical clustering. Hierarchical clustering with average linkage was performed using uncentered correlation of median centered genes and samples using Gene Cluster 3.0 [105] and visualized by Java Treeview [106]. Gene set

enrichment analysis was performed using Enrichr [107] from a candidate gene list comprised of differentially expressed genes (|log2 fold change| >1 and adjusted *P*-value <0.05).

2.4 Results

There were 404 patients identified during the study period, of which 28 (6.9%) satisfied criteria for MFD. The site of diverticulitis, interval between episodes, surgery performed (if applicable), and indication for surgery are listed for each patient in Table 2-1. One patient required radiologic guided drainage of an abscess and underwent elective resection after resolution. Four patients had evidence of perforation on CT but did not have physical exam findings or laboratory results necessitating emergent resection.

2.4.1 MFD is an Aggressive Subset of Diverticulitis

The 28 patients with MFD were compared to the remaining 376 with UFD (Table 2-2). Mean follow up after the initial episode of diverticulitis in the MFD group was 42 ± 6.5 months and in the UFD group was 72 ± 3.5 months. There were no significant differences in race, sex, BMI, or smoking history. Mean age in the MFD group was trending towards a younger age at 51 ± 2.5 versus 56 ± 0.7 in the UFD ($P = 0.063$). Pandiverticulosis, right sided diverticulitis, and family history of diverticular disease were significantly more common in MFD patients. Twenty-two of 28 (79%) MFD patients and 188 of 376 (50%) UFD patients underwent surgical management for diverticulitis (Table 2-2). The overall recurrence rate after surgery was 12 out of 210 (5.7%). Of the MFD patients, 9/22 (41%) developed an episode at a secondary location after

TABLE 2-1. Anatomic Sites of Diverticulitis and Segments Resected in MFD Patients

Patient	Initial Location	Segments Resected	Indication for Surgery	Interval* (Months)	Secondary Location^	Segments Resected	Indication for Surgery
1†	Sigmoid	Sigmoid	Recurrent episodes	156	Proximal transverse	Ascending with ileal to distal transverse anastomosis	Microperforation
2†	Distal descending	Distal descending, sigmoid	Recurrent episodes	29	Splenic flexure	Subtotal with cecal to rectal anastomosis	Recurrent episode after surgery
3†	Cecum	Terminal ileum, cecum	Right sided disease	107	Sigmoid	Total abdominal colectomy with ileorectal anastomosis	Recurrent episodes, abscess requiring drainage
4†	Sigmoid	Sigmoid	Stricture	21	Descending	No Surgery	N/A
5†	Sigmoid	Sigmoid	Recurrent episodes	102	Descending	No Surgery	N/A
6†	Sigmoid	Sigmoid	Recurrent episodes	5	Descending	No Surgery	N/A
7†	Sigmoid	Sigmoid	Recurrent episodes	32	Descending	No Surgery	N/A
8†	Sigmoid	Sigmoid	Recurrent episodes	156	Descending	No Surgery	N/A
9†	Descending	Descending	Microperforation	86	Sigmoid	No Surgery	N/A
10	Descending	No Surgery	N/A	15	Sigmoid	Descending, sigmoid	Recurrent episodes
11	Sigmoid	No Surgery	N/A	1	Descending	Descending, sigmoid	Recurrent episodes
12	Sigmoid	No Surgery	N/A	11	Descending	Descending, sigmoid	Recurrent episodes, microperforation
13	Sigmoid	No Surgery	N/A	10	Descending	Descending, sigmoid	Recurrent episodes
14	Descending	No Surgery	N/A	2	Sigmoid	Descending, sigmoid	Recurrent episodes
15	Sigmoid	No Surgery	N/A	2	Splenic flexure	Distal transverse, descending, sigmoid	Recurrent episodes
16	Descending	No Surgery	N/A	4	Sigmoid	Distal transverse, descending, sigmoid	Recurrent episodes

Patient	Initial Location	Segments Resected	Indication for Surgery	Interval* (Months)	Secondary Location^	Segments Resected	Indication for Surgery
17	Descending	No Surgery	N/A	19	Sigmoid	Distal transverse, descending, sigmoid	Recurrent episodes
18	Sigmoid	No Surgery	N/A	3	Splenic flexure	Distal transverse, descending, sigmoid	Recurrent episodes
19	Sigmoid	No Surgery	N/A	6	Splenic flexure	Distal transverse, descending, sigmoid	Recurrent episodes
20	Sigmoid	No Surgery	N/A	20	Descending	Transverse, descending, sigmoid	Recurrent episodes
21	Sigmoid	No Surgery	N/A	120	Hepatic flexure	Subtotal colectomy with cecal to rectal anastomosis	Recurrent episodes, microperforation
22	Sigmoid	No Surgery	N/A	7	Descending	Total abdominal colectomy with ileorectal anastomosis	Recurrent episodes, multiple adenomas
23	Descending	No Surgery	N/A	3	Transverse	No Surgery	N/A
24	Sigmoid	No Surgery	N/A	72	Hepatic flexure	No Surgery	N/A
25	Descending	No Surgery	N/A	22	Ascending and descending	No Surgery	N/A
26	Descending	No Surgery	N/A	35	Transverse	No Surgery	N/A
27	Descending	No Surgery	N/A	53	Sigmoid	No Surgery	N/A
28	Sigmoid	No Surgery	N/A	48	Ascending	No Surgery	N/A

*Interval represents the months between the last episode in the initial location and the first episode in the secondary location.

^The secondary location was at least 10 cm from the initial location or 10 cm from the anastomosis if surgery was performed.

†In patients 1-9 who underwent surgery to address diverticulitis in the initial location, diverticulitis only occurred in the secondary location after the initial surgery.

TABLE 2-2. Clinical Characteristics of Patient Groups

	MFD (28)	**UFD (376)**	***P*-Value**
Mean age ± SE	51 ± 2.5	56 ± 0.7	**0.063**
Male	36% (10/28)	46.8% (176/376)	0.33
Race			1.00
White	27	362	
Black or Hispanic	1	14	
Mean BMI ± SE	29.9 ± 0.9	30.7 ± 0.4	0.59
History of smoking	43% (12/28)	44% (166/376)	1.00
Mean number of episodes ± SE	2.7 ± 0.15	1.3 ± 0.03	**< 0.001**
Pandiverticulosis	50% (14/28)	24% (90/376)	**0.006**
≥ 1 episode right sided diverticulitis	25% (7/28)	2.4% (9/376)	**< 0.001**
Family history	25% (7/28)	4.8% (18/376)	**< 0.001**
Underwent surgery	79% (22/28)	50% (188/376)	**0.005**
Recurrent diverticulitis after surgery	41% (9/22)	2.1% (4/188)	**< 0.001**

Continuous variables analyzed by two-tailed Student's *t*-test and categorical variables analyzed by Fisher's exact test

their initial surgery (Patients 1-9 in Table 1). There was no evidence of an episode of diverticulitis at this secondary location at the time of the initial surgery. Thirteen patients (Patients 10-22 in Table 1) underwent surgery only after they had diverticulitis in 2 distinct locations. In this study, 8/8 (100%) MFD patients who underwent a segmental resection developed recurrent diverticulitis at a unique secondary location at least 10 cm away from the anastomotic site (Table 2-3). In contrast, of those patients undergoing an initial extended resection, only 1/13 (7.7%) developed a recurrence in a different location. Overall recurrence after segmental resection was significantly higher than after more extensive resections (8/8 vs 1/13, $P < 0.001$). Four patients underwent subtotal or total abdominal colectomy and these patients had no diverticulitis recurrences.

2.4.2 MFD Patients have a Unique Immune-Associated Transcriptomic Profile

RNA-seq is an unbiased approach to assess the transcriptome and evaluate the molecular pathways associated with disease. We identified 10 MFD patients who had been recruited into our Colorectal Disease Biobank and compared them to 11 control UFD patients in the Biobank, matched for age, sex, BMI, and smoking history. The UFD cohort utilized for RNA-seq had a significantly lower number of diverticulitis episodes as well as decreased prevalence of pan-diverticulosis, however the latter did not meet statistical significance (Table 2-4).

RNA-seq was performed from full-thickness sigmoid colon tissue obtained during sigmoid resection. Of the 17,393 protein coding genes that were evaluated, 69 (0.40%) were differentially expressed between MFD and UFD, defined as a |log2 fold change| >1 and adjusted *P*-value < 0.05 (Fig. 2-1). Of those, 43 (62.3%) genes were down-regulated and 26 (37.7%) were up-regulated. Hierarchical clustering was performed on differentially expressed genes (Fig. 2-2). This analysis led to two clusters of patients, with one

TABLE 2-3. Recurrence in MFD Based on Surgery Performed

Segments Resected	**Number of Patients**	**Recurrence**
Segmental		8/8 (100%)
Descending	1	1
Sigmoid	6	6
Terminal ileum, cecum	1	1
Extended resection		1/13 (7.7%)
Terminal ileum through transverse	1	0
Transverse through sigmoid	1	0
Distal transverse through sigmoid	5	0
Descending, sigmoid	6	1

Recurrence was significantly higher after segmental resection compared with extended resections (8/8 vs. 1/13, $P < 0.001$). As the risk for diverticulitis following subtotal/total colectomy is negligible, those 4 surgeries were not included in the analysis of segmental vs. extended resections.

TABLE 2-4. Clinical Characteristics of Patients for RNA-seq

	MFD (10)	**UFD (11)**	***P*-Value**
Mean age at diagnosis ± SE	53.5 ± 3.2	45.5 ± 4.9	0.19
Percent male	40% (4/10)	36.4% (4/11)	1.00
Mean BMI ± SE	32.7 ± 1.5	30.9 ± 2.7	0.86
History of smoking	40% (4/10)	45.5% (5/11)	1.00
Mean number of episodes ± SE	2.7 ± 0.26	1.7 ± 0.19	**0.007**
Pandiverticulosis	60% (6/10)	27.2% (3/11)	0.39

Continuous variables analyzed by two-tailed Student's *t*-test and categorical variables analyzed by Fisher's exact test

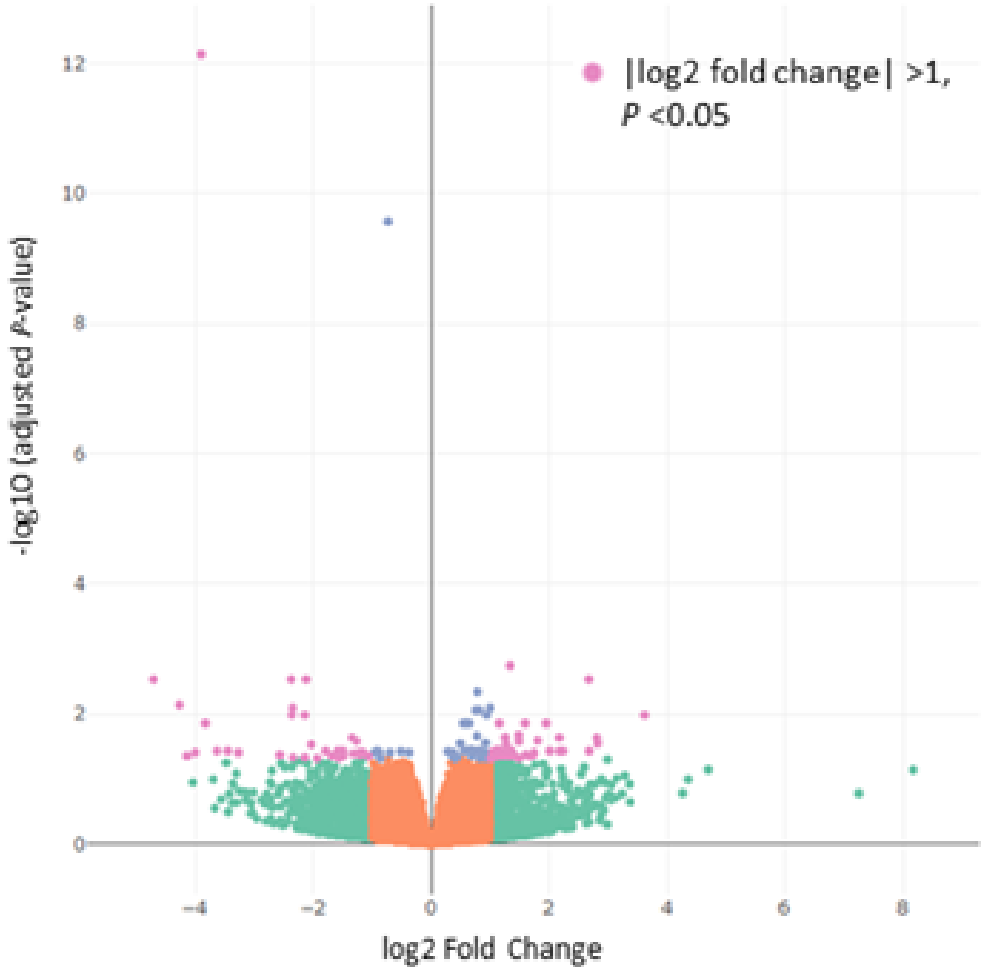

Figure 2-1: Multifocal Diverticulitis Patients Have a Unique Immune-associated Transcriptomic Profile

Volcano plot of 17,393 protein coding genes compared between multifocal diverticulitis (n = 10) and unifocal diverticulitis (n = 11). Each gene is designated by a dot with those in green indicating a |log2 fold change|>1 that was not significant, purple indicating a minor fold change with an adjusted *P*-value <0.05, pink indicating both a |log2 fold change|>1 and adjusted *P*-value <0.05, and orange are non-significant minor fold change.

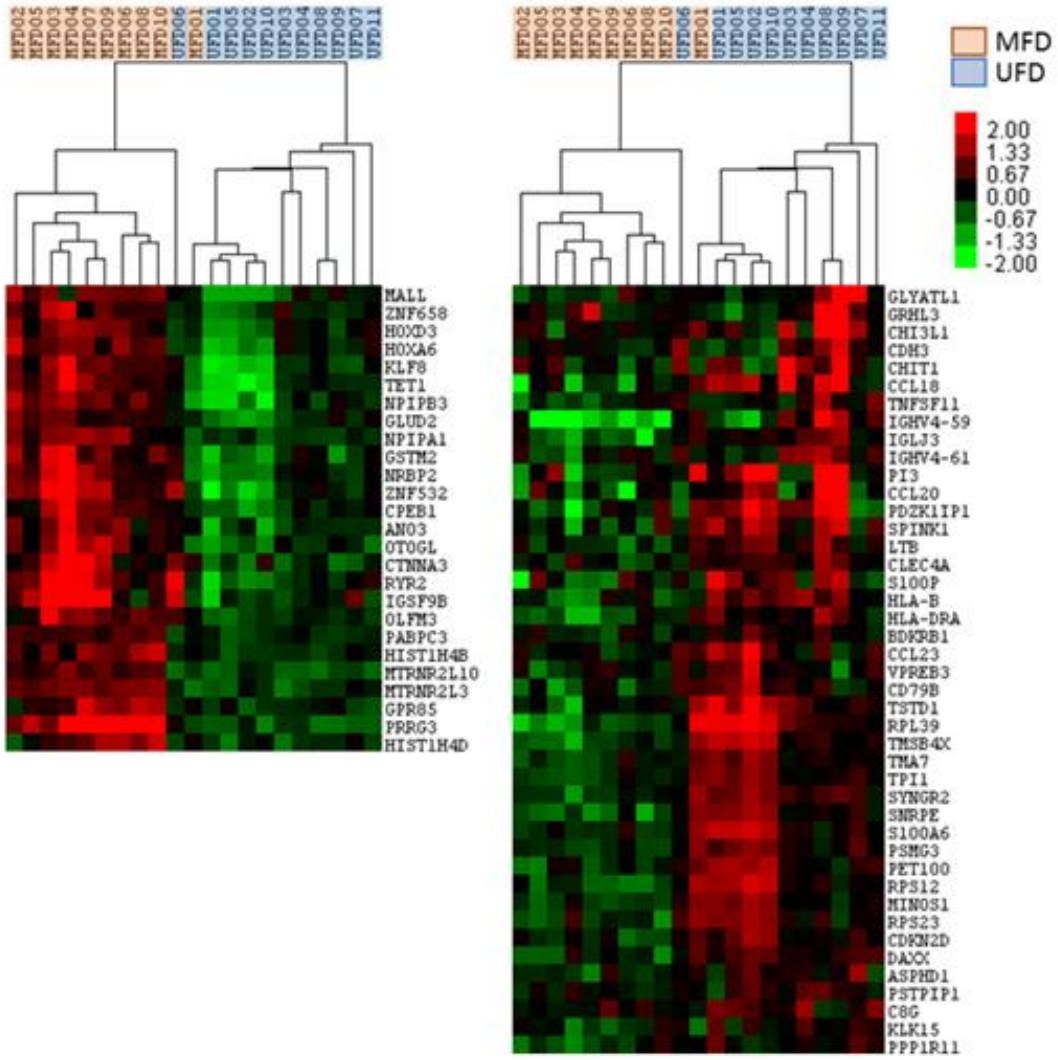

Figure 2-2: Patients Segregate by Disease Type on Hierarchical Clustering

Heatmap of 69 differentially expressed genes hierarchically clustered by average linkage of median centered genes and samples using uncentered correlation, showing segregation of UFD vs MFD patients. Red indicated high expression and green indicates low expression.

cluster of 9 MFD patients and 1 UFD patient. The second cluster was comprised of the remaining 1 MFD patient and 10 UFD patients. Gene set enrichment analysis was performed to evaluate the molecular pathways associated with MFD. Of interest to the pathophysiological mechanisms attributed to diverticular disease, MFD patients displayed a down-regulation of immune-associated genes sets (Fig. 2-3), with genes contributing to top gene sets including C-C motif chemokine ligand 18 (*CCL18),* C-C motif chemokine ligand 20 (*CCL20*), and C-C motif chemokine ligand 23 (*CCL23*). Overall, these data suggest that an altered immunological response in MFD patients may contribute to the overall risk of more aggressive diverticulitis in these patients compared to those with conventional UFD.

2.5 Discussion

Over the past decades there has been a transition to a more individualized surgical approach for diverticulitis, taking into account multiple factors including number of episodes, severity, and disease effect on lifestyle [108, 109]. The nature of these factors often requires extended follow up, which could impair quality of life in the subset that eventually undergoes surgery. The present study suggests that there is a small subset of patients with certain clinical characteristics who have a higher probability of repeated episodes and who will require more extensive surgery than a segmental colectomy due to the disease recurring at different loci. The prompt identification of such MFD patients will allow for more appropriate surgical management to prevent recurrent episodes of diverticulitis.

This study found several clinical characteristics of patients with diverticulitis that can help identify those with MFD. Patients with MFD showed a trend toward younger age of onset and more commonly had a family history of diverticulitis. They were more likely to have diverticula throughout the colon, to suffer right sided diverticulitis, to need surgery for persistent

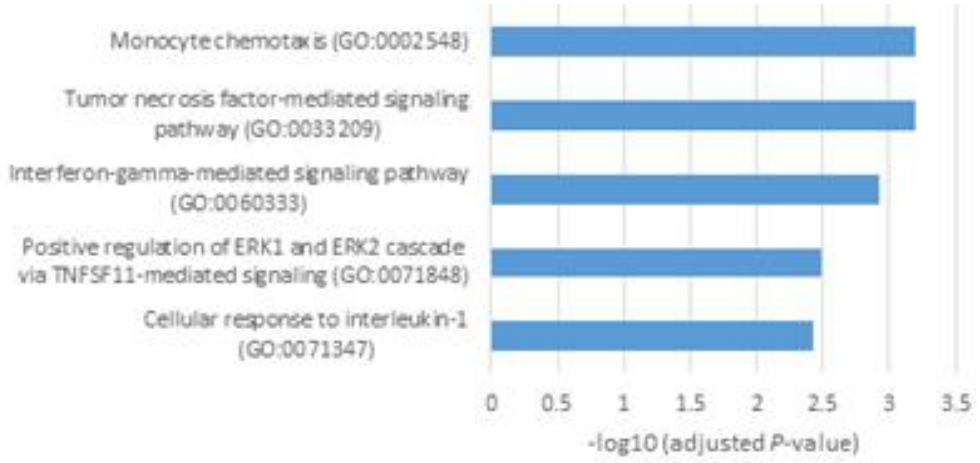

Figure 2-3: Gene Set Enrichment Analysis Shows Downregulation of Immune-Associated Sets

Gene set enrichment analysis of the 43 down-regulated genes with significant gene sets identified as adjusted *P*-value <0.05. The top 5 gene sets are shown.

symptoms, and to have a recurrence of diverticulitis after standard segmental resection. Obesity and smoking have both been shown to be environmental influences on the development of diverticular disease [44, 54], but these factors did not appear to differentiate MFD from UFD. One finding worth further study was the increased prevalence of colonic pandiverticulosis in the MFD group. There is little data in the literature on the extent of diverticulosis in patient populations. One observational study found that 72% of patients had only left sided diverticulosis, 6% right sided, and 22% on both sides [110]. In our study, 24% of patients in the UFD group had diverticula on both sides of the colon, agreeing closely with this earlier report. In contrast, pandiverticulosis was found in half of the patients with MFD, suggesting a differentiating feature.

Recurrence of diverticulitis after resection has been studied as far back as 1962, when Leigh et al. found a recurrence rate of 7% in their study cohort [111]. Since then, the cause of recurrence has been investigated several times and found to be most often related to inadequate resection. Specifically, colosigmoid anastomosis is associated with a higher rate of recurrence than colorectal anastomosis [112-114]. To our knowledge the present study is the first time recurrence of diverticulitis unrelated to inadequate resection of the sigmoid has been studied.

Many patients with MFD in our study had first undergone conventional segmental resection of the affected colon (Patients 1-9, Table 2-1). The 9 recurrences after surgery in the MFD group were all more than 10 cm away from the anastomosis and not due to inadequate sigmoid resection. There were 13 MFD patients who received more extensive resection at their first surgery due to the preoperative identification of their multifocality (Patients 10-22, Table 2-1). All patients with simple segmental resection had a recurrence, while those undergoing more extensive (extended, subtotal, or total) initial resection had a lower incidence of recurrence (8/8 vs 1/17, $P < 0.001$). Although our study size is small, these data nonetheless suggest that more extensive resection in patients identified with MFD is warranted. Whether a patient with only a

single site of disease but with other clinical factors suggesting MFD, such as pandiverticulosis or family history requires a more extensive resection after only a single site or episode of diverticulitis cannot yet be recommended, but may be a consideration in operative planning.

There has been increasing evidence for a genetic role in diverticulitis. A study evaluating 2296 twins with diverticular disease in Sweden [60] and another studying 923 twins with diverticular disease in Denmark [61] estimated a heritability of 40% and 53%, respectively. A recent GWAS identified an association between diverticular disease and intronic variants located within *ARHGAP15*, *COLQ*, and *FAM155A* [66]. However, in that study none of the variants identified appeared to affect expression of these genes or nearby genes in blood, adipocytes, or small intestinal enterocytes, leaving their exact mechanistic role in diverticulitis unclear. Our group identified a single nucleotide polymorphism located upstream of the *TNFSF15* gene associated with surgical diverticulitis [31]. *TNFSF15* is a gene involved in T-cell maturation, implying dysregulation of the immune system as a factor in the development of diverticulitis requiring surgery, a conclusion mirrored in the present study. These reports all represent a growing interest in the relationship between genetics and diverticular disease. MFD patients, by virtue of the clinical characteristics of family history, extensive diverticulosis, and earlier age of onset, may represent a unique cohort for future genetic investigation into the pathophysiology of diverticulitis.

Identifying a genetic component to disease requires a large population of patients. At the present time, we have only identified MFD in a small subset of diverticulitis patients and thus, genetic studies would be underpowered. However, using RNA-seq in an effort to differentiate between MFD and UFD, we assessed the intestinal transcriptome for underlying molecular pathways associated with disease. Although we found few genes that were differentially expressed between MFD and UFD, those that were differentially down-regulated were associated with the immune response. Our group has previously shown that conventional UFD patients have

downregulation of genes related to the nervous, muscle, and tissue systems[89]. The findings from this study suggest that MFD patients may further have a compromised immune response in addition to those changes associated with conventional diverticulitis. This may be responsible for the more aggressive disease phenotype identified clinically in these patients.

Three chemokines that mediate effects on the adaptive immune response were down-regulated amongst MFD patients, including *CCL18, CCL20,* and *CCL23*. Of interest is *CCL20*, also known as macrophage inhibitory protein 3α (MIP-3α), which functions in both homeostatic and inflammatory processes [115]. This chemokine is produced by the colonic epithelium as a chemotactic factor for C-C chemokine receptor 6 (CCR6)-expressing cells of the adaptive immune response, including immature dendritic cells and $CD45RO^+$ T cells [116]. Higher expression of CCL20 has previously been noted in inflamed human colonic tissue [116]. Not only do these chemokines suggest a pathologic basis for MFD, future studies could evaluate their use as potential biomarkers of MFD.

Our study used a strict guideline for identification of diverticulitis episodes by requiring CT confirmation of any reported episode. This created a relatively small sample size compared to what may be expected over a 10-year period at a large tertiary hospital, and contributed to a low mean number of episodes prior to surgery. This guideline may have also created a selection bias towards patients with episodes severe enough to warrant CT scanning and therefore led to a relatively high incidence of surgery in both groups. Despite these concerns, it was felt that CT confirmed episodes were necessary to determine the exact location of the inflammation, a key factor in defining and distinguishing the two study groups. This strict criterion, however, was applied uniformly to all patients in both groups to at least minimize any differential bias between the two groups. Another possible limitation could be that patients in the UFD group could subsequently develop MFD, however the longer follow up (72 months in UFD vs 42 months in MFD) mitigates this possibility.

In conclusion, we have identified a previously undescribed group of patients who are prone to recurrent bouts of diverticulitis in different colonic locations. This MFD group makes up approximately 7% of CT confirmed diverticulitis patients presenting to our tertiary care center. Clinical features of this group include early age of onset, pandiverticulosis, positive family history, and presence of right sided diverticulitis, which suggest a genetic predisposition. Using RNA-seq we have identified a potential alteration in the immune system in patients with MFD compared to those with UFD. Therefore, MFD patients have clinical and biologic features that differentiate them from conventional UFD patients. Further studies into these differences may define genetic or serum determinants that may aid in surgical decision making.

Chapter 3

Discussion

3-1 Current Diverticulitis Management

Although elective segmental resection was previously suggested after two episodes of uncomplicated diverticulitis or one episode of complicated disease [117], there has recently been a shift toward a more individualized approach to management [9]. Early surgical resection was previously recommended to prevent the need for an emergent surgery for severe disease [117, 118]. However, recent studies show that in otherwise healthy patients, recurrent episodes of diverticulitis are not associated with increased morbidity or mortality [119] and the majority of patients with complicated diverticulitis present with this at their first episode [22]. Additionally, a study of over 25,000 patients found that only 5.5% of patients who underwent medical management for diverticulitis ever required emergency colectomy and/or colostomy after initial recovery [120]. These more recent studies have lead to the shift towards more individualized management, considering multiple risk factors.

Several factors are now considered when determining whether to perform surgical resection of the diseased portion of colon. Patients who are immunosuppressed, who have collagen vascular disease, or who have chronic renal failure were found to have a five-fold higher risk of perforation after conservative management [121], so these patients should be strongly considered for surgical resection after their first episode. Currently, elective resection is recommended for patients after one episode of complicated diverticulitis (abscess, fistula, or stricture). Elective resection for patients with an episode before the age of 50 was previously recommended, but recent studies have shown no difference in severity of disease or risk of emergent surgery in younger versus older patients [122]. The majority of patients with

diverticulitis have uncomplicated episodes, and therefore other factors, such as frequency of episodes, effect of episodes on lifestyle, medical condition of the patient, or the presence of chronic symptoms, should be considered when determining whether or not to perform elective resection [9].

Unfortunately, many of these factors require an extended period of observation to identify. Since elective resection is generally avoided until inflammation has resolved from an acute episode, chronic symptoms could continue for several months before it is determined that surgical resection is necessary. Several acute episodes may occur before the pattern and severity is recognized to be lifestyle-limiting. Therefore, there would be great benefit to discovering factors that would allow identification of severe disease early, without the potential morbidity associated with a period of observation. However, our poor current understanding of the disease prevents early surgical management of the disease.

3-2 Limitations of Previous Research

Despite the prevalence of diverticulosis, the disease is still poorly understood. First, the pathophysiology associated with progression of the disease remains unclear. Previous studies have shown that diverticulosis is more common in patients with collagen vascular disorders [55-58] and that diverticulitis can be more severe in immunosuppressed patients [121]. However, identifying the presence of the disease in special populations is only the first step in identifying the disease pathophysiology. Studies that have evaluated patients with diverticulitis often suffer from a difficulty identifying adequate controls. Further investigation into diverticular disease pathophysiology is needed to help identify predictive factors of severity.

Development of an appropriate animal model for diverticular disease would be very beneficial for studying the pathophysiology of the disease. Animal models have replicated

symptoms similar to irritable bowel disease and have been used to investigate dysmotility. Post-infectious enteric neuromuscular dysfunction and hyperalgesia similar to irritable bowel syndrome has been shown in mice [123, 124]. However, attempts to develop animal models of diverticular disease itself have been limited by several factors. In the 1970s, an animal model was attempted using rabbits maintained on a low fiber diet of white bread, butter, milk, sugar, and vitamins [125, 126]. This caused constipation, increased baseline colonic pressure, and decreased motility. Stimulation of the colon with neostigmine administration in these rabbits caused temporary diverticula, but these appeared to be different than the chronic narrow-necked diverticular seen in humans [125, 126]. A follow up study found significant degradation in systemic health of the animals on the diet [127]. The largest animal study was on 1800 rats divided into 9 groups fed various amounts of fiber [128]. This study did find a correlation between fiber intake and diverticulosis, with the lowest fiber group having the highest incidence of diverticulosis. Even in this group, the incidence was 47.5%, and the first diverticula were not observed until the rats reached an age of 18 months [128]. A recent review article recommends the use of monkeys or swine as an animal model, since they are larger animals with an omnivorous diet and taenia [129]. Identification of an appropriate animal model would help test hypotheses about the development and management of diverticular disease.

Finally, current research into diverticular disease suffers from limited ability to segregate specific subsets. Diverticulitis is often separated from SUDD by the presence of inflammation, but recent studies have suggested that there may be a low level of inflammation present in SUDD [90, 92]. SUDD itself has been difficult to distinguish from irritable bowel syndrome [130]. Diverticulitis can be separated into complicated or uncomplicated disease, but this often is defined by acute episodes. It is unclear what causes a patient to develop an abscess or a free perforation during an episode as opposed to uncomplicated inflammation. Long term complications like stricture or fistula have been suggested to be related to chronic inflammation,

but there is limited evidence to support this [131]. It may be beneficial to categorize diverticulitis in a similar way to Crohn's disease. The Montreal criteria divides patients by age, location of disease, and behavior of disease [132]. Like diverticulitis, Crohn's can be penetrating, leading to abscess, perforation, and/or fistula [133]. Strictures can also develop in Crohn's disease as well [133]. Studies have shown an association between genetic mutations and severity of disease [134-136]. A similar subcategorization of diverticulitis may allow us to identify variability between patients with different complications and could potentially help identify predictive factors.

3-3 Research Included in Thesis

The research included in this thesis will benefit the study of diverticular disease in several ways. First, it identifies a distinct group of patients with MFD, who have a higher probability of repeated episodes and require more extensive surgery to address all loci of their disease. This group of patients have specific clinical characteristics including family history, pandiverticulosis, and right sided diverticulosis. The identification of these characteristics in a patient could help guide management. Additionally, the transcriptomic analysis suggests a difference in immune regulation between MFD and conventional UFD, and therefore an underlying division between the two subsets of diverticulitis.

The transcriptomic analysis also helps understand the pathophysiology of diverticular disease. A previous study by our lab comparing the sigmoid colon from patients with diverticulitis to sigmoid from controls showed dysregulation of genes associated with the immune response, nervous system, and the muscle and tissue system [89]. The addition of data associated with this more aggressive subtype further suggests the importance of the immune system in the development of diverticulitis. Differentially expressed genes involved in these pathways such as *CCL18*, *CCL20*, and *CCL23* could be further evaluated to determine their role in the

pathophysiology of disease. Additionally, by specifically measuring gene expression in the tissue, RNA-seq allows for evaluation of the interaction between genetics and the environment.

Finally, this research identifies potential biomarkers that can be used to identify MFD. CCL18, CCL20, and CCL23 are cytokines that mediate the adaptive immune response by attracting leukocytes [137]. Cytokines have been proposed as markers of disease severity. CCL18 has previously been associated with increased severity in chronic obstructive pulmonary disease [138]. CCL20 is increased in the colonic mucosa of patients with IBD [139]. Serum CCL23 levels have been associated with outcome after ischemic stroke [140]. These three cytokines could potentially identified in serum or in colonic mucosa via biopsy and be used to subcategorize diverticulitis by severity.

3-4 Future Directions

To gain a better understanding of the etiology of the disease, more effort must be put into developing an animal model. Although previous models have been limited due to the length of time and large number of animals required, these studies did show that it is possible for diverticula to develop. The previous models also involved environmental manipulation alone. The association between collagen vascular disorders and diverticular disease suggests that mutations in collagen genes could make an animal more likely to develop diverticular disease, perhaps in addition to a low fiber diet. A colon-specific genetic knockout animal could also be used to avoid systemic effects. Once developed, this model could be used to investigate the progression of diverticulosis to diverticulitis.

The three recent GWAS have provided 54 SNPs to investigate in order to determine their role in DD. Currently, the role of any of the SNPs is unclear, although several of the SNPs may fit with what we already know about the disease. Since COLQ is present in the neuromuscular

junction, the *COLQ* SNP could impact motility. The two most recent GWAS each identified several at risk SNPs in connective tissue and extracellular matrix proteins [70, 71]. For example, each found association between DD and a SNP in *COL6A1* (which encodes a collagen subunit) and a SNP in *ELN* (which encodes an elastin subunit). Further mechanistic studies are now needed to identify how these genes are involved in disease development.

Clinical studies of patients with diverticulitis could lead to the identification of genetic or laboratory markers that help predict the development of diverticulitis or segregate patients based on the severity of disease. Potential genetic markers include those previously found by GWAS and other genetic studies. Studies including more detailed information on patient disease severity may reveal SNPs that associate more with complicated diverticulitis. Laboratory markers could include cytokines identified in this study: CCL18, CCL20, or CCL23. These laboratory markers could be measured during acute episodes in an attempt to associate levels with disease severity.

There are many opportunities for further research into DD. Further evaluation of the pathophysiology is needed to understand disease development. Clinical studies are also necessary to continue identifying differences in disease presentation. Mechanistic and clinical studies can complement each other to help improve the understanding of DD. As more that is learned about risk factors and the role they play, the individualized management of DD and diverticulitis will become more effective.

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