INFLAMMATORY BOWEL DISEASES:
Genetic Markers and their Use in Predicting Disease Course, Response to Treatment, and Need for Surgery

Doctoral Dissertation

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ABSTRACT:

Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis, are well described medical conditions; however, the pathogenetic mechanisms behind them is still obscure. The three tenets of their development are environmental factors, luminal gut flora, and genetic susceptibility. While much research has been invested in deciphering the exact cause, no specific environmental factors have been found, variations in luminal gut flora have been found, and some genes have been positively identified with IBD. Certainly, much research is still needed.

This thesis consisted of two separate, yet contextually related studies that investigated the association between five genetic polymorphisms and disease behavior, response to therapy, and need for surgery, in a Hungarian IBD cohort. The specific gene variants included: *DLG5* R30Q, *MDR1* C3435T and G2677T/A, and *ABCG2* G34A and C421A. In the first study, focusing on *DLG5* R30Q, we also tested for any association with other polymorphisms in IBD susceptibility genes, *NOD2/CARD15* and *TLR4*.

In the first study, a cohort of 773 unrelated IBD patients (CD: 639, UC: 134) and 150 healthy controls were genotyped for *DLG5* R30Q, *TLR4* D299G, and *NOD2/CARD15* SNP8, SNP12, and SNP13. *DLG5* and *TLR4* variants were detected using polymerase chain reaction/restriction fragment length polymorphism, while *NOD2/CARD15* mutations were detected using denaturing high-performance liquid chromatography. In the second study, *MDR1* and *ABCG2* polymorphisms were detected using real-time polymerase chain reaction with the LightCycler equipment. The protocol was developed specifically for this study. The study's population consisted of 414 unrelated IBD patients (CD: 265, UC: 149) and 149 healthy control subjects.

There were no significant differences in carriage frequencies of *DLG5*, *ABCG2* or *MDR1* variants. However, a trend was observed for the *MDR1* G2677 allele to be associated with disease susceptibility in CD patients. The two *MDR1* variants were linked to each other in IBD, CD, UC and controls. The *MDR1* 2677TT was associated with 3435CC, 2677GT with 3435CT, and 2677GG with 3435TT. In ulcerative colitis, carriage of the *ABCG2* allele appeared to be protective against arthritis. The *DLG5* R30Q variant was significantly associated with steroid resistance. Perianal disease and frequent relapses were independently associated with steroid resistance. While no genotype-phenotype associations could be made, a trend for the *DLG5* variant was observed in extensive UC disease.

PUBLICATIONS DIRECTLY RELATED TO THIS THESIS:

1. Lakatos PL, **Fischer S** (joint first authors), Claes K, Kovacs A, Molnar T, Altorjay I, Demeter P, Tulassay Z, Palatka K, Papp M, Rutgeerts P, Szalay F, Papp J, Hungarian IBD Study Group, Vermeire S, Lakatos L. DLG5 R30Q is not associated with inflammatory bowel disease in Hungarian IBD patients, but predicts clinical response to steroids in Crohn's disease. *Inflamm Bowel Dis*. 2006;12:362-368.

2. **Fischer S**, Lakatos PL, Lakatos L, Kovacs A, Molnar T, Altorjay I, Papp M, Szilvasi A, Tulassay Z, Osztovits J, Papp J, Demeter P, Schwab R, Tordai A, Andrikovics H. The ATP-binding Cassette Transporter ABCG2 (BCRP) and ABCB1 (MDR1) variants are not associated with disease susceptibility and disease phenotype in Hungarian patients with Inflammatory Bowel Diseases. *Scand J Gastroenterol*. 2007;42:726-733.

3. Lakatos PL, **Fischer S**, Lakatos L, Gal I, Papp J. Current concept on the pathogenesis of IBD: Crosstalk between genetic and microbial factors. Pathogenic bacteria, altered bacterial sensing or changes in mucosal integrity take "toll"? *World J Gastroenterol*. 2006;12:1829-1840.

ÖSSZEFOGLALÓ

A gyulladásos bélbetegségek (IBD) két nagy csoportja, a Crohn betegség (CD) és a colitis ulcerosa (UC), mára már jól körülírt klinikai entitásnak számítanak, bár a patomechnizmusukat illetően még nem teljesen pontosak az ismereteink. A kórfolyamat elindításában szerepet játszó tényezők három nagy kategóriába sorolhatóak: környezeti faktorok, bélflóra összetétele és genetikai hajlam. Számos kutatás igyekezett feltárni a megbetegedések pontos okát. Ezek eredményeként ismerté váltak bizonyos IBD-vel összefüggést mutató gének, a bélflóra egyes jellemző variációi. Nem találtak azonban egyetlen specifikusnak mondható környezeti tényezőt sem. További kutatások szükségesek a részletek minél pontosabb megismeréséhez.

Az értekezés két különálló, de tartalmilag kapcsolódó tanulmány anyagát foglalja össze. A magyarországi IBD-populációban vizsgáltuk öt genetikai polymorfizmus és a klinikum kapcsolatát. Elemeztük az összefüggésüket a betegség lefolyásával, a terápiás válaszkészséggel és a műtéti igénnyel. A *DLG5* R30Q, *MDR1* C3435T és G2677T/A, valamint *ABCG2* G34A és C421A variáns allélek gyakoriságát vizsgáltuk. A *DLG5* R30Q-ra koncentráló első vizsgálatban, más ismert IBD-re hajlamosító gének (*NOD2/CARD15* és *TLR4*) polimorfizmusaival fennálló lehetséges kapcsolatot is kerestünk.

Az első vizsgálatban 773 (CD: 639, UC: 134) IBD-ben szenvedő betegben határoztuk meg a *DLG5* R30Q, a *TLR4* D299G és a *NOD2/CARD15* SNP8, SNP12, illetve SNP13 variánsok jelenlétét. A *DLG5* és *TLR4* polimorfizmusokat PCR/RFLP módszerrel határoztuk meg, míg a *NOD2/CARD15* mutációk gyakoriságát HPLC-vel vizsgáltuk és szekvenálással erősítettük meg. A második tanulmányban 414 rokoni kapcsolatban nem álló, IBD-s beteget vontunk be (CD: 265, UC: 149). Náluk az *MDR1* és a *ABCG2* polymorfizmusát vizsgáltuk real-time PCR technikával LightCycler készülékkel, melyhez a használt módszert magunk fejlesztettük ki.

Nem volt szignifikáns különbség a betegcsoportok és a kontorll egyének között a *DLG5*, *ABCG2* illetve az *MDR1* variánsok hordozási gyakoriságában. Tendenciaszerűen gyakoribb volt az *MDR1* G2677 aléll Crohn betegségben. Az *MDR1* két variánsa minden vizsgált csoportban kapcsolatot mutatott egymással: az *MDR1* 2677TT és 3435CC, a 2677GT és 3435CT, továbbá a 2677GG és 3435TT között egyaránt. Colitis ulcerosában az *ABCG2* allél hordozói között kisebb arányban fordult elő arthritis. A *DLG5* R30Q variáns jelenléte szignifikáns összefüggést mutatott a szeroid rezisztenciával. A perianális lokalizáció és a gyakori relapszusra való hajlam is összefüggésben álltnak a szteriod rezisztenciával. Végezetül, tendenciaszerűen gyakoribb volt a variáns *DLG5* allél kiterjedt UC esetén.

A TÉZISHEZ KÖZVETLENÜL KAPCSOLÓDÓ PUBLIKÁCIÓK:

1. Lakatos PL, **Fischer S** (joint first authors), Claes K, Kovacs A, Molnar T, Altorjay I, Demeter P, Tulassay Z, Palatka K, Papp M, Rutgeerts P, Szalay F, Papp J, Hungarian IBD Study Group, Vermeire S, Lakatos L. DLG5 R30Q is not associated with inflammatory bowel disease in Hungarian IBD patients, but predicts clinical response to steroids in Crohn's disease. *Inflamm Bowel Dis*. 2006;12:362-368.
2. **Fischer S**, Lakatos PL, Lakatos L, Kovacs A, Molnar T, Altorjay I, Papp M, Szilvasi A, Tulassay Z, Osztovits J, Papp J, Demeter P, Schwab R, Tordai A, Andrikovics H. The ATP-binding Cassette Transporter ABCG2 (BCRP) and ABCB1 (MDR1) variants are not associated with disease susceptibility and disease phenotype in Hungarian patients with Inflammatory Bowel Diseases. *Scand J Gastroenterol*. 2007;42:726-733.
3. Lakatos PL, **Fischer S**, Lakatos L, Gal I, Papp J. Current concept on the pathogenesis of IBD: Crosstalk between genetic and microbial factors. Pathogenic bacteria, altered bacterial sensing or changes in mucosal integrity take "toll"? *World J Gastroenterol*. 2006;12:1829-1840.

ABBREVIATIONS:

5-ASA:	5-aminosalicylic acid
6-MP:	6-mercaptopurine
ABC:	ATP-binding cassette
ACCA:	Anti-chitobioside carbohydrate antibodies
ALCA:	Anti-laminaribioside carbohydrate antibodies
AMCA:	Anti-mannobioside carbohydrate antibodies
ASCA:	Anti-*Saccharomyces cervisiae* antibodies
ATG16L1:	Autophagy 16-like gene 1
AZA:	Azathioprine
BCRP:	Breast Cancer Resistance Protein
CARD:	Caspase activated recruitment domain
CD:	Crohn's disease
CDAI:	Crohn's disease activity index
CRC:	Colorectal cancer
CRP:	C reactive protein
DLG5:	Discs large Homolog 5
EIM:	Extraintestinal manifestation
GWAS:	Genome-wide association scan
HLA:	Human leukocyte antigen
IBD:	Inflammatory bowel disease
IC:	Indeterminate colitis
IL:	Interleukin
IRF5:	Interferon regulatory factor 5
IRGM:	Immunity-related guanosine triphosphatase
LPS:	Lipopolysaccharide
LRR:	Leucine-rich repeats
MDP:	Muramyl dipeptide
MDR1:	Multidrug resistance gene
NAFLD:	Non-alcoholic fatty liver disease
NASH:	Non-alcoholic steatohepatitis
NF-κB:	Nuclear factor κB
NOD:	Nucleotide oligomerization domain
OCTN:	Organic cation transporter
PAB:	Pancreatic antibodies
PAMP:	Pathogen associated microbial patterns
(p)ANCA:	(perinuclear) anti-neutrophil cytoplasmic autoantibody
PGN:	Peptidoglycan
PSC:	Primary sclerosing cholangitis
PTGER4:	Prostaglandin receptor EP4
SLC:	Solute carrier
SNP:	Single nucleotide polymorphism
T_h:	T helper cell
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TPMT:	Thiopurine methyltransferase
UC:	Ulcerative colitis

1. EPIDEMIOLOGY

The field of epidemiology studies, along with various other aspects of population, **incidence**, **prevalence**, and **mortality rates**. The incidence of a disease or condition is defined as the proportion of a population or group, initially free of a condition or disease, that develops it over a set period of time, thus these are new cases. On the contrary, prevalence refers to the fraction of a population or group which is already afflicted by a given condition or has reached a clinical outcome at a stated time point.[1] While these terms comprise the basics of epidemiology, IBD studies have come a long way from merely valuating these terms. Nowadays, they attempt to determine the prognostic outcome as well as various factors that contribute to the disease process.[2]

In his paper, Binder describes three factors that are required for the completion of a **successful and useful epidemiological study**. First, the investigated population must be well-described. Next, the regional healthcare system must be organized in such a way as to allow all patients in the area access to health care and be traceable. Finally, the healthcare system should offer diagnostic and therapeutic facilities that will be able to exclude infections as well as other differential diagnoses of IBD.[2] Taking these requirements into consideration provides an explanation for the reason that the majority of studies are carried out in Western countries, where a public health system is well-rooted. Nonetheless, this chapter will examine epidemiological data from around the globe, starting from the Americas and moving in an eastward direction to Western and Eastern Europe, the Middle East and Africa, Asia and southward to New Zealand.

In the **United States**, approximately **1.4 million people** suffer from **IBD**, with 30 000 new cases diagnosed annually.[3] In a study spanning several decades, Loftus and colleagues followed the epidemiological features of IBD in Olmsted County, MD.[4] Between 1940 and 2000 there were significant increases in both CD and UC. In particular, **CD** has increased before the 1970s, while **stabilizing** later at ~ 8 cases per 100 000 person-years. In the case of **ulcerative colitis**, **stabilization in incidence** was also observed after the 1970s, at 9 cases per 100 000 person-years. Overall, the incidence rates (age- and sex-adjusted) of CD and UC in Olmsted County during the study period were 6.3 and 8.1 cases per 100 000 person-years, respectively. The prevalence was calculated as of January 1, 2001 and resulted in 214 and 174 cases per 100 000 persons for UC and CD, respectively. Comparing these results to measurements taken in 1991, CD's prevalence increased by 31%, while the prevalence of UC remained rather stable.[5]

A Canadian group[6] studied the epidemiological characteristics of IBD in five provinces, Alberta (AB), British Columbia (BC), Manitoba (MB), Nova Scotia (NS), and Saskatchewan (SK) between 1998 and 2000. Using provincial health insurance databases, they accessed physician billing claims and hospital discharge summaries to create an IBD database for each province, thus enabling them to calculate incidence and prevalence ratios for each. Their demographic choice presented populations from the Pacific (BC) and Atlantic (NS) Coasts, as well as the country's central part, the prairies (AB, SK, MB). The incidence and prevalence data from these five provinces are presented in Table 1.

Table 1. Incidence and Prevalence Rates from Five Canadian Provinces[6]

	Crohn's disease		**Ulcerative colitis**	
Province (East → West)	*Incidence*[a] *(per 100 000)*	*Prevalence*[a] *(per 100 000)*	*Incidence*[a] *(per 100 000)*	*Prevalence*[a] *(per 100 000)*
Nova Scotia	20.2	318.5	19.2	247.9
Manitoba	15.4	271.4	15.4	248.6
Saskatchewan	13.5	263.8	10.4	234.3
Alberta	16.5	283.0	11.0	185.0
British Columbia	8.8	160.7	9.9	162.1
Total	13.4	233.7	11.8	193.7
Total (without BC)	12.9	279.2	12.9	211.2

[a] Adjusted to the total population of the five investigated provinces.

Interestingly, while BC's ethnic composition is slightly different from the rest of Canada, it also presented the lowest incidence and prevalence rates of all five provinces. Subsequently, the total values were calculated both with and without BC's data.[6]

In **Latin America**, few studies have been conducted in this predominantly Hispanic population. A recently published study found **increasing incidence rates** in a population limited to the Southwest of Puerto Rico. The group identified 202 cases of IBD, comprised of 48 CD, 102 UC, and 52 indeterminate colitis (IC) patients. Over a 5-year period (1996-2000), the group noticed a four-fold increase in both, CD and IC incidence rates, and an almost doubled UC incidence rate. The incidence rate of Crohn's disease increased from $0.49/10^5$ in 1996 to $1.96/10^5$ in 2000, while that for indeterminate colitis rose from $0.61/10^5$ to $2.46/10^5$

during the same time period. Ulcerative colitis had a 1.7-fold increase from $1.96/10^5$ to $3.32/10^5$. The study's IBD prevalence rate was calculated to be 24.81 cases per 100 000.

In 1988, a large scale experiment was initiated throughout the **European Union** that saw the collection of data from 2201 IBD patients aged 15-64 between the years 1991-1993. It was termed the European Collaborative study on IBD (EC-IBD). From this total, 706 patients suffered from Crohn's disease and 1379 had ulcerative colitis. A further division was performed based on a predetermined geographical axis whereby the **Alps** served as the **"equator"**. A hypothesis was made that a North-South axis for IBD existed and the incidence rates in the north were higher. In addition, Table 2 summarizes the mean values of IBD based on the total cases as well as North-South distribution for CD and UC.[7]

Table 2. Incidence Rates as Calculated by the EC-IBD[7]

	Crohn's disease	**Ulcerative colitis**
Mean Incidence	$5.6/10^5$ per year	$10.4/10^5$ per year
Centers north of the Alps:		
Mean Incidence	$7.0/10^5$	$11.8/10^5$
Centers south of the Alps:		
Mean Incidence	$3.9/10^5$	$8.7/10^5$

Overall, **no axis was found**. This was in spite of the finding that the average incidence rate of UC was 40% higher, and CD 80% higher, in the northern centers. This can be supported by the following findings:

- The highest incidence of UC was found in Iceland ($24.3/10^5$), while the second highest value was recorded in Crete, Greece ($16.6/10^5$).
- The highest incidence rates of CD were detected in Holland and France, $9.2/10^5$ in each.

Two explanations were proposed for this failed "axial" hypothesis. The first refers to the possibility that areas with high incidence rates maintained their levels at a steady plateau while areas with lower incidence rates saw an increase in the incidence of IBD. The second maintains that predominant levels of UC are being matched or replaced by higher incidence levels of CD.[8] Farrokhyar et al.[9] have also concluded, based on the EC-IBD report, that no

axis of incidence can be identified, noting that Italy, Greece, and Northern Spain displayed IBD incidence rates that were as high or higher than countries located further north.

Table 3 presents data collected over several decades from European as well as North American studies. Examining the results, one reaches the conclusion that **UC incidence rates are higher than CD in European locations**, while being equal in North America.

Table 3. Incidence Rates in Select European and North American Locations (per 100 000)

Location	Year	Ulcerative colitis	Crohn's disease
Copenhagen, Denmark[7]	1991-1993	9.8	7.3
Florence, Italy[7]	1991-1993	8.7	3.3
Granada, Spain[10]	1979-1989	2.0	0.9
Leicestershire, UK[7]	1991-1993	10.0	3.8
North Tees, UK[11]	1995	22.1	7.36
Oslo, Norway[7]	1991-1993	15.6	7.9
Palermo, Italy[7]	1991-1993	11.0	6.6
Veszprem, Hungary[8]	1977-2001	5.89	2.23
Manitoba, Canada[12]	1989-1994	14.3	14.6
Minnesota, USA[4]	1940-2000	♂: 9.8, ♀: 6.5	♂: 6.7, ♀:6.1

In a study restricted solely to **France**, and **based on national health insurance data**, Nerich et al.[13] determined a **North-South gradient for CD** within the country. According to their geographical mapping, the axis was drawn in the middle of France. A similar gradient was not observed for UC. They noted two peak frequencies for CD, in the 20-24 years old age group and 75-79 years old. The overall incidence rate was $8.2/10^5$ for CD (men: $7.1/10^5$; women: $9.4/10^5$). In UC, with an overall incidence rate of $7.2/10^5$ (men: $7.7/10^5$; women: $6.8/10^5$), the peak was observed in the group of 30-34 years old. Thereafter, a steady decline was observed.

Incidence rates can also be used to reflect IBD's gender distribution, with respect to age. Based on the EC-IBD, it was found that both **UC and CD** have a **peak onset during late adolescence or early adulthood** while in some areas (UK, Scandinavia, USA) a second peak is seen in the age group of 50-80 years.[14,15,16,17] A more detailed inspection of data shows a peak incidence for UC in women between 25-34 years of age, followed by a rapid decline.

However, men also show a continuous risk at the older age groups. In CD, both genders show peak incidence in the age group of 15-24 years and a decline thereafter.[2]

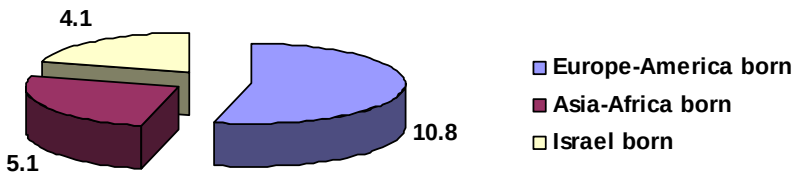
Several research groups have examined the influence of **race and ethnicity** on the development of IBD, hence its incidence, in various geographic areas. A hospital-based study was conducted in Cape Town, South Africa, with data collected between 1970 and 1980, while a second, population-based data set was collected in 1980-1984. Both studies suggested that there is a higher incidence of IBD in the Jewish population as opposed to non-Jews and is uncommon among Blacks.[18,19,20] The results are shown in Table 4.

Table 4. Incidence of IBD in Cape Town, SA; Based on Race and Ethnicity (per 10^5)

	Study period	Ulcerative colitis	Crohn's disease
Hospital-based[18,19]	1970-1980	Jews (8.5-10.4)	Jews (5.0-7.2)
		Whites (2.1-2.4)	Whites (0.9-1.2)
		Colored (1.3-1.6)	Colored (0.4-1.3)
Population-based[20]	1980-1984	Jews (17.0)	Jews (10.4)
		Whites (5.0)	Whites (2.6)
		Colored (1.9)	Colored (1.8)

A separate study[21] involving only permanent residents of Israel examined the incidence of UC in individuals of various ethnic backgrounds. The study lasted from 1961 until 1985, and investigated three ethnic subgroups, those born in Europe-America, Asia-Africa, and Israel. Of importance, the incidence rate of the Europe-America subgroup was significantly higher than for the other two. The results are shown in Figure 1 as incidence rates per 10^5 inhabitants of various backgrounds.

Figure 1. Incidence of UC per 100 000 in Israeli Jews of Various Decent (1961-1985)[21]



Another study conducted in northern Israel between 1965 and 1994 attempted to reveal race and ethnic correlation to IBD by comparing the incidence rates between Israeli Jews and Arabs.[22] **Ulcerative colitis was found to be 2.5 times greater in Israeli Jews than**

in Arabs. Two possible explanations were suggested, the first claiming that lifestyle differences may attribute to this inequality. In the last two decades, the Arab population adopted a more Western lifestyle, as opposed to the traditional agricultural lifestyle, possibly contributing to an increase in UC incidence. The second explanation proposed that genetic differences were to blame for the higher incidence of UC in the Israeli Jews; however, this was not clearly examined. Finally, more recent reports raised the possibility that IBD-associated genes seem to occur more frequently in Ashkenazi Jews.[23]

Over the past several years, some new data has emerged from East Europe. In **Veszprem**, a western province of **Hungary**, Lakatos et al.[8] examined the incidence of IBD during a **25-year period** from 1977 to 2001. The raw data displayed 560 new cases of UC and 212 new cases of CD. The **incidence rate of UC increased** from 1.66 to 11.01 (6.6-fold increase), while that of **CD increased** from 0.41 to 4.68 (11.4-fold increase) per 100 000. Additionally, they investigated the localization of UC and CD, the results of which are displayed in Figures 2 and 3. In **Croatia**, the earliest study to examine the incidence and prevalence of IBD in the 1980s revealed a steady rate of CD and UC. For the former, the incidence rate was $0.7/10^5$, while the latter's was $1.5/10^5$ inhabitants.[24,25] Interestingly, in a separate, long-term study conducted during 1973-1994, an increase in the incidence of Crohn's disease was noted. In particular, it increased from $0.34/10^5$ in 1973 to $3.47/10^5$ inhabitants in 1994.[26] From **Romania**, a single study was published on the epidemiology of inflammatory bowel disease spanning a single year, from June 2002 until June 2003. Certainly, limitations are inherent in epidemiological studies of such short duration. Nonetheless, using data from 18 secondary and tertiary centers, the authors calculated an incidence rate of $0.97/10^5$ for UC and $0.50/10^5$ for CD.[27]

Figure 2. Localization of UC in Veszprem Province, Hungary (1977-2001)[8]

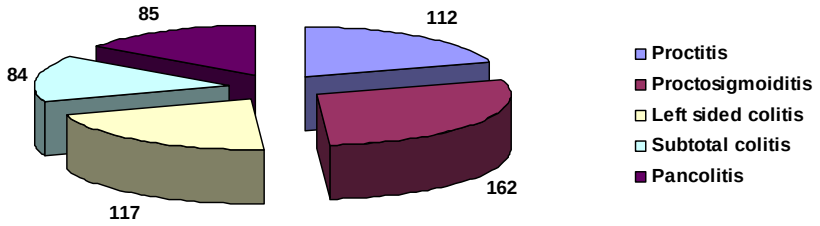
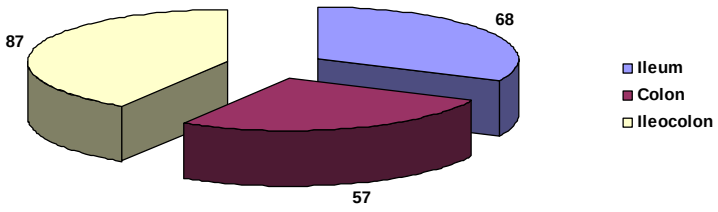


Figure 3. Localization of CD in Veszprem Province, Hungary (1977-2001)[8]



Figures 4 and 5 intend to illustrate the incidence distribution across Europe. However, the drawback behind this approach is the use of average values, which may not be representative.[28]

Shifting our focus to the Southern Hemisphere, Gearry and colleagues present data on the incidence of Crohn's disease in **Canterbury, New Zealand**.[29] Using extensive recruitment methods, 1420 IBD patients were selected, of which 715 were diagnosed with CD, 668 with UC, and 37 were diagnosed with IC. The incidence rates were calculated over a period of two years, 1994-1995, concluding that the overall IBD incidence rate was 25.2/100 000/year, while the rates for CD and UC were 16.5 and 7.6, respectively. The point-prevalence values, as calculated on June 1, 2005, were 308.3, 155.2, and 145.0/100 000 for IBD, CD, and UC, respectively. The value of this study lies in its wide breadth as a population-based study within the well-defined geographical area of Canterbury.

Figure 4. Incidence of CD in Europe, per 100 000[28]

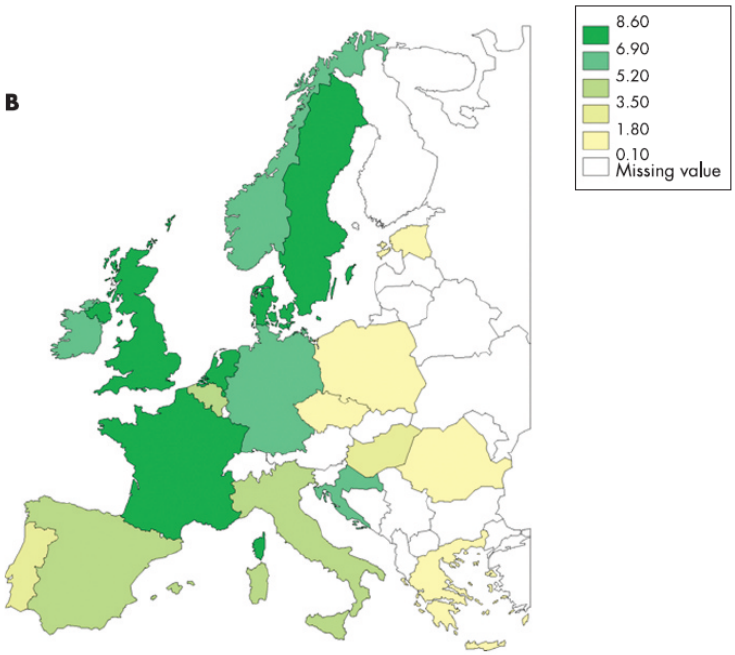
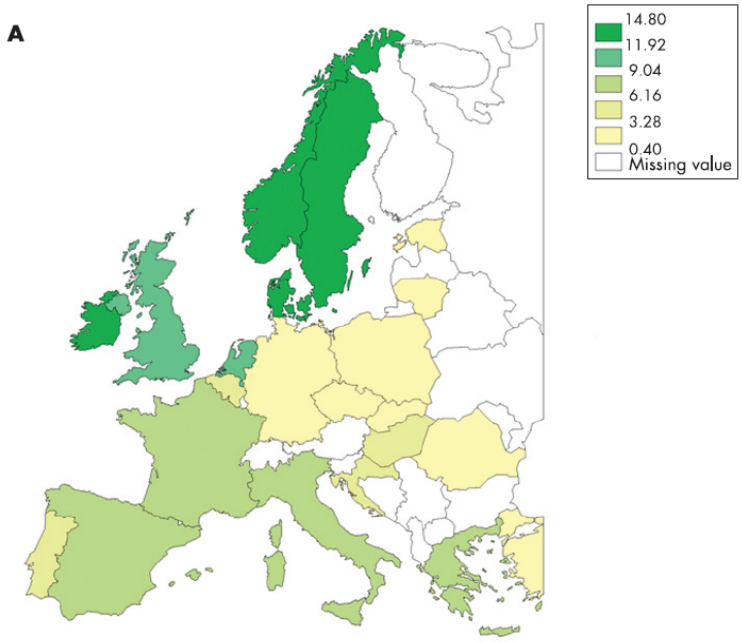


Figure 5. Incidence of UC in Europe, per 100 000[28]



Until several years ago, it has been believed that IBD is uncommon in **China**. However, in the past 15 years, there has been an **increase of at least 3-fold in the prevalence of ulcerative colitis**. Nonetheless, the rates are still not as high as those observed in Northern Europe and North America.[30] One must question the reasons behind this sharp increase. Some suggestions made by the authors, who analyzed 1560 articles and 10 218 cases, included greater awareness, better health care, improved diagnostics as well as possible changes in lifestyle and diet. In another study, Jiang et al. supported these results after finding an approximately 4-fold increase in the prevalence of IBD since the early 1990s.[31]

A chronic disease or one where survival is prolonged due to improved treatment will show an increase in the prevalence. The main problem in determining prevalence values for IBD is its chronicity combined with the ability to enter a lengthy remission period free of clinical signs. However, such patients still need to be included in the prevalence figure as they generate various monitoring and examination costs.[9] Table 5 presents prevalence data from selected European and North American locations. The overall indication is that UC is much more prevalent than CD.

Table 5. Prevalence of IBD in Europe and North America (100 000 person-years)

Location	Year	Ulcerative colitis	Crohn's disease
Copenhagen, Denmark[32]	1987	161	NA
Copenhagen, Denmark[33]	1987	NA	54
Florence, Italy[34]	1992	121	40
Granada, Spain[10]	1989	21	9
North Tees, UK[11]	1995	268	156
Veszprem, Hungary[8]	1991	59.2	17.1
Veszprem, Hungary[8]	2001	142.6	52.9
Manitoba, Canada[12]	1994	169.7	198.5
Minnesota, USA[4]	2001	213.9	173.8

NA – not assessed

Studying the epidemiology of IBD is an important aspect to investigating possible etiological bases for IBD through genetics, race, ethnicity, or environmental exposure in any given society. Nonetheless, **IBD-associated mortality** is also an important aspect of the disease for providing a better understanding and perhaps preventing fatal complications. In their study examining three regional centers in the UK, Farrokhyar et al. determined that while the mortality rates in IBD patients are not greater, the risk increases with age, comorbidity, and severity of disease.[35]

During a period of 65 years, Jess et al. included 692 patients in their long-term study of survival and cause-specific mortality. On average, the patients were followed for 14 years. They concluded that **Crohn's disease patients were more likely to die from non-malignant gastrointestinal causes** (standardized mortality ratio [SMR]: 6.4), such as complications from fistulizing disease or short bowel syndrome, and chronic obstructive pulmonary disease (SMR: 4.7).[36] Similar to a study conducted in Copenhagen County, Denmark, which found an SMR of 1.3,[37] the authors noted a standardized mortality rate of 1.2 for CD patients. Interestingly, **ulcerative colitis patients experience a lower risk for cardiovascular-related death** (SMR: 0.6), yet perforations, intestinal hemorrhage or metastatic colorectal cancer were blamed for patients' deaths.[36] Table 6 presents additional statistical data representative of European and North American centers.

Table 6. Standardized Mortality Ratios for IBD from Europe and North America

Location	Year	Ulcerative colitis	Crohn's disease
Copenhagen, Denmark[38]	1962-87	1.06	NA
Copenhagen, Denmark[39]	1979-85	NA	1.32
Florence, Italy[40]	1978-92	0.62	1.36
Stockholm, Sweden[41]	1955-90	1.37	1.51
Olmsted, Minnesota, USA[42]	1940-2004	0.82	1.2

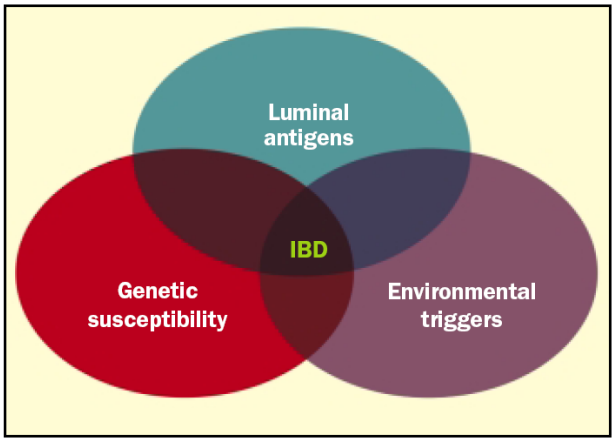
NA - Not assessed

A population-based cohort study carried out by Card et al.[43] concluded **that IBD leads to a slight increase in mortality among those afflicted**. The greatest hazard ratio (1.79) was for **UC in the 40-59 years age group**, while for **CD** the greatest hazard ratio equaled 3.82 in the younger **age group of 20-39 years**. The overall figures were as follows: 1.54 for all IBD, 1.44 for UC, and 1.73 for Crohn's disease. This further affirms previous observations of greater mortality being associated with Crohn's disease.

2. ETIOLOGY & PATHOGENESIS

Inflammatory bowel disease is a **multifactorial disease** involving **genetic susceptibility**, the **host's immune response**, and **environmental factors** (Figure 6).[44,45] It results from improper activation and maintenance of the mucosal immune system that is continuously thrown off balance by the natural bacterial flora.[46] Considering the focus of this work was on the genetics of IBD, the background information and literature review on the genes involved will be discussed at great length in the Discussion section. In this Chapter, only the host and environmenal factors will be detailed.

Figure 6. Multifactorial Involvement in IBD Pathogenesis[45]



The intestinal epithelium, aside from having the capacity to absorb nutrients, also serves as a protective barrier composed of various proteins forming tight junctions between cells. Data have been collected to show that some cells outside the lumen, specifically dendritic cells, possess processes that are able to extend through the tight junctions. It is believed that these cells "taste" the luminal content and elicit an immune response, if necessary. It is known that the intestinal epithelium has the capability to serve as an antigen-presenting cell, possessing both MHC class I and II molecules. Recent studies discovered that the epithelial cells also possess pattern recognition receptors, a group that includes intracellular NOD receptor proteins and the membrane-bound Toll-like receptor (TLR) family.[47]

2.1. Intestinal Permeability

As previously mentioned, the **intestinal epithelium has a protective function** resulting from tight junctions between the cells. Keeping this in mind, some cells, in

particular **dendritic cells**, are able to extend processes through the tight junctions, sampling the luminal milieu, and eliciting an immune response, as needed.[47]

An interesting observation was made with regard to the unity of this barrier in CD patients and even more so, their first-degree relatives. It was noted that there is an **increased permeability** in the intestines of these patients. Additionally, their **first-degree relatives**, without any signs and symptoms of CD, **showed a similar increase**.[48] This brings forth the idea that an increase in the epithelium's permeability may play a role in the pathogenesis of IBD. Certainly, increased permeability has been observed in patients prior to a flare-up, signaling the presence of a state of subclinical disease.[49]

It was also discovered that some first-degree relatives who were administered acetylsalicylic acid responded with increased epithelial permeability.[50] In some cases of CD, this may serve as a causative agent for a flare-up. The association between increased permeability and CD has offered the possibility of an exaggerated recognition of antigens, leading to an overactive immune response - as observed in CD. Additionally, this has been systematically observed by an exaggerated level of circulating, mature B cells.[51]

2.2. Intestinal Flora

For many years, researchers have been examining the **intestinal milieu** for clues over its role in the pathogenesis of IBD, without much success. In recent years, however, several advances have been made. First, one study described a **considerable decrease in the amount of anaerobic bacteria and *Lactobacillus* in active IBD cases**, in contrast to inactive cases, which showed no remarkable change.[52] It is very feasible that the resident flora of the intestine is perpetuating and sustaining the continuous inflammatory reaction seen in IBD patients. An ever-increasing amount of evidence is pointing towards the necessity in luminal flora for the progress of IBD.[47] This was clearly shown in a murine model where mutant mice, deficient in IL-10,[53] remained healthy as long as they were raised in a sterile environment. Upon the introduction of commensal flora, the mice rapidly developed colitis.[54]

Second, there is a **direct correlation between the site of an IBD lesion and the concentration of luminal bacteria at that site**, as observed by the benefit of antibiotic treatment in some patients.[55] Also, redirecting the flow of **fecal stream** and accompanying flora through a stoma seems to have a healing effect on the inflammation. A flare-up will take place if an anastomosis is connected.[56]

Finally, IBD-like enterocolitis was successfully provoked in murine models by the injection of a **purified sample of bacterial cell-wall PGN-LPS**. This was coupled with

increased levels of collagen synthesis resulting in the characteristic fibrosis, as well as increased levels of TGF-β1 and IL-6.[57] Similarly, Kennedy et al. were also able to elicit enterocolitis in IL-10-deficient mice, when exposed to intestinal bacteria, similar to the skip lesions observed in Crohn's disease.[58] Also, as seen in the human form of IBD, the inflammatory lesions in this model responded to corticosteroids.[59]

2.3. Environmental Factors

The genetics described thus far cannot account for 100% of all IBD cases; therefore, one must conclude that the **environment** is also of great importance in the pathogenesis of UC and CD. The environmental make-up of inflammatory bowel disease, or its flare-up, is quite complex and consists of external factors such as smoking, childhood infections, and non-steroidal anti-inflammatory drugs (NSAIDs), as well as internal ones such as the intestinal flora.[60] The use of oral contraceptives remains a controversial issue, and though much research has been done, little has been found to either disprove or lend support to oral contraceptives as a risk factor for IBD.[61] However, there seems to be a correlation between the use of oral contraceptives and smoking, resulting in an increased risk for IBD.[62]

Several studies were presented in recent years displaying cases of married couples that developed IBD following their move to occupy a common establishment. One such particular study was carried out in France and Belgium between 1989 and 2000 by Laharie et al.[63] The researchers grouped the subject couples into three groups, as follows:

- A – couples where both partners exhibited IBD symptoms prior to cohabitation
- B – if only one partner had IBD prior to cohabitation and both partners had it following cohabitation
- C – if neither partner displayed IBD before cohabitation yet both showed it afterwards.

In total, thirty cases were classified into these groups. Of importance, 22 of the cases were registered in group C. Such high frequency is indicative of environmental factors having an etiological role in IBD.

2.3.1. Microbial Agents

To date, **no single microbial pathogen has been identified as the causative agent of IBD** but several bacteria and viruses were investigated at one point or another. At the beginning of the last century, the first pathogen was brought under investigation, *Mycobacterium paratuberculosis*. It was observed that **Crohn's disease** displayed a

characteristic granulomatous **inflammation similar to Johne's disease of cattle**, for which *M. paratuberculosis* is a confirmed pathogen.[64] Furthermore, the pathogenic agent of leprosy, *M. leprae*, has also been targeted due to clinical, pathological and histological characteristics that are similar to CD. On the other hand, **ulcerative colitis** was correlated with a special variation of ***Escherichia coli***, with adhesive properties. This form was found in UC patients but was absent in control subjects.[65]

Among viruses, a major suspect is the **measles virus** infection at a young age.[66] The basis of this investigation is the virus' ability to target the gastrointestinal tract as well as its ability to induce granulomatous inflammation.[67] A different study examined the involvement of the measles vaccination in the pathogenesis of IBD; however, this has not been conclusively confirmed.[68] Other suspected viruses include the rotavirus and herpes virus.[64]

2.3.2. Cold Chain Hypothesis

This hypothesis is unique, as described by Hugot et al.[69] It proposes that **psychrotrophic bacteria**, for example, *Yersinia* species, are able to grow in a common household appliance, the refrigerator. These agents are then **ingested with food and mediate inflammation and diarrhea** through various mechanisms, **as seen in CD**.

First, *Yersinia* produces an invasin toxin which, along with the bacteria's ability to target M cells in lymphoid follicles, enables bacteria to penetrate cells. The notion of an **intracellular pathogen** responsible for IBD pathogenesis has also been supported by Karlinger et al.[64] It is known that CD lesions are most frequently found in lymphoid follicles of the intestine.

Next, yersiniosis produces **similar clinical signs and symptoms as CD**, which adds to the list of differential diagnoses. Among others, these include ileitis, reactive arthritis, erythema nodosum, and granuloma formation. Also, similar to *Helicobacter pylori*, the causative agent of peptic ulcer, *Yersinia* produces an urease. This enzyme is an important contributor to the formation of gastritis, and can be detected in a high number of CD patients. Moreover, these microbial pathogens have also been linked to spondyloarthropathies, which are, in turn, associated with Crohn's disease.[69]

Lastly, at the molecular level, yet not fully understood, is a connection between *NOD2/CARD15*, NF-κB, and *Yersinia* species-secreted proteins. These proteins are carried on a plasmid, pYV, including the Yop and Ysc proteins. Selected Yop genes are involved in the inhibition of NF-κB, while the possibility also exists that Yop proteins physically disrupt the

action of NOD2/CARD15 by some other pathway. Merging all of these data with the bacteria's tropism for M cells lends support to the cold chain hypothesis.[69]

2.3.3. Smoking

An enigmatic **paradox exists between smoking and the individual forms of IBD**. Smoking appears **more commonly in** those patients afflicted by **CD**, while **many UC patients are non-smokers**.[70,71] To complicate matters further, **nicotine** seems to play a curative role in UC. In one study, the application of a transdermal nicotine patch as an adjunct to typical treatment regimen has eased the symptoms of mild and moderate UC cases.[72] In another study involving 154 UC patients, 52% of the patients quit smoking within three years preceding the development of colitis.[73] An Australian study found the odds ratio to be 3.45 for smoking cessation prior to the onset of symptoms, leading the team to suggest that smoking hides the symptoms of UC. Alternatively, removing the immunosuppressive effect of smoking triggers the development of colitis in genetically susceptible individuals.[74] This may be due to nicotine's effect on Th_2 functions, which are a main constituent of UC, but lack of effect on the Th_1 functions that are present in CD.[75] Additional possible factors of nicotine and smoking include: effects on cellular and humoral immune cells,[76,77] increased colonic mucous production,[78] and reduced colonic motility.[79] Finally, smokers who show abstinence from smoking experience a milder form of disease than if these individuals had never smoked.[80]

2.3.4. Other Environmental Factors

Extrapolating on the role of the immune system in the pathogenesis of IBD brings one to the subject of **monocytes/macrophages**. In the early 1990s, immunohistochemical procedures showed that macrophages are present in the early stages of IBD in lesions formed as aphthous lesions.[81] Additionally, macrophages in IBD cases are blamed for the overproduction of **reactive oxygen and nitrogen species**, capable of causing extensive damage to cells and tissues. This is due to the inability by protective enzymes to eliminate these injurious substances.[82]

Lastly, returning to the role of bacteria in IBD pathogenesis, Lodes et al.[83] described bacterial proteins, **flagellins**, as possible culprits. These proteins are recognized for their ability to elicit an innate immune response via TLR5. Their presence is characterized by a strong IgG2a response in murine models, in addition to causing colitis when flagellin-specific

$CD4^+$ T-cells are transferred into naïve SCID (severe combined immunodeficiency) mice. Of note, there is specific recognition of these molecules in CD patients, unlike in UC.

2.4. Familial Studies

It is frequently observed that **family members of IBD probands** present the disease (CD or UC), or some other signs, indicative of an increased risk of developing it. Transmissions from parents to offspring as well as sibling-sibling associations occur. As many as **40% of first-degree relatives** of IBD patients will suffer from the disease.[84] Additional evidence to support the genetic basis of IBD comes from concordance twin studies, where monozygotic twins have a much higher concordance rate for IBD than dizygotic. These data are presented in Table 7.[85,86]

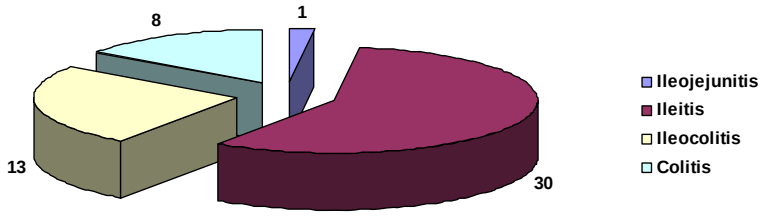
Table 7. Concordance Data in Monozygotic and Dizygotic Twins[85,86]

	Crohn's disease	**Ulcerative colitis**
Monozygotic twins	44-50%	6-14%
Dizygotic twins	0-4%	0-5%

No doubt, genetics play a role in the pathogenesis of CD, as do other factors, since the concordance rate for monozygotic twins is 50%, at best.[45]

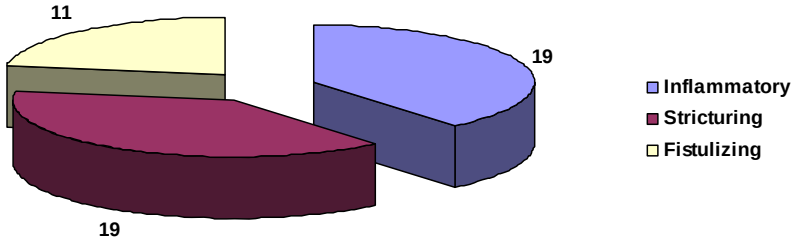
Research of whole families with at least two IBD patients presents yet another aspect to the disease – **similar disease phenotypes and location within a given family**. Bayless et al.[87] presented data indicating that within families there is a higher-than-expected concordance for disease phenotype, as expressed by the location and type of disease. A total of 554 patients were examined, of which 95 (17%) were diagnosed with CD and had positive family history for CD. The percentage of index cases having at least one first-degree relative with UC or CD defines a positive family history.[88] Of those 95 patients, 60 families were investigated for concordance relating to location and type of CD. Fifty-two (87%) families displayed concordance for disease location in at least two family members.[87] These results are illustrated in Figure 7. Values represent the number of families that display a given form of IBD.

Figure 7. Families Concordant for Disease Location[87]



Of the same 60 families, 49 (82%) families were concordant for the type of disease, which included inflammatory, stricturing or fistulizing forms. This is presented in Figure 8. Values represent the number of families displaying a given disease behavior.

Figure 8. Families Concordant for Disease Type[87]



Nonetheless, controversy concerning the strength of correlation between genotype and phenotype exists, and has been expressed in several studies. Annese et al.[89] presented a study supporting the notion that no significant correlation exists between *NOD2/CARD15* genotype and disease progression or type. Furthermore, a second study published in the same year corroborates this belief by stating that the various *NOD2/CARD15* mutations do not serve, according to the Vienna Classification, as markers for any one given form of Crohn's disease.[90]

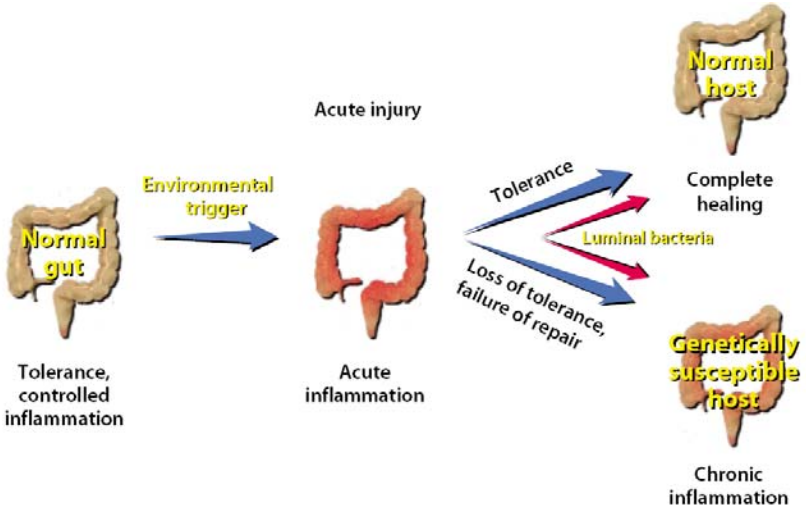
Several other studies also showed that up to 25% of index cases recalled CD patients in their family, indicating a positive familial history.[91,92] In a Belgian study performed by Peeters et al.,[88] 640 CD probands were investigated, yielding 132 (20.6%) patients with a positive family history for IBD, either UC or CD; in contrast, only 2.1% of the control group had a family member affected by IBD. Sixty-eight CD-afflicted families demonstrated the following familial demographics: 51 families had two patients each, 12 families had three patients each, and 5 families had four patients each. Another aspect examined by this study

was the age at diagnosis. It was found that offspring were diagnosed at an earlier age than their parents; however, there was no significant difference between siblings.

Some studies investigated the reason behind the apparent **earlier age-at-onset in offspring of IBD patients**. The first, suggested by Satsangi et al.,[93] indicated **genetic anticipation**. This phenomenon refers to the earlier symptomatic onset of a given inherited disease or a more severe clinical presentation of the disease.[94] Others proposed that the offspring were thoroughly examined at a younger age based on the known presence of IBD in their parents.[95] Finally, a Swedish study published in 2003 also demonstrated that genetics had a greater influence in CD pathogenesis as opposed to UC, including a similarity in disease phenotype.[96] The team found a concordance rate of approximately 50% in monozygotic twins afflicted with CD, compared with only 3.8% of CD dizygotic twins.

Figure 9 below summarizes the current, generally accepted idea of the pathogenesis of inflammatory bowel disease.

Figure 9. Overview of the Pathogenesis of IBD[45]



3. CLINICAL PICTURE & DIAGNOSIS

3.1. Clinical Picture

The clinical picture can be divided into two separate but related entities. The first involves the signs and symptoms as perceived by the patient and the clinician; the second involves the cytological changes as demonstrated in laboratory examinations. The most common complaints in both forms of IBD include **increased stool frequency** and **decreased stool consistency**, though in 5-10% of patients, constipation may be the presenting symptom. The second most common symptom is **abdominal pain**, which in some cases may help localize the site of inflammation. In patients suffering from ileal Crohn's disease, pain is commonly felt in the lower right quadrant, while UC patients often complain of pain in the left lower quadrant. In the latter case, cramping prior to and relieved by defecation is also common. In the case of **ulcerative colitis**, the presence of **tenesmus** hints towards proctitis. Finally, the presence of **gross blood** in the stool makes **UC more likely than CD**.[97] Table 8 presents the differential diagnoses that must be ruled out during the diagnostic work-up for inflammatory bowel disease.[98]

Crohn's disease, in **60%** of cases, affects the **terminal ileum** (ileitis), while colitis and anorectal inflammation are the majority of the remaining cases. A small percentage of cases will show inflammation anywhere throughout the gastrointestinal tract.[297] Importantly, with passing time, both **disease location and behaviour are unstable** and tend to change with an increasing proportion of patients developing complications.[99] Regardless of the site, CD is characterized by a **granulomatous form of inflammation**.[100]

Two characteristic findings in CD are **strictures and fistulae**. Despite research, the mechanism behind these observations is still unknown. Some support the idea of mechanical forces within the lumen of the bowel, that is, intraluminal pressure. Others claim that the inflammatory nature of CD and the associated host immune response are at work.[101] Finally, some suggest that these variations are a basis for classifying CD into perforating (fistulae formation) and non-perforating (inflammation with or without strictures) disease.[102] **Ulcerative colitis**, on the other hand, is defined as "continuous endoscopic or radiologic mucosal inflammation affecting the rectum and colon together with the typical histology of ulcerative colitis and the absence of granuloma."[9] Table 9 compares the various observations in UC and CD,[103,104,105] while Images 1 and 2 illustrate active CD and ulcerative colitis, respectively, as visualized during colonoscopy (Courtesy of Dr. Lakatos, 1st Department of Internal Medicine, Semmelweis University, Budapest, Hungary).

Table 8. Differential Diagnoses of Acute and Chronic Diarrhea, subdivided by Presence of Blood[98]

Symptom	Disease	Main differentiating diagnostic characteristic
Nonbloody diarrhea	Pancreatic insufficiency	Patient history (pancreatitis, risk factors [ie, alcohol abuse]), decreased fecal elastase, increased fecal fat
	Celiac disease	*Histology*: flat duodenal mucosa, absence of normal villi. *Serology*: endomysial/gliadin antibodies
	Bacterial overgrowth of the upper GI tract	Pathologic glucose H_2 breath test
	IBS	Normal fecal calprotectin, normal colonoscopy
	Colonic neoplasia	Colonoscopy
	Helminths (*Strongyloides stercoralis*, *Schistosoma* spp.)	Stool microscopy (fresh stools)
	Viral infection	Rotavirus, Astrovirus test from stools
	Hyperthyrosis	Serum levels of TSH (free T3 and T4)
	HIV infection/AIDS	HIV test, cutaneous (Kaposi) and gingival (hairy leukoplakia). Specialized diagnostics for *cryptosporidosis*, *microsporidosis*, *Isospora belii*, *Cyclospora cayetanensis*, *Giardia int*, CMV
	CMV infection	Typical ulcers (endoscopy), immunohistology (early antigen)
	Whipple's disease	Histology with Schiff stain
	Endocrine active tumors	Specialized hormone diagnostics, small bowel imaging
Bloody diarrhea	Inflammatory	
	Behçet's disease	HLAB51, genital and oral aphthous ulcers
	Infectious colitides (examples)	
	Campylobacter, *Salmonella*, *Shigella*, enterohemorrhagic *E coli*, *C difficile*, *Entamoeba histolytica*	Stool and biopsy cultures incl. bacterial toxins (*C difficile* toxins A and B) and parasites, microscopy of fresh stools.
	Intestinal tuberculosis	Patient history (place of birth), signs of immune suppression, histology with stain for acid-fast bacteria.
	Sexually transmitted proctitides (examples)	
	CMV, HSV, chlamydia	Virus serology, histopathology
	Neoplastic	
	Colorectal cancer	Colonoscopy, histology
	Vascular	
	Ischemic colitis	Typical presentation at colonoscopy, histology, vascular imaging, higher age at manifestation
	Iatrogenic	
	Irradiation colitis	Patient history, colonoscopy
	NSAIDS	Patient history, colonoscopy

AIDS, acquired immunodeficiency syndrome; CMV, cytomegalovirus; GI, gastrointestinal; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IBS, irritable bowel syndrome; NSAID, nonsteroidal anti-inflammatory drug; TSH, thyroid-stimulating hormone.

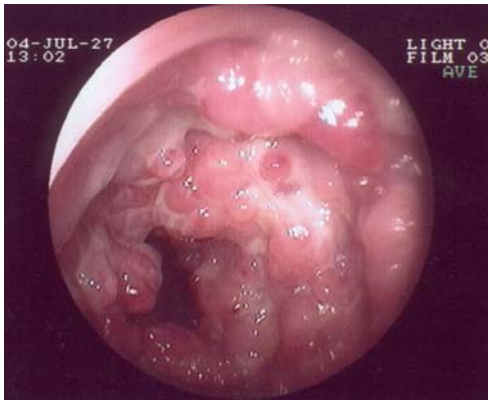


Image 1. Deep ulcers, stricturing, and the cobblestone effect seen in Crohn's disease

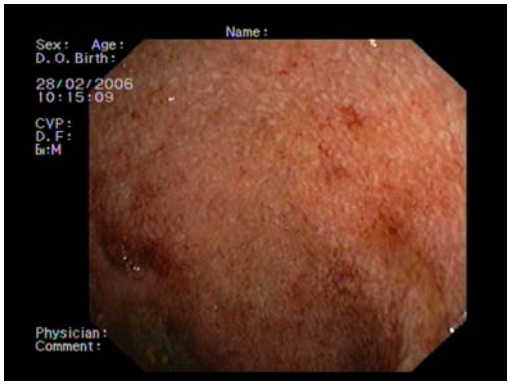


Image 2. Mucosal erosions visible in ulcerative colitis

Table 9. Comparison of Ulcerative Colitis versus Crohn's Disease

Method	Ulcerative colitis	Crohn's disease
Anatomy	Colon, rectum	Any part of GI tract. Most common: ileocolitis.
Clinical	Diarrhea with/without rectal bleeding; Abdominal pain and/or rectal cramping; Weight loss, fatigue, and fever.	Varies with site, similar to UC; Strictures and fistulae may cause severe pain, nausea, and vomiting.
Radiology	Fine granularity; small superficial erosions. Symmetry and continuity of involvement.	Narrowing of the intestinal lumen; rigidity, asymmetric lesions, mucosal destruction, cobblestone-appearance, discontinuous lesions, fistulae.
Endoscopy	Almost always involves rectum, extends proximally varying distances. Inflammation is diffuse and continuous.	Rectum often spared, discontinuous (skip) lesions; aphthoid erosions; deep, irregular ulcers; cobblestone appearance, segmented lesions.
Histology	Mainly involves mucosa: irregularity, ulceration, increased chronic inflammation in lamina propria; Goblet cell mucin depletion; glandular disarray	Compatible histology; granulomas; discontinuous inflammation; lymphoid aggregates.

3.2. Immunological Changes

Examining the inflammatory process at the cellular and molecular levels, one can identify several different elements. First, it is initiated and aggravated by a **disarrayed mucosal immune response to various antigens**,[267] which are still under close scrutiny. **Originally**, it was thought that **Crohn's disease** is elicited and perpetuated by **$CD4^+$ lymphocytes with Th_1 phenotype**, while $CD4^+$, **atypical Th_2 lymphocytes** are present in **ulcerative colitis** mucosa. While Th_1 cells secrete interferon-γ (IFN-γ) and IL-2, the Th_2 cells secrete transforming growth factor β (TGF-β) and IL-5.[264] Of note, IBD-affected intestines are

infiltrated by a large number of immune cells without any indication of inflammation, which is particularly true in the case of Crohn's disease.[44] Furthermore, the cytokines released by Th_1 helper cells activate macrophages that release additional cytokines and proinflammatory substances, such as IL-12, IL-18, and macrophage migration inhibitor factor. However, as will be explained in the Discussion, it is **now believed** that **IL-23**, which was only identified in 2000, is responsible for the inflammatory response observed in **Crohn's disease**. Due to its structural similarity to IL-12, it is clear why such initial "confusion" is possible, simultaneously over-estimating the actual role of IL-12 in IBD.

Finally, there are also changes in the profile of B-cells. This is mirrored by exaggerated production of mainly IgG, but also IgA and IgM.[106]

3.3. Diagnosis

As depicted in Table 10, specific criteria have been established over the years for diagnosing IBD. In 1998, the **Vienna Classification** was devised for use with Crohn's disease. The Vienna Classification is detailed in Table 10.[107] In the case of ulcerative colitis, this disease can be subclassified by location into: proctitis, left-sided colitis, and pancolitis.[103]

Table 10. Vienna Classification of Crohn's Disease[107]

Age at diagnosis	A1 … < 40 years old
	A2 … > 40 years old
Location	L1 … Terminal ileum
	L2 … Colon
	L3 … Ileocolon
	L4 … Upper gastrointestinal
Behavior	B1 … Non-stricturing & non-penetrating
	B2 … Stricturing
	B3 … Penetrating

Much earlier, prior to the creation of Vienna Classification, a separate need was identified by several professionals - the need to quantify disease activity during and following trials of various therapeutic forms. This led to the formation **Crohn's Disease Activity Index** (CDAI) in 1976. In its calculation, 8 variables (9 in the modified version) are taken into account including, but not limited to, bowel movements, extraintestinal manifestations and other symptoms. The modified CDAI is calculated by scoring for number of daily stools,

presence of pain, well-being, manifested symptoms, use of antidiarrheals (Lomotil), presence abdominal mass, hematocrit, body weight, and albumin level.[108] The **Harvey-Bradshaw Index** was devised to provide a short and simple index (Table 11).[109]

Table 11. Harvey-Bradshaw Index for Crohn's Disease[109]

Parameter	Score
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	n
Abdominal mass	0 = none 1 = dubious 2 = definite 3 = definite and tender
Complications	Arthralgia, uveitis, erythema nodosum, aphthous ulcers, pyoderma gangrenosum, anal fissure, new fistula, abscess (1 point per item)

For the **assessment of ulcerative colitis**, several activity indices exist, including the Powell-Tuck Activity Score,[110] the Simple Clinical Colitis Activity Index,[111] and the modified **Truelove-Witts severity index**. The first consists of more than ten items which need to be accounted for, while the second includes only six diagnostic criteria:

- Bowel frequency (day)
- Bowel frequency (night)
- Urgency of defecation
- Blood in stool
- General well being
- Extracolonic features

Finally, the parameters, and their scores, included in the modified Truelove-Witts severity index are presented in Table 12.[112]

Similarly, indices exist for endoscopic evaluation and grading of CD (Table 13),[113] while in UC, the endoscopist must evaluate the following parameters: vascular pattern, erythema, edema, granularity, blood in lumen, erosions, ulcerations, friability and grade of inflammation.[114]

Table 12. Modified Truelove-Witts Severity Index[112]

Symptom	Score
Diarrhea (no. of daily stools)	
0-2	0
3 or 4	1
5 or 6	2
7-9	3
10	4
Nocturnal diarrhea	
No	0
Yes	1
Visible blood in stool (% of movements)	
0	0
< 50	1
≥ 50	2
100	3
Fecal incontinence	
No	0
Yes	1
Abdominal pain or cramping	
None	0
Mild	1
Moderate	2
Severe	3
General well-being	
Perfect	0
Very good	1
Good	2
Average	3
Poor	4
Terrible	5
Abdominal tenderness	
None	0
Mild and localized	1
Mild to moderate and diffuse	2
Severe or rebound	3
Need for antidiarrheal drugs	
No	0
Yes	1

The maximum score is 21. A score of less than 10 on two consecutive days was considered to indicate a clinical response.

Table 13. Simple Endoscopic Score for Crohn's Disease[113]

Variable	0	1	2	3
Size of ulcers	None	Aphthous ulcers (Ø 0.1-0.5 cm)	Large ulcers (Ø 0.5-2.0 cm)	Very large ulcers (Ø > 2 cm)
Ulcerated surface	None	< 10%	10-30%	> 30%
Affected surface	None	< 50%	50-75%	>75%
Presence of narrowing	None	Single, can be passed	Multiple, can be passed	Cannot be passed

3.4. Serological & Other Disease Markers

Along with the above mentioned criteria, **serological markers** are deployed as possible means for diagnosis. These range from antibodies and autoantibodies,[115] to various products secreted by cells[116] or their metabolites.[117] Samples include serum[115] as well as urine.[117] It is possible that some of these markers can also be used for the **monitoring of disease progression** as well as the **detection of subclinical disease** in susceptible families.[115] Surely, identifying markers in IBD is a difficult task for several reasons. First, different markers might be specific for either form of IBD, or common to both. Second, several markers may be indicative of disease phenotype, course or progression. Lastly, non-specific markers of inflammation might also be present.

The list of markers isolated from patients' sera is a comprehensive one with over twenty antibodies and autoantibodies, some of which include: ASCA, anti-neutrophil cytoplasmic autoantibody (ANCA), antigoblet cell (GAB) and antipancreas autoantibodies (PAB).[115] Table 14 presents the prevalence of various serological markers in IBD and their clinical relevance.

Saccharomyces cerevisiae is **yeast** frequently used in baking and the making of many foods, including bread and wine.[118] Several studies were able to isolate Anti-*Saccharomyces cerevisiae* antibodies (ASCA) from **CD patients** at far greater frequency and consistency than from UC patients or healthy controls. One author pegged a value, for circulating ASCA, at 70% of CD patients, 10-15% of UC patients, and 0-5% in control subjects.[119] Darroch et al.[120] found **extreme increase in the titers of IgG and IgA ASCA in CD cases**. The same study group also correlated the **IgG titer** with **small bowel inflammation**, while the **IgA titer** had a direct relationship with **disease duration**. Another study related the presence of ASCA with disease location - this correlation occurred more often in proximal disease.[121]

In a Hungarian study, Papp et al. used an ELISA assay for detecting antibodies against **porin protein C of *E. coli*** (OmpC) and ASCA, while indirect immunofluorescence was used for ANCA detection. A total of 653 IBD patients were investigated for the antibodies as well as characterized for *TLR4* and *NOD2/CARD15* mutations. The results demonstrated an increased risk for CD with the presence of ASCA (OR: 7.65) and anti-Omp antibodies (OR: 1.81). In addition, these two markers were also independently associated with ileal and non-inflammatory disease. Finally, a serology dosage effect was detected.[122]

Table 14. Prevalence of Serological Markers in IBD and their Clinical Relevance[123]

	CD (%)	UC (%)	Healthy subjects	Clinical significance
Atypical pANCA	2-28	45-82	1-7	Assists in differentiation between CD and UC Atypical pANCA$^+$/ASCA$^-$: UC Atypical pANCA$^-$/ASCA$^+$: CD CD: ASCA+: ileal involvement, complicated disease course, early need for surgery
ASCA	41-76	5-15	5	Atypical pANCA$^+$: left-sided colitis, good therapeutic response, uncomplicated disease course UC: atypical pANCA$^+$: severe left-sided colitis, refractory to medical therapy, early need for surgery
Anti-OmpC (IgA)	24-55	5-11	5	Identify ASCA$^-$ CD patients Penetrating disease Faster disease progression Early need for surgery
Anti-I2 (IgA)	54	10	4	Inflammatory enteritis (19%) Stricturing form Early need for surgery
Anti-CBir1 (IgG)	50	6	8	Flagellin (CBir1) induces colitis in animal models of IBD Leads to a pathological immune response in IBD patients Differentiation between atypical pANCA$^+$ CD and UC Small bowel involvement Penetrating and stenosing form
Antiglycan antibodies (ALCA ACCA)	36	<10	0	44% in ASCA$^-$ patients ALCA-penetrating; ACCA-stenosing form (small differences)
PAB	27-39	2-6	0-2	High specificity, low sensitivity Significance? Distinct CD subgroup?

The concept of **anti-glycan antibodies** for the diagnosis or confirmation of IBD was first proposed and tested by Dotan et al. in 2006. The group investigated, using two different techniques, the presence of **antibodies against mannan, laminaribioside and chitobioside** in CD, UC, and control subjects. They noted that the presence of one of these antibodies predicted CD with a **sensitivity of 77.4%** and **specificity of 90.6%**. The latter increased to 99.1% in the presence of at least two antibodies. Furthermore, higher levels of laminaribioside or mannan were significantly associated with small bowel disease in CD ($p = 0.03$ and $p < 0.0001$, respectively).[124] Subsequently, a Hungarian group tested the serological properties of 652 IBD patients for anti-OmpC, ASCA IgG (gASCA), pANCA, anti-mannobioside carbohydrate IgG (AMCA), anti-laminaribioside carbohydrate IgG (ALCA), and anti-chitobioside carbohydrate IgA (ACCA). Their research concluded that gASCA or its combination with pANCA is most useful for the differentiation between the two forms of

IBD. Furthermore, a greater antibody response against gASCA, ACCA, ALCA, AMCA, and OmpC were significantly associated with complicated disease behaviour and need for surgery in Crohn's disease ($p < 0.0001$ and $p = 0.023$, respectively).[125]

Perinuclear anti-neutrophil cytoplasmic autoantibodies (pANCA), in general, are used for the diagnosis of various vasculitides. Specifically, they target the granule constituents of neutrophils.[115] An **atypical pANCA pattern** is observed in some conditions, including **UC**.[126] As a result, it can serve as a useful marker to discriminate between UC and CD. It was shown to react with epitopes such as: *Escherichia coli*,[127] *Bacteriodes caccae*,[127,128] *Mycobacteria*[129] and others, further supporting the opinion that microbial agents play a role in the pathogenesis of IBD and the subsequent abnormal immune response.[130] In a study from Belgium, these results are confirmed and further noted that **a combination of gASCA and pANCA** can be effectively used for the **differentiation between Crohn's disease and ulcerative colitis**.[131] To date, ANCA has been recognized as the marker of UC in the task of **distinguishing between the forms of IBD**.[115]

pANCA is also detected in **65-75% of primary sclerosing cholangitis** (PSC) patients.[118] PSC is the most frequent chronic liver disease found in IBD patients, and in particular, in **ulcerative colitis**. It is found in 2-7% of UC patients, affecting more men than women. [132,133] At the cellular level, Terjung et al.[134] found a 50-kilodalton nuclear envelope protein, which is the target of pANCA in PSC, as well as in UC.

Pancreatic autoantibodies are **extremely specific for CD**, demonstrating **97-100% specificity** in comparison with UC; however, a much poorer **sensitivity of only 27-38%**.[135,136] In a study published by Seibold et al., 27% of CD patients were shown to have PAB, while none of the controls were positive for these autoantibodies. The team concluded that PAB is a specific marker in CD.[135] Another group, Folwaczny et al., demonstrated a higher number of patients with circulating PAB, 38%, and concluded it was insufficient as a genetic marker for IBD.[136] Finally, in a more recent Belgian study, encompassing 575 subjects (289 IBD patients, 108 unaffected first-degree relatives, 78 subjects with non-IBD gastrointestinal disorders, and 100 healthy control subjects), similar rates were noted. The prevalence of pancreatic autoantibodies was 32%, 23.3%, and 22.2% in CD, UC, and unaffected first-degree relatives, respectively. Of importance, the prevalence of PABs in the non-IBD gastrointestinal disorders group and the healthy controls was 1.3% and 0%, respectively. Furthermore, the study group noticed two staining patterns, intracellular and extracellular, and detected a significant association between the extracellular patter and CD,

as compared with UC patients (p = 0.014). However, the intracellular pattern was significantly associated with both, familial UC (p = 0.0003) and familial CD (p = 0.0009).[137]

In 2002, Kayazawa et al. published their study on possible markers collected from whole gut lavage fluid.[116] As it is known that neutrophils are involved in inflammatory reactions, the researchers examined four different proteins released from neutrophilic granules to assess their correlation with disease activity in UC and CD. For UC, the disease activity was graded by colonoscopy, while CD patients were graded according to the CDAI. The four proteins examined included lactoferrin, polymorphonuclear neutrophil elastase (PMN-E), myeloperoxidase (MPO), and lysozyme. The concentration of each protein was assessed in whole gut lavage fluid (WGLF) collected at the beginning of the colonoscopy procedure.[116]

It was found that in ulcerative colitis, patients' **lactoferrin** levels showed the best relationship with colonscopic grading of disease status. However, in Crohn's disease, in addition to a strong correlation between lactoferrin and CDAI, MPO was also elevated accordingly. It seemed that lactoferrin was most suitable as a marker and was quite stable in WGLF.[116]

A different method uses fecal matter to test for **calprotectin**, serving as a marker for inflammatory bowel disease. Calprotectin is a calcium-binding protein found in neutrophils and on the surface of monocytes. Following the activation of neutrophils or adhesion of monocytes to endothelium, calprotectin is released and can be detected in serum or other bodily fluids. Pelli et al. demonstrated that patients with active IBD were all positive for this protein, as opposed to 18% of healthy individuals. [138,139]

4. COMPLICATIONS & EXTRAINTESTINAL MANIFESTATIONS

Inflammatory bowel disease is confined, by definition, to the gastrointestinal tract; however, extraintestinal manifestations (EIM) are far-reaching. Many organs and organ systems are affected including the eye, skin, liver and biliary system, musculoskeletal system, and the hematopoietic system.[132] Some conditions arise by nature of the disease process itself,[64] while others, such as uveitis and some forms of arthritis, have a genetic background closely associated with HLA genes.[45] A range of values are available for the frequency of EIMs. According to one, geographically-localized study by Lakatos et al.,[140] 36.6% of CD patients and 15.0% of UC had extraintestinal manifestations in the province of Veszprem, Hungary. Various authors report different values for the occurrence of the extraintestinal manifestations, making it is somewhat difficult to identify the most prevalent extraintestinal manifestation.

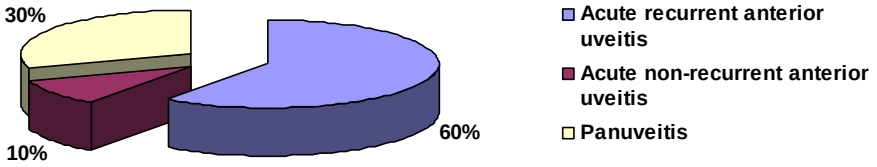
4.1. Ocular Manifestations

IBD-related ocular disease was already described in 1925 by Crohn.[141] Uncertainty exists as to the real incidence value, but estimates range from 3.5% to 11.8% with some gender-related variation.[142] **Data** about the prevalence of uveitis within each inflammatory disease **is conflicting**. Bernstein et al.[132] found it more common in UC than CD, reporting a 3.8% incidence rate. Lakatos et al., however, reported of equal incidence rates of ocular complications in UC and CD. Finally, Orchard et al. observed a greater incidence rate (6.4%) in CD than in UC (3.1%).[153] Nonetheless, agreement exists that ocular manifestations are **more common in women**.[132,140,143] The study by Lakatos et al.[140] further asserts that most ocular manifestations **develop during the earlier years of the disease**. This is supported by the observation that occasionally, ocular EIMs are diagnosed prior to the inflammatory bowel disease itself.[144]

The three most common ophthalmic conditions encountered in IBD patients are **uveitis**, **scleritis**, and **episcleritis**. Almost 50% of IBD patients with ocular manifestations will have more than one ocular complication, but as many as 68% of ophthalmic patients will have other extraintestinal manifestations not involving the eyes. Other disorders, which usually appear alongside ocular manifestations, include arthritis and ankylosing spondylitis. The latter two, as well as uveitis, are **associated with HLA-B27**.[145] Episcleritis has been reported in as many as 29% of IBD patients with ocular complications, while only 18% display scleritis.[142] Of note, **episcleritis correlates well with disease activity** and as such, has been **used as a marker**.

Uveitis is a form of ocular inflammation, occurring in as many as 17% of IBD patients, **often in association with sacro-iliac joint abnormalities and arthritis**.[142,146] The most prevalent form of uveitis in IBD is that of low-grade non-granulomatous iritis.[143] Figure 10 illustrates the distribution of the various forms of uveitis as reported by Banares et al.[147]

Figure 10. Distribution of Various Forms of Uveitis[147]



Finally, two other HLA genes associated with ocular inflammation are HLA-B58 and HLA-DRB1*0103.[148]

4.2. Dermatological Manifestations

The skin is another organ affected by HLA genes, which mainly manifests itself in the form of **erythema nodosum** (EN) and **pyoderma gangrenosum** (PG).[140] EN is a "rash consisting of painful, raised erythematous papules, predominantly on the extensor surfaces of the arms and legs."[148] Orchard et al. also associated the TNF-α_{-1031C} mutation with EN.[148] As in ocular inflammation, there appears to be preponderance toward the **female gender** in developing EN.[132] In the study published by Lakatos et al.,[140] cutaneous lesions were found in 10.2% of CD-afflicted patients and in 3.9% of UC patients.

A second dermatological complication in IBD, albeit rare, is **pyoderma gangrenosum** (Image 4; Courtesy of Dr. Lakatos, 1st Department of Internal Medicine, Semmelweis University, Budapest, Hungary), which displays an ulcerative form of skin inflammation affecting 1-2% of IBD patients.[149] Another study performed in Spain by Zazo et al. found 47.6% of IBD subjects to display skin lesions. While pyoderma gangrenosum was found in 2.8% of affected subjects, erythema nodosum was seen in 12.1% of patients and 60.7% of patients had aphthous ulcers. Overall, Crohn's disease patients (36.6%) were more commonly affected than ulcerative colitis (25.1%).[150]

Finally, **pyoderma gangrenosum shows a correlation with disease flare-up**, although it does **not necessarily coincide with improvement** of inflammation following treatment.[151]

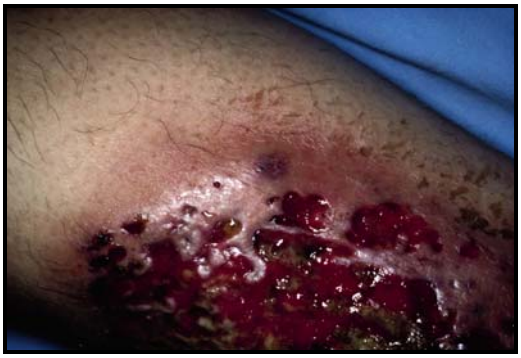


Image 3. Pyoderma gangrenosum in IBD

4.3. Musculoskeletal Manifestations

Arthritis is an interesting extraintestinal manifestation of IBD because of its proven **genetic association and basis**, and common occurrence. An early correlation was already made in 1930 by Bargen.[152] **Two forms of arthritis** might present in IBD, as described by Orchard et al,[153] **axial** and **peripheral**, with peripheral arthritis being further divided into two subtypes. The **axial form** is identified as **idiopathic ankylosing spondylitis** with a **weak HLA-B27 association**. There seems to be a common mechanism to the pathogenesis of arthritis, uveitis, and erythema nodosum in IBD. One of the many theories suggests the presence of a **common antigen found in joints, the eye, and skin** that triggers an autoimmune reaction. Specifically, an isoform of tropomyosin has been isolated.[154] Peripheral arthritis is classified into type 1 and type 2.[153]

Type 1 is defined as affecting less than 5 joints and involving the large joints, while **type 2 arthritis** affects 5 or more joints, is symmetrical, bilateral and affects small joints.[153] In addition, the behavior of type 1 arthritis is quite contradictory to that of type 2. While **type 1 arthritis is acute and self-limited**, **type 2 has a chronic nature**. Furthermore, **type 1 arthritis runs its course along with IBD**, as opposed to **type 2 arthritis**, which **progresses independently** of IBD.[140] As type 1 is strongly associated with other EIMs such as erythema nodosum and uveitis, **type 2 arthritis is solely associated with uveitis**. Finally, while the axial form of arthritis, ankylosing spondylitis, displays positive association with HLA-B27, no such correlation has been made concerning the peripheral forms.[153]

Musculoskeletal complications are the **most common extraintestinal manifestations** encountered in IBD, with an incidence rate of 11-20%.[155] Orchard et al. reported an incidence rate for CD (32.9%) that was twice as large as UC (15.8%).[153] They

consistently found larger values for type 1 and type 2 arthritis affecting CD and UC patients. Type 1 peripheral arthritis affected 3.6% and 6.0% of UC and CD patients, respectively. Type 2 arthritis was seen in 2.5% and 4.0% of UC and CD patients, respectively. Of significance, some patients will display arthropathic signs prior to a diagnosis of IBD, or arthropathic processes might already be present at the time of diagnosis. This phenomenon is more common for type 1 peripheral arthritis than type 2, as the former can also be used to gauge disease activity.[153] On the contrary, spinal involvement in IBD does not reflect the stage or severity of the disease.[155]

4.4. Thromboembolic & Hematologic Manifestations

Two common problems affecting IBD patients are **thromboembolism** and **anemia**, with the former leading to substantial disease and death in IBD patients.[156] A study by Grip et al.[157] found an **increased risk for venous thrombosis** at a **younger age among IBD patients** as opposed to the general population at risk for venous thrombosis. Interestingly, **inherited diseases of coagulopathy**, such as hemophilia, seem to have a **protective effect against IBD**, as the odds of developing UC or CD in such patients is lower than healthy subjects with normal coagulation pathways.[158] Additionally, while most EIMs are encountered during active bouts of IBD, a study found 77% of peripheral venous thromboses occurred during remission.[159] The mechanism or mutation behind thrombus mutation is still debated, as is the role of Factor V Leiden.[156,160]

The second most common cause of thrombus formation is described as a point mutation in the prothrombin gene of patients with deep-vein thrombosis (6.2%).[161] Heterozygote carriers express 30% higher prothrombin levels than healthy subjects.[162] However, no studies have found conclusive evidence linking this mutation to an increased risk of thrombosis in IBD patients.[163]

Anemia is defined, by most studies, as a hemoglobin level lower than 135.0 g/L in men and 115.0 g/L in women.[167,168] A hematocrit level below 0.40 also reflects an anemic status.[164] In IBD, anemia can arise as the result of **two main mechanisms**. The first and more important one is that of iron deficiency caused by reduced dietary intake or malabsorption. In addition, it might also be caused by increased loss through chronic gastrointestinal bleeding. Of note, although iron absorption is usually normal in IBD patients, the loss of iron may be overwhelming. The second mechanism involved in IBD anemia is a result of the chronic disease process. This type results from the general activation of the proinflammatory cytokine

system, finally resulting in a reduced red blood cell lifespan as well as decreased and/or inefficient erythropoiesis.[165,166]

It is estimated that the incidence of anemia in IBD varies from 8.8% to 73.7%; however, reports are conflicting and, at times, no definition for anemia is given.[164] One study published by Harries et al.[167] examined 55 CD patients in England. The patient pool was divided into two groups based on the nourishment level of patients, which was a reflection of the prevalence rate of anemia among the patients. Of the 25 undernourished patients, 64% showed signs of anemia, while only 26.7% of well-nourished patients were anemic. In another British study of 63 hospitalized CD patients, 63.5% were anemic.[168] When considering the prevalence rate of anemia in UC patients, a broad range of values is found depending on the study. An Israeli study found only 8.8% anemia cases among 147 patients.[169] On the high side, another study conducted in Israel found 66.6% prevalence among the females examined and 27.5% among the males.[170]

4.5. Hepatobiliary Manifestations

Primary sclerosing cholangitis (PSC) is at the forefront of EIMs affecting the hepatobiliary system, **predominantly in UC**. One study found a prevalence rate of 7.5%.[171] PSC is a condition affecting both intra- and extrahepatic bile ducts.[172] It is an idiopathic disease featuring regions of inflammation in the biliary tree, which may advance to fibrosis and strictures.[173] PSC, like many other EIMs, displays a **gender preference for females**,[132] and is proposed to be an independent risk factor and a **precancerous condition** for colorectal cancer in UC patients.[174,175] This risk increases with the progress of the disease as demonstrated by Broomé et al., who found that the average duration of disease until detection of malignancy or dysplasia was 18 years. They also found that none of the twelve patients who underwent colectomy due to dysplasia or carcinoma, died as a consequence of the cancer. Finally, UC patients with PSC had 9% risk of developing colorectal dysplasia or carcinoma after 10 years of disease, 31% after 20 years and 50% after 25 years with UC.[175]

Lakatos et al.[140] collected extensive data about the distribution of hepatobiliary complications in UC and CD. They classified three conditions affecting the hepatobiliary system: PSC, small duct PSC (if ERCP could not confirm PSC finding), and non-alcoholic fatty liver disease (NAFLD)/non-alcoholic steatohepatitis (NASH). The majority of hepatobiliary EIM cases in CD and UC consisted of NAFLD/NASH. Table 15 summarizes the results, for both for CD and UC. As shown in the table, PSC is twice as common in UC as it is CD, while the opposite can be concluded for NAFLD/NASH.

Table 15. Hepatobiliary Complications in the Western Province of Veszprem, Hungary[140]

	PSC	Small duct PSC	NAFLD/NASH
Crohn's disease	0.8%	2.4%	19.3%
Ulcerative colitis	1.6%	1.3%	9.4%

NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis

4.6. Other Complications

Inflammatory bowel disease can also present itself in the form of **surgical emergencies**. These may include: toxic colitis, massive hemorrhage, free perforation, and an abscess accompanied by sepsis.[176] Toxic megacolon is an emergency situation in UC. In fact, in up to 30% of patients, toxic megacolon may be the presentation of the disease itself.[177] It is well known that gastrointestinal bleeding is a common feature in IBD; however, severe bleeding is a rare occurrence. Most studies estimate the incidence value at 2-3%, while it is responsible for about 10% of emergency colectomy procedures in ulcerative colitis.[178] Free perforation is a rare emergency occurring in only 2% of UC patients, usually occurring together with a megacolon or toxic colitis.[179] Finally, in Crohn's disease, abscesses occur most commonly in the right lower quadrant, neighboring the terminal ileum.[176]

4.7. Colorectal Cancer in Inflammatory Bowel Disease

The notion of **colorectal cancer** (CRC) was originally documented in 1925, very shortly after the first report on Crohn's disease.[180] Until now, much of the knowledge concerning CRC has been derived from **UC patients** where it is found more frequently, and as stated above, **the risk increases with a longer disease duration**.[173] Other risk factors include greater anatomic extent,[181] presence of primary sclerosing cholangitis, dysplasia found in specimen,[182] a family history of CRC, and degree of bowel inflammation.[183] Furthermore, colitis-associated CRC has a **younger age of onset**.[183] Accordingly, British and American guidelines indicate that surveillance endoscopy needs to be initiated in the UC patient population 8-10 years after diagnosis, or in the case of extensive or left-sided colitis, after 15-20 years.[184]

5. TREATMENT

Over the years, it has become clear that the absence of a clear etiology for IBD necessitated the use of treatment regimens that remedy the symptoms alone.[100] The arsenal of possible methods includes 5-aminosalicylic acid (5-ASA), corticosteroids,[185] immunomodulatory agents,[264] chimeric monoclonal antibodies,[186] antibiotics,[272] probiotics[187] and gene therapy.[188] Here, aspects of each treatment are presented. The majority of treatments aim to control or change the immune response and the composition of the intestinal flora.[272] Consequently, all treatment options have undesirable side-effects.[189]

5.1. Aminosalicylates

This family of drugs is at the **forefront of treatment**, with the primary agent being sulfasalazine. These are appropriate for the treatment of **moderate ulcerative colitis and Crohn's disease**; however, different drug forms exist depending on the site of disease and the method of administration.[264] Two pharmaceutical forms of 5-ASA are available: sulfa-containing and sulfa-free 5-ASA.[190] These agents appear to have **immunomodulatory functions**, even though their mechanism has not yet been fully elucidated. 5-ASAs are capable of halting the production of prostaglandins and leukotrienes, thus preventing neutrophil chemotaxis. Also, they scavenge reactive oxygen metabolites and might play a role in the inhibition of NF-κB.[264]

It is important to select the proper form of 5-ASA as well as **mode of delivery**; these will affect efficacy and the resultant side-effects. The newer sulfa-free 5-ASAs pose a substantial improvement in this class of drugs, since they do not lead to the side effects seen with drugs such as sulphasalazine,[190] in particular, agranulocytosis, neutropenia, male infertility, and neuropathy.[191] Finally, depending on **disease location**, the form of administration needs to be appropriately chosen, for example, azo-compounds, microspheres, or pH-dependent compounds.[190] The latter, for instance, lead to 5-ASA release in the distal ileum while slow-release compounds release 5-ASA in the proximal small intestine.[264]

Several intolerance reports have been completed that included headache, dyspepsia, nausea, vomiting, anorexia, and fatigue. These signs and symptoms might appear in as many as 45% of patients, which is an extremely high fraction of the treated population. Of note, the prevalence of these side effects is dose-dependent and also genetically determined, owing to the hepatic degradation of a given side chain on the drug molecule.[192] It is important to realize that sulfa-free 5-ASAs (for example, mesalamine) do not come without their own arsenal of

side-effects, including paradoxical exacerbation of colitis, pancreatitis, pneumonitis, hepatitis, pericarditis, and nephritis.[193]

The **ASCEND I** trial investigated the safety and efficacy of **two different delayed-release mesalamine dosages** in 301 patients suffering from mildly to moderately active UC. The cohort was randomly assigned to delayed release 2.4 g/day or 4.8 g/day mesalamine and followed for 6 weeks, with complete remission or clinical improvement from baseline serving as primary endpoint. In the mildly active UC group, there was no significant difference between the two dosages, with 51% of patients assigned to the delayed-release 2.4 g/day mesalamine and 56% of patients in the 4.8 g/day group reached the efficacy endpoint (p = 0.441). However, in the moderate UC group, a significant difference was observed between the delayed-release 2.4 g/day mesalamine and 4.8 g/day. In the former, 57% of patients reached the efficacy endpoint compared with 72% of patients in the latter group (p = 0.0384).[194]

A recent meta-analysis showed a **significant reduction in the CDAI in patients receiving mesalamine** compared with a cohort that received a placebo (p = 0.04), concluding that mesalamine 4 g/day is superior to placebo in decreasing the CDAI, yet the clinical improvement is still unclear.[195] Since there is insufficient information on the benefit of any particular subgroup, the European Crohn's and Colitis Organisation Consensus states that 5-ASA "should be considered clinically no more effective than placebo for active ileal or ileocolonic CD."[196] Maintenance therapy may also be considered for 5-ASA though data regarding its use and efficacy is conflicting.[196]

5.2. Corticosteroids

This group is **one of the longest used drugs in IBD treatment**, their efficacy cannot be denied, and despite their side effects, are still needed.[196,197] Topical corticosteroids, in the form of **enema**, may be used to treat patients with ulcerative colitis or distal UC, who have shown adverse effects to 5-ASA. **Oral forms** of corticosteroids, such as budesonide, 9 mg/day, may be administered to patients with mild, moderate or severe forms of UC or CD.[196] Other proven options include prednisone (0.5-0.75 mg/kg/day)[198] and 6-methylprednisolone (1 mg/kg/day).[199] Finally, an **intravenous route** of administration may be used for hospitalized patients with severe stages of the disease. Even in this latter group of patients, improvement is already observed within 7-10 days. Importantly, these agents should be used only as long as necessary to control an acute phase of the disease, as they have no indications for maintenance therapy.[200]

Their effects have been shown to act through the **inhibition of proinflammatory cytokine production**. These include, but are not limited to: IL-1, IL-2, IL-4, IL-6, and IFN-γ.[201] Possibly, a disruption in the activity of NF-κB is responsible for this broad inhibitory function by corticosteroids.[202]

Corticosteroids, with their popularity and efficiency in reducing immunologic and inflammatory reactions, produce **severe side effects**. As with most therapeutic agents, these effects **depend on dosage and duration** of treatment. Some of the more severe conditions include osteoporosis, hypertension, diabetes, as well as a detrimental effect on the hypothalamic-pituitary-adrenal axis.[203,264] In addition, **dependence** also accompanies corticosteroid use. Moreover, negative effects might be observed in the hepatic, cardiovascular, mental or musculoskeletal systems. Corticosteroids administered for a short period of time can also result in fluid and electrolyte imbalances, acid-base disturbances, and metabolic abnormalities. Long-term corticosteroid therapy will induce changes in fat distribution, ecchymoses, and abdominal striae.[192] Corticosteroid administration suppresses the immune system, thus exposing the patient to a greater risk of opportunistic infections.[264]

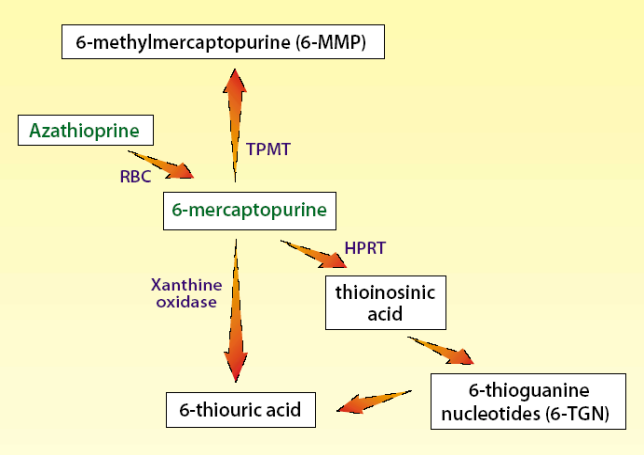
5.3. Immunomodulatory Agents

As discussed previously, and is well known, inflammatory bowel disease results from an aberrant immune response and it is only logical that some treatment regimens will attempt to control the immune system, thus affecting the extent and duration of disease. One very important compound in this class is **azathioprine** (AZA), which is a thioguanine derivative with the **active metabolite** being **6-mercaptopurine** (6-MP).[204] This derivative is the **most commonly used** immunosuppressive agent in IBD treatment.[264] It is a highly successful drug, as two-thirds of IBD patients enter remission following treatment. Furthermore, 80-90% of patients who are able to tolerate AZA are capable of enduring a long-term treatment period.[204] Another advantage for using AZA is its capacity to **wean off corticosteroid-dependent patients**. It serves as a continuation therapy, while the corticosteroid dose is simultaneously and gradually decreased. In an important recent trial, Ardizzone et al. provided the evidence to support this notion. They investigated 72 individuals who were steroid-dependent UC patients, and the result from administering 2 mg/kg/day azathiopurine or 3.2 g/day of 5-ASA. The results showed that AZA was significantly better than 5-ASA at inducing remission as well as discontinuing the use of steroids, during 6 months of therapy.[205] The pathway in which these drugs function is still unknown; however, one theory exists whereby a specific and long-lived subgroup of T-cells is suppressed.[264] In general, AZA 1.5-2.5 mg/kg/day or

mercaptopurine at a dose of 0.75-1.5 mg/kg/day may be used for patients who have relapsed.[196]

These agents are not free from **adverse effects**, which include allergic reactions, hepatitis, leukopenia, and thrombocytopenia. Also, pancreatitis is the most common form of allergy observed with 6-MP, in 3-15% of patients, but it falls short of triggering a case of chronic pancreatitis.[192] Interestingly enough, there is a **strong correlation between a patient's genetic code and their tolerance of AZA and 6-MP**. The former is metabolized into 6-MP in a non-enzymatic step. It is then converted to one of three products, 6-methylmercaptopurine (6-MMP), thioinosinic acid, and 6-thiouric acid.

Figure 11. Metabolism of Azathioprine[45]



RBC – red blood cell; TPMT – thiopurine methyltransferase;
HPRT – hypoxanthine-guanine phosphoribosyl transferase

Figure 11 illustrates the metabolic pathway of azathioprine and the functional importance of the enzyme **thiopurine methyltransferase** (TPMT), the levels of which are determined by a person's genetic code. The ratio between 6-methylmercaptopurine (6-MMP) and the active derivatives, 6-thioguanine nucleotides (6-TGN), is crucial in determining a person's tolerance to these drugs.[45] Only 0.3% of the population is homozygous TPMT-deficient, while 11% is heterozygous TPMT-deficient. Clearly, a lack of TPMT will shift the balance of reactions towards 6-TGN, leading to bone marrow suppression and severe leukopenia, resulting in infections.[206] TPMT genotyping can be performed for enzyme activity and alternate therapies may be proposed.[45] However, not all leukopenic patients are TPMT-

deficient. One study showed that 73% of patients suffering from bone marrow suppression displayed no known TPMT mutations.[206]

While the next section illustrates the efficacy of various chimeric and human monoclonal antibodies, one must still always consider azathioprine as first-line therapy because of its proven track record for the treatment of both CD and UC.

5.4. Chimeric Monoclonal Antibodies

5.4.1. Infliximab and Crohn's Disease

Infliximab is the prototypical **anti-TNF-α monoclonal antibody**.[207] It falls under the category of biological treatment, since it focuses on inhibiting or downregulating specific cytokines or chemokines involved in the immune response.[186] At a dose of 5 mg/kg it is an effective tool in patients with **active CD**.[196] Similarly to other treatment modalities, the exact mechanism of action is not fully known. By one account, infliximab binds soluble TNF-α[186,264]; by another account, shown *in vitro*, it acts by inducing apoptosis in lymphocytes and other cells.[46,208] It is possible that its efficacy is a result from a combination of these two functions. Structurally, infliximab is composed of a human IgG_1 constant region attached to a murine antigen-binding variable segment, hence forming a chimera.[264] By composition, it is about 75% human, while the rest is of murine origin.[209]

Two clinical trials, **ACCENT I and II** were conducted to test the efficacy of infliximab. ACCENT I had a complex design, whereby patients were allowed to use other treatment regimens in addition to the chimeric antibody. This made result interpretation somewhat complicated but nonetheless useful results were produced. There was sufficient evidence linking remission with a maintenance therapy of intravenous infliximab, where 24% of patients remained in remission, in contrast to 9% of placebo-treated CD patients.[210] The ACCENT II randomized trial inspected the response of patients' enterocutaneous disease to infliximab treatment, with 69% of patients demonstrating a positive outcome.[211]

Despite infliximab's clinically significant record, it does harbor several **significant side effects**, consisting of acute and delayed infusion reactions, autoimmunity and autoantibodies, and an increased risk of infections.[212] Infusions administered too quickly may lead to symptoms of an acute infusion reaction, in particular, dyspnea, chest pain, headache, and fever. Also, since infliximab is not 100% human protein, development of antibodies is a feasible and concrete phenomenon.[213] A delayed infusion reaction might occur if a time gap larger than 12 weeks occurs between subsequent infusions. Increasing levels of antinuclear

and anti-double-stranded-DNA antibodies have also been reported in long term use of infliximab but only in rare cases does a lupus-like syndrome develop.[212,214]

5.4.2. Infliximab and Ulcerative Colitis

Until recently, infliximab was only indicated for Crohn's disease without proven efficacy in patients with ulcerative colitis. Two studies, **Active Ulcerative Colitis Trials 1 and 2** (ACT 1 and 2), set out to determine the efficacy of **infliximab** in inducing and maintaining remission of ulcerative colitis. These two randomized, double-blind, placebo-controlled studies investigated 364 patients each, with **moderate-to-severe UC** despite medical therapy. The subject received either intravenous infliximab (5 or 10 mg/kg body weight) at weeks 0, 2, and 6, or a placebo. In ACT 1, following the first three doses, the patients received placebo or infliximab every 8 weeks until week 46, while in ACT 2 the administration continued every 8 weeks until week 22. All groups were followed for eight additional weeks following the last administration. Response to therapy was defined as a decrease in the Mayo Score[215] of at least 3 points, or 30%, as well as a decline in the subscore of rectal bleeding by 1 point or an absolute subscore of 0 or 1 for rectal bleeding.[216]

Both studies presented statistically **significant improvements** in the patient groups. In the first study, 69% of patients who received 5 mg infliximab and 61% of those patients who received 10 mg showed a clinical response at week 8. This was in comparison with 37% of the placebo group ($p < 0.001$) (Figure 12). In ACT 2, 64% of patients who received 5 mg and 69% of those who received 10 mg infliximab demonstrated a clinical response at week 8, compared with 29% of the placebo group ($p < 0.001$) (Figure 12). Furthermore, patients in both study groups were more likely to show a clinical response at week 30, as compared with the placebo-controlled group ($p \leq 0.002$).[216]

Finally, Järnerot and colleagues conducted a randomized, placebo-controlled study on the use of infliximab in patients with severe or moderately severe UC. The group reasoned that such patients often become candidates for colectomy and was searching for alternative rescue therapy when steroid use was ineffective. They enrolled 45 patients and randomized them into two separate arms – placebo and infliximab. The primary endpoint was colectomy or death at 3 months after randomization. Secondary endpoints included clinical and endoscopic remission after 3 months in patients who did not undergo a colectomy. None of their patients died, while 7 in the infliximab group and 14 in the placebo group underwent colectomy (OR: 4.9; $p = 0.019$). Thus, they concluded that infliximab is indeed an appropriate rescue therapy in patients not responding to conventional therapy.[217]

5.4.3. Adalimumab

Similar to infliximab, this IgG_1 monoclonal antibody is a **TNF antagonist**, though its structure varies from that of infliximab; it is **completely of human origin**.[218] Numerous controlled studies have demonstrated adalimumab to be safe and effective in treating immune-mediated conditions such as rheumatoid arthritis, psoriatic arthritis, psoriasis, and ankylosing spondylitis.[219,220,221,222,223] Shifting the focus to Crohn's disease, several trials have been conducted to test the efficacy of adalimumab in this condition. The CLASSIC I and II trials investigated the efficacy and safety of adalimumab in inducing and maintaining clinical remission in CD, respectively. The CHARM trial tested the efficacy and safety of adalimumab in maintaining response and remission in CD patients with moderate to severe disease. Finally, the GAIN study examined the use of adalimumab in CD patients who were previously treated with infliximab and either did not respond or could not tolerate the treatment regimen.

5.4.4. Certolizumab Pegol

Certolizumab pegol is a **pegylated humanized Fab' fragment** capable of binding TNF-α with high affinity. While functioning as a TNF antagonist, its structure lacks the Fc segment, thus being unable to induce *in vitro* complement activation, antibody-dependent cellular cytotoxicity, or apoptosis.[224,225] The **PRECISE trials** (I and II) set out to investigate the efficacy of Certolizumab pegol for the **induction and maintenance of remission** in moderate to severe Crohn's disease.

In conclusion, while the efficacious mechanism behind TNF antagonists is a sound solution for IBD, substantial research is still required to evaluate their long-term effects and complications.

5.5. Other Therapeutic Options

Several studies have already established a role for the intestinal flora in the pathogenesis of IBD.[52,53,54,55,56] Consequently, attempts have been made in regulating the presence of these bacteria with the hope of curbing the disease. Two options are available following this train of thought: the first is **antibiotics**, while the second is **probiotics**. The former, while efficient in CD, shows little improvement in UC patients. A commonly used antibiotic in CD is **metronidazole**, which has shown efficacy in treating patients with perianal fistulae, yet the required dose is quite high and can lead to neurotoxicity.[264]

Probiotics constitute a biological way for treating Crohn's disease as well as maintaining a clinical remission in ulcerative colitis.[187] They are live, non-pathogenic organisms that improve the gut's microbial balance leading to a healthier status. These can be divided into two groups[226]:

- *Bacteria* – usually lactic acid producers, such as lactobacilli strain GG[227] or *Escherichia coli* Nissle.[228]
- *Yeast* – eg, *Saccharomyces boulardii*.

It is important that probiotics adhere to strict criteria in order to be **safe and effective**. In addition to safety concerns, the following criteria must also be met[229]:

- be of human origin
- resist bile and acid
- have the ability to adhere, colonize, and survive in the human intestine.

Nonetheless, the majority of data supporting probiotics comes from animal models, whereby colitis-induced mice are cured by certain *Lactobacillus* strains. In humans, trials have been conducted using a **non-pathogenic strain of *Escherichia coli***.[230] Finally, a published study tested the use of **helminthic ova** in altering the immune response of the human gut. The ova of *Trichuris suis*, pork whipworm, were used to shift the Th_1 profile observed in CD to Th_2. In this study, three of the four tested CD patients entered remission, while the three UC subjects had a decrease of 43% in their Clinical Colitis Activity Index. This method certainly seems like a feasible solution with much needed research.[231]

The ever-expanding field of **gene therapy** is yet another possible solution for the treatment of IBD. The theory behind gene therapy in IBD, and other chronic inflammatory diseases, is the expression of proteins that are important in the downregulation of the pathogenic immune response. On the contrary, gene therapy may also lead to expression of proteins that will confer protection against an illicit immune response. To date, there are no human clinical trials involving specific gene therapy protocols for IBD, though animal models are being investigated.[232]

6. BACKGROUND & AIMS OF THE STUDIES

6.1. Multidrug Resistance-1 Gene and Breast Cancer Resistance Protein

The multidrug resistance-1 (*MDR1/ABCB1*) gene is located on chromosome 7 and consists of 28 exons. Its transmembrane protein product, P-glycoprotein 170 (**Pgp-170**), belongs to the ATP-binding cassette (ABC) transporters and is thought to be responsible for the **removal of xenobiotics** and **resistance to multiple chemotherapeutic agents**. It performs this function by limiting the absorption from the intestinal tract, while encouraging the removal of these compounds through the bile and urine.[233] Supporting the role of Pgp-170 is its co-localization with CYP3A4, an important enzyme for drug metabolism.[234] *MDR1* was first identified in **cancer cells**, followed by its discovery also in **normal tissue** including liver, kidney, brain, testis, and placenta.[235,236,237]

MDR1, through its product Pgp-170, is thought to play an important role in response to treatment of IBD as well as disease susceptibility. First, it is known that Pgp-170 has a **wide range of substrates**, including but not limited to, anticancer drugs, cardiac drugs, immunosuppressants, antiemetics, antibiotics, and steroids.[233] It follows accordingly that Pgp-170's capacity to remove steroids, for example, dexametahosone,[238] and immunosuppressants, such as cyclosporine[239] and methotrexate,[240] from cells could also be the basis for some cases of failed medical therapy in IBD necessitating surgical approaches. Farrell and colleagues have indeed found a significant elevation ($p < 0.0001$) of peripheral blood lymphocytes' *MDR1* in CD patients who failed medical therapy and required bowel resection. Similarly, a significant increase ($p = 0.001$) was also found in UC patients who underwent proctocolectomy due to failed medical therapy.[241]

The role of *MDR1* in bacterial-host interaction arose from a knockout mouse model. As it became known that a **possible susceptibility locus for IBD** was located on chromosome 7q21.1,[324] Panwala et al. created the mdr1a$^{-/-}$ knockout mouse model. They demonstrated that these knockout mice, raised in pathogen-free conditions, developed colitis with features of both CD and UC, which could be reversed with antibiotics. Subsequently, they concluded that the intestinal flora was necessary for the inflammatory process. Therefore, it seems that ***MDR1* not only has a protective function against xenobiotics but also bacterial products**.[242] Nonetheless, its exact function has not yet been completely defined.

One must also consider the role of mutated Pgp-170 in the susceptibility of IBD, as at the moment, at least 50 single nucleotide polymorphisms (SNPs) and 3 insertion/deletion mutations have been described.[243] The first SNP was found in exon 26 (**C3435T**), a **silent mutation** that does not change the encoded amino acid (Ile). Nonetheless, it displays a

functional variance in individual homozygous for the variant T allele; Pgp-170 expression was lower.[244] A second mutation, **G2677T/A** in exon 21, has also been correlated with **reduced Pgp-170** expression levels.[245]

The breast cancer resistance protein (***BCRP/ABCG2***) is also a member of the ATP-binding cassette transporters superfamily, which has been implicated and proven to play a role in **multidrug resistance**. It is a half-transporter with a wide range of substrates and is located in the small and large intestines as well as in the kidney and liver. The protein, which was originally described in a multidrug-resistant breast cancer cell line, also secretes drugs and xenotoxins into breast milk, posing a risk to the infant.[246,247,248]

Due to the protein's close association with *MDR1*, and having a similar function and localization, it is plausible that *ABCG2* **may also have an effect on medical therapy in IBD**, or its failure. Of note, while half-transporter are normally located to intracellular membranes, and full-transporters to the plasma membrane, this particular transporter is also found at the plasma membrane.[247]

6.2. Discs Large Homolog 5

In 1999, using a genome-wide scan, Hampe et al. discovered a susceptibility locus on chromosome 10q23.[336] Five years later, the **discs large homolog 5** (*DLG5*) gene was added to the growing list of IBD susceptibility loci by Stoll et al. who used positional cloning for this task.[249] *DLG5* is a member of the membrane-associated guanlyate kinase (MAGUK) family and, as a **scaffold protein**, has an important role in **maintaining epithelial cell integrity**.[250,251] Consequent to its location at cell-cell contact sites, it **maintains cell shape and polarity**.[252,253]

The *DLG5* SNP G113A results in the **amino acid substitution R30Q**, possibly having functional consequences.[249] It is interesting to note that **ethnic variation** has been uncovered in the transmission of this polymorphism among IBD patients, though it is not surprising and unusual. A similar trend has been observed with *CARD15* in Caucasian versus Asian populations.[254] Likewise, an analysis of Japanese CD patients revealed no association with the *DLG5* R30Q polymorphism; however, a different variant, rs3758462, was detected in this particular group.[255] Further support for genetic heterogeneity of the R30Q variant comes from a Greek CD study population, where the variant was completely absent.[256]

Recently, a **protective role** for *DLG5* R30Q polymorphism **in females** has been published. In the first such study, Biank and colleagues identified a significant negative association (OR: 0.39; $p = 0.006$) between CD and female children. Studying a group of 281

CD patients against 479 control subjects, they initially found no association between the R30Q variant and Crohn's disease. In a second statistical step, they stratified the groups by gender and found a protective effect for *DLG5* R30Q in female children but not males (OR: 0.39; $p = 0.006$).[257]

6.3. Aims of Study I

In view of the limited data on the prevalence of DLG5 mutations in East European countries, our aim was to investigate the presence of the DLG5 R30Q variant allele in a large cohort of Hungarian patients with IBD. We also aimed to investigate a possible association between genotype and clinical phenotype, need for surgery and response to medical therapy; as well, the possible association between DLG5, NOD2/CARD15 and TLR4 D299G polymorphism in CD patients was studied. As mentioned previously, *DLG5*'s function as a scaffolding gene provides a plausible argument for its role in IBD secondary to intestinal barrier dysfunction. Our secondary aim was to specifically investigate whether there is an association between the efficacy of infliximab therapy and the DLG5 variant.

6.4. Aims of Study II

The *MDR1* gene encodes an ATP-binding cassette transporter (Pgp-170) that confers resistance to multiple drugs from a very broad range of classes including antibiotics, anti-cancer drugs, cardiac drugs and steroids. However, it addition to its role, research has shown that its localization to the intestinal epithelial layer may be also protective against bacterial products. Subsequently, a dysfunction in the Pgp-170 protein may play a role in IBD pathogenesis and resistance to medical therapy. In view of the limited data on the prevalence of MDR1 and the absence of data on the relatively frequent (G34A and C421A) ABCG2 variants in East European countries, our aim was to investigate the presence of the variant alleles of these genes in a large cohort of Hungarian patients with IBD. We also aimed to investigate a possible association between genotype and clinical phenotype, need for surgery and response to medical therapy.

7. PATIENTS & METHODS

7.1. Patient Group - Study I

Our study cohort consisted of 773 unrelated IBD patients of which 639 patients were afflicted by CD. The average age of all IBD patients was 38.1 ± 10.3 years with a disease duration of 8.8 ± 7.5 years. More specifically, our CD patients (m/f = 309/330) had a duration of 8.4 ± 7.1 years, while among the 134 UC patients (m/f = 63/71) disease duration was 10.6 ± 8.9 years. In addition, we also had 150 healthy control subjects, who did not suffer form any gastrointestinal or liver disorders. They were age- and gender-matched with an average age of 37.6 ± 10.3 years and a male-to-female ratio of 72/78.

7.2. Patient Group – Study II

This study consisted of 414 unrelated IBD patients (265 CD, 149 UC) and 149 healthy control subjects. The average of our CD cohort was 35 ± 12.1 years and for UC 44.5 ± 15.4 years. The male-to-female ratio was 129/136 and 73/76 for CD and UC, respectively. For our CD cohort, disease duration was 8.7 ± 7.6 years, while in UC it was 10.7 ± 8.9 years.

7.3. Methods – Study I

7.3.1. Clinical Data Collection, Statistics, Ethics

The patients' diagnoses were made according to the Lennard-Jones criteria.[258] Background demographic and clinical information was collected by reviewing their medical charts and a questionnaire. This data included: age, age at onset, presence of extraintestinal manifestations (arthritis, ocular manifestations, dermatological lesions, primary sclerosing cholangitis), frequency of flare-ups, therapeutic effectiveness (prescribed medication and efficacy, steroid resistance), surgery associated with IBD, familial IBD cases, and smoking habits. Additionally, CD patients were questioned about perianal involvement. The Vienna Classification was used to determine disease phenotype (age at onset, duration, location, and behavior).[107] Our cohort's clinical data are presented in Tables 20 and 21 for CD and UC, respectively.

The Shapiro-Wilk's W test was used for testing the normality of our sample, while the *t* test with separate variance estimates, χ^2 test, and χ^2 with Yates' correction were used for calculating differences within IBD subgroups and between IBD and controls. A logistic regression was used to find an association between clinical and genetic data, expressed as odds ratio (OR) with 95% confidence interval (95% CI). We considered a p value < 0.05 as statistically significant.

Each patient was informed of the nature of the study and signed the informed consent form. Furthermore, the protocol was approved by the Ethical and Science Committee of the Ministry of Health (ETT-TUKEB, 321-81/2005-1018EKU) and the Semmelweis University Regional and Institutional Committee of Science and Research Ethics (81/2003).

7.3.2. Genetic Methods

Detection of the DLG5 R30Q polymorphism

To detect the R30Q variant of *DLG5*, we followed the process described by Török et al.,[314] using polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) with the *Msp*I restriction enzyme. For this purpose, genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini Kit (QIAgen GmbH, Germany). The forward primer was 5'-GGA AGG CGC AGT CCC CAC CAC CCC TCC TCA C, while the reverse primer was 5'-AAG GCC AGG CGC TTG CGG AGC TCG TTT CTC TCC TGG. 3% agarose gel was used for detection.

Detection of NOD2/CARD15 and TLR4 polymorphisms

The *NOD2* SNP 8 (R702W), 12 (G908R), and 13 (3020insC) polymorphisms have already been genotyped for 475 CD patients, as has the *TLR4* D299G.[259] *NOD2* mutations were detected using denaturing high-performance liquid chromatography (Wave DNA Fragment Analysis System, Transgenomic Ltd., Cramlington, UK), followed by sequencing of both strands to confirm variations. Sequencing reactions were performed with the ABI BigDye v1.1 Terminator Cycle Sequencing Kit followed by electrophoresis on the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). The D299G polymorphism was detected using the PCR/RFLP technique and visualized on 3% agarose GTG gel (NuSieve, BMA, Rockland, ME).

7.4. Methods – Study II

7.4.1. Clinical Data Collection, Statistics, Ethics

As in the previous study, the diagnosis was based on the Lennard-Jones criteria[258] with the demographics and clinical data collected through a medical chart review and a questionnaire. These parameters included age, age at onset, presence of extraintestinal manifestations (arthritis, ocular manifestations, dermatological lesions, primary sclerosing cholangitis), frequency of flare-ups, therapeutic effectiveness (prescribed medication and efficacy, steroid resistance), surgery associated with IBD, familial IBD cases, and smoking

habits. In addition, CD patients were asked about perianal disease. Similarly, the Vienna Classification was used to confirm disease phenotype according to age at onset, duration, location, and behavior.[107] Our cohort's clinical data are presented in Tables 24 and 25.

In addition to the Shapiro-Wilk's W test for normality, we also used *t*-tests with separate variance estimates and χ^2, both with and without Yates' correction to compare data between the IBD group and the control subjects, and within the IBD subgroups. Logistic regression was used to link the genetic with the clinical data, with the results being expressed as OR with 95% CI.

As already noted in the previous study, each patient was informed of the nature of the study and those in agreement signed an informed consent form. The protocol was approved by the Ethics and Science Committee of the Ministry of Health and the Semmelweis University Regional and Institutional Committee of Science and Research Ethics.

7.4.2. Genetic Methods

Whole DNA was processed using QIAamp DNA Blood Mini Kit (QIAgen GmbH, Germany) in order to retrieve genomic DNA. For each of the transporter genes, *MDR1* and *ABCG2*, we tested for the presence and frequency of two variants. For the former, G2677T/A (Ala893Ser/Thr on exon 21) and C3435T (a silent base substitution of I1145I on exon 26) were examined. For *ABCG2*, we investigated G34A (Val12Met, exon 2) and C421A (Gln141Lys, exon 5). The hybridization probes for both *MDR1* polymorphisms, used with the LightCycler (Roche Diagnostics, Basle, Switzerland) for genotyping, were previously published.[260,261] **The technique described here was developed particularly for this study** for investigating these polymorphisms. For the G2677T/A, the forward and reverse primers were 5'-GCA GGA GTT GTT GAA ATG AAA ATG and 5'-CGC CTG CTT TAG TTT GAC TCA, respectively.[261] The forward and reverse primers for C3435T were 5'-TGT TTT CAG CTG CTT GAT GG, and 5'-AAG GCA TGT ATG TTG GCC TC, respectively.[260] Additional amplification primers and hybridization probes were designed by the LightCycler Probe Design software (Roche Diagnostics) and Primer3.[262] Tables 16 and 17 present the reaction mixture and details for the *MDR1* variants, while Tables 18 and 19 summarize the reaction mixtures used for the *ABCG2* variant amplification.

Table 16. Reaction Mixture for *MDR1* Polymorphism Detection

Component	**Amount per reaction**
2× PCR Master Mix (Promega)	5 µL
Distilled water	1.6 µL
2677 primer mix	1.0 µL
3435 primer mix	1.0 µL
Finnzyme *Taq* DNA polymerase (2 U/µL)	0.8 µL

Table 17. Reaction Details for *MDR1* by Polymorphism

G2677T/A asymmetric PCR (F:R = 8.3:1)	**C3435T asymmetric PCR (F:R = 1:5)**
50 µl MDR1-2677-F (100 pmol/µl)	6 µl MDR1-3435-F (100 pmol/µl)
6 µl MDR1-2677-R (100 pmol/µl)	30 µl MDR1-3435-R (100 pmol/µl)
10 µl MDR1-2677-SENS (100 pmol/µl)	10 µl MDR1-3435-SENS (100 pmol/µl)
10 µl MDR1-2677-ANC (100 pmol/µl)	10 µl MDR1-3435-ANC (100 pmol/µl)
324 µl distilled water	344 µl distilled water

Table 18. Reaction Mixture for the *ABCG2* V12M Polymorphism Detection

Component	Amount per reaction
2× PCR Master Mix (Promega)	5 µL
Distilled water	2.15 µL
$MgCl_2$ (25 mM)	0.35 µL
MXR-V12M-LCF (10mM)	0.5 µL
MXR-V12M-R-LCR (10 mM)	0.15 µL
MXR-V12M-ANC (10 mM)	0.25 µL
MXR-V12M-SENS (10 mM)	0.25 µL
Finnzyme *Taq* (2 U/µL)	0.35 µL

Table 19. Reaction Mixture for the *ABCG2* Q141K Polymorphism Detection

Component	Amount per reaction
2× PCR Master Mix (Promega)	5 µL
Distilled water	0.75 µL
$MgCl_2$ (25 mM)	0.2 µL
MXR-Q141K-LCF (10µM)	0.2 µL
MX-E166Q-R-LCF (10 µM)	2.0 µL
MXR-Q141K-ANC (10 µM)	0.25 µL
MXR-Q141K-SENS (10 µM)	0.25 µL
Finnzyme *Taq* (2 U/µL)	0.35 µL

An asymmetric system was used, as described by Szilvási et al., in order to achieve a higher efficiency for the melting peak analysis.[263] For the simultaneous amplification of *MDR1* variants, 12.5:1.5 pmol forward (F):reverse (R) and 1.5:7.5 pmol F:R were used for G2677T/A and C3435T, respectively. Likewise, the asymmetric PCR for *ABCG2* consisted of 5:1.5 pmol F:R for G34A and 2:20 F:R for C421A, in separate amplification reactions. The

cycling conditions were almost identical in the amplification reactions of both genes (Figure 12). The decline in fluorescence was continuously monitored with melting peaks displaying distinct patterns for the wild-type and variant alleles (*MDR1*: Figures 13 and 14; *ABCG2*: Figure 15). Two independent investigators reviewed the results.

Figure 12. PCR Temperature Cycling Protocol

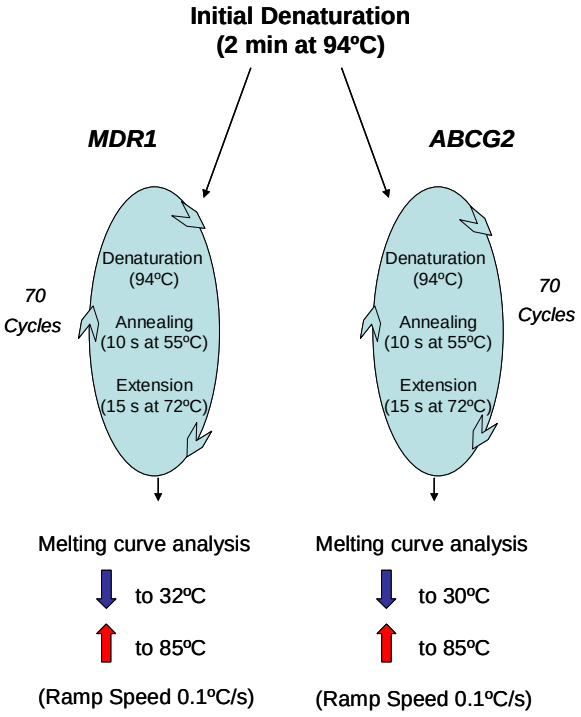


Figure 13. LightCycler Melting Curve of *MDR1* G2677T/A

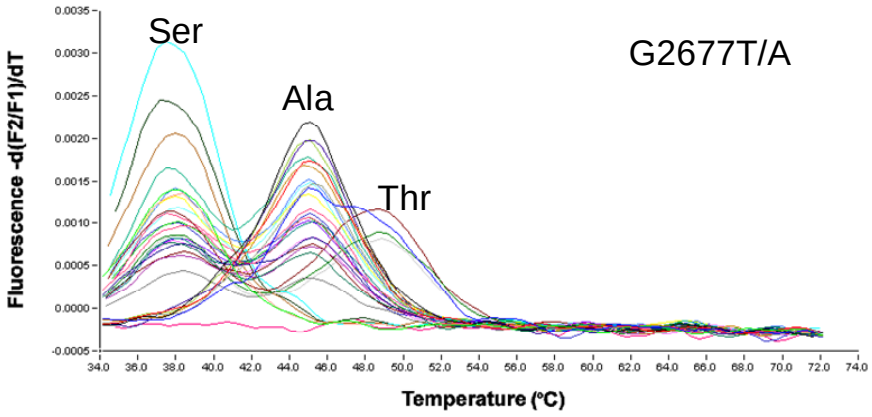


Figure 14. LightCycler Melting Curve of *MDR1* C3435T

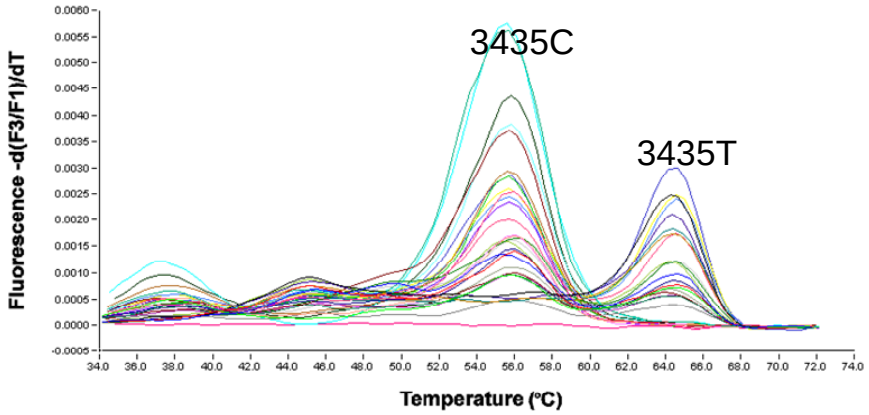
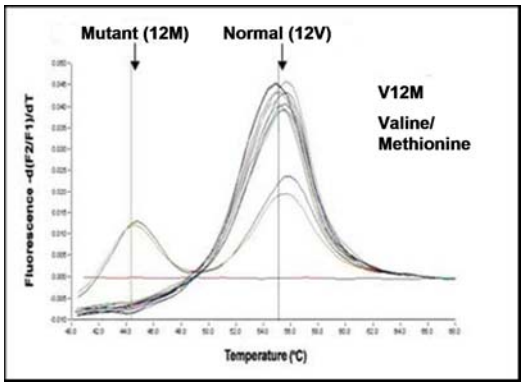
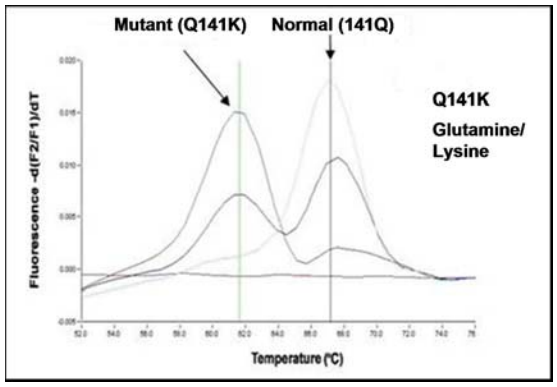


Figure 15. LightCycler Melting Curves of *ABCG2* V12M and Q141K





8. RESULTS

8.1. STUDY I: *DLG5* R30Q is not associated with IBD in Hungarian IBD patients but predicts clinical response to steroids in Crohn's disease

Our results demonstrated no significant difference in the frequency of the *DLG5* R30Q morphism between IBD (22.0%), CD (20.8%), and UC (27.6%) compared with the healthy control group where the frequency was 28.0%.

Tables 20 and 21 present the cohort's clinical data as well as genotypic distribution and demographics.

Table 20. Clinical Data of Crohn's Disease Patients divided by Presence of *DLG5* R30Q

	All (n = 639)	***DLG5* R30Q carrier (n = 133)**	***DLG5* R30Q non-carrier (n = 506)**
Male/female	309/330	66/67	243/263
Age (years)	36.8 ± 12.7	37.7 ± 13.5	36.6 ± 12.5
Age at presentation (years)	28.4 ± 11.4	28.9 ± 12.1	28.2 ± 11.2
Duration (years)	8.4 ± 7.1	8.5 ± 7.9	8.4 ± 6.9
Familial IBD	78 (12.2%)	14 (10.5%)	64 (12.6%)
Location (n)			
L1	152	26	126
L2	166	35	131
L3	314	71	243
L4	7	1	6
Behavior (n)			
B1	257	52	205
B2	159	30	129
B3	223	51	172
Perianal disease	181(28.3%)	37 (27.8%)	144 (28.5%)
Frequent relapse	244 (38.2%)	55 (41.3%)	189 (37.3%)
Arthritis	203 (31.7%)	39 (29.3%)	164 (32.4%)
Occular	45 (7.0%)	10 (7.5%)	35 (6.9%)
Erythema nodosum/ Pyoderma gangrenosum	51 (7.9%)	12 (9.0%)	39 (7.7%)
PSC	23 (3.5%)	5 (3.7%)	18 (3.5%)
Steroid use/refractory	499 (78.1%) / 47 (9.4%)	104 (78.2%) / 17 (16.3%)*	395 (78.1%) / 30 (7.6%)*
Azathioprine use	407 (63.7%)	87 (65.4%)	320 (63.2%)
Operation	268 (41.9%)	50 (37.6%)	218 (43.1%)
Smoking habits (n)			
no	383	72	311
yes	199	46	63
previous	57	15	41
***NOD2* (n = 475)**			
carrier	153	35	118
non-carrier	322	64	258
***TLR4* (n = 475)**			
carrier	48	12	36
non-carrier	427	87	340

*OR: 2.4; 95% CI: 1.3-4.5; $p = 0.013$

Table 21. Clinical Data of Ulcerative Colitis Patients divided by Presence of *DLG5* R30Q

	All (n = 134)	*DLG5* R30Q carrier (n = 37)	*DLG5* R30Q non-carrier (n = 97)
Male/female	63/71	18/19	45/52
Age (years)	44.1 ± 15.1	44.5 ± 15.5	44.1 ± 14.9
Age at presentation (years)	33.9 ± 14.1	34.1 ± 14.7	33.8 ± 13.9
Duration (years)	10.6 ± 8.9	10.3 ± .18	10.7 ± 9.3
Familial IBD	8 (5.9%)	2 (5.4%)	6 (6.1%)
Maximum extent (n)			
proctitis	5	1	4
left-sided	77	17	60
extensive	52	19*	33*
Chronic continuous	25 (18.7%)	7 (18.9%)	18 (18.5%)
Arthritis	35 (26.1%)	9 (24.3%)	26 (26.8%)
Ocular	6 (4.5%)	3 (8.1%)	3 (3.1%)
Erythema nodosum/ Pyoderma gangrenosum	6 (4.5%)	2 (5.4%)	4 (4.1%)
PSC	5 (3.7%)	2 (5.4%)	3 (3.1%)
Steroid use/refractory	69 (51.5%) / 10 (14.5%)	20 (54.1%) / 2 (10.0%)	49 (50.5%) / 8 (16.3%)
Azathioprine use	27 (20.2%)	8 (21.6%)	19 (19.6%)
Operation	5 (3.7%)	3 (8.1%)	2 (2.1%)
Smoking habits (n)			
no	103	28	75
yes	15	3	12
previous	16	6	10

*OR: 2.1; 95% CI: 0.95-4.4; $p = 0.07$

The following table presents the carriage distribution of R30Q in all groups, both in absolute values and by percentage.

Table 22. Carrier Rates of *DLG5* R30Q in CD, UC and Control Subjects

	DLG5 R30Q Genotype, n (%)				
	Non-carrier	**All carriers**	**Heterozygous**	**Homozygous**	**Variant allele frequency**
CD (n = 639)	506 (79.2)	133 (20.8)*	122 (19.1)	11 (1.7)	144 (11.3)*
UC (n = 134)	97 (72.4)	37 (27.6)	35 (26.1)	2 (1.5)	39 (14.6)
Control (n = 150)	108 (72)	42 (28)†	39 (26)	3 (2)	45 (15)†

n (%)

*OR = 0.67; 95% CI: 0.45-1.013; $p = 0.06$ vs. control subjects

†OR = 0.72; 95% CI: 0.50-1.03; $p = 0.07$ vs. control subjects

A tendency was observed in the CD group toward a protective role by 113A (OR: 0.67; 95% CI: 0.45-1.013), as well as an association with steroid resistance (16.3% carriers

vs. 7.6% non-carriers; OR: 2.4; 95% CI: 1.3-4.5; $p = 0.013$). In addition, steroid resistance was independently associated with penetrating disease (OR: 2.0; $p = 0.046$), perianal manifestations (OR: 2.5; $p = 0.07$), and frequent relapses (OR: 4.2; $p < 0.00001$) but not with location, familial disease, or need for surgery in univariate analysis. Using a logistic regression, steroids resistance was independently associated with R30Q carriage, perianal disease, and frequent relapse. In addition, after correcting for various confounding factors (Table 23) by logistic regression, we noted a significant association between the *DLG5* 113A allele and steroid resistance. Again, disease location was not associated with the *DLG5* polymorphism using logistic regression. As noted in Table 20, 78.1% of patients received steroid treatment during the course of their disease. Finally, no association was found between carriage of the *DLG5* R30Q and any of the *NOD2* and *TLR4* mutations genotyped in this cohort. In ulcerative colitis patients, we noted a trend towards a more aggressive form of disease (51.4% vs. 34.0% in controls; OR: 2.1; 95% CI: 0.95-4.4; $p = 0.07$) with no additional associations.

Table 23. Logistic Regression: Association between Clinical Phenotype, Carriage of *DLG5* R30Q Variant Allele, and Steroid Resistance in Patients with Crohn's Disease

Factor	**Coefficient**	***P* value**	**OR**	**95% CI**
Female gender	0.09	0.78	1.09	0.57-2.09
DLG5 113A allele	**0.807**	**0.02**	**2.24**	**1.14-4.43**
Perianal disease	**0.84**	**0.02**	**2.31**	**1.15-4.67**
Penetrating disease	-0.08	0.82	0.92	0.45-1.87
Frequent relapses	**1.30**	**0.001**	**3.66**	**1.71-7.84**
Azathioprine use	1.35	0.03	3.85	1.14-12.93
Current smoking	0.48	0.14	1.62	0.85-3.11

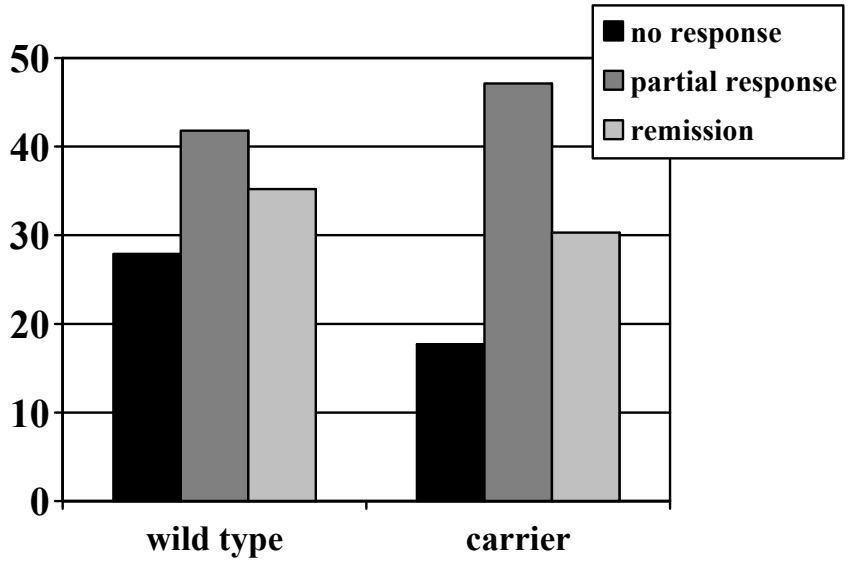
In a separate part of our study, we also investigated the association between *DLG5* R30Q and response to infliximab therapy (5 mg/kg body weight). The sub-study's cohort consisted of 25 male and 35 female CD patients with an average age of 34.3 ± 11.1 years and disease duration of 7.9 ± 5.1 years (Table 24). In just over half of the patients (32), the disease was localized to the ileocolon, while 22 had colonic disease and 6 patients displayed ileal form. Three patients had upper gastrointestinal disease. The disease was classified as

penetrating in 33 patients and inflammatory in 27, with perianal involvement in 33 patients. Approximately half of the patients (51.7%) had some arthritic manifestations, while the rate of ocular and dermatological manifestations was also elevated (16.7% and 13.3%, respectively). The greater majority of the cohort was prescribed steroids (95.0%) or azathioprine (93.3%). While 23 patients received infliximab treatment for fistulizing disease, 37 patients were subscribed infliximab for inflammatory disease that did not respond to conventional therapy. Within the infliximab group, 17 patients were carriers of the 113A variant. Assessing the response in the short-term, at week 8, no association was found between carriers and non-carriers with regards to treatment response (Figure 16).

Table 24. The Clinical Phenotypes of CD Patients in Infliximab Sub-study

Male/female		**25/35**
Age at presentation (years)		28.4 ± 11.4
Age (years)		36.8 ± 12.7
Duration (years)		8.4 ± 7.1
Location	**L1**	6
	L2	22
	L3	32
	L4	(3)
Behaviour	**B1**	27
	B2	-
	B3	33
Perianal involvement		33 (55%)
Extraintestinal manifestations:		
- Arthritis		31 (51.7%)
- Ocular		10 (16.7%)
- Cutaneous		8 (13.3%)
- PSC		2 (3.3%)
Steroid use/refrectory		57 (95%) / 17(29.8%)
Azathiprine use		56 (93.3%)
***DLG5* R30Q**		17 (28.3%) vs. controls (28.0%)

Figure 16. Association between the Presence of *DLG5* R30Q and response to Infliximab Induction Therapy (5 mg/bwkg at weeks 0, 2, and 6) Assessed at Week 8 in CD Patients



partial response: CDAI decrease by ≥70 points and/or ≥50% decrease in the number of draining fistulae, remission: CDAI<150 or closure of all fistulas

There was no significant association between *DLG5* R30Q and CD or UC; however, a protective trend was observed in Crohn's disease. No interaction was observed between *DLG5* R30Q and either *NOD2* or *TLR4* polymorphisms in our cohort. In CD patients, a positive association was observed between *DLG5* R30Q carriage and steroid resistance. In UC patients, a positive trend was observed between *DLG5* R30Q carriage and extensive disease. No association was found between *DLG5* R30Q carriage and response to short-term infliximab treatment in a sub-cohort of CD patients.

8.2. STUDY II: The ATP-binding cassette transporter *ABCG2* (*BCRP*) and *ABCB1* (*MDR1*) variants are not associated with disease susceptibility, disease phenotype, response to medical therapy or need for surgery in Hungarian patients with inflammatory bowel disease

Our cohort's clinical data as well as demographic and genotypic distribution are presented in Tables 25 and 26.

Table 25. Clinical Data of Patients with Crohn's Disease divided by *ABCG2* and *MDR1* C3435T

	All (n = 265)	***ABCG2* carrier (n = 72)**	***ABCG2* non-carrier (n = 193)**	***MDR1* 3435CC (n = 66)**	***MDR1* 3435 CT or TT (n = 199)**
Male/female	129/136	36/36	93/100	27/39	102/97
Age (years)	35.1 ± 12.1	36.0 ± 13.1	34.9 ± 11.8	34.1 ± 11.5	35.5 ± 12.3
Age at presentation (years)	26.6 ± 10.5	27.2 ± 11.6	26.4 ± 10.1	26.8 ± 10.8	26.6 ± 10.4
Duration (years)	8.7 ± 7.6	8.9 ± 8.1	8.6 ± 7.3	7.4 ± 6.0	9.1 ± 7.9
Familial IBD	31 (11.7%)	7 (9.7%)	24 (12.4%)	8 (12.1%)	23 (11.6%)
Location (n)					
L1	59	17	42	16	43
L2	67	18	49	19	48
L3	133	36	97	30	103
L4	6	1	5	1	5
Behavior (n)					
B1	93	24	69	18	75
B2	73	19	54	18	55
B3	99	29	70	30	69
Perianal disease	97(36.6%)	26 (36.1%)	71 (36.8%)	29 (43.9%)	68 (34.1%)
Frequent relapse	118 (44.52%)	32 (44.4%)	86 (44.6%)	32 (48.5%)	86 (43.2%)
Arthritis	108 (40.7%)	29 (40.2%)	79 (40.9%)	29 (43.9%)	79 (36.7%)
Steroid use/refractory	192 (72.4%)/ 36 (18.7%)	51 (70.8%)/ 10 (19.6%)	141 (73.1%)/ 26 (18.4%)	45 (68.1%)/ 10 (22.2%)	147 (73.9%)/ 26 (17.6%)
Azathioprine use	184 (69.4%)	45 (62.5%)	139 (72.0%)	50 (75.7%)	134 (67.4%)
Surgery	115 (43.4%)	33 (45.8%)	82 (42.5%)	31 (46.9%)	84 (42.2%)
Response to infliximab					
remission	24	7	19	9	15
partial	17	3	12	6	11
no effect	6	1	5	1	5
Smoking habits (n)					
no	156	40	116	33	123
yes	88	26	62	25	63
previous	22	16	6	8	14

Table 26. Clinical Data of Patients with Ulcerative Colitis divided by *ABCG2* and *MDR1* C3435T

	All (n = 149)	***ABCG2* carrier (n = 45)**	***ABCG2* non-carrier (n = 104)**	***MDR1* 3435CC (n = 39)**	***MDR1* 3435 CT or TT (n = 110)**
Male/female	73/76	24/21	49/55	19/20	54/56
Age (years)	44.4 ± 15.5	43.2 ± 16.9	44.9 ± 14.8	49.2 ± 17.2[#]	42.8 ± 14.5[#]
Age at presentation (years)	34.1 ± 14.6	31.2 ± 14.6	35.3 ± 14.5	37.2 ± 15.7	33.0 ± 14.1
Duration (years)	10.7 ± 8.9	12.1 ± 10.9	10.2 ± 7.9	12.2 ± 11.3	10.3 ± 7.9
Familial IBD	11 (7.3%)	2 (4.4%)	9 (8.6%)	3 (7.7%)	8 (7.2%)
Maximum extent (n)					
proctitis	8	2	6	2	6
left-sided	85	27	58	25	60
extensive	56	16	40	12	44
Chronic continuous	29 (19.5%)	8 (17.7%)	21 (20.2%)	8 (20.5%)	21 (19.1%)
Arthritis	40 (26.8%)	7 (15.5%)*	33 (31.7%)*	12 (30.7%)	28 (25.4%)
Steroid use/refractory	82(55.0%) / 12 (14.6%)	24 (53.3%) / 2 (8.3%)	58 (55.7%) / 10 (17.2%)	17 (43.6%) / 1 (5.9%)	65 (59.1%) / 11 (16.9%)
Azathioprine use	30 (20.1%)	12 (26.6%)	18 (17.3%)	9 (23.1%)	21 (19.1%)
Colectomy	5 (3.3%)	3 (6.6%)	2 (1.9%)	1 (2.6%)	4 (3.6%)
Smoking habits (n)					
no	114	38	76	31	83
yes	17	3	14	2	15
previous	18	4	14	6	12

[#]$p = 0.04$; *OR = 0.39; 95% CI: 0.16-0.98; $p = 0.046$

We found no significant differences in the frequency of *ABCG2* variant alleles G34A and C421A between IBD (4.9% and 10.6%, respectively), CD (5.5% and 9.6%), UC (4.0% and 11.7%) and the control subjects (4.0% and 9.4%). Similarly, no significant differences were found between *MDR1* C3435T and G2677T/A, and IBD or its individual forms. However, the G2677 allele displayed a tendency in CD towards an association with disease susceptibility (58.7% vs. 52.4%, OR:$2677_{\text{G vs. T/A}}$: 1.29; 95% CI: 0.97-1.72; $p = 0.08$). Table 27 presents the distribution of *MDR1* C3435T and G2677T/A, according to CD, UC or control group.

Table 27. *MDR1* C3435T and G2677T/A Genotypes and Allele Frequencies in Hungarian IBD Patients versus Control Subjects

	CD (n = 265)	UC (n = 149)	Controls (n = 146)
C3435T			
C	264 (49.8%)	154 (51.6%)	148 (50.7%)
T	266 (50.2%)	144 (48.4%)	144 (49.3%)
CC	67 (25.3%)	39 (26.2%)	40 (27.4%)
CT	130 (49.1%)	76 (51.0%)	68 (46.6%)
TT	68 (25.6%)	34 (22.8%)	38 (26.0%)
G2677TA			
G2677	311 (58.7%)*	162 (54.4%)	153 (52.4%)*
2677T	205 (38.7%)*	123(41.3%)	131 (44.8%)*
2677A	14 (2.6%)*	13 (4.3%)	8 (2.8%)*
GG	94 (35.5%)	41 (27.5%)	46 (31.5%)
GT	118 (44.5%)	71 (47.6%)	59 (40.4%)
TT	39 (14.7%)	24 (16.1%)	33 (22.6%)
GA	5 (1.9%)	9 (6.0%)	2 (1.4%)
TA	9 (3.4%)	4 (2.8%)	6 (4.1%)

n (%), * $p = 0.08$, $OR_{2677G \text{ vs. } T/A}$: 1.29, 95% CI: 0.97-1.72

The genotype distribution of the *ABCG2* variants, G34A and C421A, are presented in Table 28.

Table 28. Genotypic Distribution of *ABCG2* G34A and C421A in Hungarian IBD Patients versus Control Subjects

	***ABCG2* G34A**		***ABCG2* C421A**		**All carriers**
	Heterozygous	**Homozygous**	**Heterozygous**	**Homozygous**	
CD (n = 265)	29 (10.9%)	0	47 (17.7%)	2 (0.7%)	72 (27.2%)
UC (n = 149)	12 (8.1%)	0	35 (23.5%)	1 (0.7%)	45 (30.2%)
Controls (n = 149)	12 (8.1%)	0	28 (18.8%)	0	40 (26.8%)

p – not significant

In addition, no association was found between the *MDR1* and *ABCG2* SNPs investigated in our cohort; however, the two *MDR1* SNPs were associated to each other in the IBD, CD, UC and control group. The *MDR1* 2677TT genotype was associated with 3435CC (56.6% vs. 1% in non-3435CC), 2677GT with 3435CT (70.6% vs. 20.7% in non-3435CT), and 2677GG with 3435TT (85.3% vs. 15.4% in non-3435TT) (Table 29). Examining the genotype and EIMs, using univariate analysis, we noted a protective effect for *ABCG2* against arthritis in UC (OR: 0.39; 95% CI: 0.16-0.98; $p = 0.046$); yet no associations were found

between other extraintestinal manifestations (ocular, dermatological, or PSC) and either *MDR1* or *ABCG2* carriage.

Table 29. Association between *MDR1* C3435T and G2677T/A in Hungarian IBD Patients

	G2677T/A				
MDR1	GG	GT	TT	GA	TA
C3435T					
CC	6	35	60	1	4
CT	42	146	3	6	9
TT	87	8	0	7	0

As in Study I, here we also examined for possible associations between *MDR1* and *ABCG2* polymorphisms and response to infliximab induction treatment (5 mg/kg body weight) assessed at week 8. A total of 47 CD patients (24 males, 23 females) at an average age of 33.2 ± 11.6 years and disease duration of 7.6 ± 4.7 years were included in this sub-study. Disease location was identified as ileocolonic in 25 patients, colonic in 19 patients, ileal in 2 patients, and 3 patients with upper gastrointestinal disease. An inflammatory disease was present in 17 patients, 30 patients had a penetrating form of disease, and perianal involvement was observed in over half of patients (27; 57.4%). The greater majority of patients were prescribed azathioprine (44/47, 93.6%) and steroids (44/47, 93.6%). We found no associations between any of the polymorphisms tested and short-term response to infliximab at week 8. The results are detailed in Table 25.

No significant associations were observed between the *ABCG2* variants, G34A or C421A, *MDR1* variants, C3435T or G2677T, and IBD. However, a positive trend was observed for *MDR1* G2677 allele to increase susceptibility in CD patients. The expression of the *MDR1* variants was linked. Carriage of the *ABCG2* presented a statistically significant protective effect against arthritis in UC patients. Other extraintestinal manifestations were not associated with *MDR1* or *ABCG2* variants. No association was found between any of the *MDR1* or *ABCG2* variants, and short-term infliximab therapy.

9. DISCUSSION

9.1. Genetic Factors

Many groups have established the existence of **IBD susceptibility loci** in the human genome[264]; however, no clear cause-and-effect has been identified with certainty. The genes associated with IBD can be divided into three different subgroups: 1. genes involved in bacterial sensing and autophagy; 2. Mucosal integrity and transport; and 3. cytokine genes. In this thesis, we investigated members from the second group, as well as possible associations between the studied *DLG5* variant and *NOD2/CARD15* or *TLR4*.

The primary susceptibility locus was localized to chromosome 16q and named **NOD2** (*n*ucleotide *o*ligomerization *d*omain)/**CARD15** (*c*aspase *a*ctivation *r*ecruitment *d*omain).[265] Mutations in the wild-type NOD2/CARD15 proteins are associated with early onset,[266,267] involvement of the terminal ileum,[268,269] and a fibrostenosing disease form.[270] Additionally, *NOD2/CARD15* mutations have a functional effect by **decreasing the activity of NF-κB** in response to LPS.[271] In general, it seems that **CD has a greater genetic predisposition** than UC, as expressed by a familial pattern of inheritance.[272] However, **some susceptibility loci** (eg, on chromosome 6p) **appear in both CD and UC**, indicating a small overlap in IBD's genetic etiology.[273,274,275] In **ulcerative colitis**, **HLA genes** are associated with **disease pathogenesis** as well as **extraintestinal manifestations**.[44,46] Finally, evidence is accumulating for the involvement of various **cytokines** (eg, IL-2, IL-6, IL-12, IL-17, TNF) in the pathogenesis, maintenance or aggravation of IBD.[44,264,276] Figure 17 illustrates the identified chromosomal loci with their respective IBD genes.[46]

9.1.1. Genes Involved in Bacterial Recognition

9.1.1.1. *NOD2/CARD15*

In 2001, three independent groups identified the **first susceptibility gene for CD**; it was localized to chromosome **16q (IBD1)** and named *NOD2* – later renamed as *CARD15*.[277,278,279] The ***NOD2/CARD15*** gene belongs to the Apaf-1/CARD superfamily of cytosolic proteins involved in apoptosis and activation of **nuclear factor-κB** (NF-κB).[280]

The wild-type *NOD2/CARD15* protein consists of 1040 amino acids and is composed of two CARDs on the N-terminus, a nucleotide binding domain (NBD), and a sequence of leucine-rich repeats (LRR) on the carboxy-terminus,[270] as depicted in Figure 18.[281] The LRR are structurally similar to a portion of the Toll-like receptors (TLR) and might serve a similar function in the **recognition of exogenous microbial components**, for example, lipopolysaccharide (LPS). The NOD2/CARD15 protein is expressed with the highest

concentration in **circulating monocytes** as well as **epithelial cells of the small intestine** and in **Peyer's patches**.[280]

Figure 17. Chromosomal Loci Identified with IBD Susceptibility Genes[46]

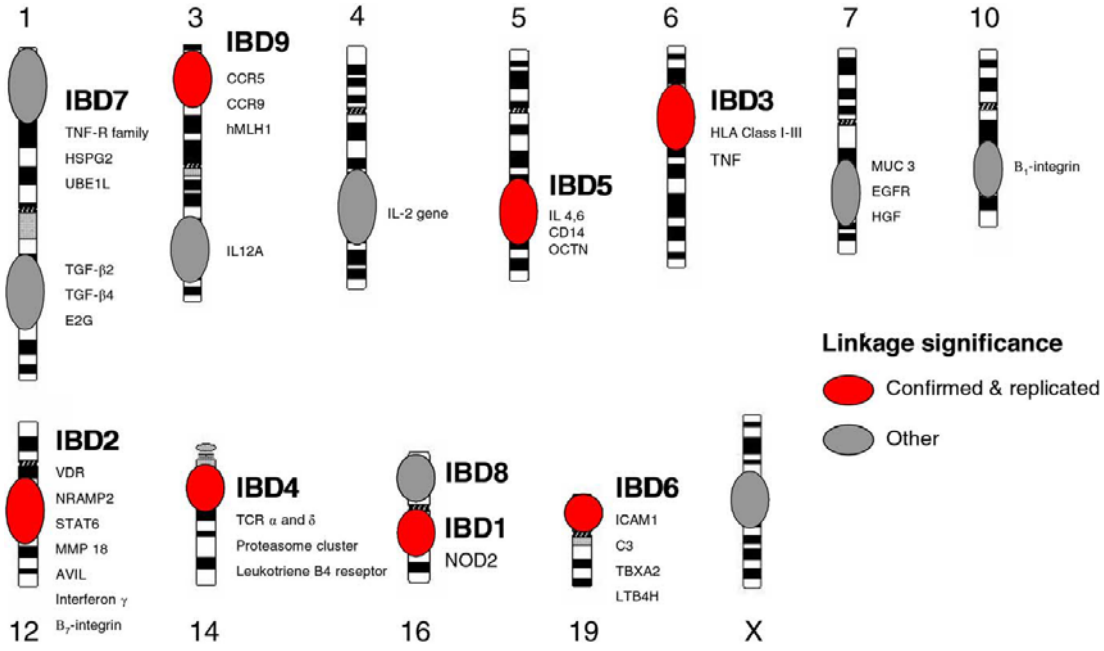
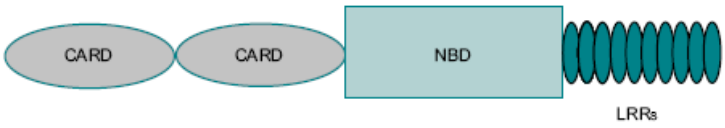


Figure 18. Schematic Organization of NOD2/CARD15[281]



Two pathways leading to NF-κB activation are depicted in Figure 19.[267] The left-hand pathway is initiated by TLR, while the second by a NOD receptor protein. Further explanation on the TLRs is provided in a separate section. As previously mentioned, these receptors are able to **recognize bacterial LPS**, which belongs to a larger group of **pathogen associated microbial patterns** (PAMPs), also including **peptidoglycan** (PGN) and **muramyl dipeptide** (MDP).[282] Unlike LPS, which is present only in the cell wall of Gram negative ($Gram^-$) bacteria, PGN is present in both $Gram^-$ and Gram positive ($Gram^+$) bacteria. After the initial recognition of PAMP, the NOD2/CARD15 protein will form a homodimer with another

NOD2/CARD15 unit, interact with the cytosolic serine-threonine kinase RICK, leading to further protein activation and culminating in a **functional NF-κB**.[280] This latter protein will **initiate proinflammatory reactions**.[283] It is important to note that there is still some contradiction in the literature as to the true ligand of NOD2/CARD15. It stems from the notion that some researchers believe that PGN, through MDP, is the only ligand for NOD2/CARD15.[284]

Mutations of the NOD2/CARD15 gene and the subsequent malformed protein are strongly associated with the development of **Crohn's disease**, yet lack any association with ulcerative colitis.[46] Specifically, there are three mutations that are most frequent and strongly associated with CD. These are:

- SNP8 (Arg702Trp - tryptophan substituted for arginine at codon 702)
- SNP12 (Gly908Arg - arginine substituted for glycine at codon 908)
- SNP13 (Leu1007fsinsC - frameshift mutation that cuts off the last 3% of protein; also known as 3020insC)[277]

Figure 19. Pathways in the Activation of NF-κB[267]

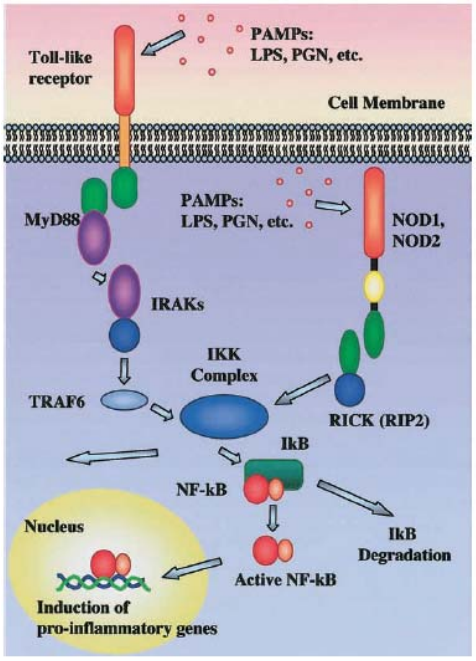
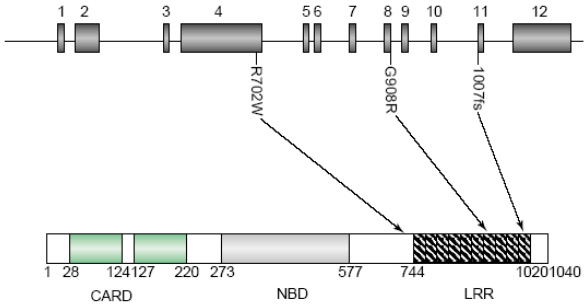


Figure 20 below illustrates the relative location of these mutations.[285] One should note the proximity of all three alterations to the LRR. In a study by Lesage et al.,[266] evidence is presented to corroborate this large number of variations in the LRR region. After the

summation of all sequence variations detected in 612 IBD patients, 93% of the variations were found in the distal third of the gene, doubtlessly corresponding to the LRR region. Finally, while the severing effect of SNP13 on the terminal 3% of the protein is known, the functional effects of SNP8 and SNP12 remain a mystery.[286]

According to several researchers, **up to 30% of CD patients** are **heterozygotes** for one of the three mutations while only **15% might show homozygosity** for a mutation. Included in these latter 15% are also those patients who may present compound heterozygosity for a mutation, whereby a different mutation is present on each chromosome 16.[277,278,279] Compared with a healthy population, 8-15% demonstrate heterozygosity, while **only 0-1% of healthy controls are homozygotes**.[46]

Figure 20. Three Most Common NOD2/CARD15 Mutations[285]



Of the three most common *NOD2/CARD15* variations present, some believe that **SNP13 is the most common type**.[265,284] It results in the shortening of the protein's C-terminus by 3%,[277] or the equivalent of 33 amino acids.[287] Regardless of the gene's significant role in the pathogenesis of CD, several studies identified families with homozygote individuals who are healthy individuals. On the other hand, some ethnic groups do not possess this mutation at all, but are nonetheless afflicted by CD. Van der Linde et al.[287] presented data from the Netherlands supporting the presence of healthy homozygotes within afflicted families. In one family, which had a SNP13-homozygous CD patient, it was also found that two sisters as well as the father and his sister, were all homozygous for this mutation; none of them presented any clinical signs.

Examining **ethnic prevalence of the SNP13 mutation**, one finds that although it might be present by as many as 50% of central Europeans,[266] the distribution is quite different in other parts of the world.[288] For example, Japanese[289] and African American[267] probands

lack these mutations, while a study by Guo et al.[271] confirmed the absence of the SNP13 mutation in Chinese patients.

As mentioned previously, LRR is responsible for binding the PGN of $Gram^+$ and $Gram^-$ bacteria,[284] and possibly also binds the LPS of $Gram^-$ bacteria as indicated by some studies.[282] Certainly, a **mutation in the LRR** will be reflected in its ability to detect and bind PGN or any other PAMP. Nonetheless, it seems that CD involves an elevation of NOD2/CARD15 transcription in monocytes and epithelial cells of involved colonic tissues.[290] Two interesting, yet contradictory, observations have been made regarding the effect of NOD2/CARD15, as a whole, on the activation of NF-κB. The first was an *in vitro* decrease of NF-κB activity caused by common *NOD2/CARD15* mutations.[286,291] These mutations proved to effect a loss-of-function characteristic to the protein.[46] On the other hand, NF-κB in IBD tissue was found to be elevated.[292] The question remains as to how the **discrepancy between enhanced and loss-of-function protein** is possible. One possibility, put forth by Inohara et al.,[283] is that altered NOD2/CARD15 fails to elicit the protective mechanisms that are in place for the purpose of dealing with bacterial products such as MDP. These protective pathways are most likely regulated by NF-κB.

Susceptibility for CD based on *NOD2/CARD15* should also focus on the specific cells that express this protein. Sufficient evidence exists to conclude that **NOD2/CARD15 is present in monocytes, dendritic cells, and intestinal epithelial cells**.[280,293,294] Additionally, recent studies have gathered circumstantial evidence for the role of **Paneth cells in the pathogenesis of CD**.[46] These cells are known to have an antimicrobial effect through their secretions which include: lysozyme, phospholipase A_2, and α- and β-defensins.[295] Additionally, Paneth cells show their highest concentration in the **terminal ileum**, known to be the most susceptible site for CD.[46,296] As mentioned earlier, terminal ileitis is strongly associated with *NOD2/CARD15* mutations. Finally, recent findings are suggestive of reduced expression of α-defensin coupled with a mutated *NOD2/CARD15* gene, thus leading to a defective response to bacterial antigens.[288]

Using various laboratory techniques, Lala et al.[297] demonstrated a high concentration of Paneth cells in ileal crypts as well as, more importantly, the presence of NOD2/CARD15 protein.

Image 4. Lysozyme staining: positive, dark-stained cell bases. It is already known that Paneth cells are capable of sercreting lysozyme, among other antimicrobial products.

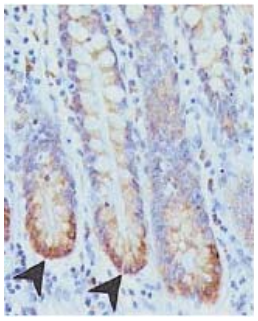


Image 5. Staining of NOD2/CARD15 RNA: identified by the brown staining (arrowheads) of NOD2/CARD15 antisense strands in terminal ileal crypts afflicted by CD.

Furthermore, Image 3 illustrates the presence of NOD2/CARD15 protein, in CD-affected terminal ileum, after staining using histochemical methods with an antibody against NOD2/CARD15.

Image 6. Staining of NOD2/CARD15 protein: indicated by brown staining within the cells.

In conclusion, NOD2/CARD15 has a significant role in the pathogenesis of CD, but not UC, and may also have some influence on the disease phenotype, thus influencing prognosis.

9.1.1.2. *NOD1/CARD4*

A second member in the NOD/CARD family displaying homology to NOD2/CARD15 is the NOD1/CARD4, with the latter's locus being established on chromosome 7p14. It is similar in structure to the NOD2/CARD15 protein, except for the presence of **only one CARD** and it too, **activates NF-κB**, **induces apoptosis** and **detects bacterial products**. In particular, the NOD1 protein **detects diaminopimelic acid found in Gram-negative bacteria peptidoglycan** and mediates a response through its LRR.[282,298]

While it is obvious that *NOD1/CARD4* is a candidate for further investigation in association with IBD pathogenesis, **results thus far have been confusing and conflicting**. In a 2003 study by Zouali et al., the group established no association between *NOD1/CARD4* and genetic susceptibility for IBD based on the investigation of 381 IBD families, some even containing multiple affected probands, and the detection of nine nucleotide changes.[299] Two years later, McGovern et al. published findings that established an association between *NOD1* and both forms of IBD.[300] Following the investigation of 556 IBD trios, they concluded that a *NOD1* variant, located on intron IX, was significantly associated with the age at diagnosis of Crohn's disease. In a large Hungarian study investigating 434 CD patients, the authors found a significant association between the *NOD1* G796A polymorphism and disease susceptibility. Carriage of this variant was highly associated with Crohn's disease compared to healthy control subjects ($p < 0.0001$) and non-inflammatory bowel disease control subjects with chronic gastritis ($p = 0.008$). Also, the A allele was more frequently observed in CD patients than in either control group ($p < 0.0001$ and $p = 0.019$).[301] Further studies are required to determine whether *NOD1/CARD4* variants are associated with disease susceptibility and the existence of geographic variation.

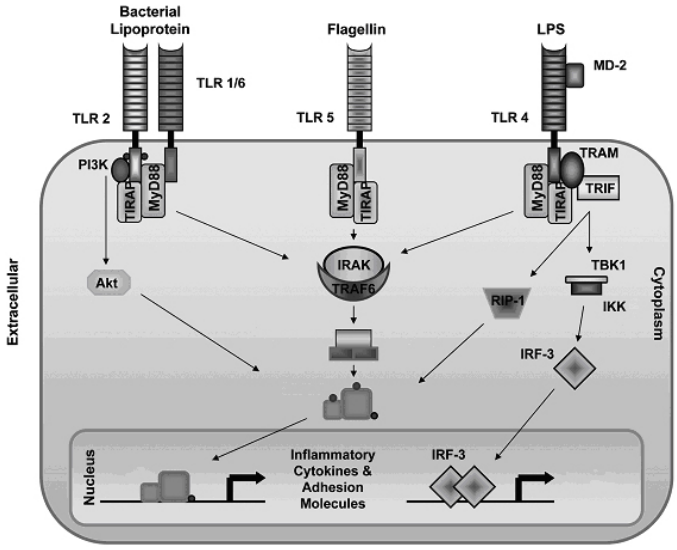
9.1.1.3. Toll-like Receptors

This group of receptors belongs to the **same pattern recognition receptors as the NOD** family with the difference that these are **transmembrane proteins**. At this position, they come in contact with the various bacterial products, including LPS and PGN.[302] While it is not yet known how each TLR recognizes the various PAMPs, quite a bit is known about

their individual targets. For example, TLR4 detects LPS, whereas TLR5 detects flagellin.[277,303] Furthermore, TLR2 and TLR9 detect PGN and CpG DNA, respectively.[303]

Of interest in IBD is **TLR4**, which has been shown to **induce pro-inflammatory cytokine production**.[304] In the human body, LPS derived from Gram$^-$ bacteria binds LPS-binding protein in the serum. Subsequently, this complex is recognized by the CD14 molecule located on peripheral blood monocytes and macrophages. With the aid of the secreted protein MD-2, TLR4 is now able to recognize CD14 molecule with the attached LPS.[305] Figure 21 illustrates the detailed activation mechanisms of Toll-like receptors responding to various PAMPs, culminating in NF-κB translocation into the nucleus and an inflammatory response.[302]

Figure 21. Activation of Toll-like Receptors[302]



Two separate groups studied Belgian[306] and Greek[307] cohorts, and established an **association between the *TLR4* Asp299Gly polymorphism and both CD and UC**. The polymorphism in *TLR4* predisposes to infections with Gram negative bacteria, consequent to the protein's role in the recognition of LPS. The Belgian study was the first to establish an association between the *TLR4* Asp299Gly polymorphism and IBD. In this study, the variant was investigated in concert with the presence of *NOD2* mutations (Arg702Trp, Gly908Arg, and Leu1007fsinsC). The Greek study concurred with these results, but also found a CD14 polymorphism, -159 (C/T), to be significantly elevated in Crohn's disease (T allele, OR: 1.73, $p < 0.0048$). A third group noted the possibility for disease phenotype in association with *TLR4* polymorphism carriage state. In their cohort, 47.4% of patients who were Asp299Gly$^+$/NOD2$^-$ experienced a stricturing disease phenotype compared to 10.1% of

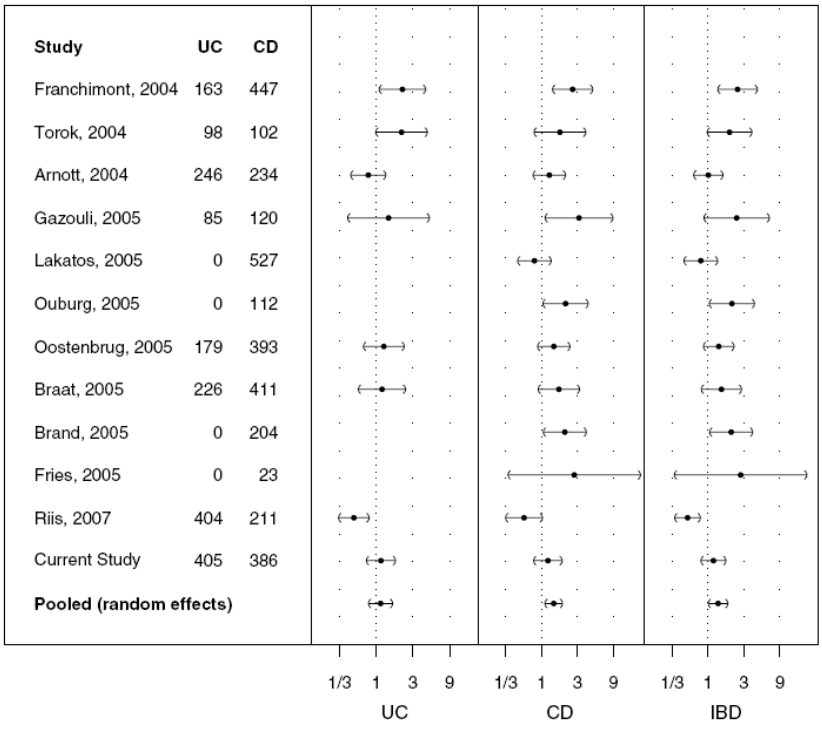
patients with Asp299Gly$^-$/NOD2$^+$ (p = 0.0009). Furthermore, patients with Asp299Gly$^-$/NOD2$^+$ tended to show greater prevalence of penetrating disease (p = 0.059).[308]

A group from New Zealand conducted its own association study between the *TLR4* Asp299Gly and Thr399Ile variants and IBD. In addition, it also performed a meta-analysis of similar studies involving the Asp299Gly polymorphism. Their own results found no association between *TLR4* polymorphisms and IBD, yet the meta-analysis showed a significantly greater carriage frequencies of 299Gly in CD patients (OR: 1.45) and IBD (OR: 1.36). In addition, no genotype-phenotype associations were detected.[309] The results of their meta-analysis, presenting the odds ratio of *TLR4* Asp299Gly, are divided to CD, UC or IBD, in Figure 22.

Finally, in a study published in 2006 by Pierik et al., several *TLR* variants were identified that influenced disease phenotype. In particular, a positive association has been established between *TLR1* R80T and pancolitis in UC (OR: 2.844, p = 0.045), as was the *TLR2* R753G polymorphism (OR: 4.741, p = 0.027). In Crohn's disease, a negative association was found between *TLR1* S602I and ileal disease (OR: 0.522, p = 0.03).[310]

Contradictory studies also exist supporting the lack of association between *TLR4* and IBD susceptibility. One group studied Scottish and Irish Crohn's disease patients for various *NOD2/CARD15*, *TLR4*, and *CD14* polymorphisms. Similar to pervious studies, they noted a significant association between two of the three common *NOD2* mutations (R702W, p = 0.269; G908R, p = 0.008; 1007fsinsC, p = 0.003) but failed to find an association between *TLR4* or *CD14* mutations and their cohort.[311] In another study, a Hungarian group noted an association between *NOD2/CARD15* variants and IBD, but only a modifier effect was observed for *TLR4* D299G – it lowered the age at presentation (p = 0.06).[312] Interestingly, Oostenbrug et al. investigated 781 patients and concluded that while *TLR4* polymorphisms are significantly associated with both forms of IBD, the Asp299Gly and Thr399Ile variants were not involved.[313] Finally, in a German study, a significant association was found between the Thr399Ile variant and ulcerative colitis (p = 0.014), yet no association between the Asp299Gly variant and IBD was established.[314]

Figure 22. Odds Ratio of *TLR4* Asp299Gly According to CD, UC or IBD[309]



In the present thesis, we did not find a statistically significant association/linkage between the *DLG5* variant and either *NOD2/CARD15* or *TLR4* mutations. This suggests that these genes are susceptibility factors independent of each other. Although our main goal was not to study the frequency of the *NOD2/CARD15* and *TLR4* variants, in the present study, the prevalence of these variants was within the range previously reported by our group in Hungairan patients.[312]

9.1.2. Genes Involved in Mucosal Transport and Integrity

OCTN1/SLC22A4, OCTN2/SLC22A5

The **IBD5 locus** on chromosome 5q31 is only the second locus to be clearly associated with increased risk for **Crohn's disease**. The initial linkage was found in a Canadian population, only to be followed by further research to identify a 250 kb risk haplotype within the IBD5 locus. This locus was found to have significant association with CD.[274,327] Among the several genes found in this stretch of DNA, solute carrier family 22, member 4 (***SLC22A4***) and member 5 (***SLC22A5***), also known as organic cation transporter 1 and 2 (OCTN1, 2), respectively, were identified.[315] OCTN2 is a **carnitine transporter**, which is important for the transport of long-chain fatty acids from the cytosol to the mitochondria.

These fatty acids are then β-oxidized[316] and a disturbance in this pathway might hamper the **oxidation burst-mediated mechanisms** responsible for pathogen destruction.[317] On the other hand, OCTN1 has a lower affinity for carnitine but transports ergothioneine, which is exclusively synthesized by mycobacteria and fungi; the transporter's physiological function has not yet been completely discovered.[318]

In 2004, Peletkova et al. proposed that the polymorphism L503F in *OCTN1* and the promoter variant G-207C in the *OCTN2* gene were associated with IBD5 findings. Homozygotes for the so-called TC haplotype had an odds ratio of 3.43-5.14, while patients carrying both the TC haplotype and a *CARD15* risk allele had an odds ratio of 7.28-10.50.[328]

In a later study, Noble et al. tested three SNPs, IGR2096, IGR2198, and IGR2230, as well as the *OCTN1* L503F (C1672T) polymorphism and *OCTN2* G-207C.[319] All five polymorphisms are within the extended IBD5 haplotype first described by Daly et al.[320] In CD patients, the group found a linkage disequilibrium between each investigated SNP and the disease. Significant associations were found between the *OCTN1* (p = 0.0008) and *OCTN2* promoter variant (p = 0.0092) and homozygosity for the TC-haplotype with CD. TC-haplotype homozygosity was observed more often in the CD group than in the control subjects (80% vs. 68.5%, OR: 1.8, p = 0.0016).[319] Contrary to previous reports,[274,321] they found no association between *OCTN1/2* polymorphisms and earlier age at onset, disease phenotype, or disease behavior.[319] However, a significant association was noted between the need for surgery due to CD complications and IGR2198 (OR: 1.91; p = 0.037), *OCTN1* (OR: 2.7; p = 0.0007), *OCTN2* (OR: 1.77; p = 0.031), and the TC haplotype (OR: 2.2; p = 0.0023). No associations with UC could be made.[319]

9.1.3. Other IBD Susceptibility Loci

Following the discovery of *NOD2/CARD15*, it was clear that other susceptibility loci must be present, as its mutations accounted for no more than 20% of CD patients.[322] Table 8 lists other susceptibility loci as well as the form of IBD to which they are linked. Although nine genetic loci are depicted in Figure 17, only the first seven loci (IBD1-7) have been confirmed by strong linkage analysis obeying strict criteria.[323] As demonstrated in Table 30 below, **some loci are exclusively linked to either CD or UC**, while others play a role in both forms of IBD. It is also quite clear that each locus codes for different genes that play a role in the pathogenesis of IBD.

Table 30. IBD Susceptibility Loci

Locus	Chromosome	Associated IBD Form	Associated Genes
IBD1	16q12	CD	NOD2[277]
IBD2	12q13	UC	VDR, IFN-γ[324]
IBD3	6p13	CD, UC	MHC, TNF-α[325]
IBD4	14q11	CD	TCR α/δ complex[326]
IBD5	5q31-33	CD	IL-3, -4, -5, -13, CSF-2,[274,327] OCTN1/2,[328] IRGM[329]
IBD6	19p13	CD, UC	ICAM-1,[330] C3, TBXA2R, LTB4H[274]
IBD7	1p36	CD, UC	TNF-R family, CASP9[331,332]
IBD8	16p12	CD	Unknown[333]
IBD9	3p26	CD, UC	Unknown (CCR5, CCR9)[324,334]
Other loci	1p31	CD, UC	IL-23R[335]
	10q23	CD, UC	DLG5[336]
	2q37.1	CD	ATG16L1[337]
	7q32	CD, UC	IRF5[338]
	5p13.1	CD	PTGER4[339]

VDR – vitamin D Receptor; IFN – interferon; HLA – human leukocyte antigen; TNF – tumor necrosis factor; TCR – T-cell receptor; IL – interleukin; CSF – cerebrospinal fluid; ICAM – intercellular adhesion molecule; TBXA2R – thromboxane A_2 receptor; LTB4H – leukotriene B_4 hydroxylase; TNF-R – TNF receptor; CASP – Caspase; CCR – chemokine receptor; OCTN – organic cation transporter; DLG5 – discs large homolog 5; ATG16L1 – autophagy-related 16-like 1; IRF5 – interferon regulatory factor 5; PTGER4 – prostaglandin receptor EP4; IRG(M) - immune-related guanosine triphosphatase.

The **IBD2** locus, mapped to chromosome 12q13,[324] was strongly associated by linkage analysis with the occurrence of ulcerative colitis, rather than Crohn's disease.[267] Also, in 2000, Parkes et al.[340] published work supporting Bonen and Cho,[267] stating that although IBD2 has far greater influence on the occurrence of UC, it plays a minor role in CD with the possibility of locus heterogeneity.

IBD3 is located on the short arm of chromosome 6. Included in this region is the MHC (HLA class II), which has a role in the phenotype of both CD and UC.[46] Aside from the HLA genes, this region also includes TNF-α, presenting a mutation (TNF_{-857C}) that is closely associated with IBD.[325]

The **IBD4** locus, located on the long arm of chromosome 14 and identified as 14q11-12, is exclusively associated with CD.[326]

9.1.3.1. HLA Genes & IBD

The first studies to suggest genetic susceptibility in IBD investigated the association between certain HLA regions and IBD. Some of the most thoroughly researched genes of IBD included those of the major histocompatability complex (MHC), particularly the human leukocyte antigen (HLA) cluster on **chromosome 6**. The HLA genes are divided into two classes:

- Class I: found in all cell types and includes gene products of HLA-A, -B, and -C.
- Class II: found only on specialized immune cells and expresses products of the genes HLA-DP, -DQ, and -DR.

It is the latter, HLA class II, which is of particular interest in the pathogenesis of IBD and especially of **UC**.[267] A 1999 study by Stokkers et al. [275] found evidence for the following associations between IBD and HLA genes:

- Crohn's disease:
  - Positive association with HLA-DR7, -DRB3*0301, -DQ4.
  - Negative association with HLA-DR2 and -DR3.
- Ulcerative colitis:
  - Positive association with HLA-DR2, -DR9, -DRB1*0103.
  - Negative association with HLA-DR4.

This study proved especially powerful as it spanned over three decades of publications concerned with IBD and HLA.

Another aspect in the investigation of HLA genes, involving both classes, is the **correlation with disease phenotype, severity, and extraintestinal manifestations** (EIM) of IBD (eg, arthropathies).[46,341] A common EIM is arthritis, of various forms. The first, migratory pauciarticular large-joint arthritis has been shown to be related to HLA-DRB1*0103, while chronic, small-joint, symmetrical arthritis is associated with HLA-B*44. HLA-DRB1*0103 is also coupled with uveitis, as is HLA-B*27.[342] In addition, the presence of the DRB1*0103 allele, though rare, is related to a severe state of disease. This observation is supported by an early need for colectomy.[343]

9.1.3.2. Interferon Regulatory Factor 5

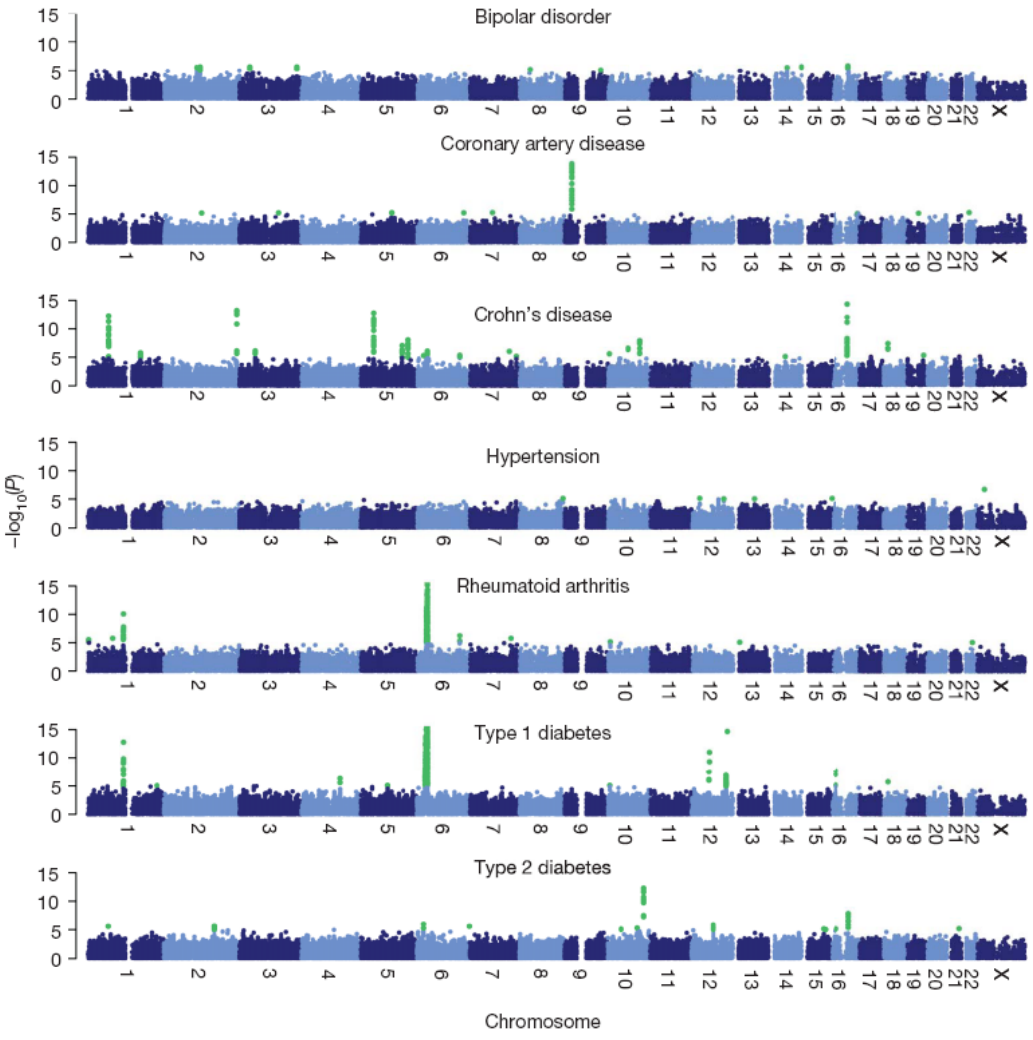
The interferon regulator factors (IRFs) constitute a family of nine transcription factors possessing several functions, including **defense against pathogens**, **hematopoietic development**, and **cellular survival**.[338] Structurally, they are all similar at the N-terminal DNA-binding domain; however, they differ in the activating signals and interaction with DNA regulatory elements.[344] While some IRFs are activated by viral infections, such as IRF 3 and 7, others, for example, **IRF5**, do not.[344,345,346] The latter, is **activated by Toll-like receptors** and stimulates the **release of pro-inflammatory cytokines**, in particular, IL-6, -12, and TNF-α.[338,347] Given the established association between cytokines and IBD as well as the role of IRF5 in other autoimmune disorders, systemic lupus erythematosus and rheumatoid arthritis,[348,349] Dideberg et al. investigated a possible link between IRF5 and IBD. The group studied two separate Belgian IBD and control cohorts (Wallonia – CD: 748; UC: 254; control: 241; Leuven – CD: 488; UC: 192; control: 311) against 12 polymrphisms in the IRF5 gene, and found a **significant association** between a 5bp insertion-deletion mutation (CGGGG) in the promoter region and IBD in the Wallonia cohort. The same result was confirmed in the Leuven patient group. While this polymorphism was associated with both forms of IBD, it was far stronger in UC (OR: 2.42; $p = 5.3 \times 10^{-8}$) than in CD ($p = 6.8 \times 10^{-4}$). Overall in the Wallonia cohort, the OR equaled 1.81 ($p = 1.9 \times 10^{-5}$). In the second cohort, from Leuven, the OR value was 1.59 ($p = 3.2 \times 10^{-5}$).[338] It is believed that this particular mutation leads to an additional, third binding site for the SP1 transcription factor.[350]

9.1.3.3. Genome-wide Association Scan

The genome-wide association scan (GWAS) is a **novel method** used to find associations between certain diseases or conditions and SNPs. It requires a **large number of affected and control subjects**, who are genotyped for thousands of polymorphisms across the entire genome. One condition that must be met is the identification of probable SNPs related to the investigated condition. Statistical tools are then used to sift through the data and locate significant associations.[351] The Wellcome Trust Case Control Consortium has exemplified such a wide-breadth and extensive study involving 14 000 patients, 2000 for each of seven conditions/diseases and 3000 control subjects. Using GWAS, the Consortium investigated coronary artery disease, hypertension, diabetes mellitus types 1 and 2, bipolar disorder, rheumatoid arthritis, and Crohn's disease. Twenty-four independent associations were found among 6 of the 7 studied patient groups, with **Crohn's disease** having **nine associations**, the most of any of the investigated diseases or conditions. The figure below

illustrates the distribution of these associations across the entire genome for each condition.[352] Using the same technique, Hampe et al. identified an association between the **autophagy 16-like 1 gene** (*ATG16L1*) on chromosome 2q37.1 and CD,[337] while Duerr and colleagues identified the ***IL-23R*** gene's association with IBD.[335]

Figure 23. Findings of the Wellcome Trust Case Control Consortium, by Disease[352]



9.1.3.4. IL-23R

The first study to discover the *IL-23R* association with IBD, was conducted on a large North American population consisting of 547 non-Jewish, ileal CD patients and 548 controls, testing 308 332 autosomal SNPs. The motivation behind limiting the cohort to Crohn's disease patients by ethnicity and localization was to prevent pathogenic heterogeneity. While

they found **several significant associations**, only three were of far greater significance than the rest. The first two included known *CARD15* polymorphisms, while the third was the **Arg381Gln** polymorphism in the *IL-23R* gene ($p = 1.56 \times 10^{-3}$). Nine other markers were found both in the *IL-23R* gene and in the intergenic region, adjacent to the IL-12 receptor, β-2 gene. All had significant association values of $p < 0.0001$. Of interest, the glutamine allele of Arg381Gln is rarer than the arginine allele, being present in 1.9% of the non-Jewish, ileal CD cohort versus 7.0% of the control subjects, implying a **protective effect**.[335] In a subsequent study, 1902 CD, 975 UC, and 1345 control subjects were genotyped for eight SNPs identified in the North American study. Similarly to the previous study, the research group also found a **significant association between Arg381Gln and CD**, versus controls ($p = 1.1 \times 10^{-12}$; OR: 0.38). Furthermore, by examining all phenotypes of CD and UC, and finding no associations to any single form, they concluded that the *IL-23R* variant influences susceptibility but not disease phenotype. Finally, the study further affirmed the power and viability of genome-wide association scans.[353]

9.1.3.5. Autophagy Genes

For the detection of autophagy 16-like 1 gene (*ATG16L1)*, a protein in the **autophagosome pathway that processes intracellular bacteria**, Hampe et al. screened 735 CD and 368 control subjects for 19 779 nonsynonymous SNPs. Next, they narrowed their search to 72 SNPs which demonstrated a significance level of $p \leq 0.01$. Subsequently, they were able to find a significant association to the *ATG16L1* locus ($p = 4.0 \times 10^{-8}$), for the T300A SNP.[337] A subsequent study by Prescott and colleagues revealed a similar and **significant association between T300A and CD** ($p = 2.4 \times 10^{-6}$), as detected in 1236 cases. In addition, the group also found a specific and significant association with the ileal form of Crohn's disease. Finally, the study found a modest but **significant association between *ATG16L1* T300A genotype and ulcerative colitis**.[354]

The ***IRGM*** gene located on chromosome 5q33.1 was identified through a genome-wide association scan to be **significantly associated with Crohn's disease** ($p = 6.6 \times 10^{-4}$). Like *ATG16L1*, it is also involved in the autophagy pathway. Nonetheless, the exact SNPs that are responsible for the increased disease susceptibility still need to be elucidated.[329]

9.1.3.6. Prostaglandin Receptor EP4

The prostaglandin receptor EP4 (*PTGER4*) was **associated with Crohn's disease** using a genome-wide association scan. In the study, Libioulle and colleagues first tested 547 CD patients and 928 controls for over 300 000 SNPs. They found three chromosomal regions with significant *p*-values ranging from 10^{-6} to 10^{-9}. Two of the three loci were the *IL-23R* and *CARD15* genes, while the third was located to a gene desert on 5p13.1. The group further tested the results involving 5p13.1 on a second cohort consisting of 1266 CD patients, 559 control subjects, and 428 trios, again, establishing a *p*-value of $< 4 \times 10^{-4}$.[339]

9.1.3.7. Cytokines

Cytokines are substances produced by various cells and tissue types, even epithelial cells,[355] and have immunomodulating and proinflammatory functions, including the ability to attract leukocytes, through a chemoattractant gradient, to various tissues.[356,357] Over the years, numerous cytokines and chemokines have come under scrutiny for their role in the pathogenesis of IBD. This wide array of cytokines includes: IL-1,[358] IL-2,[44] IL-6,[322] IL-8,[356] IL-12,[276] IL-16,[359] IL-17,[276] IL-23,[360] and TNF-α.[361]

IL-2 is a mainstay immunoregulatory cytokine.[44] Circulating IL-2 levels are below detection levels during health and disease states; however, IBD T-cells display significant variation in their response to IL-2. CD T-cells will show exaggerated response while UC T-cells will respond only slightly.[362] Moreover, the IL-2 receptor has also been found to be upregulated in active cases of IBD.[363]

A second important cytokine in the pathogenesis of IBD has been identified to be **IL-8**, which has been extensively studied for its role. The cytokine is expressed by neutrophils, macrophages and intestinal epithelial cells, and has been found to be **upregulated in both forms of active disease**. Daig et al., using mucosal biopsy specimens, noted a significant increase in IL-8 expression in macroscopically inflamed bowel segments in CD ($p < 0.0001$), UC ($p < 0.001$) and inflammatory control subjects ($p = 0.010$).[364] Additionally, IL-8 was also significantly elevated in un-inflamed CD biopsy samples ($p < 0.001$). Finally, over a decade ago, Mazzucchelli et al. demonstrated a non-specific but important association of IL-8 with the histological grade in both forms of active IBD disease.[365]

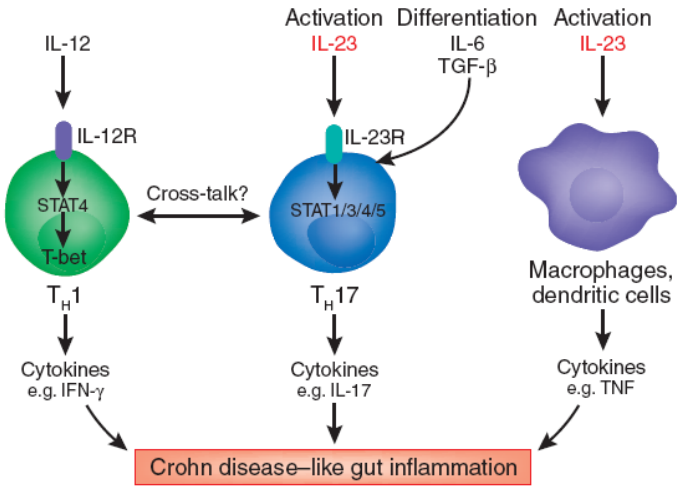
IL-12 and **-17** are another pair of immunoregulatory cytokines, as reported by Nielsen et al.[276] While their exact role is unknown, mounting evidence suggests an **increase in their mRNA concentration in IBD**. Fujino et al. conducted a small-scale study that consisted of 40 tissue samples, 20 each from CD and UC patients, retrieved endoscopically or surgically.

They also included five infectious colitis, eight ischemic colitis samples, and 15 normal colon samples. Elevated IL-17 levels were only detected in the mucosa of CD and UC samples, in $CD3^+$ T cells or $CD68^+$ monocytes/macrophages, but not in the control inflammatory samples or healthy tissues.[366] Consequently, it is possible to conclude that IL-17 may have a specific association with IBD inflammation but further extensive research is needed on a larger scale.

At the turn of this century, Oppmann et al. discovered **IL-23**. At the structural level, the IL-23 is similar to IL-12, both possessing the p40 subunit, yet only IL-23 contains the p19 subunit. This composition gives IL-23 both common and distinct functions compared with IL-12. Among its various functions, it is able to bind IL-12Rβ1 and stimulate secretion of IFN-γ.[360] Also of importance, **IL-23 activates a subset of T-cells that secrete IL-17**, hence termed **Th_{17}**. Animal models have shown that this subset **mediates chronic inflammation and autoimmune diseases**.[367] IL-17, being a pro-inflammatory cytokine, enhances T cell priming. In addition, it promotes the production and release of numerous other pro-inflammatory mediators by various cell types. Together, fibroblasts, macrophages, epithelial and endothelial cells produce IL-1 and -6, TNF-α, metalloproteases, nitric oxide synthase 2, and chemokines.[368] As noted in Table 8, using the GWAS technique, an **association** has been **established** between the **IL-23 receptor and Crohn's disease**. Several mechanisms have been proposed for the role of IL-23 in this form of IBD. Through its expression on the cell membranes of macrophages and dendritic cells, IL-23 may affect the intestinal barrier function and immune response to gut flora. T-cell deficient animal models support this mechanism, whereby IL-23 is necessary for the induction of gut inflammation.[369,370]

Last year, Seiderer et al. identified a **new member in the IL-17 family**, termed **IL-17F**. Subsequent investigation of its association with IBD led to several interesting results. The cytokine's mRNA levels in the intestine were **positively correlated with disease activity in CD** (4.4-fold higher in inflamed colonic mucosa versus non-inflamed CD biopsies), but not in UC ($p = 0.016$). However, the **average level of IL-17F mRNA expression was higher in UC** compared with CD ($p < 0.0001$).[371] The figure below summarizes the role of IL-23 and Th_{17} versus IL-12 in inducing Crohn's disease-like gut inflammation. There has been a clear shift from the belief that IL-12 is responsible for the Th_1 subpopulation observed in CD, towards a role for IL-17-secreting Th_{17} cells, induced by IL-23.

Figure 24. The Shifting Paradigms in the Pathogenesis of IBD[369]

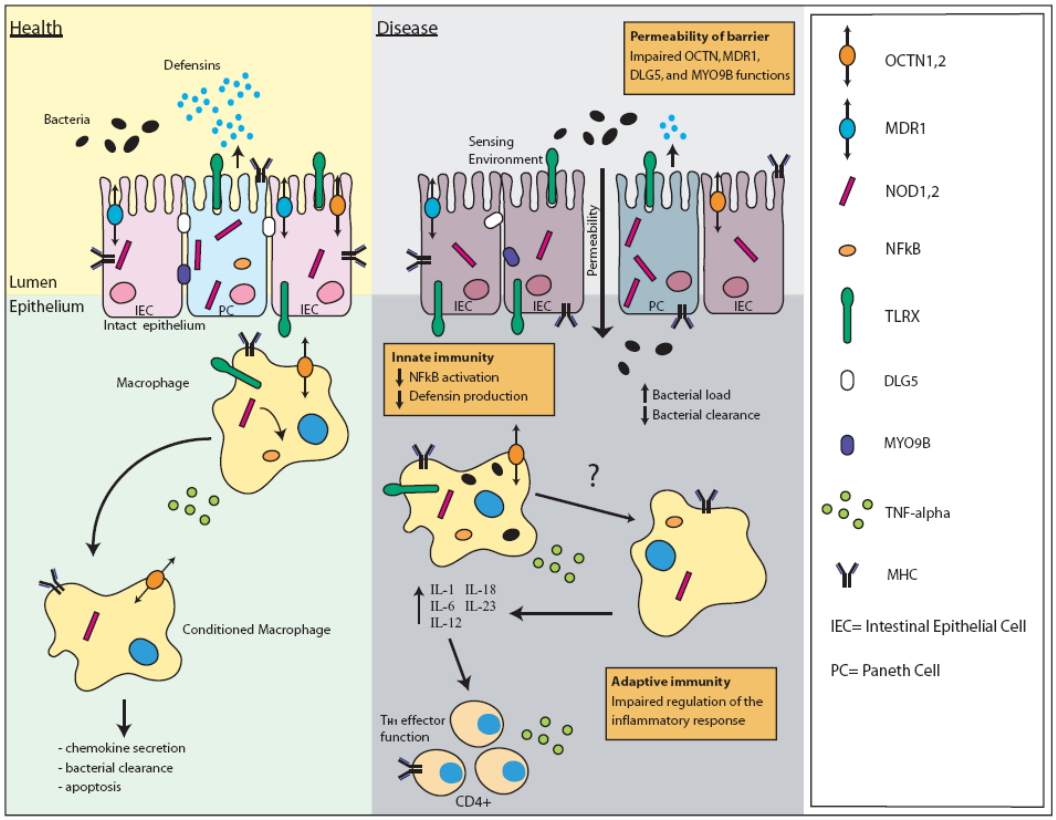


TNF-α is an important **susceptibility gene** located on chromosome **6p21**, within the HLA class III gene cluster, and consequently, within the **IBD3 locus**.[267,343] As TNF-α is a **proinflammatory cytokine**, and its concentration is **increased in CD patients' mucosa**,[361] there is an increased focus on its role in disease pathogenesis. The response of CD patients to infliximab, a TNF-α antagonist, provides further indirect support for the cytokine's role in this form of IBD. In 2002, a British group published their findings after investigating four TNF-α mutations, -1031T/C, -863C/A, -857C/T, and -308G/A. Their findings included a significant association between the -857C/T promoter variant and both forms of IBD. Importantly, the association with CD was only made after considering CD-patients who did not possess any *NOD2* mutations. None of the other three tested polymorphisms displayed any significant association with either form of IBD.[372]

An Australian team demonstrated in 2003 a significant association (p = 0.00041) between TNF-α_{-857C} and CD, further supporting the role of IBD3. Additionally, they also noted a strong and significant association (p = 0.00016) between the abovementioned mutation and families that carried one or both *NOD2* polymorphisms (SNP8, SNP13).[373] This was contrary to the British group's findings. Finally, a Chinese team made a significant association (p = 0.02) between TNF-α_{-308A} and ulcerative colitis, in an ethnically distinct population, the Chinese Han. However, the team did not enroll any Crohn's disease patients.[374]

In conclusion, the etiology and pathogenesis of IBD, at the cellular level can be summarized in Figure 25.

Figure 25. Key Molecular Mechanisms in the Pathogenesis of IBD[375]



9.2. The Importance of the *DLG5*, *MDR1* and *ABCG2* Mutations in Hungarian Patients with IBD

These were the first studies to be published on the frequency of *DLG5*, *MDR1*, and *ABCG2* variant alleles in Hungarian IBD patients. In total, we tested five polymorphisms against several variables including: disease susceptibility, genotype-phenotype correlation, response to infliximab therapy or steroids, epistasis with *NOD2/CARD15* risk alleles, and need for surgery. We established numerous positive and negative associations and, while some concurred with previous studies, others were in stark contradiction to formerly published results.

In our studies, we found no genetic susceptibility associations between either form of IBD and any of the tested polymorphisms, *DLG5* R30Q, *MDR1* C3435T or G2677T/A, and *ABCG2* G34A or C421A. The *MDR1* C3435T and G2677T/A did not display any significant association with disease susceptibility in our cohort; however, a tendency was observed for the G2677 variant allele to be overtransmitted in CD patients versus control subjects (OR:

1.29; 95% CI: 0.97-1.72,; $p = 0.08$). These results are in agreement with Brant et al. who have noted a significant association between the common allele (893Ser) and IBD in a case-control analysis ($p = 0.002$) and a pedigree disequilibrium test ($p = 0.00020$-0.00030). Similar to our results, the group did not associate C3435T with IBD.[376] However, our results do not concur with two other studies conducted in Scottish Caucasian and Spanish Caucasian populations. In the former, Ho et al. found a significant association between the 3435TT genotype and ulcerative colitis (34.6% vs. 26.5%; OR: 1.60; 95% CI: 1.04-2.44; $p = 0.04$) versus control subjects, yet no association with CD was detected. Furthermore, they correlated this variant allele with extensive UC (42.4% vs. 26.5%; OR: 2.64; 95% CI: 1.34-4.99; $p = 0.003$). Unlike in our study, the G2677T SNP was not associated with either form of IBD.[377] In the Spanish population, a different conclusion was reached. A significant association ($p = 0.021$) was made between C3435T and CD patients versus the control group. In addition, no associations were made with either disease behavior or location.[378] Schwab et al. also reported of increased frequencies of the T-allele ($p = 0.049$) and TT-genotype ($p = 0.045$) in 149 UC patients but found not association with CD, in their German cohort.[379] While all of these studies have reached different results, the possibility also exists for other *MDR1* gene variants to be associated with IBD.

An important meta-analysis was conducted by Annese and colleagues in 2006, where they examined data concerning the two *MDR1* polymorphisms of IBD, C3435T and G2677T/A. The group collected data on the distribution of C3435T from six studies and on G2677T/A from five studies. The data for C3435T are presented in Table 31. Taking these data into consideration, as well as that for G2677T/A (not shown), the group calculated the *p* values and odds ratios for the alleles and genotypes (Table 32).

Evaluating the pooled data in Table 32, one can observe a significant difference in the association between C3435T, and CD and UC. In Crohn's disease, no association was found with this variant (T vs. C: OR: 0.968; $p = 0.519$). On the contrary, ulcerative colitis studies demonstrated a significant difference between the T and C alleles (OR: 1.170; $p = 0.002$), as well as between the TT and CC genotypes (OR: 1.332; $p = 0.008$). This is in partial contradiction to our study, where no association was found between C3435T and IBD. With regards to the G2677T/A variant, no significant association was found with either form of IBD. Nevertheless, our study presented a trend for greater frequency of the G2677 variant allele in CD.

Table 31. Distribution of C3435T Alleles in the Existing Literature[243]

	C (n [%])	T (n [%])
Glas[380]		
UC (n = 123)[1]	111 (45)	135 (55)
CD (n = 135)	130 (48)	140 (52)
IBD (n = 258)	241 (47)	275 (53)
HC (n = 265)	272 (51)	258 (49)
Schwab[379]		
UC (n = 149)[a]	129 (43)	169 (57)
HC (n = 149)	154 (52)	144 (48)
CD (n = 126)	134 (53)	118 (47)
HC (n = 126)	128 (51)	124 (49)
IBD (n = 275)	263 (48)	287 (52)
HC (n = 275)	282 (51)	268 (49)
Ho[377]		
UC (n = 335)[b]	280 (42)	390 (58)
CD (n = 268)	252 (47)	284 (53)
IBD (n = 603)	532 (44)	674 (56)
HC (n = 370)	354 (48)	386 (52)
Palmieri[381]		
UC (n = 468)	488 (52)	448 (48)
CD (n = 478)	503 (53)	453 (47)
IBD (n = 946)	991 (52)	901 (48)
HC (n = 450)	470 (52)	430 (48)
Potočnik[382]		
UC (n = 144)	134 (47)	154 (53)
CD (n = 163)	161 (49)	165 (51)
IBD (n = 307)	295 (48)	319 (52)
HC (n = 355)	376 (53)	334 (47)
Urcelay[378]		
UC (n = 311)	317 (51)	305 (49)
CD (n = 303)[1,c]	369 (61)	237 (39)
IBD (n = 614)	686 (56)	542 (44)
HC (n = 324)	344 (53)	304 (47)

HC: Healthy controls; [1] Subjects not in Hardy-Weinberg equilibrium; [a] $p = 0.045$ (T vs. C), $p = 0.049$ (TT vs. CC); [b] $p = 0.02$ (T vs. C), $p = 0.04$ (TT vs. CC); [c] $p = 0.006$ (T vs. C), $p = 0.01$ (TT vs. CC).

Table 32. Odds Ratios and 95% CIs for Different Outcomes Obtained through the Meta-analysis for *MDR1* Variants[243]

		Fixed effects		Random effects	
		***P* value**	**OR (95% CI)**	***P* value**	**OR (95% CI)**
	Outcome CD				
C3435T	T vs. C (6 studies)	0.519	0.968 (0.878-1.068)	0.772	0.974 (0.842-1.126)
	TT vs. CC (5 studies)	0.297	0.892 (0.720-1.106)	0.472	0.900 (0.675-1.200)
G2677T/A	G vs. T (4 studies)	0.635	1.027 (0.920-1.145)	0.635	1.027 (0.920-1.145)
	GG vs. GT TT (3 studies)	0.338	1.092 (0.912-1.308)	0.338	1.092 (0.912-1.308)
	Outcome UC				
C3435T	T vs. C (6 studies)	0.002	1.170 (1.062-1.289)	0.003	1.178 (1.058-1.311)
	TT vs. CC (5 studies)	0.008	1.332 (1.080-1.644)	0.017	1.367 (1.057-1.768)
G2677T/A	G vs. T (4 studies)	0.843	0.989 (0.887-1.103)	0.862	0.986 (0.836-1.162)
	GG vs. GT TT (3 studies)	0.947	0.994 (0.830-1.190)	0.947	0.994 (0.830-1.190)
	Outcome IBD				
C3435T	T vs. C (6 studies)	0.083	1.074 (0.991-1.165)	0.135	1.083 (0.976-1.201)
	TT vs. CC (5 studies)	0.225	1.116 (0.935-1.332)	0.274	1.135 (0.904-1.426)
G2677T/A	G vs. T (5 studies)	0.351	1.041 (0.957-1.132)	0.448	1.047 (0.930-1.178)
	GG vs. GT TT (4 studies)	0.366	1.065 (0.929-1.221)	0.366	1.065 (0.929-1.221)

The second ATP-binding cassette transporter to be examined was the *ABCG2*, which in our study proved to have no association with IBD. However, the variant alleles investigated in this study, 34A and 421A, showed a protective effect against arthritis in ulcerative colitis (OR: 0.39; $p = 0.046$). Also, while the ABCG2 protein product might be suspected of affecting response to therapy, steroids or infliximab, thus altering the need for surgery, this was not the case in our study. We found no significant association between the variant alleles of both, *ABCG2* and *MDR1*, and the response to infliximab (in CD), steroids, or subsequent need for surgery, in either Crohn's disease or ulcerative colitis. Concerning disease behavior, our study found a tendency for the *MDR1* 3435CT/TT genotypes to be present in patients

with fistulizing disease (fistulizing: 30.3% vs. non-fistulizing: 21.7%; OR: 1.57; 95% CI: 0.92-2.76; $p = 0.09$). However, this was the only association we found for C3435T and could not replicate a previous association with extensive UC.[377]

The DLG5 protein is believed to be a scaffolding protein, playing a role in the intestinal barrier function. In our study, we found no association between the *DLG5* R30Q variant and IBD, or its sub-types. Rather, we noted a lower carriage frequency in CD patients, contrary to Stoll and colleagues who reported overtransmission of this variant in IBD as a whole, and in CD patients.[249] Daly et al., in a joint Canada-UK study, succeeded in replicating the *DLG5* R30Q association with IBD using a Canadian/Italian case-control cohort, yet a second cohort from the UK failed to present the same association. Finally, a third Canadian family cohort demonstrated significant overtransmission of the risk allele ($p = 0.018$).[383] Two additional studies, one in a Scottish population[384] and another in a German cohort,[385] were not able to establish the variant allele's overtransmission, as noted in our study. Importantly, the variant allele's frequency in the control group was higher (15%) than in previous reports.

As has been demonstrated for *NOD2/CARD15* mutations in Japanese patients,[386] it is possible that ethnic variation is a factor in *DLG5* polymorphisms. While this has not yet been proven, it can be observed in the frequency differences between Caucasian patients and other ethnic groups. For example, in one study that examined 484 Japanese CD patients and 345 control subjects, the G113A polymorphism was completely absent, yet a significant association ($p = 0.023$) was noted for another *DLG5* variant (rs3758462).

We were unable to identify any significant genotype-phenotype associations in CD, in agreement with a previous study by Török et al.[385] Still, carriage of G113A, while not associated with response to infliximab, was independently associated with steroid resistance. One possible explanation for this observation may be derived from reviewing *DLG5*'s function. The protein is believed to be a scaffolding protein responsible for maintaining epithelial cell structure and integrity, as well as intracellular signal transduction an intercellular contact. Consequently, it may be hypothesized that *DLG5* is involved in the dysfunction of the epithelial barrier function, thus influencing intestinal permeability and the response to luminal antigens. As such, it is likely that response to steroid treatment is also influenced in this manner. *In silico* research has suggested that the G113A variant prevents the protein's proper folding. Similarly to the observations made in CD, no genotype-phenotype associations were made between the *DLG5* variant and UC, yet a trend towards more extensive disease was noted (OR: 2.1; 95% CI: 0.95-4.4; $p = 0.07$). Finally, contrary to the findings by two other groups,[249,387] we could not identify epistasis between *DLG5*

polymorphisms and any of the three most common *NOD2/CARD15* mutations, SNP8, 12, and 13 or *TLR4* D299G. Still, further studies are necessary to elucidate the ethnic differences in the expression of *DLG5* polymorphisms as well as genotype-phenotype associations.

In a large study published in early 2008, Browning et al. examined numerous, previously researched adult cohorts for an association with the R30Q variant, and stratified for gender. In total, 4707 CD patients and 4973 controls were included, across 12 cohorts. While a protective trend was observed for male CD patients compared with control subjects, it was not statistically significant (OR: 0.87; 95% CI: 0.74-1.01; p = 0.058). However, in females, the 30Q allele was found to be significantly associated with decreased risk of CD (OR: 0.86; 95% CI: 0.76-0.97; p = 0.010).[388] Figures 26 and 27 illustrate the wide variability in the odds ratio of the *DLG5* R30Q in CD patients, stratified by gender.

Figure 26. Odds Ratios of *DLG5* R30Q in Female CD Patients versus Female Control Subjects[388]

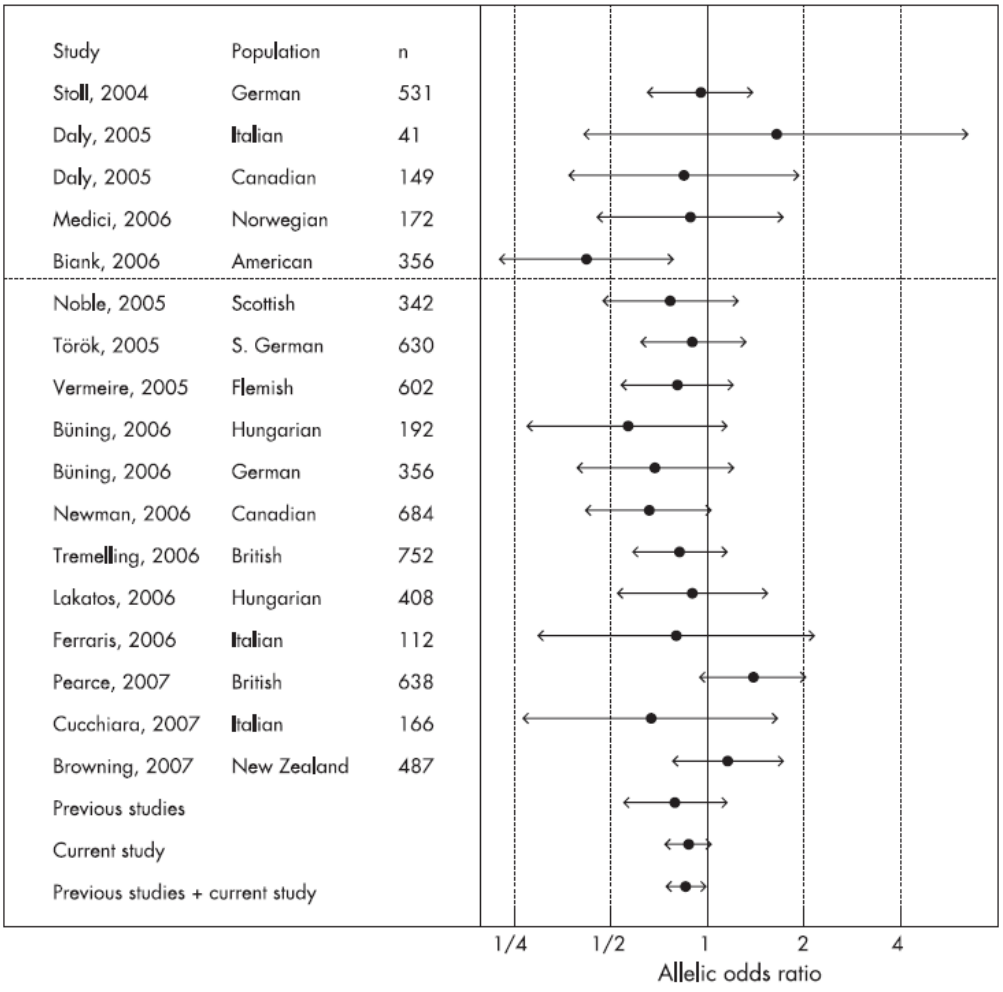
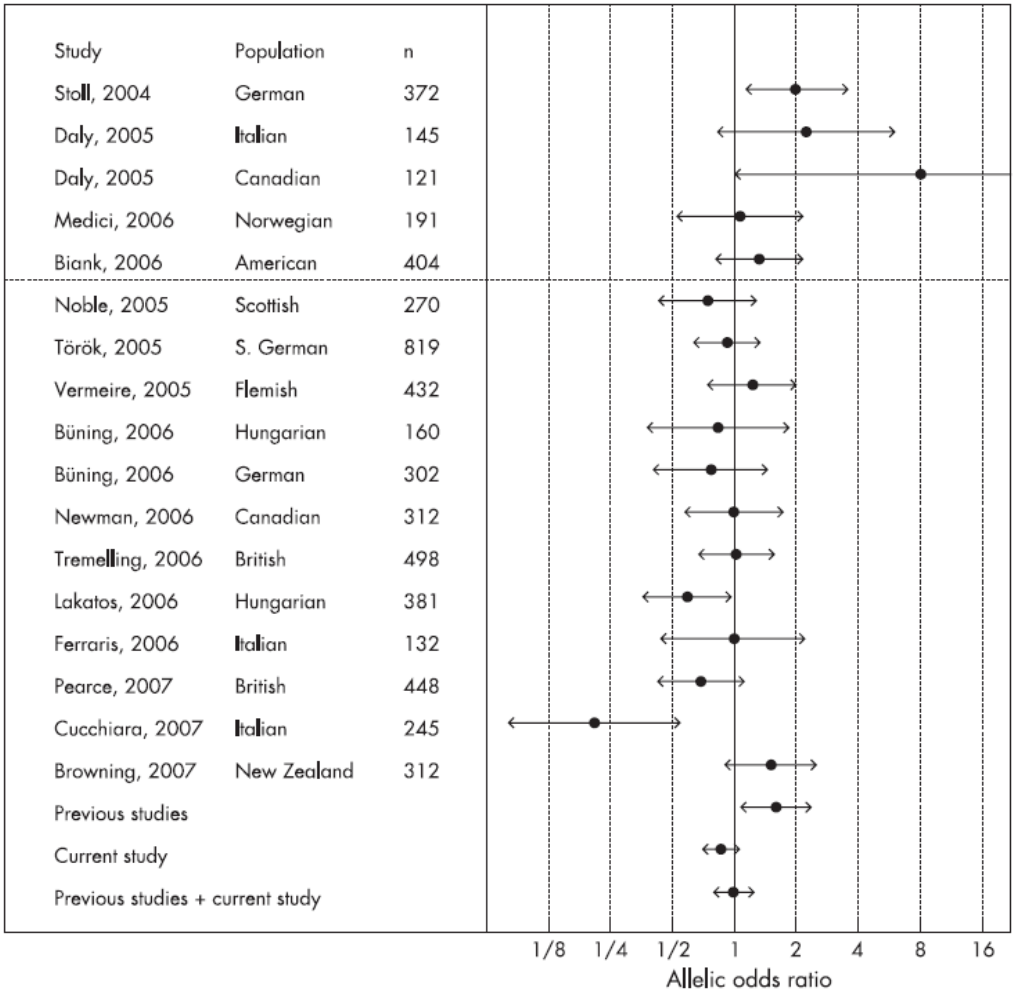


Figure 27. Odds Ratios of *DLG5* R30Q in Male CD Patients versus Male Control Subjects[388]



10. SCIENTIFIC CONTRIBUTIONS TO THE FIELD:

1. These are the first studies to examine the *DLG5*, *ABCG2*, and *MDR1* polymorphisms in the Hungarian IBD population. In one respect, it is yet another addition to the growing body of knowledge concerning ethnic differences in IBD susceptibility.
2. In our cohort, we did not find any association between the variants of *ABCG2*, *MDR1* or *DLG5* and disease susceptibility. However, one variant of the *MDR1* gene, G2677, showed a trend for association with Crohn's disease (OR: 1.29, $p = 0.08$).
3. Carriage of *ABCG2* showed a significant protective effect against arthritis in patients with ulcerative colitis (OR: 0.39; $p = 0.046$).
4. *DLG5* 113A carriage appeared to have a protective effect in Crohn's disease patients (OR: 0.67; $p = 0.06$).
5. *DLG5* demonstrated a trend for association with extensive disease in patients with ulcerative colitis (OR: 2.1; $p = 0.07$). However, no other genotype-phenotype associations could be made.
6. *DLG5* R30Q was associated with steroid resistance in patients with Crohn's disease (OR: 2.4; $p = 0.013$). After correcting for various confounding factors, the 30Q variant was still significantly associated with steroid resistnace ($p = 0.02$). In addition, perianal disease and frequent relapses were independently associated with steroid resistance ($p = 0.01$ and $p = 0.001$, respectively). Contrary to other reports, we could not associate *MDR1* polymorphisms with steroid resistance.
7. None of the tested variants demonstrated any association with clinical response to short-term infliximab therapy in the sub-cohort of CD patients.

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PUBLICATIONS DIRECTLY RELATED TO THIS THESIS:

1. Lakatos PL, **Fischer S** (joint first authors), Claes K, Kovacs A, Molnar T, Altorjay I, Demeter P, Tulassay Z, Palatka K, Papp M, Rutgeerts P, Szalay F, Papp J, Hungarian IBD Study Group, Vermeire S, Lakatos L. DLG5 R30Q is not associated with inflammatory bowel disease in Hungarian IBD patients, but predicts clinical response to steroids in Crohn's disease. *Inflamm Bowel Dis*. 2006;12:362-368.

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ABSTRACTS:

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