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CHAPTER 1

GENERAL INTRODUCTION

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Introduction

Crohn's disease (CD) and Ulcerative Colitis (UC) which together form the main entities of the inflammatory bowel diseases (IBD), are chronic inflammatory disorders of the gastro intestinal (GI) tract.

In UC, first described in 1859 by Wilks [1], the mucosa and submucosa of the large bowel are involved. The inflamed area is continuous and uniform without interspersing areas of unaffected tissue. In contrast in CD, as such identified by Crohn et al in 1932 [2], the whole gastrointestinal tract can be involved, however most commonly it is confined to the distal part of the ileum and colon. The inflammation affects all layers of the bowel wall and has a patchy appearance with areas of ulceration separated by narrow areas of healthy tissue. In a subgroup of patients granulomas are found. Frequently observed complications in CD are stenoses and strictures while in longstanding UC and Crohn's colitis there is increased risk for colon carcinoma.

The incidence of IBD is not evenly distributed around the world and varies between 0.08 to 10 per 100,000/year for CD [3, 4] and from 0.5 to 24.5 for UC [3, 5]. The highest incidences are reported in the western countries, whereas the incidence in Asia is low [3] and although accurate data are lacking, this is most probably also true for the developing countries [6].

Over the last decade, major progress has been made in unraveling the complex etiology of IBD. It is now well established that, although clinically related, CD and UC are different disease entities with a different genetic and immunological basis. It is even questionable whether CD and UC comprise only two disease entities or represent a spectrum of clinically related disorders with different etiologies.

There is no curative treatment for IBD available on the moment, therefore therapy is aimed at treating acute disease and then achieving and maintaining remission. Options for treatment are determined by the localization of the disease, its severity and the presence or absence of extraintestinal manifestations. Besides direct therapy, adjunctive treatment with antidiarrheal agents and supplementation of minerals and vitamins can be important [7].

For a long time, the only treatment options were anti-inflammatory drugs (aminosalicylates), steroids and surgical resection. Later, the immunosuppressants azathioprine, 6-mercaptopurine, methotrexate, and cyclosporine were added to this arsenal. With the introduction of anti-TNF treatment in the mid-nineties of the last century, a new era in the treatment of

IBD was introduced. The recent advances in understanding the etiology of IBD have resulted in the development of an impressive number of new biological treatments.

Etiology of IBD

While the exact pathogenesis of IBD remains enigmatic it is clear that disease occurs as the consequence of a complex interplay between genetic susceptibility and a disrupted barrier function of the gastro intestinal epithelium which in conjunction with one or more environmental triggers leads to an uncontrolled immune response in the intestine.

In the following we will summarize the existing evidence for this thesis and highlight some of the key players in disease pathogenesis that have received awareness over the last years and form the basis of this thesis.

Environmental factors

Since there is a clear increase in incidence of IBD in the western world over the last decades [8] the culprit has been sought in nearly every new compound that has been introduced in the past 100 years; these include such items as toothpaste, chewing gum and even soft toys. However strong arguments for involvement of any of these substances are not met mainly because most of these studies suffer from a lack of statistical power and sufficient methodology [8].

Up till now smoking is the only generally accepted external risk factor for IBD. Smoking not only increases the risk for CD [9], it also has an aggravating effect on the course of disease, with more complications and a higher rate of surgery. Interestingly, the opposite is the case with UC where smoking seems to be protective [10]. Notably, incidence rates for UC are highest among former smokers.

Oral tolerance

The GI tract faces the challenge of (a) absorption of food nutrients; (b) being unresponsive to the gut commensal flora, but (c) at the same being reactive against harmful invading pathogens. One mechanism by which the gut might cope with these conflicting goals is through oral tolerance, the immune mechanism by which the mucosal immune system maintains unresponsiveness to mucosal antigens which might otherwise induce immune responses. Disturbances

in oral tolerance and the inability to remain unresponsive to food antigens or commensal flora could form an underlying cause for IBD. Oral tolerance is mediated by several distinct, yet interacting mechanisms including the generation of regulatory T cells producing suppressive cytokines and the induction of clonal deletion and/or anergy. That indeed regulatory T-cells could play a role in IBD was demonstrated in an animal model of colitis where adoptive transfer of T-cells expressing FoxP3 (a transcription factor critical for development of regulatory T-cells [11-13]) prevented weight loss and inflammation [14]. Such CD4⁺CD25⁺FoxP3⁺ regulatory T-cells were subsequently reported to be differentially distributed when comparing IBD patients with non-IBD inflammatory controls, with the numbers of cells being significantly lower in IBD lesions [15]. Recent publications however indicate that the role of regulatory T-cells in IBD might not be the consequence of a quantitative effect [16] but could be the consequence of the transition of these cells under inflammatory conditions from a regulatory to an effector phenotype [17].

Disrupted barrier function

For an inflammatory response to take place, the immune system has to get in contact with potentially hostile and threatening stimuli. In the gut the epithelium forms this interface between the mucosal immune system and the luminal contents of the intestinal tract. This interface is not meant to be an impenetrable wall but rather functions as a sentinel, allowing beneficial molecules like nutrients to pass, while at the same time providing an effective barrier against toxins and pathogens. Therefore, an improperly functioning epithelial defense might be an underlying cause of IBD.

The gut has several defense mechanisms at its disposal. These depend on exclusion of commensal bacteria by secretion of antimicrobial peptides such as defensins, the scavenging and binding of luminal organisms by mucins, a relatively impermeable mucosal barrier where tight and adherens junctions between the epithelial cells regulate passage of potentially pro-inflammatory molecules and the presence of a sensing mechanism that is able to react and regulate immunological responsiveness once the barrier has breached.

A defect in one of these defensive mechanisms can result in enhanced microbial antigenic exposure which could lead to pathogenic T-cell responses which finally can develop in chronic intestinal inflammation. [18].

Indeed, defective epithelial barrier function, which can be measured as increased intestinal permeability, has been implicated in Crohn's disease and Ulcerative Colitis. This increased permeability precedes relapses in disease, implicating that it concerns an important pathophysiological event [19, 20]. These observations are paralleled by the findings in animal models where increased mucosal permeability is seen in preclinical stages of colitis in IL-10^{-/-} mice [21] and ileitis in Samp-1/Yit mice [22]. Increased permeability either spontaneously or after exposure to nonsteroidal anti-inflammatory drugs is also present in a subset of unaffected first degree relatives of patients with Crohn's disease [23, 24] indicating the involvement of a genetic component.

Taken together it is clear that unraveling the mechanisms of barrier function and permeability could provide important insights in IBD pathogenesis.

Abnormal permeability refers to an increased influx of small water-soluble compounds across the epithelial lining of the intestine. This flux is regulated primarily by the tight and adherens junctions [25]. A tight junction (TJ) is a structure that connects epithelial cells at the apical site. These structures seal off the intercellular space and regulate passive diffusion of solutes and macromolecules through the paracellular space to the underlying lamina propria. In intact gastrointestinal epithelia, it is the passage of ions through the tight junction that determines the overall epithelial permeability of the intestine [25]

Tight junctions are made up of a complex of integral membrane proteins, which are anchored to proteins in the cytoplasmic plaque [26]. These integral membrane proteins consist of different molecules including occludin; members of the claudin family and the junctional adhesion molecule (JAM). Of these, occludin was the first to be shown to be an integral part of the tight junction complex [27] later JAM was identified [28]. With the discovery of the claudins it became apparent that these molecules in fact form the main tight junction proteins. The claudins form a multigene family, consisting of about 24 members in humans and mice [29] and are expressed in a tissue-specific manner [30]. It is this tissue specific expression that can dictate local differences in epithelial permeability [31]. How these integral proteins coalesce to form tight junction fibrils is currently unknown.

The tight junction membrane proteins interact with scaffolding proteins (zona occludens 1, 2, 3; e.g. ZO-1, ZO-2, ZO-3) which together form the cytoplasmic plaque. These ZO proteins in turn bind to actin [32], thereby anchoring the tight junction to the cytoskeleton

and in this way connecting them with various signal transduction and transcriptional pathways involved in the regulation of tight junction function.

Increased permeability can be the result of an alteration in the protein composition of the junctions or their regulatory systems. Strong evidence that TJ barrier function is altered in IBD comes from studies from Zeissig et al. [33] where they assessed the expression of 12 claudin isoforms in patients with Crohn's disease. They found that expression of the pore-forming claudin 2 was increased, particularly in the crypt epithelium while the expression of the sealing claudins 5 and 8 on the other hand was downregulated. Together this led to an altered tight junction structure and a pronounced barrier dysfunction already in mild to moderately active Crohn's disease. However none of these changes were detected in patients with inactive IBD, suggesting that this altered claudin expression is a secondary effect rather than a primary one. Such a secondary effect could be the consequence of cytokine action. IL-13, which is elevated in UC was shown to increase claudin 2 expression in epithelial cells from patients [34]. Also two other cytokines which are critical to IBD pathogenesis, TNF and interferon- γ , could increase paracellular permeability, however here it was accompanied with a reduced claudin 2 expression suggesting that the mechanisms by which interferon- γ and TNF increase permeability differ from that of IL13 [35].

The importance of an intact epithelial barrier is unequivocally demonstrated in an animal model employing N-cadherin transgenic mice [36] which shows that, provided the disruption is significant, normal commensal organisms can induce an immune response fierce enough to initiate an inflammation. Such a scenario is not likely to be the case in humans as is exemplified by the altered permeability in healthy first degree relatives of IBD patients. The exact relevance of a deranged epithelial barrier function to the pathogenesis of IBD remains to be elucidated. Nevertheless the data suggest that inappropriate interactions between luminal antigens and the mucosal immune system form an important step in the genesis of IBD.

Microbiological factors

Studies in experimental colitis models have shown that disease in general does not develop when mice are reared in a germ-free environment and furthermore that colitis can occur as the consequence of a genetically determined aberrant immune response to constituents of the microbial flora [37, 38]. This strongly suggests that the normal microflora is necessary to initiate

or maintain the inflammatory process, presumably by providing one or more antigens or co-stimulatory factors that drive the immune response in a genetically susceptible host. Extrapolating these findings to human IBD, it is tempting to speculate that disease in humans is mediated by either an uncontrolled immune response to the normal intestinal flora or, alternatively, to a pathogenic microbe. In favor of the latter, there are some striking similarities between a granulomatous disease in cattle, Johne's disease, which is caused by infection with *M Paratuberculosis* and Crohn's disease in humans [39] and to this day there is controversy whether *M Paratuberculosis* might be involved in a subgroup of CD patients [40]. It is noteworthy in this respect that occasionally slow-growing mycobacteria can be identified in the gut from patients with Crohn's disease, although these data are so far conflicting [41]. The same holds true for measles virus infection [42]. Therefore it must be concluded that despite an exhaustive search, to date no compelling evidence exists that supports an infectious etiology.

Consonant with the observation that disease does not develop in germ-free animals, there is emerging circumstantial evidence that the normal microbial flora is a major antigenic source that drives the uncontrolled immune response in IBD. As alluded to above, this may or may not be the consequence of a defect in oral tolerance and/or a primary defect in mucosal barrier function.

There is substantial recent interest in some specific *E. Coli* strains that can be isolated from ileal lesions of patients with CD. These strains are able to adhere and invade epithelial cells hence its designation Adhering Invasive E. Coli (AIEC) [43, 44]. They are found in ileal specimens in a substantially higher proportion of CD patients as compared to controls (37% vs. 6% respectively). As can be concluded from the fact that these strains are also observed in control subjects, these AIEC strains do not represent specific pathogens exclusively found in CD. This implies that they may belong to transient normal flora which can preferentially colonize CD ileal mucosa. Other indications for the involvement of the normal commensal flora arise from the fact that IBD patients have increased serum and mucosal antibody responses to commensal bacteria, and by the observation that IBD patients lose tolerance to their flora [45-48]. In addition, there are reports that diverting the fecal stream (and diminishing the antigenic microbial load), and antibiotics have a beneficial effect in IBD patients. Also there are reports that probiotics improve the course of disease in a subgroup of IBD patients. Finally, the recent

identification of mutations in NOD2 a gene that controls immune responses to bacterial cell wall compounds in a subgroup of CD patients further emphasizes the intense interplay between genetics, microbial flora and the immune system in IBD.

Immunological factors

The widely adopted view for IBD is that it concerns an exaggerated response of the immune system on normal constituents of the gastrointestinal tract. For its defense against potential harmful invaders the immune system relies on two systems, the evolutionary older innate immune system and the adaptive immune system. The first line of defense is formed by cells of the innate system to which belong the macrophages, dendritic cells, neutrophils, eosinophils, mast cells and natural killer cells. On recognition of a pathogen they respond by secretion of immune active molecules as cytokines and chemokines which can alter vascular permeability and attract and activate other immune cells such as neutrophils and monocytes (precursors of macrophages). This recognition is non-specific, in that it does not recognize properties of individual microbes but instead conserved molecular microbial patterns or PAMPS (pathogen associated molecular patterns) which are shared by large arrays of microbes. Recognition is performed by pattern recognition receptors (PRRs) such as the membrane bound Toll-like receptor (TLR) family and the cytosolic NLR (nucleotide-binding domain, leucine-rich-repeat-containing) family.

In contrast to the innate response the adaptive response relies on recognition of individual pathogens. In addition to this specific recognition it provides memory by long-lived memory cells that can recognize their target long after the primary infection has subsided. The cells performing these tasks are the T-lymphocytes to which the cytotoxic CD8 T cells (CTLs) and CD4 or Thelper (Th) cells belong. CTL's scan every cell in the body for the presence of infectious or malignant signals and when encountering such cells kill them by the release of cytotoxic molecules. The CD4 cells can after recognition of their specific antigen develop into either Th1 or Th2 cells depending on the cytokine milieu in which recognition takes place. In the presence of Interleukin-4 (IL-4) the Th2 lineage will develop. These Th2 cells induce B-cell proliferation and in this way are key to the humoral immune response which relies on antibody action. Interleukin-12 (IL-12) is the cytokine that is indispensable for the full de-

ployment of the Th1 lineage. Mature Th1 cells produce Interferon- γ (IFN- γ) which has strong antiviral, immunoregulatory properties [49].

The innate and adaptive system do not work separately but are tightly interconnected with a central role at this interface for the IL-12 cytokine family of which IL-12 was the first discovered member [50]. IL-12 is primarily synthesized by the (innate) macrophages and dendritic cells and its primary targets are natural killer cells (innate) and T cells (adaptive). With the latter one it promotes the forming of the Th1 lineage whilst at the same time inhibiting polarization towards the Th2 lineage.

IL-12, as already mentioned, is a member of the IL-12 type family of heterodimeric cytokines that includes IL-12, interleukin-23 (IL-23), interleukin-27 (IL-27) and interleukin-35 (IL-35). IL-12 is formed by a 35-kDa light chain (known as p35 or IL-12 α) and a 40-kDa heavy chain (known as p40 or IL-12 β) [51]. The two genes coding for these chains are located on separate chromosomes (5q for the p40 subunit and 3p for p35 subunit in man and chromosomes 11 and 6 respectively in mice) [52]. The homology of p35 with single-chain cytokines and the homology that the p40 subunit shares with cytokine receptors has led to the concept that IL-12 could have evolved from a primordial cytokine, presumably of the IL-6 family, and one of its receptors [53]. Regulation of its synthesis is complex. Ligation of TLRs with a pathogen leads to the activation of transcription factors of the NF κ B and IRF (Interferon Regulatory Factor) pathways. The genes for the p35 and p40 subunit employ different members. IRF5 is involved in the IL-12p40 synthesis while IRF1, IRF3 and IRF7 promotes exclusively the expression of the IL-12p35 gene. The primary synthesis of IL-12 after TLR stimulation is then further stimulated by a strong feed-back loop via IFN- γ . Binding of this cytokine to its receptor induces IRF8 which enhances expression of both the p40 and p35 genes, and it induces IRF1 which further boosts p35 expression (for a review see [54]). Once IL-12 is synthesized it exerts its effect through binding to its receptor. This receptor is mainly synthesized by activated T-cells and NK cells. It is composed of two chains, IL-12R β 1 and IL-12R β 2 [55]. Binding to this receptor activates the Janus kinase (JAK)–STAT (signal transducer and activator of transcription) pathway of signal transduction which in turn leads to the transcriptional activation of numerous genes involved in prototypic Th1 responses, such as the synthesis of tumor necrosis factor (TNF) and particularly, IFN- γ [56, 57].

Studies employing mouse models of IBD demonstrated that mucosal inflammation can manifest itself as a Th1 (IL-12 driven, IFN- γ and TNF- α mediated) or Th2 -driven final common pathway (characterized by high expression of IL-5 and IL-13). These different immunological profiles are accompanied by a specific histological picture. Thus, Th1 mediated colitis is usually associated with a transmural inflammation resembling Crohn's disease, whereas a mere Th2 profile is associated with superficial mucosal inflammation which is more associated with Ulcerative Colitis [37]. The model of an IL-12 driven disease agrees in many aspects with the human situation. The IL-12 p70 heterodimer secretion is enhanced in cells cultured ex-vivo from mucosal tissue biopsies of CD patients [50, 58-60], the expression of the IL-12 receptor β 2 chain is increased in active CD [61, 62] and mRNA for both IL-12 p35 and p40 is upregulated in mucosal tissue samples from CD patients [50, 58]. Most compelling evidence however is derived from studies with monoclonal antibodies specific for the IL-12p40 subunit. In animal models of IBD it was shown that usage of these antibodies could block intestinal inflammation [63]. Subsequent clinical trials with CD patients showed that anti IL-12 treatment was an effective therapeutic approach in CD with response and remission rates as high or higher as realized with an other established antibody therapy against the pro-inflammatory cytokine TNF [64] .

This Th1/Th2 model and the role of IL-12 in IBD has undergone substantial adaptations as a consequence of the discovery in 2000 of IL-23 and IL-27 [65, 66]. IL-23 is also a heterodimer consisting of the p40 subunit in conjunction with an IL-23 specific p19 chain. Realising that anti IL-12 antibodies are directed against the p40 subunit, implied that any effect of this anti IL-12 treatment may in fact have to be attributed to anti IL-23 activity. Subsequent studies in mouse models of IBD showed that indeed IL-23 plays a key role in chronic intestinal inflammation. Mice that were unable to produce IL-23 through genetic depletion of its p19 subunit were highly resistant to colitis whereas on the other hand mice that were not able to synthesize IL-12 (by genetically inactivating the p35 gene), developed severe colitis. Also treatment of mice with an antibody specific for the p19 subunit resulted in highly attenuated intestinal pathology [67, 68].

Recently it has been suggested that IL-23 could exert its effect yet in another way than through IL-17, namely by overriding immunosuppressive pathways. It was shown that the number of FoxP3+ regulatory T-cells increased in the colon in the absence of IL-23. On the

other hand, in the absence of FoxP3, IL-23 deficient mice developed colitis indicating that IL-23 is not essential for intestinal inflammation when FoxP3 is lacking [69].

That IL-23 is probably also a key player in human IBD stems not only from the observation that lamina propria macrophages from CD patients display elevated IL-23 synthesis rates [60] but can also be inferred from a completely different source. Genome wide association studies revealed that the gene for the IL-23 receptor (IL23R) is strongly associated with resistance or susceptibility to CD [70, 71].

Similar to IL-12 it is not IL-23 itself that acts as the inflammatory effector, but it mediates its effect through a recently discovered distinct lineage of pro-inflammatory CD4 T cells, the T helper 17 (Th17) cells which are able to secrete high levels of the pro-inflammatory cytokine IL-17 [72-74].

Given the apparent importance of the IL-23, Th17, IL-17 axis in inflammatory responses one could conclude that the IL-12, Th1, IFN- γ axis is of minor importance in immune pathology. However, this is probably an oversimplification. In human CD for instance both IL-12 and IL-23 are elevated [60]. In mice that are unable to generate Th1 cells, large numbers of Th17 cells are present but these mice are resistant to experimental autoimmune encephalomyelitis (EAE) [75, 76]. Also p19 knock out mice, which are unable to synthesize IL-23, develop severe colitis in a chemically induced colitis model, however they could be rescued by blockade of IL-12 synthesis [77]. Together these observations suggest that both pathways are required for the development of intestinal inflammation.

As already mentioned earlier a more superficial mucosal inflammation is seen in patients suffering from UC. Such a superficial inflammation is associated with a Th2 profile, however absence of elevated levels of the prototypical Th2 cytokine IL-4 has made it difficult to classify the inflammation seen in UC. There is, however, increased secretion of IL-5, another Th2 cytokine. Additional circumstantial evidence comes from the notion that UC is associated with the production of various autoantibodies such as anti-neutrophil cytoplasmic antibody (pANCA) and anti-tropomyosin, which is indicative for a Th2 rather than a Th1 response (38). Most compelling evidence comes from studies where it is shown that the lamina propria of Ulcerative Colitis patients but not Crohn's patients contain NK-T cells that produce significantly elevated amounts of IL-13, yet another Th2 cytokine [78, 79]. IL-13 shows overlap in its biological activities with IL-4 [80] which is in part due to the use of common recep-

tors [81]. In subsequent studies it was shown that IL-13 can profoundly influence epithelial cell function, including stimulation of apoptosis, impaired epithelial woundhealing and tight junction biosynthesis [34]. Thus mechanistically the observed high IL-13 levels could account for much of the phenotype seen in UC. Epithelial apoptosis and impaired woundhealing would facilitate erosion and ulcer type lesions. Altered tight junction formation would be responsible for the loss of water and ions into the intestinal lumen (diarrhea).

Genetic factors

Epidemiological and family studies have shown that there is a strong genetic predisposition to develop IBD [82], in particular CD. Most compelling evidence for this thesis is provided by twin studies showing that concordance rates in identical twins with CD are as high as 58% while concordance rates in dizygotics are similar to siblings [83].

Transmission of disease does not follow a simple Mendelian inheritance pattern but instead has a complex mode of inheritance. This means that disease is not attributable to either a recessive trait or a major gene with a large effect, but instead to many genes each with their own genetic contribution.

Candidate gene approaches and family based linkage studies during the 1990s have identified nine loci, termed IBD 1–9 which have been replicated in independent data sets (table 1). Some of these are specifically associated with CD (IBD1) while others are specific for UC (IBD2) and still others for both.

The methodology employed in these studies were either hypothesis driven (a gene could be a candidate based on a presumed role in IBD pathology) or difficult to realize as they required large cohorts of families with affected siblings. With the introduction of a novel strategy to dissect complex genetic traits, *a priori* knowledge on gene function was no longer needed, nor was the recruitment of patient cohorts dependent on the presence of multiple affected family members. This technique, known as genome wide association studies (GWAS), became available after the discovery that the human genome contains about 10 million single nucleotide polymorphisms (SNPs), implying that the whole human genome could be divided in 10 million “chunks” which could be individually “tagged” by their SNP. By comparing the distribution of SNPs between affected and unaffected individuals a higher incidence of specific SNPs in a genomic region in the affected group can indicate that a gene in this region is

involved in disease pathology. Stringent statistical criteria to separate true from false positives and replication of the findings in independent cohorts are prerequisites in this type of studies.

Table 1 Major susceptibility Loci implicated with candidate gene and family based linkage studies (adapted from [140]).

Locus	Chromosomal location	Study	Diagnosis
IBD1	16q12	Hugot [89]	CD
IBD2	12q13	Satsangi [133]	UC
IBD3	6p13	Hampe [134]	CD, UC
IBD4	14q11	Ma, Duerr [135, 136]	CD
IBD5	5q31–33	Rioux [137]	CD
IBD6	19p13	Rioux[137]	CD, UC
IBD7	1p36	Cho [138]	CD, UC
IBD8	16p	Hampe [139]	CD, UC
IBD9	3p26	Satsangi [133]	CD, UC

To date seven of such scans were performed and have uncovered at least ten new genomic regions associated with susceptibility to Crohn’s disease (table 2) [84], very recently, several others were added to this list [85]. The genes emerging from these studies confirm the importance of pathological pathways already unveiled in the earlier studies as innate immunity and barrier integrity but has also found new routes with the discovery of the autophagy related genes ATG16L and IRGM.

The above mentioned GWAS were performed in Crohn’s disease patients and to date such a study has not been reported for UC, however recently two studies one by Fisher et al. and the other by Franke et al.[86, 87] have tested variants from the CD studies for association with UC. These studies confirmed that the IL-23 receptor gene is indeed associated with susceptibility in both diseases and showed that there also exist strong associations with the IL-12B (5q33), the MST1 (3p21) and NKX2-3 (10q24) genes. Franke reported also an association of UC with a “gene desert” at 10q21 but this was not confirmed in the study of Fisher et al.. In a more or less similar approach an association was reported for the myosin IXB gene and UC [88]. In this case the primary association with the gene at 19p13 (IBD6) was not reported with CD but with celiac disease, a food antigen driven intestinal inflammatory disease.

In the next paragraphs the genes that have been studied in detail so far will be further discussed.

Table 2 Overview of the Genome-Wide Association studies performed to date in IBD
(adapted and updated from [146])

Population and study	Novel gene or region identified	Gene or region from other study confirmed
Japanese, British [141]	TNFSF15	TNFSF8
German, British [132]	ATG16L1	NOD2 SLC22A4
N America (european)[70, 142]	IL23R 10q “gene desert” PHOX2B NCF4 FAM92B	ATG16L1
Belgium [143]	5p “gene desert”	NOD2 IL23R ATG16L1
British [71, 122]	IRGM NKX2-3 PTPN2 3p21 1q “gene deserts” IL12B FLJ45139	NOD2 IL23R ATG16L1 IBD5
German [144]	NELL1	NOD2 5p “gene desert” SLC22A4
Quebec [145]	4p 16 17 q 11 17 q 23	NOD2 IBD5 IL23R ATG16L1 IRGM 1q “gene desert” 5p “gene desert”

NOD2 on chromosome 16

Polymorphisms in the NOD2 gene.

A French and an American group co-reported in 2001 for the first time a strong association between Crohn’s disease and mutations in NOD2 (also known as Caspase-recruitment domain protein 15 [CARD15]) on chromosome 16 which encodes an intracellular molecule that is thought to be involved in the recognition of bacteria [89-93]. Initially, three major polymorphisms which were associated with increased susceptibility to Crohn’s disease but not to UC were identified. These polymorphisms give rise to changes in the NOD2 molecule, in two

cases leading to an amino acid change (Arg702Trp, Gly908Arg) and in the other to a truncated protein (Leu1007fsinsC). Later, a large number of additional, rare polymorphisms have been added to this list.

The disease associated polymorphisms show a marked gene dose effect. Thus, individuals with one risk allele have a three times higher risk of developing CD while this is increased up to 20 to 40 fold when they carry two copies of the risk alleles. What should be kept in mind however is that these mutations are only found in a subgroup of patients and that the prevalence differs widely among ethnic groups. For instance in a Japanese CD population it is virtually absent [94] while in a group of European pediatric patients 45% possessed one or two of the mutations [95]. Altogether, in the Caucasian population 10-20% of CD patients carry mutations on both chromosomes (i.e., are homozygous or compound heterozygous). Additional genotype-phenotype studies report associations between NOD2 mutations and an earlier age of onset and a more pronounced ileal involvement [96-99].

Structure and function of the NOD2 molecule.

NOD2 is a member of the so-called CATERPILLAR (caspase recruitment domain <CARD> transcription enhancer R <purine>, pyrin lots of leucine repeats) family of proteins [100]. It consists of 2 N-terminal CARD domains which are involved in protein-protein interactions, a central nucleotide oligomerization (NOD) domain and at the C-terminal end multiple leucine-rich repeat (LRR) domains. These structures are homologue to the LRR's that are involved in pathogen recognition of the Toll like receptors (TLRs), cell surface molecules that play an essential role in initiating immune responses against pathogens [101-103]. The LRR's are now known to bind muramyl dipeptide (MDP), a bacterial cell wall component derived from peptidoglycan. The presence of these repeats suggested that functionally, the NOD proteins could be involved in the recognition of bacterial products. Notably, the reported mutations in the NOD2 gene are all located in or around these recognition areas.

Initially it was thought that expression of NOD2 was confined to the cytosol of monocytes/macrophages but it is now clear that it is also synthesized in dendritic cells and notably in epithelial cells of the small and the large intestine [104, 105].

In the latter NOD2 is predominantly expressed in Paneth cells, specialized epithelial cells which are thought to be involved in host defense by secreting anti-bacterial agents in response to a bacterial challenge [106].

After binding to its ligand, intact NOD2 combines with RICK (serine threonine kinase receptor-interacting CLARP-associated kinase). This then is enabled to inactivate the inhibitor of NF- κ B by phosphorylation. The now activated NF- κ B migrates to the nucleus where it induces expression of proinflammatory target genes [107].

How the mutations found in this gene lead to Crohn's disease has only partly been resolved. The consequence of the mutations in patients with CD would be that the NOD2 molecule fails to recognize its ligand and is not able to respond with a defensive action on a bacterial challenge [82]. The mutations therefore would lead to a so-called "loss of function phenotype". However the assumed lowered NF- κ B activation is inconsistent with the CD phenotype, which is characterized by marked upregulation of NF- κ B.

Several hypotheses derived from studies on animal models, as reviewed by Eckman and Karin [108] are proposed to cope with these contradicting observations.

In the first model the NOD2 mutations would lead to diminished epithelial responses. Paneth cells of NOD2 deficient mice were shown to have lowered expression of the anti-microbial peptides Defcr4 and Defcr-rs10 [109]. These mice also showed defective defense against orally administered *Listeria monocytogenes*. In the human situation NOD2 is also highly expressed in Paneth cells [110] and synthesis of α -defensins in CD patients is diminished and especially in patients with NOD2 mutations [111, 112]. Thus in this model the NOD mutation would primarily lead to a diminished synthesis of epithelial defense molecules – a loss of function mutation. This lack of defense would then lead to an overgrowth of inflammation causing bacteria and this would, secondary, give rise to a mucosal inflammatory response. Very recently this scenario has come under fire by work of Simms et al. where they show that reduction in α -defensin expression is independent of NOD2 status and that the reduction seen is a consequence of epithelium loss due to the inflammatory process rather than the inciting event [113].

The second model is based on data obtained by Maeda et al. [114]. Murine macrophages expressing the analogue of the human Leu1007finsC allele showed elevated expression of mature Interleukin-1 β (IL-1 β) as well as increased NF- κ B activation after stimulation

with MDP. Moreover mice expressing this mutant NOD2 allele showed increased colonic inflammation when challenged with sodium dextran sulphate (DSS). This induced colitis can be diminished by treatment with the inhibitor of IL-1 β , the IL-1 receptor antagonist (IL1-RA). Thus enhanced IL-1 β release would be the consequence of a mutated NOD2 gene (a gain of function mutation). The elevated IL-1 β release in turn would then induce an increased expression of pro-inflammatory cytokines which ultimately leads to an uncontrolled inflammatory response in the intestine. Recently evidence was reported that the enhanced secretion of IL-1 β is not a consequence of an altered interaction with the NF- κ B route, but rather a consequence of enhanced processing of the precursor molecule of IL-1 β through the CARD domain of NOD2 [115].

A third theory is provided by the data of Watanabe et al. [116, 117]. Consistent with human CD, macrophages from NOD2 deficient mice showed an elevated production of IL-12 and IL-23 when stimulated with TLR2 ligands. In wild-type, but not mutated macrophages this overproduction could be suppressed by incubation with the NOD2 ligand MDP. This suggests that stimulation of TLR2 can be modulated by the action of intact NOD2. Recently the same group has expanded the modulating capacity of NOD2 to other TLRs (TLR3, 4, 5 and 9) and also showed evidence that this modulation involves IFN regulatory 4 (IRF4) action. Thus deficient NOD2 leads to a dysregulated IL-12/23 [118] production which in turn promotes the Th1 mediated inflammatory response seen in human CD [37].

The contradictory observations (loss or gain of function) might be a consequence of tissue related expression of NOD2. In Paneth cells ineffective sensing by NOD2 would lead to diminished defensin synthesis followed by bacterial overgrowth from the lumen (loss of function). This overgrowth will subsequently provoke an inflammatory signal and recruit circulating macrophages to the intestinal mucosa. These, unlike resident macrophages, express the full array of TLR and NLR receptors. Sensing with these receptors would deploy the complete arsenal of proinflammatory mediators, however, downmodulation via NOD2 – as proposed in the scenario of Watanabe et al - at an appropriate stage would not be possible with NOD2 deficiency, leading to sustained inflammatory responses (gain of function).

The IL23R gene on chromosome 1

As IL-23 is a pivotal cytokine in the differentiation of T-helper cells, especially their differentiation into Th17 T cells, genes that are involved in its signaling pathway are strongly indicated in IBD pathology.

Duerr et al. [70] reported in a North-American study a strong association of multiple SNPs in the interleukin 23 receptor gene (IL23R) with CD. The strongest association in IL23R was found with a rare, non-synonymous SNP (Arg381Gln), which was reduced in CD cases, suggesting a protective effect for this polymorphism. Frequencies of other more common SNPs in the introns of IL23R were elevated in CD, indicating that they increase disease risk. Perhaps these variants have opposite effects on the expression or function of IL23R and, thus, opposite effects on disease risk. In a separate family-based association analysis also linkage with UC was demonstrated.

The IL12B gene on chromosome 5

Interleukin-12 and interleukin-23 are both heterodimeric cytokines that share the p40 chain. As discussed before, both cytokines are implicated in IBD pathology. Therefore the gene encoding this subunit would be an ideal candidate gene. Indeed, association of its gene has been reported for type1 diabetes, severe asthma and psoriasis [119-121]. However, linkage with IBD could not be established until the introduction of GWAS. Although initially its association was modest, it could be replicated in an independent cohort [122] and recently it was confirmed in two other studies which also reported its association with UC [86, 87]. It should be noted however that these associations were reported for a snp (rs6887659) located at approximately 65000 bp upstream of the IL12B gene implying that it must concern a trans-acting regulatory sequence in the vicinity of this snp, or even an other, unknown, gene. The study reporting this snp found a strong association of this snp with risk on developing psoriasis, it also reported a strong but independent contribution of another snp rs3212227. This snp is located in the IL12B gene (rs3212227) itself. Interestingly, it is this latter snp that was reported in earlier studies not to be associated with IBD [123]. This implies either that the involvement of IL-12B in psoriasis and CD manifests itself along different pathways, or indeed the involvement of an other gene.

The MYO9B gene on chromosome 9

The MYO9B gene codes for Myosin IXB. The gene was first implicated for susceptibility in celiac disease [124], a disorder of the small bowel that is caused by intolerance to gluten. Testing for association with IBD identified it as being also implicated in IBD with the strongest association found in UC [88].

Human myosin IXB is expressed in several tissues and cell types - including intestinal and other epithelial cell lines [125]. *MYO9B* variants could, hypothetically, influence intestinal permeability through its Rho-guanosine triphosphatase activation domain as Rho kinases can effect tight junction assembly [126]. Its association with IBD became controversial as a Norwegian study could not support its role as a susceptibility gene in IBD [127]. However replication has subsequently been reported in Spanish and Italian studies [128, 129]. An explanation for these conflicting results might lie in the heterogeneous nature of IBD.

The ATG16L1 gene on chromosome 2

Autophagy, or autophagocytosis, is involved in the degradation of a cell's own components through the lysosomal machinery. It plays a normal part in cell growth, development, and homeostasis, helping to maintain a balance between the synthesis, degradation, and subsequent recycling of cellular products [130]. It is also involved in clearance of pathogens as is demonstrated by its critical role for inhibition of *Mycobacterium tuberculosis* survival in infected macrophages [131].

German and British studies demonstrated association with a coding variant of ATG16L1 (autophagy-related 16-like 1) gene, on 2q37.1, thereby implicating the autophagy pathway of the innate immune system in IBD susceptibility for the first time [132]. How it is involved in IBD pathogenesis is not clear yet. It is expressed in the colon, small bowel and intestinal epithelial cells. However when comparing expression of ATG16L in tissue from CD patients with controls, no significant differences could be established so far [132].

Conclusion and perspectives

New developments in therapy for IBD will no doubt reflect the complex nature of this disease. It is a disease which seems to consist of several subphenotypes in which each subphenotype is the result of an intricate interplay of several factors that mediate or predispose to the inflam-

matory response in IBD. These factors are of an immunogenic, environmental, microbiological or genetic nature. It is this wide array of one and another mutually influencing factors which is causative for the large variance observed in the responsiveness to different therapies.

A better understanding of the genetic background of IBD will certainly provide us with tools for better clinical management such as prediction of disease course, treatment tailored to disease subtype and anticipating for adverse drug reaction.

Enhanced knowledge of the immunological mechanisms which are involved in the pathogenesis of the disease have already and will further reveal new pathways for intervening in the immunological response.

Finally, the emerging insight on the importance of the intestinal microflora in the pathogenesis of this disease shows new perspectives for treatment. This could be either focused on the side of the host by trying to strengthen the defense mechanisms at the site of interaction, the epithelial barrier, or by modulating the gut flora by stimulating the presence of protective commensal bacteria.

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