Gut mucosal gene expression in inflammatory bowel disease

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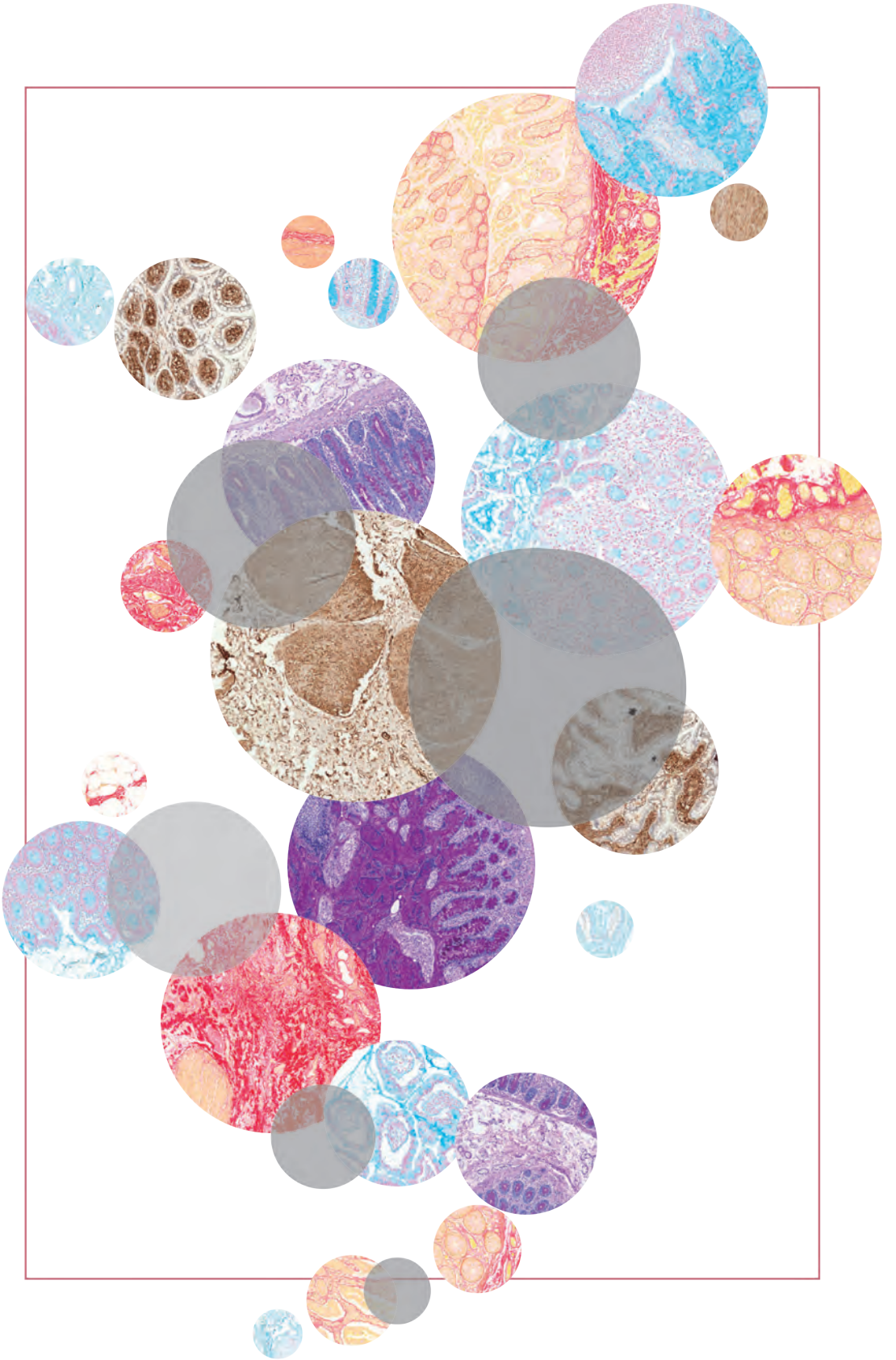

chapter TWO

The genetic background of Inflammatory Bowel Disease: From correlation to causality

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ABSTRACT

Recent studies have greatly improved our insight into the genetic background of Inflammatory Bowel Disease (IBD). New high throughput technologies and large-scale international collaborations have contributed to the identification of 200 independent genetic risk loci for IBD. However, in most of these loci it is unclear which gene conveys the risk for IBD. More importantly, it is unclear which variant within or near the gene is causal to the disease. Using targeted GWAS, imputation, re-sequencing of risk loci and in silico fine-mapping of densely typed loci, several causal variants have been identified in IBD risk genes, and various pathological pathways have been uncovered. Current research in the field of IBD focuses on the effect of these causal variants on gene expression and protein function. However, more elements than only the genome must be considered to disentangle the multifactorial pathology of IBD. The genetic risk loci identified to date only explain a small part of genetic variance in disease risk. Currently, large multi-omics studies are incorporating such factors, ranging from the gut microbiome to the environment. In this review we present the progress that has been made in IBD genetic research and stress the importance of studying causality to increase our understanding of the pathogenesis of IBD. We highlight important causal genetic variants in the candidate genes *NOD2*, *ATG16L1*, *IRGM*, *IL23R*, *CARD9*, *RNF186* and *PRDM1*. We describe their downstream effects on protein function and their direct effects on the gut immune system. Furthermore, we discuss the future role of genetics in unravelling disease mechanisms in IBD.

INTRODUCTION

Inflammatory Bowel Disease (IBD) is a chronic relapsing immune-mediated disease, characterized by inflammation and ulceration of the gut mucosa. IBD roughly consists of two subtypes: ulcerative colitis (UC) and Crohn's disease (CD). In UC, the disease is generally limited to the colon, whereas in CD, inflammation can occur in the entire gastrointestinal tract. UC patients have continuous inflammation limited to the mucosal layer. In CD, the inflammation is discontinuous and involves all layers of the gut.

The incidence of IBD in the western world is around 1 per 1000 individuals and is increasing[1]. In the clinic, IBD patients present with recurrent episodes of abdominal pain, diarrhea, bloody stools and weight loss. Furthermore, patients can suffer from extra-intestinal manifestations of the skin, joints, eyes, and less frequently in abdominal organs such as the biliary tract[2]. Medical treatments are expensive, and often surgical resection of parts of the intestine is necessary. Although the efficacy of medical therapies is improving, up to 20% of UC patients and 50% of CD patients require surgery within 10 years of diagnosis[3].

The aetiology of IBD is complex, and is most likely based on a combination of microbial, environmental and genetic factors. Smoking and appendectomy, for instance, are known to be risk factors for CD, although both seem to have a protective effect in UC[4-7]. Diet is a shared risk factor for both UC and CD: frequent intake of fast-food predisposes to both diseases[8]. Breastfeeding is associated with lower risk for IBD[9]. Twin studies show that there is an hereditary component in IBD risk, which is stronger in CD than in UC[10].

Hypothesis-driven genetic studies in CD revealed the first susceptibility genes[11,12]. Subsequently, more in-depth and large-scale research on the genetic background of IBD has been carried out through Genome-Wide Association Studies (GWAS). To date, these studies have identified 200 genetic risk loci, or genetic regions, associated with IBD[13]. For the majority of these genetic regions, the disease associated genes or the causal genomic variants or remain unknown. In order to search for disease mechanisms and pathways, current research focuses on identifying these causal genes and causal variants **(Figure 1)**. In the search for these, various strategies can be applied: first, sometime causal variants are identified in the GWAS, as happened in the first GWAS for CD (e.g. R381Q in *IL23R* and T300A in *ATG16L1*)[14,15]; second, when not identified by the original GWAS data analysis, causal variants can be found through re-sequencing of candidate genomic regions surrounding an associated common variant (e.g. IVS11+1C>G in *CARD9*, R179X in *RNF186*)[16-18]; third, in case of the availability of dense genotyping of genomic

regions, causal variants can be identified by in silico fine-mapping analyses (e.g. W620R in *PTPN22* and I170V in *SMAD3*)[19]; and fourth, whole-exome sequencing (WES) and whole-genome sequencing (WGS) of large cohorts of IBD cases and population matched healthy controls are currently being performed by US and UK groups. These studies are expected to yield more in-depth knowledge on causal variants in genomic IBD risk loci[20,21].

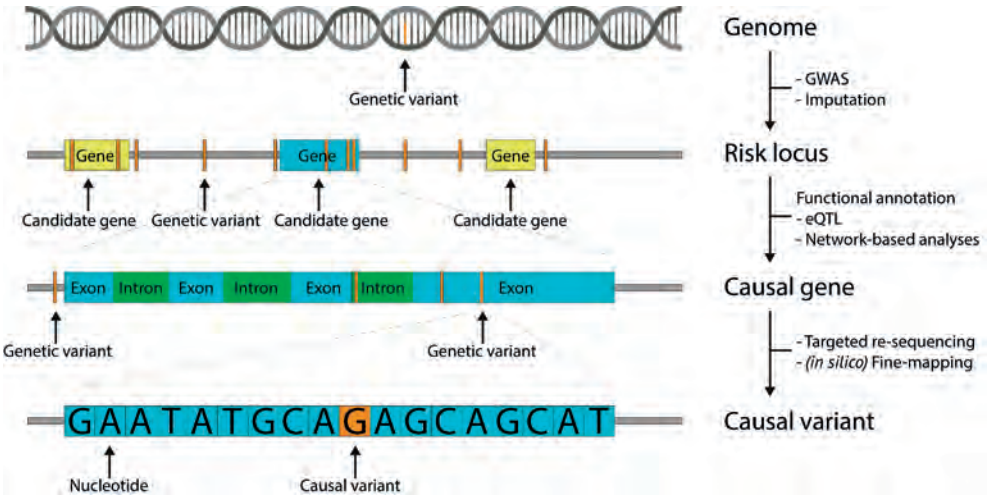
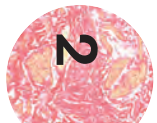


Figure 1. The identification of a causal genetic variant.
From top to bottom. **Genome**: Genome-wide association studies (GWAS) with or without imputation methods identify signals that are highly significantly associated to a certain trait. The identified region is called a risk locus. **Risk locus**: a risk locus on the genome may harbor one or more candidate gene(s) and/or genetic variant(s). Functional annotation may identify the causal gene within the risk locus. **Causal gene**: the gene displayed here contains several introns and exons. Genetic variants can be located in both introns and exons but also up- or downstream of the causal gene. The actual genetic causal variant may still be unknown, and techniques such as re-sequencing or fine-mapping can be used to identify the causal variant. **Causal variant**: an example of nucleotides located in an exon of the candidate gene. The actual causal variant (G: guanine) in orange. This variant can be subjected to functional studies.

This review highlights the recently identified causal genetic variants for IBD, as discovered through the above-mentioned methods (**Table 1**). Furthermore, we describe the disease pathways that have been revealed through these findings. The focus of this review is on adult-onset IBD, as (very) early-onset IBD generally has an oligogenic nature and is reviewed elsewhere[22,23].

Table 1. Overview of causal genetic variants discussed in this review article.
Method refers to the method by which the causal genetic variant was identified. References refer to the first reported discovery. Odds ratio (OR) and minor allele frequencies (MAF) derive from these references.

Method	Genetic variant	Type	Chromosome	Gene	Effect	OR	MAF	Reference
Hypothesis-driven	fs1007insC	Nonsense	16	*NOD2*	Risk for CD	2.1	0.04	Hugot et al., 2001 Ogura et al., 2001
	R702W	Missense	16	*NOD2*	Risk for CD	2.13	0.04	Hugot et al., 2001
	G908R	Missense	16	*NOD2*	Risk for CD	2.32	0.01	Hugot et al., 2001
	rs10870077	Intronic	9	*CARD9*	Risk for IBD	1.21	0.436	Zhernakova et al., 2008
Genome-wide association	R381Q	Missense	1	*IL23R*	Protective for IBD	0.26	0.070	Duerr et al., 2006
	T300A	Missense	2	*ATG16L1*	Risk for CD	1.45	0.533	Hampe et al., 2007
Targeted re-sequencing	G149R	Missense	1	*IL23R*	Protective for IBD	0.60	0.0043	Rivas et al., 2011 Momozawa et al., 2011
	V362I	Missense	1	*IL23R*	Protective for IBD	0.72	0.0152	Rivas et al., 2011 Momozawa et al., 2011
	c.313C>T	Synonymous	5	*IRGM*	Risk for CD	-	0.3037	Brest et al., 2011
	CNVdel	Deletion	5	*IRGM*	Risk for IBD	1.5	0.10	McCarrol et al., 2008
	L105	Synonymous	5	*IRGM*	Risk for CD	-	0.3037	Parkes et al., 2007
	A64T	Missense	1	*RNF186*	Risk for UC	1.49	0.0069	Beaudoin et al., 2013
	R179X	Nonsense	1	*RNF186*	Protective for UC	0.30	0.00-0.0078	Rivas et al., 2015
	c.IVS11+1G>C	Splice-site	9	*CARD9*	Protective for IBD	0.29	0.0071	Rivas et al., 2011
Whole-exome sequencing	S354N	Missense	6	*PRDM1*	Risk for CD	1.23	0.01835	Ellinghaus et al., 2013
	L450F	Missense	6	*PRDM1*	Protective for UC	0.37	0.01135	Ellilnghaus et al., 2013



GENETICS: GWAS STATE OF THE ART

Since the first GWAS was published in 2002[24], many genetic variants, or single nucleotide polymorphisms (SNPs), have been shown to be associated with a specific trait or disease in individuals. Over the entire length of the human genome, SNPs occur with a frequency of approximately one per 300 nucleotides. In GWAS, differences in frequencies of certain SNPs are compared between cases, usually patients with a specific disease, and healthy controls. When the frequency of a SNP is statistically significantly different (usually a $p < 5x10^{-8}$ is considered to be genome-wide significant) between patients and healthy controls, then the region on the genome surrounding this SNP, hereafter called a "risk locus", is associated with the disease. GWAS have become an important tool to study the aetiology of complex inflammatory diseases, including IBD.

Early GWAS and subsequent meta-analyses in CD revealed CD risk loci with candidate genes, encoding proteins involved in immunological pathways such as autophagy (*ATG16L1* and *IRGM*), bacterial handling (*NOD2*) and T cell signaling (*IL23R*)[14,15,25,26]. GWAS and GWAS meta-analyses of UC and CD showed substantial overlap between UC and CD risk loci (*IL23R*, *IL12B*, *NKX2-3* and *MST1*), but also identified some UC specific (*IL10*, *HLA*) and CD specific (*NOD2*, *ATG16L1* and *IRGM*) risk loci[27-31]. Increasing the sample size by combining both genetic and clinical data from databases of the members of the International IBD Genetics Consortium (IIBDGC) substantially increased the number of identified IBD risk loci[25,26,31,32].

Of the above-mentioned IBD risk loci, the majority of risk loci overlap with those of other, mainly immune-mediated diseases[33,34]. This knowledge was applied in the design of the ImmunoChip: a chip composed of all genetic variants correlated to immune-mediated diseases[35,36]. The ImmunoChip project has several limitations, such as lack of dense coverage of all loci, lower sensitivity in non-European ethnic groups, and limited coverage of the genome. Nonetheless it has contributed greatly to our knowledge of IBD risk loci: a meta-analysis of all GWAS and ImmunoChip data from a total of 75,000 individuals increased the number of risk loci to 163[37]. Most of these genetic risk loci contribute to both UC and CD, and the vast majority of loci associated with only one disease showed the same direction of effect in the non-associated disease. This suggests that nearly all biological mechanisms involved in one disease have a similar role in the other. However, some shared loci show a risk effect in opposite directions for each disease. For example, the presence of the risk variant R620W, located in the *PTPN22* gene, increases risk for UC, but is protective for CD[37,38]. The mechanism underlying this inverse risk effect remains yet elusive.

The 163 Immunochip-identified genetic IBD risk loci have all been discovered in cohorts of European descent, presumably introducing a major ethnic bias[37]. Recently, GWAS have been performed in Asian populations in which completely new risk loci for IBD were discovered[39-42]. Subsequent meta-analyses were performed and several risk loci associated with IBD in European populations were found to be associated with IBD also in these non-European cohorts. The largest GWAS and ImmunoChip data meta-analysis in the field of IBD to date combined genotype data from cohorts of different ancestry[13]. This study comprised genetic data from 96,486 individuals including 9,846 individuals of non-European ancestry and allowed for the identification of 38 additional risk loci for IBD, increasing the total number of IBD risk loci to 200. Of these 200 IBD risk loci in cohorts from different ancestry, 138 show overlap with other complex diseases, including other immune-mediated diseases[43]. One of the key findings of this study is that differences in effect size of variants between ethnicities are minimal. It shows that most of the apparent differences in genetic risk between populations can be explained by differences in allele frequencies between populations, or in sample size differences between the cohorts. These findings suggest that trans-ethnicity studies can be used to identify new risk loci in complex diseases.

FROM GWAS "TAG SNPS" TO FUNCTIONAL "CAUSAL" VARIANTS

GWAS has made genetic research much more affordable, as it only covers tag SNPs as markers for genetic loci, representing a proportion of the common genetic variation. However, a large part of the genetic variation is left untagged.

Imputation methods deal with the problem of untagged missing genetic variation, by using the principle of linkage disequilibrium (LD). LD is the linked transmission of multiple SNPs within a genomic region, which can be used to infer the alleles of SNPs that are not directly genotyped. Imputation processes extrapolate SNPs in LD from densely characterized reference panels (such as reference panels from the 1000Genomes project or the Genome of the Netherlands (GoNL) project[44,45]) to a sparsely genotyped study sample. By this means, it is possible to estimate unobserved genotypes with a high accuracy, thus increasing chances of finding true associations[46].

Many risk loci contain more than one gene. To identify the candidate gene in such genomic regions, a number of analyses can be applied **(Figure 1)**. First, network-based analysis tools can calculate the enrichment of connectivity between genes in associated loci and can thereby prioritize specific genes within a risk locus[37,47,48]. Second, expression quantitative trait loci (eQTL) can be used to identify candidate genes[49]. eQTL are genetic

variants whose variation is associated to gene expression levels. If an IBD associated variant or a variant in LD with an IBD associated SNP is a known eQTL, the gene whose expression is regulated by the eQTL is the most likely candidate gene within a genomic region.

When candidate genes are identified using significantly associated SNPs, the actual causal genetic variant for the disease often remains unknown. In order to find this causal variant, genomic regions harbouring these candidate genes can be re-sequenced, revealing the complete genomic sequence of this region. The first causal variants identified using this method were located in genetic regions of *NOD2*, *IL23R* and *CARD9*[16,50].

If genetic associations are identified in densely genotyped genomic regions, high-resolution in silico fine-mapping can be used to identify causal variants. In silico fine-mapping can identify which associated risk variants are statistically convincing causal variants. To date, 18 variants with a >95% probability of causality have been identified using this method[19]. In the last few years, causal genetic variants have been identified through a combination of targeted GWAS, imputation, re-sequencing and fine-mapping. After causal variants have been identified, functional studies are necessary to show how these causal genetic variants lead to disease[17,51].

IBD candidate genes that encode proteins can roughly be categorized in three major disease-associated pathways: regulation of inflammatory responses (e.g. *IL23R*), regulation of autophagy (e.g. *ATG16L1*), and microbial sensing to activate autophagy (e.g. *NOD2*). Genetic variants in genes encoding proteins involved in the above-described pathways have been identified to be associated to IBD, either conferring risk for or protection from the development of IBD[52,53]. It is known that host genetics can alter immunological responses to commensal microbes. Moreover, genetics can influence the gut microbiome[54,55]. Compared to healthy controls, IBD patients show a dysregulated microbiome. This dysregulation may contribute to the disease, as microbes play an important role in regulation of the immune system[56,57].

In the sections below, we will describe current knowledge on important causal genetic variants and illustrate how these variants contribute to the pathogenesis of IBD.

NOD2

From all genetic variants that are assumed to play a role in the pathogenesis in IBD, the most striking are those in the region of the *Nucleotide-Binding Oligomerization Domain Containing 2 (NOD2)* gene, increasing the risk for CD. Three of the most common variants located in *NOD2* (fs1007insC, R702W and G908R) had been identified prior to the GWAS era, by hypothesis-driven methods[11,12]. Re-sequencing and fine-mapping studies confirmed these variants to be causal and identified novel causal variants located in *NOD2*[16,19].

NOD2 plays an important role in the innate immune system by sensing muramyl dipeptide (MDP), a peptidoglycan derived from bacterial lipopoylysaccharides (LPS)[58]. *NOD2* variants conferring the largest genetic risk are located in the leucine-rich repeat (LRR), causing either a frameshift mutation (fs1007insC) leading to a truncated LRR, or amino acid changes (R702W and G908R)[11,12,59]. Cells that express these variants fail to activate NFκB upon stimulation with the NOD2 ligand MDP[60,61]. Although the function of NOD2 and the influence of the genetic variants on NOD2 function have been studied intensively, there is yet no consensus on how this altered NOD2 function eventually leads to disease[62].

Currently, there are two major hypotheses of NOD2-related pathogenesis, based on different aspects of NOD2 function and mechanisms of disease in CD[62]. First, NOD2 has an important role in host defence in epithelial cells (*i.e.* Paneth cells), most likely through its role in the production of α-defensin[63-65]. *NOD2* deficiency is associated with an altered microbiome composition, causing UC-resembling colitis in Dextran Sulfate Sodium (DSS)-treated mice, in which massive entry of luminal bacteria takes place[66-68]. Contrarily, *NOD2* deficiency prevents CD-resembling colitis by supressing inflammation in 2,4,6-Trinitrobenzenesulforic acid (TNBS)- colitis models, having milder entry of luminal bacteria[69]. A second hypothesis is that *NOD2* deficiency leads to colitis because of an inappropriate Toll-like receptor (TLR) stimulation[62,70,71]. In both mouse and human dendritic cells (DC), pre-stimulation with MDP leads to downregulation of TLR ligands and stimulants, which does not happen in *NOD2*-transgenic mice[72,73]. *NOD2*-expressing mice are protected from colitis upon MDP stimulation, whereas *NOD2*-deficient mice are not[73].

It is likely that both alteration of α-defensin expression and TLR downregulation play a role in the pathogenic effects of the *NOD2* risk variants, and that *NOD2* risk variants increase the inflammatory potential of the microbiome through mucosal immunodeficiency, leading to increased inflammation[62].

ATG16L1

Many studies have linked genetic variants in *Autophagy Related 16-Like 1 (ATG16L1)* to CD[14,74,75]. The missense variant T300A is strongly associated with CD in European populations and has remained one of the most significant variants in CD.

ATG16L1 encodes a protein essential for autophagy, a cellular lysosomal degradative process for recycling of proteins in the maintenance of homeostasis[76-78]. Both extra- and intracellular stress, signals such as starvation, growth factor deprivation, endoplasmatic reticulum (ER) stress, and pathogen infection can upregulate autophagy[77].

Numerous studies have investigated the role of ATG16L1 to autophagy and inflammatory signalling[79-86]. ATG16L1 can limit cytokine production, and both mice and patients carrying the *ATG16L1* risk variant have defective microbial clearance and altered cytokine response toward both viruses and bacteria, suggesting the involvement of host-microbe interactions[55,79,81,82,88,97]. In fact, biopsies from inflamed parts of the terminal ileum from CD patients carrying the T300A risk variant contain more *Bacteroides fragilis*, *Fusobacteria* and *Escherichia coli* in comparison to those from patients carrying the protective T300 variant[88]. *B. fragilis* has beneficial immunomodulatory properties and protects against experimental colitis[55,89-94]. The bacterium uses an ATG16L1-dependent pathway which induces regulatory T cells to generate this mucosal tolerance. Human immune cells from CD patients carrying the T300A variant are unable to induce regulatory T cells in response to *B. fragilis*, and show impaired killing of pathogens[55,88].

Only recently the mechanisms underlying the *ATG16L1* T300A variant have been elucidated. Turnover of ATG16L1 is dependent of caspase-3 and -7[95,96]. T300A is located in the cleavage site for these caspases. Cellular stress enhances cleavage, and the presence of T300A leads to increased cleavage resulting in protein instability. This consequently leads to diminished autophagy and increased inflammatory cytokine responses. Smoking is a risk factor for developing CD. Interestingly, it has been shown that cigarette smoke has a bacterium- and *ATG16L1* allele-specific effect on the xenophagic activity of human monocytes. This promotes survival of pathogenic adherent invasive *E. coli* (AIEC), thereby linking a common occurring risk variant of a gene to an environmental risk factor[97].

In summary, *ATG16L1* T300A may contribute to CD by both impaired killing of pathogens and an inability to respond to beneficial microbes.

IRGM

Genetic risk variants in the region harbouring the *Immunity-Related GTPase family M (IRGM)* gene have been strongly associated with CD and, to a lesser extent, to UC[31,37,98-101]. The IRGM gene encodes an autophagy protein that plays an important role in innate immunity against intracellular pathogens like *Mycobacterium tuberculosis*, *Salmonella typhimurium* and AIEC bacteria (associated with CD)[102-104]. The fact that *IRGM* is a distinctly human gene makes it more difficult to study its function[105]. IRGM indirectly activates AMP-activated protein kinase (AMPK), a key regulator of cellular energy homeostasis, stabilizing autophagy regulators (such as ULK1 and ATG14)[106,107]. Furthermore, IRGM directs co-assembly of autophagy machinery, and connects this machinery to bacterial pattern recognition receptors such as NOD2, leading to enhancement of autophagy. This IRGM-NOD2 interaction is thought to prevent excessive inflammation.

The genetic variant L105 that lies immediately upstream of *IRGM*, is strongly associated with CD[98,108]. After re-sequencing exons of *IRGM*, coding sequence-variation within *IRGM* was excluded as a source of this association[98]. A deletion allele of a copy number variant (CNVdel) upstream of *IRGM*, in perfect LD with L105, is associated with altered *IRGM* expression and CD[101,102]. In turn, reduced *IRGM* expression reduces the efficacy of anti-bacterial autophagy.

However, a different study showed that the exonic risk variant *IRGM* rs10065172 (c.313C>T), also in perfect LD with L105 and the CNVdel, could be a causal variant in *IRGM*[109]. c.313C>T alters the microRNA (miRNA) binding site of miRNA-196, a miRNA overexpressed in the inflamed intestinal epithelium in CD patients. Normally miRNA-196 downregulates *IRGM*, but this downregulation is lost in the presence of c.313C>T. Loss of this downregulation results in compromised autophagy of AIEC bacteria in patients carrying the variant. IRGM induces autophagy to eliminate intracellular mycobacteria and this genetic variant is of particular interest since it has been associated with susceptibility to tuberculosis[110-112]. This fact supports the theory that mycobacteria may be important in the pathogenesis of CD[103]. Taken together, these results suggest that the association of IRGM with CD arises from an alteration in *IRGM* regulation affecting the efficacy of autophagy.

INTERPLAY BETWEEN NOD2, ATG16L1 AND IRGM

NOD-receptors detect bacteria and induce an autophagy-mediated immune response. MDP stimulation in primary human DCs activates autophagy, resulting in increased bacterial killing and the generation of $CD4^+$ antigen-specific T cell responses[82]. NOD2 (and NOD1) can recruit ATG16L1 to the plasma membrane at the bacterial entry site[113]. Cells with *NOD2* genetic variants (R702W, G908R and fs1007insC) fail to recruit ATG16L1 to the plasma membrane and show impaired pathogen-induced autophagy and impaired $CD4^+$ antigen-specific responses[82,113,114]. In contrast, there is no consensus regarding NOD2 signalling in the presence of *ATG16L1* T300A. Two independent studies show that MDP-stimulated autophagy is impaired in DC and lymphoblastoid cells from CD patients carrying *ATG16L1* T300A[82,113]. However, in another study using cells from healthy donors, the *ATG16L1* T300A variant impaired NOD2-dependent anti-bacterial function in colonic epithelial cells, but not in monocytic cells[114]. These seemingly conflicting results from functional studies indicate that the manner in which *ATG16L1* and *NOD2* contribute to disease may be tissue- and situation specific. Thus, the interaction between *ATG16L1* and *NOD2* and its contribution to the pathogenesis of IBD may very well be related to the process of autophagy[115], but could also act in an autophagy-independent manner[85].

A third well known IBD risk gene, *IRGM*, may play an additional important role in the interaction between NOD2 and ATG16L1: in human cells IRGM is in complex with NOD2 and ATG16L1, enhancing the assembly of this complex[106]. IRGM stimulates phosphorylation and activation of important autophagy regulators (such as ULK1 and Beclin 1) and connects them to ATG16L1[106,116-118]. Polyubiquitination of IRGM is under control of NOD2 and stabilizes autophagy initiation complexes. NOD2 enhances association of ubiquitination-competent IRGM with ULK1 and Beclin 1, but not in mutant IRGM[106]. In contrast, mutant IRGM remains its ability to bind ATG16L1 equally well as the ubiquitination-competent IRGM. Our increased knowledge on NOD2, ATG16L and IRGM dysfunction in IBD this has up to now not resulted in therapeutic options. Manipulation of the microbiome or small molecules can directly or indirectly modulate autophagy and innate immunity, an effect that could be explored in patients carrying these risk alleles[119].

IL23R

Multiple variants within the locus harbouring the *Interleukin 23 Receptor (IL23R)* gene are strongly associated to IBD[15,25,26]. The *IL23R* variant R381Q, conferring protection against IBD, has been identified through GWAS[15], while the protective G149R and V362I *IL23R* variants have been identified by targeted re-sequencing[16,18]. The *IL23R* risk locus plays a role in an important pathway for IBD: T-helper 17 (Th17) signalling[120]. The *IL23R* gene encodes the IL23 receptor complex (IL23R), which is a receptor for the heterodimeric pro-inflammatory cytokine IL23, composed of p19 and p40 subunits. p40 is also part of the IL12 cytokine[121]. Binding of IL23 by IL23R results in activation of Janus Kinase 2 (JAK2), subsequent phosphorylation of signal transducer and activator of transcription 3 (STAT3) and STAT4, leading to the transcription of other pro-inflammatory cytokines[122]. This increased transcription of pro-inflammatory cytokines causes differentiation of $CD4^+$ T cells into pro-inflammatory Th17 cells, that are critical in antimicrobial defences. Genetic variants in or near the *IL23R* gene are thought to generate an inappropriate immune response to commensal bacteria in the gut, and dysregulation of the IL23-Th17 pathway[120,123]. Moreover, loci within genes encoding proteins downstream in the IL23-Th17 pathway have also been associated with IBD (*JAK2* and *STAT3*)[25].

Several studies have investigated functional consequences of genetic variants in *IL23R*. The R381Q variant, conferring protection against IBD, results in loss of function: cells expressing this variant have decreased levels of phosphorylated STAT3 and STAT4. This consequently leads to the reduced production of pro-inflammatory cytokines with reduced Th17 effector functions[124-127].

As outlined above, the IL23-Th17 pathway plays an important role in mediating intestinal inflammation and is therefore a promising target for IBD medication. Ustekinumab, an anti-IL12/23p40 monoclonal antibody, prevents human IL12 and IL23 from binding to the IL12Rβ1 receptor chain of IL12 (IL12Rβ1/β2) and IL23 (IL12Rβ1/23Rα) receptor complexes on the surface of natural killer (NK) and T cells. A recent phase 3 study in patients with moderate-to-severe CD refractory to anti-Tumour Necrosis Factor alpha (TNFα) showed favourable response and remission to Ustekinumab[128]. Specific blockade of the IL23 pathway through use of a monoclonal antibody against p19 was effective in both the prevention and treatment of a murine model of $CD4^+$ T cell–mediated colitis[129]. Similarly, in a phase 2a trial, CD patients refractory to anti-TNF showed favourable response to anti-IL23p19[130].

CARD9

In 2008, the first *caspase recruitment family member 9 (CARD9)* disease association has been discovered through a custom SNP array for innate immunity. The identified variant rs10870077 in the *CARD9* genetic locus on chromosome 9, confers risk for IBD. Another variant, S12N, in perfect LD with rs10870077, increases expression of *CARD9*[26,131,132]. Moreover, this variant is associated with other immune-mediated diseases such as ankylosing spondylitis, immunoglobulin A nephropathy and primary sclerosing cholangitis[145-147].

The *CARD9* locus encodes an innate immune system pattern recognition receptor, which is necessary for the immune response against bacterial and fungal infections. Currently, all clinical reports concerning humans that have loss of function of CARD9, or deficient *CARD9* expression, show a higher susceptibility to fungal infections than subjects with normal CARD9 function and expression. Also, reduced numbers of inflammatory cells are seen[133-135]. After its genetic association with innate immunity, the mechanisms by which *CARD9* is involved in protection from infections have also been studied intensively. *CARD9*-deficient mice show lower pro-inflammatory cytokine production than *CARD9*-competent mice and are more susceptible to DSS-induced colitis[136-138]. The functional pathway involved is the recognition of microbes through a surface receptor, with subsequent binding of CARD9 and activation of NFκB in macrophages and neutrophils[139]. In addition, CARD9 interacts with NOD2 in response to bacteria[138-140].

Recently, CARD9 was found to be a mediator in the IL22 pathway activation, which initiates immune responses to both bacteria and fungi through antimicrobial peptides[141,142]. CARD9 augments IL22 activity, through microbial stimulation of the aryl hydrocarbon receptor (AHR)[143]. In IBD patients, reduced AHR-ligand production is seen. An AHR-agonist, such as the strain lactobacillus, could counter this reduced ligand production. This creates therapeutic possibilities for personalized medicine[144].

In contrast, more recently a rare splice-site variant, c.IVS11+1G>C, conferring protection against IBD has been identified[16,18]. This variant leads to a truncated protein which causes an ineffective pro-inflammatory response with diminished cytokine production through defective microbial sensing[134].

Altogether, the effects of *CARD9* variants may be bidirectional: c.IVS11+1G>C can cause loss of function of CARD9 resulting in diminished immune responses, whereas the risk variant S12N may lead to overexpression of *CARD9*. This overexpression can result in a hyper reactive immune state and increasing susceptibility to IBD[148].

RNF186

Several GWAS have identified risk loci for UC in the region of the *Ring Finger Protein 186 (RNF186)* gene[29,42,131]. However, the function of this gene, the causal variant(s) within these loci and eventually its consequences for protein function have long remained elusive. The *RNF186* gene encodes the ring finger E3 ligase which localizes at the endoplasmatic reticulum (ER) and regulates ER stress-mediated apoptosis[149]. Deep re-sequencing of GWAS loci recently allowed for the identification of a likely causal variant in *RNF186*: A64T[18]. This variant confers risk for the development of UC. The A64T variant is located in the E3 ubiquitin-protein ligase domain of RNF186[18]. The ligases encoded regulate key adaptors of pro-inflammatory pathways[150-152]. *RNF186* expression is higher in human intestinal tissue as compared to other human tissue, with the highest levels of expression located in the transverse colon[131,153]. *Shigella* infection in mice induces upregulation of RNF186 in the intestinal epithelium[154,155]. A recent study conducting a search for protein truncating variants (*i.e.* loss of function variants), identified a novel genetic variant in *RNF186*, R179X, which confers protection against UC[17]. Interestingly, R179X truncation does not inhibit protein expression, and the mechanism of action of this variant is as yet unclear.

PRDM1

The *Positive Regulatory Domain 1 (PRDM1)* gene on chromosome 6 encodes a zinc finger transcriptional repressor, B Lymphocyte-Induced Maturation Protein 1 (BLIMP1), which is involved in plasma cell differentiation, immunoglobulin secretion, and is strongly upregulated in apoptosis[156-161]. *PRDM1* is mainly expressed in mucosal T cells and plasma cells[51]. However, mutations in *PRDM1* also affect various other immunological cell types such as NK-cells, DC and macrophages. Through WES studies, two missense mutations in *PRDM1* (S354N and L450F) associated with IBD have been identified[51]. The variant S354N confers risk for CD, and L450F is protective for UC. In both healthy controls and CD patients carrying S354N but not L450F, T cells exhibit increased proliferation, cytokine secretion and activation markers upon stimulation. T cells from CD patients also exhibit increased expression of L selectin. L selectin is necessary for peripheral blood lymphocyte migration into the intestinal tissue, which is thought to contribute to the pathogenesis of IBD[162-164]. In addition, the common risk allele of rs7746082, representing the strongest association to CD in this locus, lowers expression of *PRDM1* in ileal biopsies of CD patients[25-51].

Currently, mice with a T cell specific BLIMP1 deficiency are used as a model for CD. These mice develop severe colitis and have more peripheral effector T cells upon colitis induction, as compared to WT mice[165-166].

Moreover, in DC from subjects carrying the common risk allele of rs6911490, representing the strongest association to UC in this locus, *PRDM1* expression is significantly lower as compared with DC from subjects not carrying the risk allele[31,167]. Moreover, these cells show exaggerated immune responses upon bacterial stimuli[167].

Various immune cell subtypes are influenced by *PRDM1*. BLIMP1 has been shown to be an essential transcription factor to activate Th17 cells, the dysregulation of which contributes to the pathogenesis of IBD[168]. Moreover, mouse *PRDM1*-knockout colonic DC show higher pro-inflammatory cytokine production and lower levels of the anti-inflammatory cytokines. Blocking of the pro-inflammatory cytokines with anti-IL1β and anti-IL6 diminishes the exaggerated immune response in the *PRDM1*-knockout cells. This provides future possibilities for targeted treatments[167]. In addition to this, the BLIMP1-dependent production of IL10 protects against inflammation and may be a target for future therapies as well[169].

CONCLUSION AND FUTURE PERSPECTIVES

In this review we have outlined the progress that has been made in the understanding of IBD through the use of genetic studies. We have discussed the role of GWAS in studying the genetics of IBD and also the importance of moving from genetic correlation to causality and from genetic correlation to functional consequences. Further, we have highlighted recently identified causal genetic variants in candidate genes and their downstream effects on protein function. These recent advances greatly contribute to our understanding of how genetic variants can lead to disease. In addition, these recent advances show potential opportunities for future therapies.

The genetic risk loci for IBD identified thus far only explain a small part of the genetic variance in disease risk: 13-13.5% in CD and 7.5-9% in UC[37,170]. Most likely, the number of identified genetic risk loci will grow further over the coming years and within the identified loci a growing number of causal genetic variants will be discovered. The identification of causal variants is extremely important for making a more adequate estimation of the genetic variance explained. Identifying causal variants is also instrumental for drug development and repositioning[171,172]. In order to better understand disease mechanisms and to develop more specific therapies to target inflammatory pathways, the effects of causal variants on protein function will be a principal subject of research in the field of IBD. Identifying causal variants through techniques such as re-sequencing and fine-mapping alone will be insufficient to completely elucidate mechanisms leading to disease.

A promising approach to study the effect of specific candidate genes and causal variants are disease models, such as the TNBS/DSS-treated mouse models and human-derived cells. Studies on the function of genetic variants were initially performed in complete knockout models of the candidate gene, but have more recently been studied in specific causal variant models, which provide a more realistic representation of the clinical effect of these causal variants.

In order to increase the chances of identifying causal variants in IBD risk loci, there are efforts to enlarge numbers of cases in studies. Currently, large numbers of samples of IBD patients in both the US and the UK are sequenced using whole-exome and whole-genome technologies, respectively[20,21]. These efforts will likely result in the identification of additional causal variants.

In some IBD risk loci the causal variant will be extremely difficult to detect. This can be due to variable effect size or effect direction of a variant amongst patients; variants in currently known risk loci can be both protective and risk-promoting (e.g. *CARD9*). Within other risk loci the causal variants are located in non-coding areas of the genome, which makes them difficult to detect. Functional studies on IBD risk loci in which no causal variants have been identified, can be performed by obtaining an approximation of the functional effects within this risk locus. This can be done with eQTL studies, which show the effect of the associated risk variant on gene expression.

As the possibilities to analyse and integrate large data sets are evolving, future research opportunities emerge. Considering the multifactorial origin of IBD, consisting of, amongst others, the microbiome, the environment and the proteome, the study of IBD should be approached more widely. Although on the one hand these factors have a clear effect on disease, they can also be affected by and have an interaction with genetic variants. Using a multi-omics approach, these factors must be incorporated in the study of IBD. Multi-omics studies integrate different levels of data that contribute to disease. Multi-omics studies have so far revealed important interplays between the host's genetics and other factors, such as genetic variation and clinical disease behaviour and the composition of the microbiome[173] (Imhann *et al*, *unpublished data*). GWAS studies have shown genetic susceptibility to adverse drug responses to IBD medication[174,175]. From oncologic immunotherapy studies we have learned that the gut microbiome can affect efficacy of these immunotherapies[176,177]. These findings stress the importance of integrating multi-omics data from large cohorts in order to find therapies targeting patients with specific risk profiles. Multi-omics studies provide a basis for the next step in IBD research: translating findings into clinical practice and more specifically into personalized treatment strategies

The current progress that has been made in IBD research has paved the way for translation of basic scientific findings to clinical practice. With the ongoing identification of causal genetic variants, both functional and multi-omics studies will greatly improve our understanding of IBD and subsequently the treatment of IBD patients over the coming years.

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SUPPLEMENTARIES

Sequence variant nomenclature used in this review. Please note that we used nomenclature widely used in IBD literature though not always HGVS approved.

Substitution: a sequence change where, compared to a reference sequence, one nucleotide is replaced by one other nucleotide.
Example at DNA level: c.313C>T, substitution of base C (cytosine) for T (thymine) at position 313. (c. refers to a substitution in coding DNA).
Example at protein level: R381Q, substitution of amino acid R (arginine) for Q (glutamine) at position 381.

Splice-site mutation: mutation at specific site at which splicing takes place during the processing of precursor messenger RNA into mature messenger RNA.
Example at DNA level: c.IVS11+1G>C, IVS11 refers to intervening sequence (i.e. intron) 11, this mutation indicates a substitution of G (guanine) for C (cytosine) at position 1 of the given intron.

Insertion: a sequence change where, compared to the reference sequence, one or more nucleotides are inserted
Example: c.3020insC: a C (cytosine) nucleotide insertion at position 3020 in coding DNA. This may or may not lead to a frame-shift mutation.

Frame-shift mutation: a sequence change between the translation initiation (start) and termination (stop) codon where, compared to a reference sequence, translation shifts to another reading frame.
Example: fs1007insC, the mutation c.3020insC, in exon 11 of the *NOD2* gene, results in a frameshift at the second nucleotide of codon 1007 and a leucine-1007-to-proline substitution in the tenth leucine-rich repeat, followed by a premature stop codon. fs1007insC is widely used to denote this mutation in IBD literature.

Copy-number variations (CNV): deletions and duplicates of chromosomal segments.
Example: CNVdel, denotes in this review a deleted allele of a 20kb CNV upstream of *IRGM*.

rs number: reference SNP cluster identification tag assigned by the NCBI.
*Example: r*s1120926, this rs number refers to a G (guanine) to A (adenine) corresponding to c.1142G>A at DNA level and R381Q at protein level.