TOPIC HIGHLIGHT

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Role of genetics in the diagnosis and prognosis of Crohn's disease

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Abstract

Considering the epidemiological, genetic and immunological data, we can conclude that the inflammatory bowel diseases are heterogeneous disorders of multifactorial etiology in which hereditability and environment interact to produce the disease. It is probable that patients have a genetic predisposition for the development of the disease coupled with disturbances in immunoregulation. Several genes have so far been related to the diagnosis of Crohn's disease. These genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and most replicated associations with Crohn's disease have been demonstrated with *NOD2*, *IL23R* and *ATG16L1* genes. Many genes have so far been implicated in the prognosis of Crohn's disease and many attempts have been made for classification of genetic profiles in Crohn's disease. *CARD15* seems to be not only a susceptibility gene, but also a disease-modifier gene for Crohn's disease. Enriching our understanding of Crohn's disease genetics is of value, but when combining genetic data with functional data the outcome could be of major importance to clinicians.

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EVOLVING ROLE OF GENETICS IN CROHN'S DISEASE

Despite decades of research the etiology of inflammatory bowel diseases (IBD) remains largely unexplained, but considering together the epidemiological, genetic and immunological data, we can conclude that IBD are heterogeneous disorders of multifactorial etiology in which hereditability (genetic) and environment (microbial, behavior) interact to produce the immunological background of the disease. It is probable that patients have a genetic predisposition for the development of the disease

coupled with disturbances in immunoregulation. The disease can then be triggered by any of a number of different unknown environmental factors and sustained by an abnormal immune response to these factors. Accordingly, the intensive interaction between intestinal epithelial cells and immune competent cells is critical to maintain and perpetuate the chronic inflammatory process characteristic for IBD[1].

Early epidemiologic evidence for the role of genetic factors in the pathogenesis of Crohn's disease (CD) came from studies demonstrating higher rates of CD among individuals of Caucasian and Jewish ethnicity, familial aggregation of CD and higher concordance rates for both twins developing CD in monozygotic compared with dizygotic twins. The search for specific CD susceptibility genes, however, has been difficult due to complex genetics, including factors such as the lack of simple Mendelian inheritance patterns, involvement of several genes, and the influence of environmental factors and intestinal microflora on disease development. More than 30 distinct genomic loci encode genes involved in a number of homeostatic mechanisms and have been suggested to be involved in CD etiopathogenesis and prognosis[2].

Until very recently, two main approaches could be undertaken to identify genes in complex diseases: the positional cloning approach, based on linkage analysis, and the candidate gene approach, based on association studies. Linkage analysis studies the co-segregation of the disease with a marker within families. The candidate gene approach uses case-control cohorts or trios of affected offspring with both parents. Here, a specific gene with known or potential interest for the disease is studied. The allelic frequencies (in the case of case-control study) or the transmission of a single nucleotide polymorphism (SNP) towards affected offspring (in the case of trios) are analyzed, and differences between patients and controls, or distortion of transmission towards affected children, will point towards implication of the gene in the pathogenesis of the disease under investigation.

Despite the large numbers of genome-wide association studies (GWAS) established to date, most diseases have only managed to explain some additional percentage of the hereditability estimates. In an attempt to explain some of this missing hereditability, researchers have adopted several complementary strategies. Larger cohorts of cases are being collected, through either further patient recruitment or collaborations. The meta-analysis data generated to date have demonstrated how increasing the cohort sample size generates additional statistical power to detect smaller and smaller odds ratios[3]. Advances in technology, and particularly bioinformatics, have now made it possible to perform GWAS using common copy number variation probes. Many groups are looking to high-throughput sequencing technology, with the aim of sequencing candidate gene regions identified by GWAS, to hopefully identify either the causal or rare variants[4,5]. Several GWAS have been published in the last decade and have identified many genes associated with Crohn's disease (Table 1). Among these, there are recognition-related genes such as *NOD1* and *TLRs*, other susceptibility genes including *DLG5, OCTN* and *HLA,* and the newest susceptibility genes in CD resulting from GWAS: *IL23R* gene, *ATG16L1* gene and *IRGM* gene[6].

THE ROLE OF GENES IN THE DIAGNOSIS OF CROHN'S DISEASE

Several genes have so far been related to the diagnosis of Crohn's disease. These genes are related to innate pattern recognition receptors, to epithelial barrier homeostasis and maintenance of epithelial barrier integrity, to autophagy and to lymphocyte differentiation. So far, the strongest and most replicated associations with CD have been demonstrated with *NOD2, IL23R* and *ATG16L1* genes.

Genes related to innate pattern recognition receptors
NOD2/CARD15 gene: *NOD2/CARD15* (Caspase Recruitment Domain Family member 15) acts as a pattern recognition receptor (PRR); this locus has been characterized as the *IBD1* locus on 16q12-13[7].

Fine mapping of the *IBD1* locus identified the underlying gene on chromosome 16 as the *CARD15* (previous *NOD2*) gene. *CARD15* represents homology with the *R* genes in plants; genes that confer resistance to infection[8]. Thirty nonconservative polymorphisms have been identified within the gene, which are associated with CD, but only three are common (Arg702Trp, Gly908Arg and Leuc1007insC). These three common variants account for approximately 82% of the mutated alleles. *CARD15* is associated with CD only and not with ulcerative colitis. *CARD15* codes for a protein expressed in monocytes, macrophages, dendritic cells, epithelial cells and Paneth cells. *CARD15* is involved in the recognition of bacterial peptidoglycan-derived muramyl dipeptide through the leucine-rich repeat region. Of importance, the frameshift mutation 1007fsinsC that leads to a truncated protein lacking the 33 distal amino acids was associated with impaired activation of the transcription factor NF-kappa B after stimulation.

It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. Although nucleotide-binding oligomerization domains (NODs) are expressed at low levels in absorptive and secretory intestinal epithelial cells, Paneth cells in the small intestine have been recognized as the predominant site of expression of *NOD2* in the epithelium. Furthermore, *NOD2* mutations have been associated with decreased expression of antimicrobial peptides, the α-defensins, by Paneth cells. In addition, a distinct gene polymorphism resulting in low β-defensin 2 gene copy number has been associated with a predisposition to colonic Crohn's disease. In addition, *NOD2* plays important roles in the promotion of antibacterial T-helper-17 (Th-17) cells in the IL-23-IL-1-IL-17 axis.

CARD15 variants are found in 35% to 45% of white

Table 1 Genetic polymorphisms related to Crohn's disease	
Diagnosis of CD	
Innate pattern recognition receptors	*NOD2/CARD15, OCTN, TLR*
Epithelial barrier homeostasis	*IBD5, DLG5*
Molecular mimicry and autophagy	*ATG16L1, IRGM, LRRK2*
Lymphocyte differentiation	*IL23R, STAT3*
Secondary immune response and apoptosis	*MHC, HLA*
Prognosis of CD	
Age of CD onset	*TNFRSF6B, CXCL9, IL23R, NOD2, ATG16L1, CNR1, IL-10, MDR1, DLG5, IRGM*
CD behavior	
Stenotic/stricturing behavior	*NOD2, TLR4, IL-12B, CX3CR1, IL-10, IL-6*
Penetrating/fistulizing behavior	*NOD2, IRGM, TNF, HLADRB1, CDKAL1*
Inflammatory behavior	*HLA*
Granulomatous disease	*TLR4/CARD15*
CD location	
Upper gastrointestinal	*NOD2, MIF*
Ileal	*IL-10, CRP, NOD2, ZNF365, STAT3*
Ileocolonic	*ATG16L1, TCF-4 (TCF7L2)*
Colonic	*HLA, TLR4, TLR1, -2, -6*
CD activity	*HSP70-2, NOD2, PAI-1, CNR1*
Surgery	*NOD2, HLA-G*
Dysplasia and cancer	*FHIT*
Extraintestinal manifestations	*CARD15, FcRL3, HLADRB*103, HLAB*27 HLA-B*44, HLA-B*35, TNFα-308A, TNF-1031C, STAT3*
Pharmacogenetics in CD	*CARD15, NAT, TPMT, MDR1, MIF, DLG5, TNF, LTA*

NOD: Nucleotide binding and oligomerization domain; CD: Crohn's disease; LRR: Leucine-rich repeat; OCTN: Organic cation transporter; TLR: Toll-like receptor; DLG5: Discs large homolog 5; LTA: Lymphotoxin α; CARD: Caspase recruitment domain; STAT3: Signal transducer and activator of transcription 3; MHC: Major histocompatability complex; PAI-1: Type 1 plasminogen activator inhibitor; FHIT: Fragile histidine triad; NAT: N-acetyltransferase; MIF: Macrophage migration inhibition; HLA: Human leucocyte antigen; TPMT: Thiopurine methyltransferase; MDR1: Multidrug resistance gene 1; TNF-α: Tumour necrosis factor-α; Tcf: T-cell factor; Atg: Angiotensinogen; FCRL: Fc receptor-like; ZNF: Zinc finger; TNFRSF: Tumor necrosis factor receptor superfamily; IRGM: Immunity-related GTPase family, M; HLA: Human leukocyte antigen; LRRK2: Leucine-rich repeat kinase 2; CNR: Cadherin-related neuronal receptor.

CD patients, with the exception of Scandinavian, Irish and Scottish patients[9,10], in whom the prevalence is much lower. Genotype relative risks of 3 (simple mutation) and 10-44 (double mutations) have been reported in European Caucasians[9,10]. However, *CARD15* mutation is not frequent or even absent in African-American populations, and in Indian, Chinese and Japanese populations[11-13]. Other *CARD* related genetic loci that have been associated with CD diagnosis are the *CARD4* (*NOD1*), *CARD8* and *CARD9* loci[14,15].

Organic cation transporter genes: Organic cation transporters (OCTNs, 5q31-33) are membrane transporters for drugs and positively charged endogenous metabolites. The novel OCTN subfamily may also transport carnitine, which is essential for metabolism of lipids and is involved in transport of light chain fatty acids into mitochondria for beta-oxidation. Early studies in this field reported on two functional mutations in the carnitine/*OCTN* cluster on *5q31* (the *IBD5* locus) that were associated with Crohn's disease. As membrane transporters of organic cations, OCTNs are therefore important in the maintenance of intracellular homeostasis. In humans *OCTN1* and *OCTN2* map to *IBD5* on *5q31*. An *OCTN3* has recently been described in humans[16].

Toll-like receptor genes: Host response to microbial pathogens includes self-defense mechanisms such as defensins, PRRs, pathogen-associated molecular patterns and toll-like receptors (TLRs). TLRs recognize conserved motifs on pathogens that are not found in higher eukaryotes and initiate "innate" (rapid and non-specific) immune responses[17]. Subsequently, specific receptors recognizing chemo-attractant molecules mobilize phagocytic leukocytes and induce their migration to inflammatory sites. There, leukocytes encounter the invading microorganisms and ingest them through the activation of phagocytic receptors that mediate the uptake process. Innate immune responses are linked to the generation of corresponding adaptive immune responses and studies of genetically engineered or cellularly manipulated animal models have generated a great deal of new information[18].

Leucocyte-epithelial interactions are of special interest, as exposure of epithelial TLRs to microbial ligands has been shown to result in transcriptional upregulation of inflammatory mediators, whereas ligation of leucocyte TLRs modulates specific antimicrobial responses[19]. It has been shown that Paneth cells play an important role in innate host defense *via* their ability to secrete antimicrobial peptides and proteins. In addition, it has been shown that *NOD2* mutations lead to loss of negative regulatory effects on TLR signaling while activation of the *CARD* domain results in activation of NF-κB[20].

TLRs are the most important receptors of the innate immune system. They are expressed by immune cells and by intestinal epithelial cells in IBD patients. In humans, at least 10 different TLRs are described and each recognizes a specific pathogen-associated molecular pattern. A trans-

mission disequilibrium test on Belgian IBD trios with CD demonstrated preferential transmission of the *TLR4* Asp299Gly polymorphism from heterozygous parents to affected children[21]. *TLR9* modulates CD susceptibility and there is interaction between other polymorphisms such as *NOD2*, *IL23R* and *DLG5*[22,23].

Genes related to epithelial barrier homeostasis

The gastrointestinal tract uses a system of tolerance and controlled inflammation to limit the response to dietary or bacteria-derived antigens in the gut[24]. When this complex system breaks down, by means of either a chemical or pathogenic insult in a genetically predisposed individual, the resulting immune response may lead to IBD[25]. Genes or loci involved in the maintenance of epithelial barrier integrity and associated with Crohn's disease are the *IBD5* and the Discs large homolog 5 (*DLG5*)[26].

The *DLG5* gene is a 180-kb protein containing 1900 amino acids. DLG5 protein harbors a caspase activation and recruitment domains (*CARD*), is a further CD susceptibility gene of the *CARD* family and contributes to *CARD*-mediated mechanisms of host defense. In fact, the *DLG5* gene associated protein is a member of the MAGUK (Membrane Associated Guanylate Kinase) family of scaffolding proteins. Scaffolding proteins organize protein complexes at cellular junctions to integrate the tethering of adhesion molecules, receptors and intracellular signaling enzymes. Of interest is a population variation regarding *DLG5* variants. For example, the *DLG5* R30Q variant has not been confirmed in other European studies[27,28]. Other genes of potential importance in the same panel are the *PTGER4*, *ITLN1*, *DMBT1*, *BPI* and *XBP1* genes[29].

Genes related to molecular mimicry and autophagy

The innate immune system is the first line of defense against infection. Of interest, virulence factors from bacteria and viruses have been identified that manipulate host innate immune signaling pathways through molecular mimicry. These microbial proteins contain signaling domains that bear sequence and structural similarity to their host targets, thereby potentially sabotaging host immunity by hijacking crucial signaling pathways and uncoupling receptor activation from effector induction. Several protein families have evolved to function as receptors or sensors of pathogen invasion. There are two types of signaling domains for the above receptors: the TIR domain for the TLRs, and the Pyrin domain or *CARD* for the *NOD*-like receptors (*NLRs*) and retinoic acid-inducible gene 1-like receptors or helicases.

Molecular mimicry has been invoked as one of the mechanisms responsible for the activation of autoreactive cells by microbial peptides that have structural similarities to self peptides, but there is also evidence that antigenically unrelated infections or specific inflammatory signals can result in autoaggressiveness and induction of organ-specific autoimmunity, including the gut. The extent and severity of this loss of tolerance is still being defined,

as it has been demonstrated that loss of tolerance in IBD patients is not exclusive for bacterial antigens and occurs also for orally administered soluble proteins[30]. This subversion of innate immune signaling through molecular mimicry is closely related to the phenomenon of autophagy. Autophagy is the tightly orchestrated cellular 'housekeeping' process responsible for the degradation of damaged and dysfunctional cellular organelles and protein aggregates, and is well recognized to play an important role in maintaining cellular homeostasis under physiological and pathophysiological conditions. Regulated degradation and turnover of subcellular components is essential for normal cellular function, growth and development. The major catabolic pathway responsible for the disposal of obsolete or damaged organelles and protein aggregates is autophagy (i.e., "self-digestion"). During this process organelles and proteins are encircled in a double-membrane vesicle (the autophagosome), delivered to lysosomes and the substrates for adenosine-triphosphate generation, and products can be recycled to synthesize new proteins, high-energy phosphates and other cellular components. Autophagy has evolved as a conserved mechanism for cell survival under conditions of starvation and stress. In addition to (macro)autophagy, characterized by the sequestration of organelles and proteins within an autophagosome, there are two additional subtypes of self-digestion: microautophagy which refers to protrusion of the lysosomal membrane *per se* around a region of cytoplasm; and chaperone-mediated autophagy in which degradation is restricted only to those proteins with a consensus peptide sequence recognized by specific chaperone complexes[31]. Autophagy is now considered to be important for host defense against intracellular microorganisms. The associations of the autophagy-associated genes with Crohn's disease strongly support the hypothesis that abnormal innate immune responses to intracellular pathogens contribute to the pathogenesis of Crohn's disease. In fact, the pathological characteristics of human Crohn's disease represent "granuloma" formation. The mechanisms of granuloma formation remain unclear. Recent studies have demonstrated functional roles for IL-23 in the differentiation and promotion of Th-17 cells. Autophagy genes that have been related to CD diagnosis are the *ATG16L1*[32,33], *IRGM* and the *LRRK2* genes[34]. Unraveling the mechanisms of such molecular mimicry is crucial to our understanding and clinical intervention of infectious diseases and inflammatory disorders of unknown etiopathogenesis, including Crohn's disease.

Genes related to lymphocyte differentiation

***IL23R* gene:** Dysregulated cytokine production by mucosal lymphocytes and macrophages has been implicated in the pathogenesis of CD. In fact, an exclusive increase of CD4^{+} T cells in inflammatory bowel disease and their recruitment as intraepithelial lymphocytes has been demonstrated[35]. CD4^{+} T cells secreting interleukin-17 [T helper type 17 (Th-17) cells] have emerged as a key effector population driving colitis in animal models previously

associated with exaggerated T helper type 1 responses.

Of the genes involved in the differentiation of Th-17 lymphocytes the *IL23R* gene has been proved of great importance and has been related to Crohn's disease[36,37].

IL23R, consisting of an *IL-12β1* and an *IL23R* chain, is highly expressed on memory T cells. *IL23* is a novel cytokine formed *via* the binding of *IL12p40* to a p19 protein. After binding to the *IL23* receptor, *IL23* preferentially activates memory T cells. IL23 does exhibit some similar biological activities to *IL-12*; however, *IL-12* is more involved in the differentiation of naïve T-cells into Th1 lymphocytes and subsequent *IFNγ* production. *IL23*, on the other hand, mediates proinflammatory activities in part by the production of *IL17* through activation of Th17 lymphocytes[38].

Signal transducer and activator of transcription 3 gene: Signal transducers and activators of transcription 3 (*STAT3*) play an important role in various autoimmune disorders including IBD[39,40]. *STAT3* was initially identified as an acute phase response factor, an inducible DNA binding protein that binds to the *IL-6* responsive element within the promoters of hepatic acute phase protein genes, and is involved in IL-6 dependent T-cell proliferation through prevention of apoptosis. Subsequent studies indicate that *STAT3* becomes activated in response to a wide variety of cytokines and growth factors. Recent studies have revealed that *STAT3* activation plays distinctly different roles between innate immune responses and acquired immune responses in colitis. *STAT3*-mediated activation of acquired immune responses plays a pathogenic role in colitis by enhancing the survival of pathogenic T-cells. In contrast, *STAT3*-mediated activation of innate responses contributes to the suppression of colitis. Emerging data indicate that *STAT3* is one of the crucial targets for the treatment of IBD. However, as the receptors of these cytokines and growth factors are present in both innate and acquired cells, activation of *STAT3* is likely to occur in both cell types. Therefore, as the function of *STAT3* is a double-edged sword, careful attention should be directed toward the cell population that is being targeted when one contemplates *STAT3* inhibition or activation in human IBD[41]. Within the same panel, other than *STAT3* genes, and with probable importance, are the *TNFSF15, JAK2, CCR6* and *ICOSLG* genes[42-44].

Genes related to secondary immune response, apoptosis and other pathways
Chemokines play a central role in the pathogenesis of IBD as they are able to trigger multiple inflammatory actions including leukocyte activation and chemoattraction, granule exocytosis, production of metalloproteinases for matrix degradation and upregulation of the oxidative burst[45]. Therefore, further support is given for genes that relate to secondary immune response, apoptosis and other pathways. For example, in the *IBD4* locus 4 several interesting candidate genes, which may be relevant in the pathogenesis of CD, lie within this region (e.g., genes regulating apoptosis, signal transduction proteins, chemo-

kine receptors, T cell receptors, metalloproteinases).

Gene expression profiles from colon lamina propria fibroblasts have demonstrated several functional changes in some proteins coded from the corresponding genes: collagen types Ⅰ, Ⅳ, XIV; matrix metalloproteinase 1; cathepsin K; stroma cell-derived factor-1; chitinase3-like-1; and many others[46]. The major histocompatibility complex (MHC) has been extensively investigated. Human leucocyte antigen (HLA) class Ⅱ molecules present partially digested antigen to the T-cell receptor and play a central role in the immune response. In CD, the MHC and HLA studies have yielded conflicting and heterogenous results. *HLADR1* has been implicated in CD[47].

Many other genes, loci and chromosomes involved in CD have also been advocated in several studies that, however, still require wide replication and association with clinical practice. These include *CNR1, MCP-1*[48], *PTPN2* (protein tyrosine phosphatase)[49], *PTPN22, NKX-3, IL-18 RAP/IL-18R1, IL12/IL23* pathway[50], *PTGER4, MST1/BSN/MST1R*[50-52], *IL-2/IL-21*[53], *TYK2, JUN, NAT2*[54], *IL-10, NELL1, NKX2-3*[55], *Cyclin Y*, Hect domain, 1q24, 10q21, 5p13, RCC1-like domain, *ICOSLG, CDKAL1*[56], 13q13.3, 1p35.2, 3p29, 5p13.1[57,58], X chromosome[59], *NLRP3*[60], Vitamin D receptor (*VDR*) polymorphisms[61] and many others as well.

Genes in family and ethnic group studies
Linkage studies performed in complex genetic disorders such as CD frequently use model-free analytic methods, which are non-parametric analyses that do not assume Mendelian recessive or dominant models of inheritance.

The strongest risk factor for IBD is having a relative with the same disease. First-degree relatives of patients with CD have a 12-to-15 times greater risk of developing CD than do people of comparable age in the general population[61]. Familial clustering can also result from exposure to common environmental risk factors. Twin studies are very useful to determine the degree of genetic *vs* non-genetic etiologies for a trait. Today, there is no evidence of a separate entity of familial IBD[62,63]. Based on the current literature, phenotypic differences between familial and sporadic cases of IBD are weak. Available data are to be accepted with caution, however, as they are mostly retrospective and may be biased. *CARD15* explains around 20% of the genetic predisposition to Crohn's disease[64]. The relative risk of developing CD in the presence of one mutation is 2-4, but increases dramatically in the case of two mutations (compound heterozygous or homozygous).

Although *NOD2* provides no clear familial predisposition, unaffected relatives do carry an increased rate of *CARD15* variants (37.1%) compared to controls, and it would be interesting to see if they will eventually develop symptoms[65-67]. In addition, maternal transmission of *CARD15* variants seems protective with a lower ratio of affected/unaffected children when compared to fathers[68,69]. In the light of the foregoing data, it seems that genetic counseling should be carried out with caution. In addition, families should not receive genetic counsel-

ing/information about age at onset and disease severity. Ethnic group studies and ethnic variation were firstly demonstrated in Jewish populations, and those studies are of major importance in this context[70].

THE ROLE OF GENES IN PROGNOSIS OF CROHN'S DISEASE

This is a major issue that greatly concerns patients. Many genes have so far been implicated in the prognosis of Crohn's disease and numerous attempts have been made to classify the genetic profiles in Crohn's disease. Of interest, *CARD15* seems not only a susceptibility gene, but also a disease-modifier gene for CD. Of the many studies published on the clinical relevance of *CARD15* mutations, there are several providing data on disease location, and the majority of them support a significant association of *CARD15* mutations with ileal disease site, while some demonstrate a connection with the absence of colonic location. Some studies also provide data supporting the relevance of *CARD15* variants with stricturing disease behavior, and also penetrating behavior. Other pertinent studies have revealed an association with early onset of the disease. These investigations also support the theory that pediatric Crohn's is a "more genetic disease" consistent with other polygenic disease models. Other reports provide data on an increased risk or need of surgery related to CD[71].

Differences among studies are difficult to explain, and we could argue about the low number of patients in some of the studies, the disease variability among Caucasians and, finally, differences regarding disease assessment and interobserver agreement. Whether the described relationship between the *CARD15* variants and both stenosing phenotype and increased need for surgery in CD patients is a true association, or only reflects the high proportion of ileal CD developing bowel stenosis and, therefore, requiring surgery, is still a matter of controversy.

Genes related to age of Crohn's disease onset
With respect to age of CD onset and more specifically to childhood or early-onset Crohn's disease, many genes/loci have been implicated: *TNFRSF6B*, *CXCL9*[72], *IL23R*[73,74], *NOD2*[75], *ATG16L1 rs2241880*[76], *CNR1*[77], *IL-10*[78], *MDR1*[79]. Of interest, *DLG5* seems protective for female children[80], while there are also studies not supporting the relationship of genes and early onset of CD[81] or supporting the relation of *IL-10* and *IRGM* with adult onset[82].

Genes related to Crohn's disease behavior
Genes related to stenotic/stricturing behavior in CD are: *NOD2/CARD15*[83], *TLR4*[84], *IL-12B*[85] and *CX3CR1*[86,87]. Of importance, *NOD2/CARD15* has been also related to acute intestinal obstruction[88]. *IL-10* and *IL-6* are also potentially related to stenotic/structuring behavior in CD, while genetic variants of several metalloproteinases and their inhibitors would be excellent candidate genes, since these molecules are considered to play a key role in the

abnormal fibrogenesis that underlies the development of bowel stenosis in CD patients. Genes related to penetrating/fistulizing behavior in CD are as follows: *NOD2*, *IRGM*, *TNF*[89], *HLADRB1*[90], the C-allele in *CDKAL1* rs6908425 SNP is associated with *NOD2*(-) perianal fistula, whereas *OCTN* and the near *IL-12B* gene rs12704036 T-allele have a relationship with non-perianal fistula[91]. Inflammatory CD behavior has been related to *HLA* variation[92] while granulomatous disease has been related with *TLR4/CARD15* variants[93].

Genes related to Crohn's disease location
Upper gastrointestinal Crohn's disease has been related to *NOD2*[94] and *MIF* variants[95]. Ileal CD has been related to the following genes: *IL-10*[96], *CRP* gene[97], *NOD2*, *ZNF365* and *STAT3*[98]. Genes/loci associated with ileocolonic CD are 3p21, *ATG16L1*[98] and *TCF-4* (*TCF7L2*)[99]. No role for phenotype in *IL23R* gene has been demonstrated[100] while a detailed genotype-phenotype analysis revealed weak associations of the *IL23R* rs10024819 variant with ileal involvement and stenoses in carriers of the TT genotype. Finally, the *HLADRB1*0701* has been associated with ileal CD, but only in patients who have no *CARD15* variants[101]. Colonic CD has been related to the following genes: *HLA* region (associated with inflammatory colonic phenotype); and *TLR4*[102], *TLR1*, *-2*, *-6*[103]. The *TNF* gene showed a negative association with stricturing behavior or colonic location[104]. For *IBD5* and *OCTN1* and *2*, results have not been consistent but associations with perianal and ileal disease have been reported.

Genes related to Crohn's disease activity
Genes implicated in disease activity are the following: *HSP70-2* heat shock protein gene[105], *NOD2*[106], *PAI-1* (Type 1 plasminogen activator inhibitor[107]), while the combination of *NOD2* and *PAI-1* predicted complicated disease behavior[108]. Of importance, *NOD2* predicted lower weight in children[109], and *CNR1* low body mass index[110].

Genes related to surgery
The *NOD2* gene has been related to early pediatric surgery[111], stenosis and need for surgery[112], previous surgeries[113], increased number of surgeries[107] and surgical costs[114]. *NOD2* has no relation to the risk of re-operation[115]. Finally, *HLA-G* has been associated with higher risk for ileocolonic resectio[116].

Genes related to dysplasia and cancer
The *FHIT* gene (fragile histidine triad gene) located at 3p14.2 has been identified as a candidate tumor-suppressor gene. The gene spans the t(3;8) translocation breakpoint of familial renal cell carcinoma and contains the FRA3B fragile site. It encodes the human diadenosine triphosphate hydrolase, which *in vitro* cleaves the diadenosine substrate into ADP and AMP. It has been suggested that *FHIT* gene plays a role in the pathogenesis of IBD and the development and progression of a subgroup of IBD-related carcinomas at an early phase[117-119].

Genes related to extraintestinal manifestations and concomitant diseases

Extraintestinal manifestations are common in CD. Genes related to CD extraintestinal manifestations have been reported, as follows. Peripheral arthritis was related to *FcRL3*[120], *HLADRB*103, HLAB*27 HLA-B*44*, *HLA-B*35, TNFa-308A*[121]. *CARD15* has been related to spondylarthropathy[122] and uveitis[123] but not to sacroileitis[124]. *TNF-1031C* was associated with erythema nodosum while certain *HLA* alleles (*HLA-B27, HLA-B35* and *HLA-B44*) were connected with different disease behavior and extraintestinal manifestations such as arthropathy, eye and skin manifestations. Genes/loci related to other chronic diseases concomitant to CD are 10p12.2 (sarcoidosis and CD)[125], *STAT3* (multiple sclerosis and CD)[126] and a parallel genetic fingerprint between leprosy and CD[127].

Pharmacogenetics in Crohn's disease

Pharmacogenetics is of major importance in CD therapeutics and prognosis. Genes have been implicated in influencing the efficacy and side effects of drugs and reflect a complex interplay regarding absorption, elimination and transport. Future studies need to be large and prospective with uniformly phenotyped patients, and correlating genetic associations with functional data. In addition, hypotheses such as whether observations about drug response in IBD lead us to IBD etiology or whether the genes that control the drug response are related to genes that control the disease still remain unanswered. Pharmacogenetic studies to date have found no association between *CARD15* variants and prediction of response to various IBD therapies. In addition, responses to azathioprine (AZA), steroids and infliximab are not related to *NOD2*[128]. Of note, *NOD2* was only related to antibiotic failure[129]. For mesalazine, variability in drug acetylation was demonstrated many years ago with patients divided into slow and rapid acetylators, because of polymorphisms in the N-acetyltransferase (*NAT*) genes. Two isoenzymes NAT1 and NAT2 have been identified in humans and more than 50% of Caucasians are NAT2 slow acetylators. Mesalazine is acetylated in the liver by NAT1 into N-acetyl-5 aminosalicylates and excreted in the urine[47].

The clinical usefulness of pharmacogenetics in CD is limited to AZA and thiopurine methyltransferase (TPMT) at this moment. The human *TPMT* gene, consisting of 10 exons, is located on chromosome 6p22.3. The hereditary nature of *TPMT* deficiency in humans was initially identified in a study of *TPMT* activity in red blood cells (RBC). This and subsequent studies determined the distribution of *TPMT* activity in RBC to be trimodal; 90% of persons have high activity, 10% have intermediate activity and 0.3% low or no detectable enzyme activity. To date, numerous mutant *TPMT* alleles have been identified, including the three most frequent alleles (*TPMT*2, TPMT*3A* and *TPMT*3C*), which account for 80%-95% of intermediate or low TPMT enzyme activity cases. The prevalence of the most frequent SNPs in the *TPMT* gene has been reported to vary worldwide. However, it is of interest that studies on the prevalence of *TPMT* SNPs in large IBD cohorts are lacking.

Although AZA is an effective drug for maintenance of remission in IBD, it is associated with side effects. Clinically sound pharmacogenetic studies over the last two decades have shown that polymorphisms in the *TPMT* gene locus play a significant role in the occurrence of various side effects of thiopurine drugs including life-threatening bone marrow toxicity, a serious dose-related toxicity[130-134].

The G2677T variant in the *MDR1* gene predicted gastrointestinal and unspecified intolerance to azathioprine and methotrexate in inflammatory bowel disease patients. These findings suggest a role for MDR1/P-gp in the mechanism of action of azathioprine and methotrexate[135,136].

Twin studies have linked polymorphisms of the *VDR* gene with bone mineral density in healthy women, and in addition *VDR* is an important regulator of calcium metabolism and bone cell function and influences calcium absorption from the intestine. *VDR* polymorphisms have also been implicated in susceptibility to Crohn's disease[137].

The *HLADR* region has been associated with failure to budesonide[138] while *DLG5R30Q* predicted response to steroids[139]. Other genes such as *MIF* (macrophage migration inhibition)[140] and *MDR* have also been related to steroid therapy[136]. In addition, 1082 AA *IL-10* genotype was associated with steroid dependency, whereas the allele 113A of the *DLG5* gene conferred resistance to steroids.

Regarding response to infliximab, the data for the *TNF* gene are conflicting. Specifically, there are conflicting data regarding the role of *FcGR3A*, which has been supported by some authors[141,142], but was not confirmed in patients in the ACCENT I study. Response to infliximab is not related to *TNFa-308*[143] or *TNFR1* and *TNFR2*[144] or *NOD*[145] or *CRP* gene[136]. The association between the Fas ligand-843 TT genotype and lack of response to infliximab seemed to be the most relevant observation[136]. The relationship between infliximab response and the lymphotoxin alpha gene is also conflicting[144].

WHAT LIES AHEAD

Gene-to-gene crosstalk and epistasis

With new methodologies such as genome-wide association studies, microarrays and fine SNP analysis becoming available during the last decade, our investigative armamentarium has been considerably enriched. As many studies with complex statistics arise, we understand increasingly the real crosstalk present among genes and the need for a genetic panel for disease diagnosis and prognosis. It is now evident that gene-to-gene interaction and epistasis modulate disease activity and susceptibility[146]. Some data have come to light. A genome-wide scan in a Flemish population of IBD affected families supports the

Table 2 Predicted future developments in the genetics of Crohn's disease

What lies ahead in the genetics of crohn's disease
Gene-to-gene crosstalk and epistasis
Genome-wide association studies
Microarrays
Fine single nucleotide polymorphism analysis
Genetic consortium studies and genome-wide scans
Genome-wide association studies
Genetic consortium studies
Future perspectives
Functional studies to understand the mechanisms
Combining genetic data with functional data
Combination of a panel of clinical, biochemical, serological and genetic factors
Functional consequences of polymorphisms
Molecular and cellular mechanisms leading to Crohn's disease
Predict disease outcomes
Redesigning the methods of treatment

existence of *IBD4* on 14q11, and has shown additional evidence for the existence of other susceptibility loci (1p, 4q and 10p). This study has further demonstrated that epistasis and gene-to-gene interactions (*CARD15-TLR4*) are also present in IBD and that population heterogeneity is not to be underestimated[147]. Crosstalk has been demonstrated for *TLR9* with *NOD2, IL23R* and *DLG5*, and epistasis has been shown between *IL23R* and *DLG5*. Also, potential epistasis between *IL23R* variants and the three other previously described CD susceptibility genes *CARD15*, *SLC22A4* and *SLC22A5* (*OCTN 1* and *2*) has been shown[116].

Genetic consortium studies and genome-wide scans

Over the past few years, a combination of progress in high-throughput genotyping technology and growing knowledge about the human genome through the International HapMap project and the Human Genome Project have enabled GWAS for several complex diseases. To understand the approach to conducting GWAS in this setting, it is important to expound on the concept of linkage disequilibrium, which refers to the nonrandom association of alleles at nearby loci. Specifically, linkage disequilibrium refers to adjacent alleles assorting together nonindependently from generation to generation because they are tightly linked and thus less likely to become separated by recombination. Genetic consortium studies are of major importance, and homogeneity in methodology issues is of paramount value[148-151]. Appropriate study design[152], power analysis[153] and overall data analysis and meta-analysis[154] are mandatory. Accurate estimation of sample sizes required in a genetic association study is essential before commencing genotyping, to ensure that the study is sufficiently powered to detect the subtle genetic effects that contribute to most complex diseases. The extensive genetic variation and complex linkage disequilibrium across even a small genomic region will give rise to several alternative scenarios. Genetic variation across a region studied should be carefully evaluated and

consideration should be given to possible linkage disequilibrium and allelic heterogeneity when evaluating power of an association study. As larger datasets are studied and combined, as genotyping platforms provide even greater depth of coverage of the genome, and as modest hits are followed up in large independent panels, so the vast majority of true signals should be identified. These robust genetic data will truly provide a solid platform for functional studies to understand the mechanisms by which these genetic variants predispose to Crohn's disease. Finally, studies at post-transcriptional level become more and more urgent[155]. Enriching our understanding of CD genetics is important, but when combining genetic data with functional data the outcome could be of major importance. In fact, improved understanding of immune mechanisms, on which manifold genetic and environmental traits might converge, and which ultimately mediate all phenomena in inflammatory bowel disease, holds promise (Table 2).

CONCLUSION

The recent advances in the understanding of CD genetics have been tremendous[156]. Starting with the susceptibility area, whole genome linkage and association scans have already led to the identification of a number of susceptibility genes (*NOD2/CARD15, DLG5, OCTN1* and *2, NOD1, IL23R, PTGER4, ATG16L1* and *IRGM*) of which the *NOD2/CARD15* gene is the most replicated and understood at present. Although it is clear that genetic research in IBD has advanced our understanding of the clinical heterogeneity of the disease, new efforts are required and point towards the complex combination of a panel of clinical, biochemical, serological and genetic factors, in order to achieve the optimal prediction of both clinical behavior and response to therapy.

Genome-wide association studies have allowed an unprecedented rapid unraveling of the genetic basis of IBD; however there will be much more follow-up work needed in this field. First, ongoing work including meta-analysis of the CD genome-wide association studies will probably reveal additional CD susceptibility genes. It will then be essential to investigate the functional consequences of polymorphisms in these genes so that the molecular and cellular mechanisms leading to CD can be better characterized. Finally, genotype-phenotype correlation studies should help clinicians predict disease outcomes with more accuracy, including the risk for complications, need for surgery, and response to therapy, and finally lead to redesigning the methods of treatment of CD patients.

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