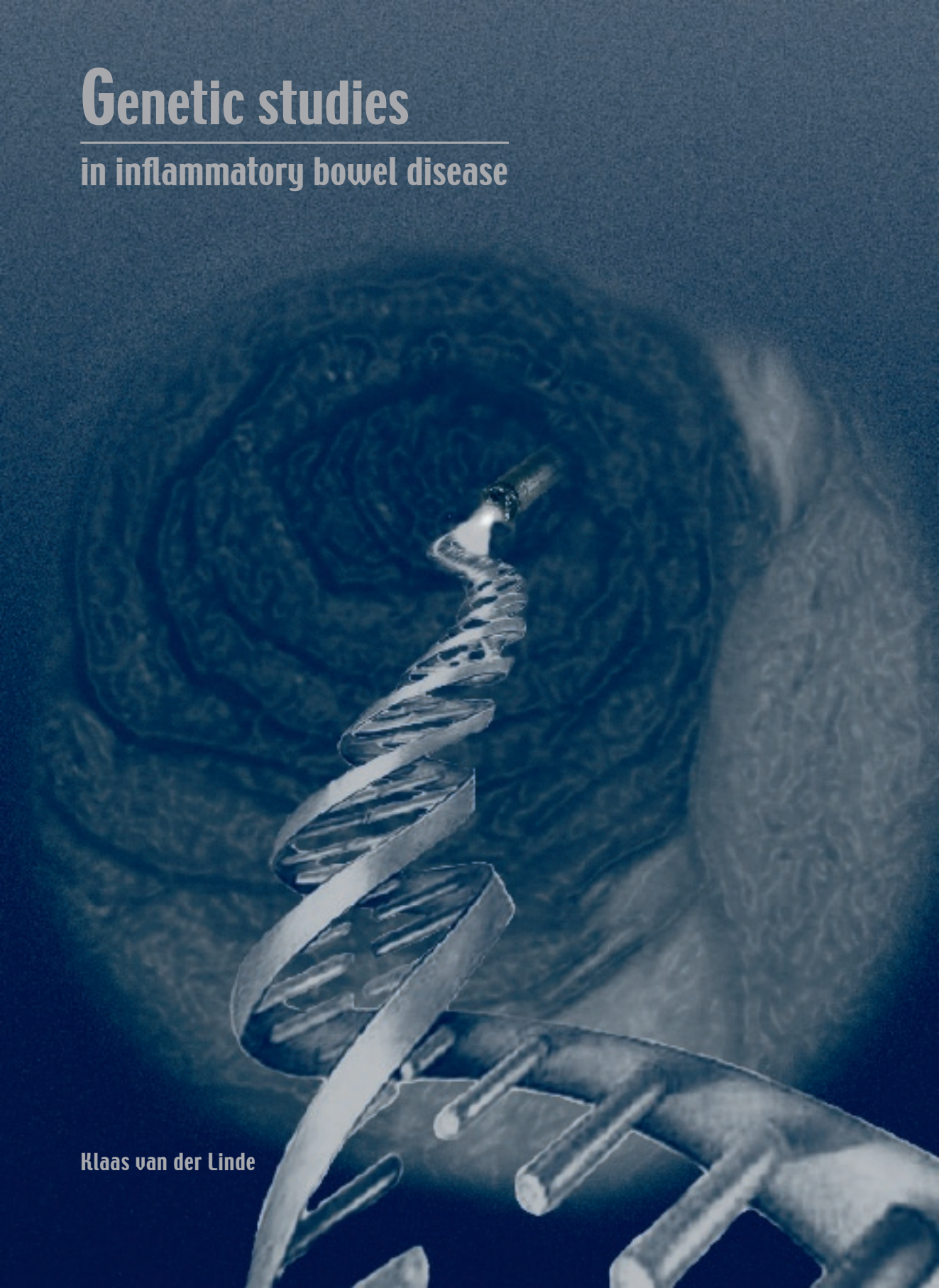
Genetic studies

in inflammatory bowel disease



Klaas van der Linde

GENETIC STUDIES IN INFLAMMATORY BOWEL DISEASE

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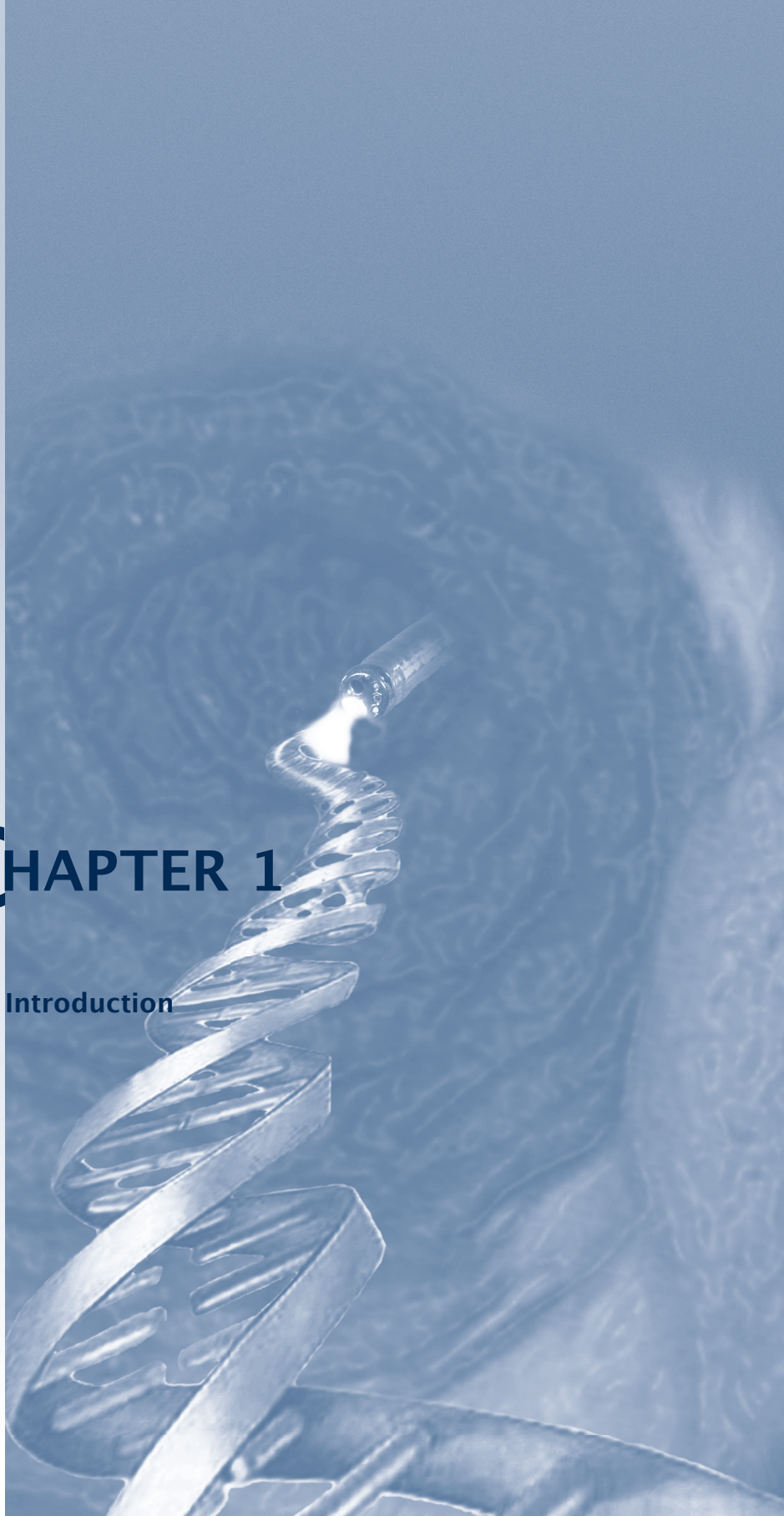

CHAPTER 1

Introduction

INTRODUCTION TO THIS THESIS

Inflammatory bowel disease (IBD) is an idiopathic chronic gastrointestinal disease with genetic susceptibility playing an important role [1]. This is further explored in this thesis. The initiative for this thesis started within the departments of Gastroenterology and Hepatology, and Internal Medicine of the Erasmus MC University Medical Centre Rotterdam (UMCR).

AIM OF THIS THESIS

The primary research goal was to explore aspects of the genetic aetiology of IBD by searching for mutations in inflammation-related genes in patients with IBD. Many IBD-affected patients and (non-)affected relatives were recruited to set up a clinical and genetic IBD database. Initially, the research protocol focused on cytokine (receptor) genes. This focus was extended to research on other polymorphic genes based on new findings in the literature. We have mainly focused on mutations within the interleukin-10 (IL-10) and caspase recruitment domain 15 (CARD15) genes, with emphasis on their prevalence, biological (dys)function, and association with disease phenotypes. Furthermore, genetic anticipation in IBD was studied.

HIGHLIGHTS OF IBD

Definition

IBD is an idiopathic chronic relapsing disorder characterised by inflammation of the gastrointestinal tract. There are two well defined subtypes, Crohn's disease (CD) and ulcerative colitis (UC).

Crohn's disease

CD was launched as a defined disease in 1932 by Crohn, Ginzburg and Oppenheimer working at the Mount Sinai hospital in New York (United States). Initially they described the disease as 'regional ileitis' [2]. With later knowledge that the disease could also affect other sites of the gastrointestinal tract, the 'Crohn's disease' became accepted.

Epidemiology CD is a typical Western disease, with a rising incidence [3, 4]. Lately, the incidence of CD also rose in non-western countries [5, 6]. The incidence is characterised by a north-south gradient. In Europe, the incidence of CD is 80% higher in northern countries compared with southern countries [7]. In The Netherlands, the incidence is 4-8 cases per 100,000 and the prevalence around 0.05% [7-9]. The disease is somewhat more common in females than in males, and the median age of diagnosis is 29 years [9]. Appendectomy and smoking are associated with an increased

risk of developing CD [10, 11]. The risk of small bowel and colorectal cancer is slightly increased in CD, but the overall survival is similar to the general population [12, 13]. However, in a recent European multicentre study an overall increased mortality was seen ten years after diagnosis, especially in patients diagnosed with CD beyond the age of 40 years and mainly due to gastrointestinal causes [14].

Clinical aspects CD is characterised by a focal or multifocal chronic transmural inflammatory process with lymphoid aggregates [15]. Non-caseating granulomas can be found in 26-37% [16, 17], and may indicate a more aggressive disease [17]. CD can affect any part of the gastrointestinal tract, of which the ileocecal region is most common (40%). In roughly one-third of patients CD is confined to the small bowel, another third has disease only affecting the colon, whereas in the remainder the disease affects both small and large bowel [18, 19]. In 1998, the Vienna classification was proposed to categorise patients with CD based on the age at diagnosis, the location of disease and the disease behaviour [20]. CD can give rise to complications like stenosis and/or fistulas. Symptoms of CD are abdominal pain, diarrhoea, fever, rectal bleeding and weight loss. These symptoms are related to the location of inflammation and the disease behaviour [21]. Several immune-related extra-intestinal manifestations are common in CD, such as arthritis, erythema nodosum, pyoderma gangrenosum, aphtous stomatitis and uveitis [22].

Diagnosis In most cases the diagnosis is based on compatible symptoms, risk factors, physical findings, typical ileocolonoscopic features (Figure 1), histology of biopsies or surgical samples, and conventional radiology studies, like enteroclysis and abdominal ultrasonography [18, 23, 24]. New diagnostic tools can be helpful, like capsule and double-balloon endoscopy, magnetic resonance imaging and computed tomography [25]. Laboratory tests, especially C-reactive protein, and detection of specific antibodies like anti-Saccharomyces cerevisiae antibody (ASCA) and perinuclear anti-neutrophil cytoplasmic antibody (pANCA), can also be helpful [26, 27]. Using serodiagnostic tests as the initial work up in patients suspected to have IBD might be cost-effective [28].

Treatment In moderate active CD, sulfasalazine is effective in left-sided disease, while budesonide is first choice in right-sided and terminal ileum disease. In more severe active disease prednisone is indicated. Infliximab, a chimeric monoclonal antibody directed against tumour necrosis factor α (TNFα), is used for steroid-resistant patients, or when CD is complicated by perianal fistula. Immunomodulators, like azathioprine, 6-mercaptopurine and methotrexate, are effective to maintain remission after induction therapy, in particular with steroids. Most patients need surgery during their disease course [29-31].

Ulcerative colitis

Early reports about UC were published by Lockhart-Mummery from St. Mark's Hospital in London (United Kingdom) in the beginning of the 20th century [32].

Epidemiology The incidence of UC in The Netherlands is 7-13 cases per 100,000

inhabitants and the prevalence around 0.06% [7, 9, 33]. Similar to CD, the incidence of UC shows a European north-south gradient and it is rising in both western and non-western countries [5-7]. Males are more often affected than females [7, 9]. In contrast with CD, appendectomy and smoking reduce the risk of developing UC [10, 34]. The overall risk for colorectal cancer is the same as in the general population, but patients with an extensive colitis have a considerably increases risk [12]. Survival of UC-affected patients does not differ from the general population [13].

Clinical aspects In UC, intestinal inflammation is continuous and superficial including the mucosa and submucosa. UC is a large bowel disease invariably involving the rectum, which can extend to the proximal colon. Based on the extend of inflammation, UC is classified as proctitis (rectum only), left-sided colitis (up to the splenic flexure) and extended or pancolitis (beyond the splenic flexure). Symptoms are rectal bleeding, urgency, diarrhoea, abdominal discomfort, loss of appetite and weight loss [15, 35]. UC can also present with extra-intestinal manifestations. Most of them are similar to the manifestations associated with CD. Primary sclerosing cholangitis is a serious extra-intestinal manifestation, which is more common in UC- than in CD-affected patients [22].

Diagnosis As UC is limited to the colon, (ileo)colonoscopy and mucosal sampling are the most important tools to establish the diagnosis. The typical endoscopic mucosal appearances of UC (continuous mucosal inflammation starting from the dentate line with loss of vascular pattern, cryptitis, erosions, normal ileocecal valve and sometimes periappendicular inflammation) are different from those found in CD (skip lesions, cobblestoning, aphthous and longitudinal ulcers, ileocecal involvement and anal lesions). Endoscopic views are demonstrated in Figure 1. In severe disease, the endoscopic features of UC and CD may be difficult to distinguish [18, 24, 36]. Non-invasive test are also used in UC [26-28].

Treatment First-line treatment in mild to moderate UC consists of 5-aminosalicylates (5-ASA, mesalazine), which can induce and maintain remission. In moderate to severe active disease steroids are effective, usually combined with azathioprine or 6-mercaptopurine. Cyclosporine can be used in patients with severe active UC who are steroid-resistant [29, 30]. Recently, infliximab was also proved to be effective (as rescue therapy) in moderate to severe active UC [37, 38]. Surgical therapy is necessary in acute toxic colitis, intractable disease or colorectal cancer. Usually a total proctocolectomy with ileostomy or an ileal pouch-anal anastomosis is performed [39].

Indeterminate colitis

In patients with exclusively colonic inflammation in who no differentiation can made between CD and UC, the disease is defined as indeterminate colitis (IC). Originally, the term IC was preserved for patients who had undergone colorectal surgery, but in whom subsequently no pathological diagnosis could be made on the resected specimen [40]. In The Netherlands, the incidence of IC is around 0.001% [9]. During follow-up of patients with the initial diagnosis IC, about 50% can be reclas-

sified as having either CD or UC. It is assumed that the clinical course of IC is more similar to UC than CD. Medical treatment is based on the anatomical distribution, the severity and the relapse rate of the disease, including 5-amino-salicylates, steroids, azathioprine or 6-mercaptopurine, and even infliximab. Surgical treatment involves total proctocolectomy with an ileal pouch-anal anastomosis. This procedure in patients with IC, however, is associated with more frequent occurrence of postoperative complications than in patients with UC [41].

Pathogenesis

Acquired immunity The traditional concept regarding the cause of IBD is an abnormal response of cells from the acquired immune system, leading to an exaggerated aggressive activity of effector lymphocytes and pro-inflammatory cytokines in response to gut-derived antigens presented by antigen-presenting cells. The disease may also result from dysfunctional control mechanisms, such as an abnormal secretion of the anti-inflammatory cytokine IL-10 and transforming growth factor-β. The pro-inflammatory cytokines are secreted by T-helper (Th) cells, of which two types are identified. Th-1 cells mainly secrete IL-12, interferon γ (IFNγ) and TNFα. It is responsible for the cell-mediated immunity. Primary mediators of Th-2 cells are IL-4 and IL-5. They mediate the humoral immunity. Until recently, CD was thought to be Th-1 driven and UC a Th-2 driven disease.

Innate immunity Recently, evidence has become available that the innate immune system is also involved in the aetiology of IBD. This immune system is a rapidly responding non-specific defence mechanism against pathogens. It recognises molecular motifs of an antigen instead of the specific antigen itself. Toll-like receptors (TLRs) are well described surface receptors of several intestinal immune cells, sensing so-called pathogen-associated molecular patterns.

Bacteria In the complex inflammatory mechanism of IBD, several responsible cofactors are identified. For example, gut flora is important for inflammatory induction as deviation of the faecal stream [42], antibiotic and probiotic treatment especially in pouchitis [43] can lead to disease remission. Animal studies using normal and knockout models, such as IL-10 deficient mice, have demonstrated that a germ-free environment leads to a quiescent immune system [44].

Epithelium Otherwise, the intestinal epithelium which is part of the innate immune system is important as it is the first (anatomical) defence level. In CD, an increased intestinal permeability has been found [45]. The epithelium harbours M-cells and dendritic cells, which are responsible for sampling components of luminal bacteria and to present these components to cells of the immune system.

Environment Environmental influences in IBD susceptibility are well known. Accepted risk and preventive factors in IBD are smoking, appendectomy, perinatal events and socio-economic background. In general, these factors have an effect on the immune response, leading to up- or down-regulation of the intestinal inflammatory mucosal response [44, 46-48]. In multiple CD-affected families, a trend was found

towards an excess of consecutive CD-affected sib-pairs as affected siblings were not randomly distributed within sibships as would be expected in case of a pure genetic disorder. Adjacent birth order of CD-affected siblings might involve a common environment. Therefore, the excess of observed affected sibling clusters supports the hypothesis of environmental involvement in CD [49].

Genes Genetic susceptibility to IBD and the search for disease-causing (altered) genes are of major interest since the first CD gene, the CARD15 gene, was identified in 2001 [50, 51]. This aspect will be described next in more detail.

Genetic susceptibility in IBD

Genetic epidemiology Initial evidence for genetic involvement in IBD was based on epidemiological studies, including ethnic (Jews versus non-Jews) [52, 53] and racial differences (Caucasians versus non-Caucasians) [54]. In five to ten percent of patients with IBD familial clustering exists, meaning that the largest risk to develop IBD during life is having a family member with IBD [55]. First-degree relatives of CD- and UC-affected probands have a 5.2 and 1.6% age-corrected lifetime risk to develop IBD, respectively. In Jewish families this risk is even higher [53]. Evidence for a genetic role in IBD comes from the observed higher concordance rate for disease type in monozygotic twins than in dizygotic twins, especially in CD (around 40 versus four percent) [56-58]. These data also support a reduced penetrance of IBD genes, as not 100% disease concordance exists in the monozygotic twins and the disease risk in dizygotic twins is similar to siblings [59]. In monozygous CD-affected twins, also high concordance rates were found for the three phenotypes as defined in the Vienna Classification [58]. Otherwise, high concordance rates for type, location and behaviour of disease is reported in multiple IBD-affected families other than twins [60], suggesting a common genetic risk factor.

Genetic anticipation Some monogenic disorders have demonstrated genetic anticipation, meaning that in following generations the disease manifests at an earlier age and/or with a more severe course. This phenomenon is thought to be the result of expansion of unstable trinucleotide repeats in the disease-causing gene. Although IBD is a multigenic disease, several studies have demonstrated remarkable differences in age at diagnosis between IBD generations. However, these retrospective studies involving disease-affected parent-child pairs included generations at different ages. This will lead to a preferential inclusion (ascertainment bias) in which the parent and child have a different completed risk period for their disease (follow-up bias). Therefore, it is likely that genetic anticipation in IBD is explained by bias within the data [60].

Genetic research Since the early 1990's, various molecular genetic studies in IBD have been performed. Initially, studies using complex segregation analysis in IBD-affected families provided evidence for the involvement of a recessive gene with incomplete penetrance in CD and a dominant or additive gene in UC [61-63]. Although simple Mendelian inheritance might be true in a subset of IBD patients (a major sin-

gle mutation among several other involved genes with a limited individual impact), studies using segregation analysis cannot discriminate between inheritance models involving multiple genes or genetic heterogeneity (i.e. different genetic diseases with similar phenotype), which has become the accepted genetic model in IBD [59]. Meanwhile, nine putative chromosomal IBD loci have been identified (IBD1-9, Figure 2) [64, 65]. The current genetic model for IBD includes a heterogeneous family of inflammatory disorders in which specific clinical disease manifestations in any individual are determined by interaction of genetic and environmental factors. In this type of complex disease, specific susceptibility genes can lead to either CD or UC. Other modifier genes are responsible for specific phenotypes once the disease is present (Figure 3) [64, 66].

GENE SEARCH TECHNIQUES

Markers

To search for DNA regions of interest including susceptibility genes or to evaluate susceptibility of already identified genes, genomic variations (alleles) are required. These DNA variations, also called polymorphic genetic markers, have to be located near or within the chromosomal region of interest or gene. These markers can for example consist of single nucleotide polymorphisms (SNPs), insertions or deletions of one or more base pairs, or microsatellites. Microsatellite markers are anonymous repeated DNA sequences with a variable length. Genetic markers are (more) useful when individuals in the study population have different alleles on the two homologous chromosomes carrying the particular marker and when a high heterozygosity exists, meaning much genetic variability. With this (flagging) technique two complementary genetic analyses are available, i.e. linkage and association studies [59, 65].

Linkage analysis

Linkage analysis is performed in multiple disease-affected families, usually affected sibling pairs or affected relative pairs. It evaluates whether a particular locus co-segregates with the presence or absence of the trait within families. One would expect that disease-causing loci are present in affected subjects. Especially, multi-point linkage analysis is informative as it tests for linkage between two or more proximal allele markers and a trait. A set of closely linked alleles inherited as a unit is called a haplotype. Two-point linkage analysis tests for segregation of only a single maker, and therefore its informative value depends highly on the heterozygosity of the marker. Linkage is based on the frequency of co-segregation of the marker with the disease within families with multiple disease-affected members. In fact, the method compares the number of alleles which are shared between relatives. The outcome is expressed in a log odd (LOD) score. A LOD score of at least three is usually accepted as significant linkage [59, 65, 67].

Association analysis

In an association based strategy, the frequency of specific alleles of a marker (indirect testing) is evaluated in patients with the disease (cases), and in (healthy) individuals without the disease. In contrast with linkage analysis, the chosen makers are usually much more close to the studied allele. Association analysis can also be performed by using a specific gene mutation (direct testing). The method is more powerful than linkage analysis with respect to gene (and any given locus) causality [59, 67, 68].

GENE FINDING

Genome-wide screening linkage mapping

In this type of genetic research the whole human genome is screened for chromosomal regions that may contain susceptibility genes. This systematic mapping approach is based on linkage analysis using a large number of (highly) polymorphic markers, that are evenly spread across the genome. Traditionally, linkage analysis for genome screening is performed with microsatellite markers. Using a dense map of closely spaced SNPs is a new approach with a higher power to detect linkage [69].

In a complex disease like IBD, affected sib-pairs are mostly used and no restrictions are made regarding the mode of inheritance (non-parametric analysis). When a chromosomal region harbours a genetic factor involved in the aetiology of the disease, affected sib-pairs will share alleles at this region more often than expected by chance (identical-by-descent). During gamete formation, randomly one of the rearranged chromosomes due to meiosis is transmitted to offspring. Therefore, when more than 50% of the affected relatives co-inherit the specific chromosomal region linkage is present.

Meanwhile, 11 genome scans in IBD have been performed using the traditional linkage method with microsatellite markers. The genome scans have identified seven chromosomal regions with confirmed and replicated IBD linkage (Figure 2). The IBD1 locus, a pericentromeric region of chromosome 16, is the best known locus and was found to be significantly linked to CD. Within this locus the CARD15 gene has been discovered as the first susceptibility gene in CD [59, 65, 67, 70].

Positional mapping

The linkage peaks identified in genome scans usually cover large regions which may contain hundreds of biologically plausible candidate genes (see below). Positional mapping is a refining technique, in which a dense set of markers (SNPs) is tested for (the strongest) association throughout the linkage region. Its results depends on whether linkage dysequilibrium (LD) exists with the causal genetic variant. LD is a population genetic phenomenon by which alleles at different but closely

located genetic loci remain together on chromosomes throughout generations. Hopefully, ultimately a causal variant in a susceptibility gene is discovered. This technique has a greater power than linkage studies to identify genes with a small disease effect [59, 67].

Candidate gene analysis

Testing candidate genes is another approach to find disease genes in complex traits. It focuses on genes that are selected based on knowledge about their biological function that is likely to have an influence on the disease susceptibility. This approach is usually conducted in a case-control association study, but linkage analysis with candidate genes is also possible [59, 67, 68].

Highly polymorphic and well studied candidate genes are those of the human leukocyte antigen (HLA) region on chromosome 6. These genes play an important role in the presentation and recognition of antigens. Associations between several major histocompatibility complex (MHC) class II HLA alleles and CD and/or UC, specific IBD phenotypes, and extraintestinal manifestations have been found [71]. The MHC complex is part of the IBD3 locus, which is one of the IBD-linked chromosomal regions found by genome screening [72]. Other examples of associations with IBD have been found for promoter polymorphisms of the TNF gene [71], a specific allele of the variable number of tandem repeats sequence in intron 2 of the IL-1 receptor (IL-1R) antagonist gene [73], and polymorphisms of the TLR-4 gene [74]. Associations between polymorphisms in the IL-10 gene will be discussed below. In addition, candidate gene studies with negative results have been published, showing no association between the studied gene and IBD. For example, the multidrug resistance 1 gene [75], the IL-4R gene [76, 77] and the chemokine receptor CCR5 gene [77].

CARD15 GENE

Gene finding In 2001, the first susceptibility gene in CD was discovered. This CARD15 gene (formerly called nucleotide binding oligomerisation domain 2 (NOD2)) is located on chromosome 16q12. It was found through fine mapping of the IBD1 locus by one consortium group [50], and by a candidate gene approach with direct sequencing and transmission analysis within IBD families [51, 78, 79] by two other groups. CARD15 is a member of the Ced4 superfamily, a group of apoptosis regulating molecules, including apoptotic protease activating factor 1 [80] and CARD4 [81, 82] in humans. CARD15 is expressed in monocytes, macrophages, dendritic cells and intestinal epithelial cells, especially in Paneth cells [83-86].

Mutations In 2002, 67 nucleotide variations in the CARD15 gene were identified, of which 30 non-conservative missense mutations with a putative functional effect. Only three of these mutations independently appeared to be highly associated with CD in Caucasian populations from North America and Europe. These mutations were

2104C → T in exon 4 (SNP8, R702W, Arg702Trp), 2722G → C in exon 8 (SNP12, G908R, Gly908Arg), and 3020insC in exon 11 (SNP13, L1007fs, Leu1007fsinsC). These three common mutations represent more than 80% of all mutated alleles and are located near or in the leucine-rich region (LRR) [87]. The frameshift mutation L1007fs leads to a truncated protein lacking the 33 distal amino acids. Since 2002, another 17 mutations have been reported [88-90]. Mutations within the centrally located NOD (see below) show association with Blau syndrome, a rare monogenic highly penetrant granulomatous disease [91]. No association was found between the three common CARD15 mutations and UC [87], except for one study which demonstrated a significantly increased prevalence of L1007fs in UC compared with controls [92]. Several studies in Caucasian populations from different countries have been published about the prevalences of the three common CARD15 mutations in IBD. The L1007fs is associated with the highest risk toward CD, corresponding with four-fold increased relative risk to develop CD in heterozygotes. Carriage of two mutations (homozygosity or compound heterozygosity) leads to a 17-fold increased relative risk for development of CD [93]. In Scandinavian countries like Sweden with a high incidence of CD, CARD15 mutations are less present even in (monozygous) twins, and a disease association was only found for R702W and G908R [94, 95]. CARD15 mutations were also found to be less prevalent in African-Americans and Hispanics with CD [96]. Furthermore, common CARD15 mutations are nearly absent in the Asian population [93]. In a recent Dutch multicentre study, association with CD was found for R702W and L1007fs, and carriage of at least one variant in 36% of all CD-affected patients [97].

Phenotyping CARD15 mutations may predispose to certain CD phenotypes, as associations have been reported between the common variants and specific clinical characteristics. In two large pooled studies, especially small bowel disease and to a lesser extent stricturing disease showed associations with carriage of at least one common variant. In the same analyses, a slightly increased odds ratio was found for a positive family history for IBD [93, 97].

(Dys)function CARD15 expression is increased through bacteria and pro-inflammatory cytokines [84, 85]. CARD15 is an intracellular protein containing two N-terminal caspase recruitment domains (CARDs), a centrally located nucleotide oligomerisation domain (NOD), and a C-terminal leucine rich region (LRR). The LRR functions as a recognition receptor of muramyl dipeptide (MDP), which is a small component of cell wall peptidoglycan of gram-positive and gram-negative bacteria [98-100]. It is thought to play a protective role in the innate and adaptive immunity as well [100]. All three common CARD15 nucleotide variants are considered to be disease-associated mutations. *In vitro* studies with transfection of CARD15 plasmids into human embryonic kidney cells and MDP stimulation leads to activation of transcription factor nuclear kappa-B (NFκB) in mononuclear cells [78]. Increased levels and activity of NFκB have been observed in colonic mucosa of patients with active CD, and to a lesser extent in patients with active UC [101]. NFκB comprises a family of inducible transcription factors. It is an important regulator of immune- and inflammation-related genes, such as

cytokines and anti-apoptotic genes [102]. Especially, L1007fs has been demonstrated *in vitro* to be associated with a reduced NFκB activity [98, 99, 103, 104]. However, a L1007fs knock-in mouse model showed that carriage of L1007fs was associated with an elevated NFκB activity [105]. This mouse model is more in line with the clinical observation of elevated NFκB activity in patients with active CD [101]. Therefore, it is not clear whether CARD15 mutations lead to 'gain-of-function' or 'loss-of-function' [106].

A promising hypothesis is a decreased expression of intestinal-specific antibacterial α-defensins (cryptdins) from Paneth cells [86, 100]. Paneth cells are specialised epithelial cells located in the mucosal crypts of Lieberkühn and thought to play a protective role in the host against enteric flora [107]. Paneth cells show a high CARD15 expression in CD-affected terminal ileum, but weak expression in normal ileal mucosa of CD-affected patients and non-IBD individuals [86]. Moreover, in CD-affected terminal ileum a diminished expression was found of defensin-5 and defensin-6, which are secreted by Paneth cells. CARD15 L1007fs carriage showed a further defensin deficiency [108, 109].

Meanwhile, other CARD15 involving pathways are explored, for example synergy between CARD15 and specific TLRs, which are membrane bound and similar to CARD15 recognising conserved motifs from microbial pathogens. CARD15 compound heterozygous CD-affected individuals show a loss of synergy between CARD15 and TLR-9 (expressed in Paneth cells) with respect to TNFα and IL-8 [110, 111]. Another possible disease pathway is an altered intestinal barrier function. This can be harmed due to an increased intestinal permeability, which is found in patients with CD and in their healthy relatives. Carriage of CARD15 L1007fs appeared to be associated with an increased intestinal permeability in healthy first-degree relatives [45, 112], except for one study [113].

INTERLEUKIN-10

Function IL-10 is usually expressed in response to a stimulus in macrophages, monocytes, dendritic cells, B lymphocytes and CD4+ regulatory T cells. It is an anti-inflammatory cytokine and initially named as cytokine synthesis inhibitory factor. It influences the expression of several cytokines, soluble mediators and cell surface molecules by binding to the IL-10R of cells of the myeloid origin. For example, IL-10 can down-regulate the production of pro-inflammatory cytokines TNFα, IFNγ, IL-1 and IL-6, which are first-line cytokines in a Th-1 driven inflammatory response. IL-10 also inhibits (the expression of) chemokines, prostaglandin E2 and MHC class II antigens. Furthermore, TLR-4 (LPS signal transducing receptor) is down-regulated and specific scavenger receptors are up-regulated. One of the transcriptional factors activated following stimulation of IL-10R is signal transducer and activator of transcription 3, which can inhibit NFκB. All together, IL-10 induces differentiation of macrophage-like cells limiting an ongoing immune response and inflammation, and

contributes to clearance of the infection through enhanced phagocytosis. It regulates the innate and adaptive immune response [114-116].

IL-10 is considered to play an important role in IBD based on animal studies. IL-10 gene knock-out mice spontaneously develop chronic enterocolitis, resembling CD in humans [117]. However, in a germ-free environment these IL-10 -/- mice have no signs of disease [118]. IL-10 is effective in preventing and treating spontaneous enterocolitis. Therefore, IL-10 seems to be an important cytokine for modulation of the immune response maintaining mucosal tolerance against the normal intestinal bacterial flora [115]. Subcutaneously administered recombinant IL-10 has been evaluated in four clinical trials in CD-affected patients with inconsistent results [119-122]. It has been suggested that a subgroup of CD patients might benefit from IL-10 administration [120]. Maybe treatment with genetically modified bacteria expressing IL-10 for mucosal delivery will in the future provide an alternative treatment option [123].

Gene The IL-10 gene is located on chromosome 1q32.1. Three SNPs (-1082G/A, -819C/T and -592C/A) and two proximally located microsatellite polymorphisms (IL-10.G and IL-10.R) have been defined [124-127]. The -1082G allele is associated with a higher IL-10 production of stimulated T cells and monocytes, and therefore its presence might protect against IBD [127]. In IBD patients, the frequency of -1082G was lower compared with controls [128], particular in UC-affected patients [128, 129], which fits this hypothesis. However, these results could not be replicated [130-133]. Allele -1082G was found to be associated with CD, especially with combined carriage of the IL-10.G14 allele [134], which is a high IL-10 producing allele [135]. The low IL-10 producing -1082 A/A genotype showed association with steroid dependency in CD and UC [129]. Genotype -1082 G/G was associated with complicated disease behaviour in CD [136]. Based on its function, the IL-10 gene seems to be a good candidate for susceptibility to IBD [114, 137]. However, candidacy is unlikely concerning the conflicting results with respect to the polymorphisms and the microsatellite markers. Moreover, no linkage has been found between IL-10 and IBD [138].

There is support for a relation between IL-10 production and carriage of CARD15 mutations. *In vitro* stimulation of mononuclear cells with a TLR-2 ligand results in a significantly reduced IL-10 production in patients with CD homozygous for CARD15 L1007fs compared with CD-affected patients and healthy controls homozygous for the wild-type CARD15 allele [139]. It is likely that this effect is not caused by differences in carriage of the high and low IL-10 producing alleles, as in a Spanish study with CD-affected patients no association was found between carriage of high IL-10 producing alleles (-1082G, IL-10G14 or both) and at least one common CARD15 mutation [140].

GENES UNDER RESEARCH

Based on the results of the genome screens, several new putative IBD susceptibility genes are being evaluated. The IBD5 locus (5q31), which showed to be associated with susceptibility to CD [141, 142], harbours the organic cation transporters genes OCTN1 and OCTN2. Their related proteins are transporters of carnitine and organic cations. Two mutations within these genes (1672C → T in exon 9 of OCTN1, and -207G → T in the promoter of OCTN2) are together as a two-allele risk haplotype associated with CD [143-145]. Association of nucleotide variants within the Drosophila discs large homologue 5 (DLG5) gene with CD was found by positional cloning of the IBD linkage region 10q23 [146]. DLG5 is thought to play a role in maintaining the epithelial cell structure and transmitting transmembrane signals [147]. DLG5 mutations might be involved in intestinal permeability, leading to an increased IBD risk [106]. The CARD4 gene is part of an IBD susceptibility locus on chromosome 7p14. Its encoded protein is similar in structure and function to CARD15. Genetic variants of the CARD4 gene are associated with IBD [148]. Evaluation of TLRs in IBD is based on their key role in the innate immune system. Moreover, in a colitis mouse model TLR-4 antagonist can prevent development of colitis [149]. Two polymorphisms within this candidate gene (Asp299Gly and Thr399Ile) have been evaluated in IBD with variable results [74]. Very recently, in three case-control cohorts IBD showed association with nucleotide variants of the myosin IXB (MYO9B) gene, especially in UC-affected patients. This gene is thought to play a role in intestinal permeability and bacterial invasion of the epithelium [150]. It is located on chromosome 19p13, which region is linked with IBD (IBD6 locus) [72, 142].

New methods to identify susceptibility genes are genome-wide linkage and association studies using a very large amount of SNPs (up to 500,000) [69, 74, 151]. These studies can be performed due to the outcome of the International Human Haploptype Map Project (HapMap). This project has determined common patterns of DNA sequence variations of the humane genome, including SNPs [152]. With the use of microarrays these multiple SNPs can be genotyped (SNP chips) [153]. Very recently, the first genome-wide association study in IBD have shown a highly significant association between CD and the IL-23R gene [154].

Otherwise, microarrays can evaluate the expression of many genes of interest, with studies in IBD performed on 1,000–20,000 genes [74]. Another approach is proteomic analysis. It evaluates expressed proteins, and its related genes [74]. The use of bioinformatic tools to combine large amounts of data resulting from various approaches may lead to the identification of susceptibility and modifier genes in IBD [155].

DESIGN OF THIS THESIS

Aim

The initiative for this thesis started within the departments of Gastroenterology and Hepatology, and Internal Medicine. As described above, genetic susceptibility plays an important role in the aetiology of IBD. Insight can provide a better understanding of the pathogenesis, and may lead to improvement of IBD therapy. The primary study goal of this thesis was to search of mutations in inflammation-related genes in patients with IBD. The study was approved by the Institutional Review Board of the Erasmus MC UMCR.

Participants

Patients meeting standard criteria of IBD [18] and attending the Erasmus MC UMCR, were systematically registered and asked whether they had IBD-affected relatives. In case of a positive family history, patients (probands), their affected relatives and some non-affected relatives were offered to participate in the study. Clinical information was collected and a pedigree was drawn up based on a questionnaire, which was completed by the participants. Most of the affected relatives and a few probands did not attend the Erasmus MC, but other Dutch hospitals. Their diagnosis of IBD was verified, based on medical and surgical information supplied by their treating physician. Participants were asked to provide blood for DNA testing. All participants provided written informed consent. Also healthy anonymous Caucasian individuals were included, which served as non-related non-IBD-affected controls.

IBD database

From 1998 until the end of 2002, 1193 patients with IBD were included. Most of them were Caucasian (94.1%). Details are presented in Table 1.

Research methods

For the genetic study presented in chapters 2, a linkage analysis with candidate genes and microsatellite markers was used. We evaluated specifically individual sib-pairs, focusing on those pairs who shared common alleles. In sib-pairs who share no alleles, it is unlikely that the gene of interest plays an (important) role in the disease pathogenesis. In contrast, the change of gene involvement in sib-pairs who share both alleles is a priori higher. In chapter 3, this concept (allele-sharing method) was used to search for mutations in the IL-10 gene by sequencing the IL-10 gene in the 'high change' sib-pairs. IL-10 is an IBD candidate gene because of its anti-inflammatory effect as discussed above.

The study methods presented in chapters 4, 6 and 7 are based on association analysis with candidate genes, i.e. the IL-10 and CARD15 genes.

In chapter 8, genetic anticipation is evaluated using standard statistical methods.

OUTLINE OF THIS THESIS

This thesis includes molecular and clinical genetic studies in Dutch familiar and sporadic patients with IBD. Most of the chapters have been published or accepted as a scientific paper in established gastroenterology journals.

In **chapter 2**, a linkage analysis study is presented, including inflammation-related cytokine genes in IBD-affected sib-pairs. The results were used for further detailed studies on IL-10 in those sib-pairs who showed allele-sharing.

Chapter 3 describes the finding of a Gly15Arg mutation in the IL-10 gene within a multiple CD-affected family. Its biological function was further evaluated in an *in vitro* stimulation test with mononuclear cells and a monocytic cell line. The mutation appeared to lead to a decreased IL-10 secretion.

In **chapter 4**, a multicentre survey is presented about the prevalence of the Gly15Arg mutation in a large group of Dutch patients with CD and in healthy unrelated controls.

Chapter 5 reviews the role of CARD15 in CD based on the literature published until 2002. An update of recent data is given in this chapter.

Chapter 6 focuses on the CARD15 3020insC (L1007fs) mutation, which is highly associated with CD. In the presented study, the prevalence of the mutation is evaluated in IBD-affected families and healthy unrelated controls. One family is described in detail, as non-IBD-affected relatives appeared to be homozygous carriers of 3020insC.

In **chapter 7**, the prevalence of the three common CD-associated CARD15 mutations (R702W, G908R and L1007fs) is evaluated in a large group of multiple IBD-affected families, sporadic IBD-affected patients and unrelated healthy controls.

Chapter 8 presents a study regarding genetic anticipation in patients with IBD.

Chapter 9 includes a summary and discussion of the presented studies. Furthermore, future perspectives are given.

Chapter 10 includes a summary in Dutch of this thesis.

Table 1. Characteristics of the Rotterdam inflammatory bowel disease database

	N or years of age (range)
Crohn's disease	
Males	269
Females	441
Mean age	44.3 (17.9 – 90.9)
Ulcerative colitis	
Males	239
Females	191
Mean age	45.9 (12.5 – 86.2)
Indeterminate colitis	
Males	22
Females	31
Mean age	45.9 (18.7 – 84.0)
Patients with familial IBD	
Confirmed familial disease	138
Unconfirmed familial disease [a]	127
Sporadic patients with IBD [b]	928

[a] IBD-affected relatives of which the diagnosis could not be confirmed as no medical and surgical information of their treating physician was available.

[b] Data also include probands which were not interviewed about familial disease (n = 33).

IBD, inflammatory bowel disease.

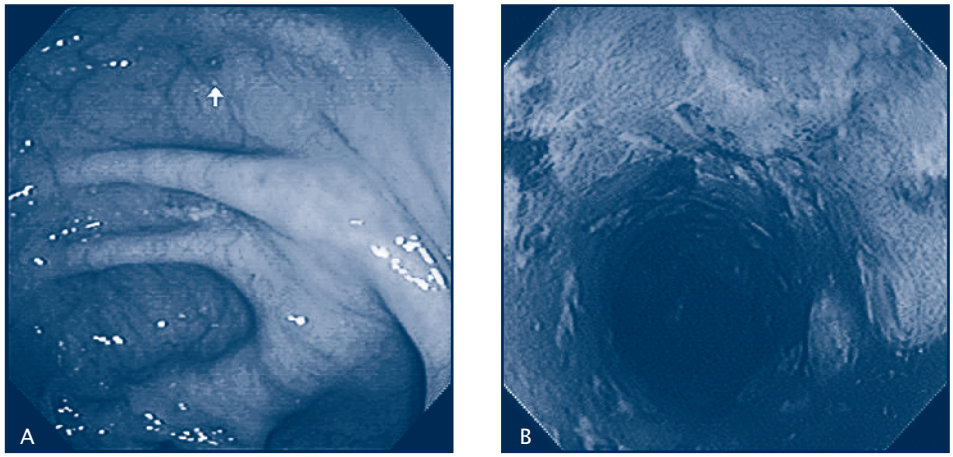

Figure 1. Endoscopic views of inflammatory bowel disease. **A**, early Crohn's disease of the colon with a small aphthous lesion. **B**, moderate severe active ulcerative colitis, characterised by fragile continuous mucosal inflammation with loss of vascular pattern and with loss of haustration.

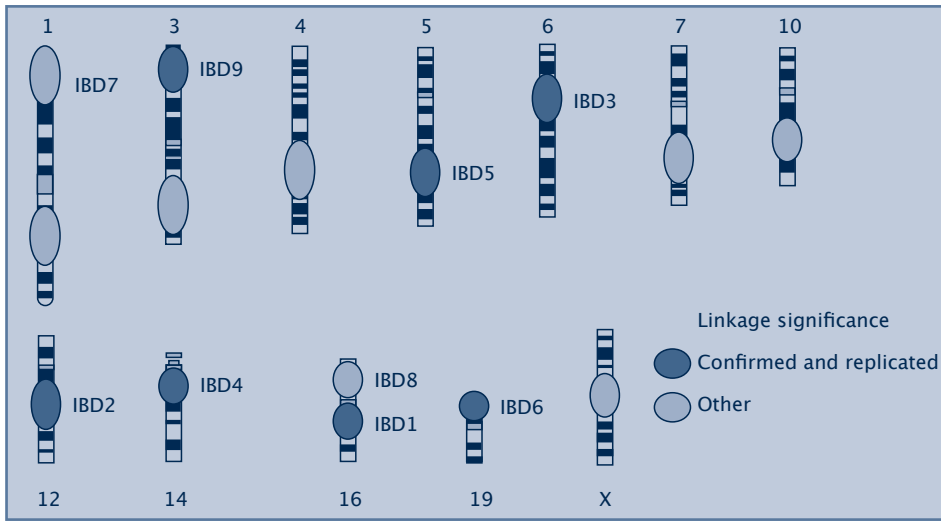

Figure 2. Linkage areas in inflammatory bowel disease [64].

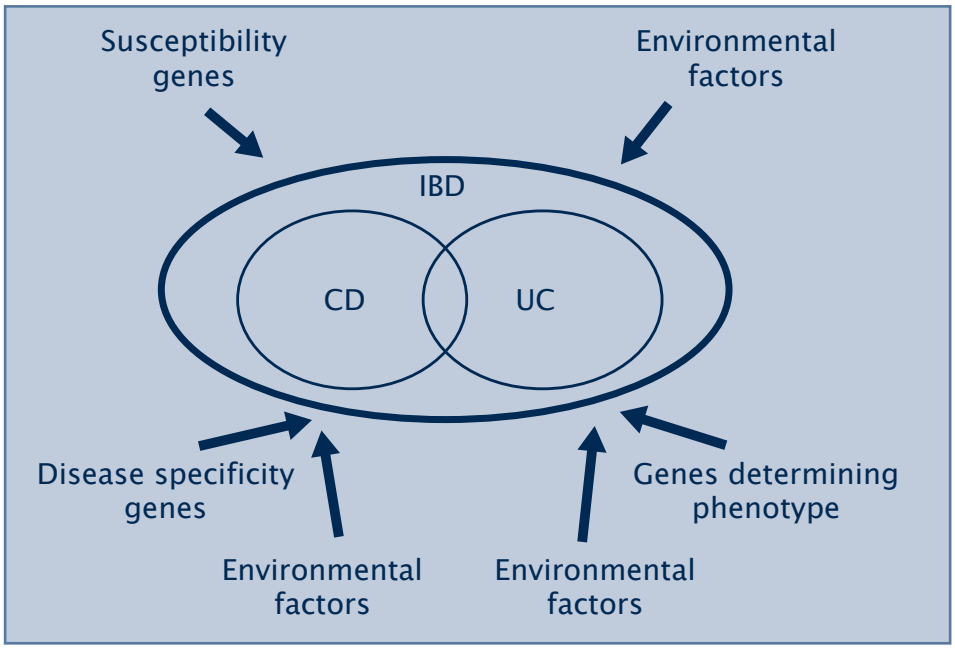

Figure 3. A schematic figure of the current genetic model in inflammatory bowel disease (IBD). Specific genes combined with environmental factors lead to either Crohn's disease (CD) or ulcerative colitis (UC). Other modifier genes are responsible for specific phenotypes once the disease is present [64, 66].

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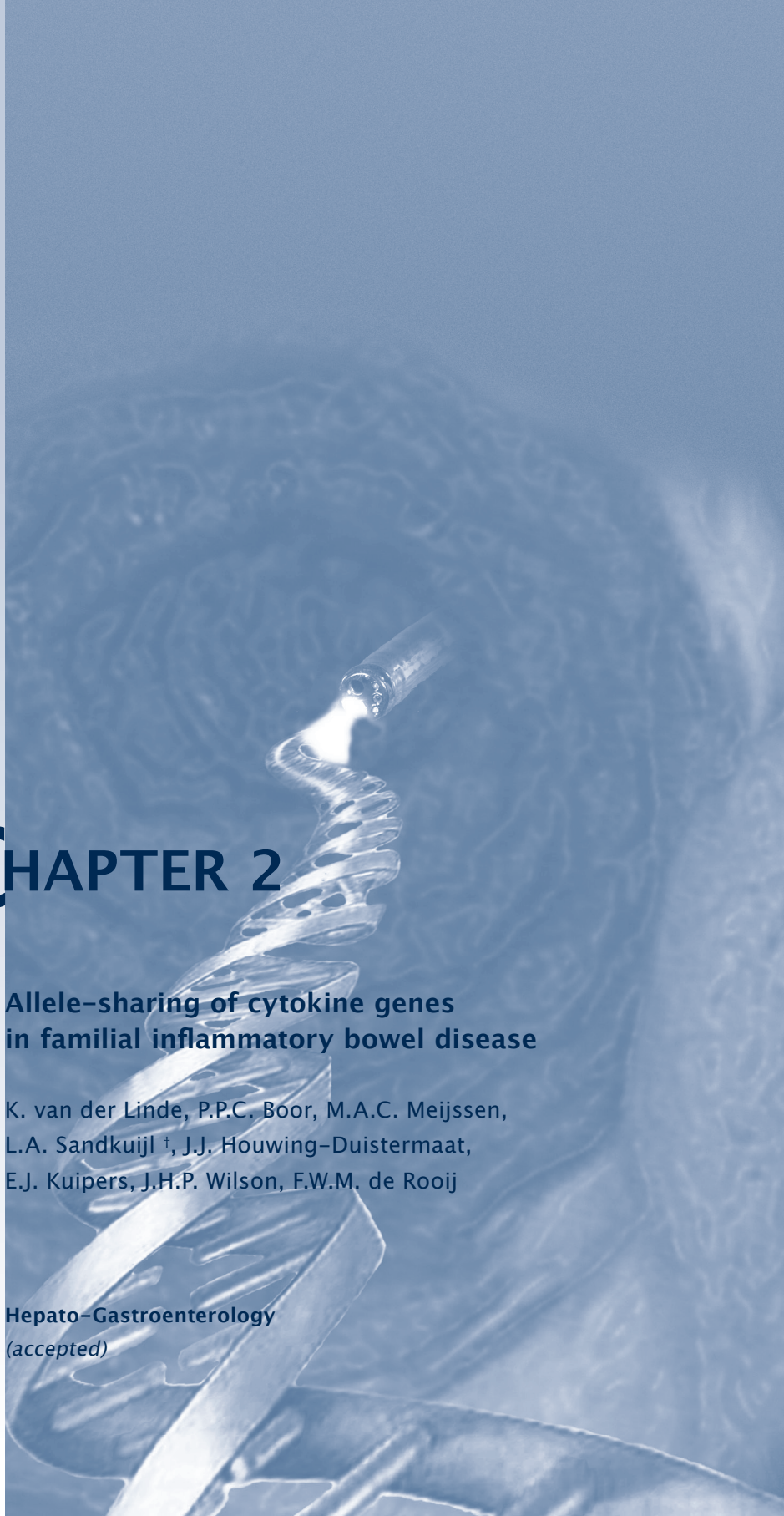

CHAPTER 2

Allele-sharing of cytokine genes in familial inflammatory bowel disease

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ABSTRACT

Background

The pathogenesis of inflammatory bowel disease (IBD) is complex, multifactorial, and involves genetic predisposition. This predisposition is likely to include various chromosomal loci, but simple Mendelian inheritance cannot be excluded in a subset of families with IBD.

Methods

We evaluated allele-sharing in 17 sib-pairs with IBD as an approach to select candidate genes for further studies in individual families. It was determined whether each sib-pair had inherited the same alleles for interleukin-2 (IL-2), IL-2 receptor β (IL-2Rβ), IL-4, IL-4R, IL-10, IL-10R, tumour necrosis factor α (TNFα), TNFα-R1 and TNFα-R2.

Results

The results were very different per individual family. The estimated probability of sharing both alleles identical-by-descent at IL-4R, IL-10, IL-10R and TNFα were 50%, 39%, 40%, and 33% respectively. The LOD score was significant for IL-4R ($P = 0.04$).

Conclusion

In this small group of sib-pairs with IBD a modestly increased allele-sharing was found for some inflammation-related genes. Different results per family may suggest genetic heterogeneity. This method can be useful as a first step to further evaluation of specific candidate genes which may play a pathogenetic role in individual families.

INTRODUCTION

The pathogenesis of inflammatory bowel disease (IBD) is complex and multifactorial. Besides imunological and environmental factors, genetic predisposition plays an important role in the susceptibility to the two major phenotypes of IBD, Crohn's disease (CD) and ulcerative colitis (UC). Epidemiological evidence for genetic involvement includes ethnic differences in the frequency of disease and familial aggregation [1]. The most convincing genetic evidence is a higher disease concordance rate in monozygotic twins than in dizygotic twins [2, 3].

The search for IBD susceptibility genes has been performed using either a genome-wide screening strategy or a candidate gene approach. Highly polymorphic anonymous genetic markers (microsatellite markers) are used to flag chromosomal regions of interest, which include susceptibility genes. In genome screens, multiple informative markers are used spanning (almost) the whole genome. In candidate gene studies, linkage is evaluated by using polymorphisms or microsatellite markers in or around susceptibility genes [4]. In most studies affected sib-pairs are used, and in the analysis no restrictions are made regarding the mode of inheritance (non-parametric). When a region or gene harbours a genetic factor involved in the aetiology of the trait, affected sib-pairs will share more alleles identical-by-descent than under random segregation [5].

At present, the genetic aetiology of IBD is based on polygenic models of inheritance with environmental stimuli. Although IBD is probably heterogenetic, simple Mendelian inheritance in subsets of IBD should not be precluded [6, 7]. The results of genome studies in IBD, which are based on the overall data of many familial pairs [1], may blur the genetic impact of certain genomic regions and corresponding genes in individuals with IBD and their IBD-affected family members.

In IBD, mucosal inflammation is characterised by an exaggerated and sustained immune response due to a dysregulated production and interaction of pro-inflammatory and anti-inflammatory cytokines and cytokine receptors [8]. In this study we evaluated identical-by-decent sharing of marker alleles at candidate cytokine (receptor) genes in a relatively small group of IBD-affected sib-pairs.

MATERIALS AND METHODS

Patients

Dutch Caucasian families with two or more siblings with either CD or UC, and their non-affected parents were recruited by interviewing IBD-affected patients attending the Erasmus MC University Medical Centre Rotterdam (UMCR). When one or both parents were not available, non-affected siblings were included. Probands, affected relatives and non-affected relatives completed a questionnaire about IBD. Based on information supplied by their physician the diagnosis was verified according to

standard criteria [9]. The study was approved by the Institutional Review Board of the Erasmus MC UMCR. All participants gave written informed consent.

DNA isolation

Genomic DNA was isolated from whole peripheral venous blood collected in ethylenediaminetetraacetic acid anticoagulated tubes (Becton Dickinson, Leiden, The Netherlands) using standard techniques. In some instances DNA was isolated from Epstein-Barr virus transformed lymphoblastoid cell lines [10], derived from peripheral venous blood collected in acid citrate dextrose solution containing tubes (Becton Dickinson).

Genotyping

Nine well defined inflammation-related cytokine genes [8] and the IBD2 locus on chromosome 12 [1, 11] were evaluated. The genes and locus are summarised in Table 1. To identify these genes for further statistical analysis, microsatellite markers located within or close to these genes were used. If the heterozygosity of a marker was < 0.80 or unknown, additional markers from the same region were tested if possible. For the interleukin-10 (IL-10) gene, only one marker was used with a heterozygosity of 72%. Amplification of the microsatellite markers was performed by polymerase chain reaction (PCR) on a PCR thermocycler (PCT 200, Biozyme, Landgraaf, The Netherlands). PCR conditions were chosen according to the literature (see Table 1), sometimes with minor modifications. PCR fragment analyses was performed with an ABI 310 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) supported by Genescan (version 2.1.1) and Genotyper (version 2.1) software program.

Allele-sharing and statistical analysis

For each pair of IBD-affected siblings the probability of sharing zero, one or two alleles identical-by-descent at the cytokine (receptor) gene was determined. When parental genotypes were missing, the allele frequencies estimated from the set of non-affected parents were used. Under random (Mendelian) segregation, the probabilities of sharing zero, one or two alleles identical-by-descent are 25%, 50% and 25%, respectively. For the IBD-affected sib-pairs in this study, the probabilities of sharing zero, one or two alleles identical-by-descent were estimated by maximising the log likelihood function under the possible triangle constraints using Mapmaker/sibs software program [12, 13].

RESULTS

Patients

Fifteen families including 17 IBD-affected sib-pairs were examined in this study. In five pairs both siblings had CD, in seven pairs both had UC, and in five pairs one individual had CD and the other UC (mixed families). One family included three UC-affected siblings of which two females were a dizygous twin. Twenty-seven non-affected parents and three non-affected siblings were included. The affected sib-pairs are summarised in Table 2.

Allele-sharing of cytokine (receptor) genes

For each sib-pair the probability of sharing alleles at the nine cytokine (receptor) genes and the IBD2 locus are presented in Table 2. As expected, the results appeared to vary between families. In Table 3 the maximum likelihood estimates of the probabilities of sharing alleles identical-by-descent are given for the tested markers. Here, IL-4 was excluded because it appeared to be non-informative. For IL-4 receptor (IL-4R), IL-10, IL-10R and tumour necrosis factor α (TNFα) the probability of sharing two alleles identical-by-descent were higher than expected, namely 50%, 39%, 40% and 33% respectively. In this group, only the IL-4R showed a significant LOD score of 0.95 ($P = 0.04$).

Allele-sharing of IBD2 locus

The allele-sharing results for the chromosomal marker D12S83, corresponding with the IBD2 locus, was not different from what could be expected based on Mendelian inheritance.

DISCUSSION

The genetic basis of IBD is considered to be heterogeneous, meaning that involvement of several altered genes or interaction between specific genes may lead to similar clinical phenotypes. The results of the recent published genome screens in IBD support this theory, and suggest that the pathogenesis is unlikely to be of monogenetic origin [1]. The same conclusion can be drawn from the results of candidate gene studies in IBD, which are focused on the involvement of specific genes in the disease pathogenesis. Well studied genes are those of the major histocompatibility complex, including the human leukocyte antigen class II molecules and TNFα [14], and several pro-inflammatory and anti-inflammatory cytokine genes [15-18].

In this study, allele-sharing of four (anti-)inflammation-related cytokine genes and their receptors were evaluated in a relatively small group of 17 affected sib-pairs with IBD. A low but near significant LOD score was found for the IL-4R gene. This gene is located within a pericentromeric locus on chromosome 16, which was

the first genomic susceptibility region of interest described by Hugot et al. in 1996. This so-called IBD1 locus appeared to be linked with CD [19]. In candidate gene studies, however, the IL-4R gene did not appear to be associated with IBD [20-22]. In the meantime, linkage of the IBD1 locus with CD has been found to be based on genetic alterations in the CARD15 gene [23, 24].

The allele-sharing method is based on the concept that when a gene is disease-involved, sib-pairs will share more alleles identical-by-descent than under no association between disease and gene. Although the statistical power of this method is low, and hence a large group of sib-pairs is necessary to achieve statistical significance, the individual results of sib-pairs may also give insight in the heterogenetic concept of the IBD pathogenesis. For example, in this study the allele-sharing results of the IL-10 gene (chromosome 1q) suggest a weak (non-significant) linkage with IBD. In 39% of the included sib-pairs both siblings shared two alleles (the maximum likelihood estimate under the triangle constraints). Although no significant LOD score was found for IL-10 in the whole group, it might still be worthwhile to look for mutations in those sib-pairs who share both alleles. In these specific sib-pairs the chance of IL-10 involvement in the disease pathogenesis is relatively high. Otherwise, in those sib-pairs who do not share any allele it is unlikely that the IL-10 will play a pathogenetic role. Therefore, searching for mutations in these individual families is probably not worthwhile. By following this strategy, we recently found a functional IL-10 mutation in two multiple CD-affected families [25, 26].

In conclusion, this study evaluates allele-sharing of several cytokine (receptor) genes in a relative small group of IBD-affected sib-pairs. A significant increased identical-by-decent allele-sharing was found for IL-4R. By using this approach to select and further analyse candidate genes in individual multiple IBD-affected families, it is possible to find (functional) mutations. The various results between sib-pairs are suggestive for the presumed heterogenetic pathogenesis of IBD. In certain families, involvement of specific cytokine genes may be more important than in other families. Functional assays and detailed DNA analysis of corresponding candidate cytokines and their genes should be focused on sib-pairs who share both parental alleles.

Table 1. Evaluated chromosomal locus and candidate genes

Gene / locus [a]	Chromosome	Function	Marker name	Heterozygosity	Sequence	Reference
IL-2	4q26-q27	pro-inflammatory	-	89%	forward '5-AAAGAGACCTGCTAACACA-3' reverse '5-CCTATGTTGGAGATGTTTAT-3'	27
IL-2Rβ	22q11.2-q12	pro-inflammatory	-	91%	forward 5'-GAGAGGGAGGGCCTGCGTTC-3' reverse 5'-CACCCAGGGCCAGATAAAGA-3'	28
IL-4	5q31.1	anti-inflammatory	-	49%	forward 5'-CTCAAAGTGCTGGGATTAGC-3' reverse 5'-AGCCATCTCGGTTGGATGGA-3'	29
				61%	forward 5'-ATGGCAGATAGGTCCACCGG-3' reverse 5'-TCATCCTCATCTGTTGTAGC-3'	30
IL-4R	16p12.1-p11.2	anti-inflammatory	D16S313	57%	forward 5'-TGACTCTTTTCGTCAACACAGC-3' reverse 5'-TATTCAAATACAAGCAACCGTG-3'	31
			D16S298	79%	forward 5'-TTCCTCATGTATAAATTGGGTGTGGCCA-3' reverse 5'-ACCTCCCGGCCCAATCCCAATGCTT-3'	32
IL-10	1q31-q32	anti-inflammatory	IL-10G	72%	forward 5'-GTCCTTCCCCAGGTAGAGCAACACTCC-3' reverse 5'-CTCCCAAAGAAGCCTTAGTAGTGTTG-3'	33
IL-10R	11q23.3	anti-inflammatory	D11S939	68%	forward 5'-TCAGTTTCCTCATCCACCAA-3' reverse 5'-AAAAGCACATATTTTGCTCAGAGTC-3'	internet [b]
			D11S1340	unknown	forward 5'-TGGCCTCTTGTCACTCCTA-3' reverse 5'-TGCTCTTCAAAGCAAAATG-3'	internet [b]
			D11S1341	66%	forward 5'-GCTGAATGAGTCCTGAGTAATAA-3' reverse 5'-GGCCTAGACGTTCTTTTGTG-3'	internet [b]
TNFα	6p21.3	pro-inflammatory	-	unknown	forward 5'-GCCTCTAGATTTCATCCAGCCACA-3' reverse 5'-CCTCTCTCCCCTGCAACACACAC-3'	34
			-	unknown	forward 5'-CATAGTGGACTCTGTCTCCAAAG-3' reverse 5'-AGATCCTTCCCTGTGAGTTCTGCT-3'	34
TNFαR1	12p13.2	pro-inflammatory	D12S1673	58%	forward 5'-GAGGTGCATTTTCCAGGG-3' reverse 5'-GGGTCCAGCATGTTCATC-3'	internet [b]
			D12S1695	76%	forward 5'-TGTGAGCCACTGCACC-3' reverse 5'-GCCTTCAAAACCATAGTACAATAAC-3'	internet [b]
TNFαR2	1p36.2	pro-inflammatory	D1S434	61%	forward 5'-AGCTAATTTACATTACCCAAAAAGA-3' reverse 5'-GCAGGTGGCACAGTGA-3'	35
			-	54%	forward 5'-GTGATCTGCAAGATGAACTCAC-3' reverse 5'-ACACCACGTCTGATGTTTCA-3'	35
IBD2	12	-	D12S83	80%	forward 5'-TTTTTGGAAGTCTATCAATTTGA-3' reverse 5'-TAGCAGAGAAAGCCAATTCA-3'	internet [b]

[a] IBD2, IBD2 locus; IL, interleukin; TNF, tumour necrosis factor; R, receptor.
[b] Website www.genethon.fr

Table 2. Allele-sharing identical-by-descent in sib-pairs with inflammatory bowel disease

Allele-sharing (%) in IBD-affected sib-pairs (n = 17) [b]																		
Gene / locus[a]	Number of shared alleles	CD-CD	CD-CD	CD-CD	CD-CD	CD-CD	UC-UC	UC-UC	UC-UC	UC-UC	UC-UC	UC-UC	UC-UC	Mixed	Mixed	Mixed	Mixed	Mixed
IL-2	0	19	0	9	0	0	0	0	0	100	0	0	0	100	0	100	0	0
	1	81	11	91	100	100	100	100	100	0	50	50	50	0	100	0	1	0
	2	0	89	0	0	0	0	0	0	0	50	50	50	0	0	0	0	100
IL-2Rβ	0	100	0	27	50	0	0	0	100	0	0	0	0	100	0	100	0	100
	1	0	100	73	50	100	0	0	0	100	50	50	50	0	0	0	100	0
	2	0	0	0	0	0	100	100	0	0	50	50	50	0	100	0	0	0
IL-4	0	24	24	20	25	25	25	25	50	50	25	25	25	50	50	25	25	50
	1	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
	2	26	26	30	25	25	25	25	0	0	25	25	25	0	0	25	25	0
IL-4R	0	0	0	18	0	0	0	50	0	100	0	0	0	98	0	50	50	98
	1	15	100	82	2	0	0	50	0	0	0	0	0	2	0	50	50	2
	2	85	0	0	98	100	100	0	100	0	100	100	100	0	100	0	0	0
IL-10	0	0	0	26	0	100	0	50	100	50	0	0	0	0	0	25	100	100
	1	100	34	74	100	0	0	50	0	50	0	0	0	0	0	50	0	0
	2	0	66	0	0	0	100	0	0	0	100	100	100	100	100	25	0	0
IL-10R	0	-	82	8	2	0	0	0	0	0	0	0	0	0	0	-	0	-
	1	-	18	92	98	100	100	2	100	100	0	100	100	100	0	-	98	-
	2	-	0	0	0	0	0	98	0	0	100	0	0	0	100	-	2	-
TNFα	0	50	2	0	0	98	0	0	48	0	0	0	0	0	0	100	98	0
	1	50	98	8	2	2	0	100	4	100	0	100	100	0	100	0	2	100
	2	0	0	92	98	0	100	0	48	0	100	0	0	100	0	0	0	0
TNFαR1	0	0	10	20	98	76	86	0	88	88	0	98	98	0	0	0	77	0
	1	100	90	80	2	22	14	0	12	12	2	2	2	98	12	50	22	50
	2	0	0	0	0	2	0	100	0	0	98	0	0	2	88	50	1	50
TNFαR2	0	100	50	98	0	2	0	0	2	50	0	98	98	0	0	0	0	0
	1	0	50	2	100	98	100	50	96	50	2	2	2	4	100	98	0	98
	2	0	0	0	0	0	0	50	2	0	98	0	0	96	0	2	100	2

[a] IL, interleukin; TNF, tumour necrosis factor; R, receptor.

[b] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; Mixed, one sibling affected with CD and the other with UC.

Table 3. Allele-sharing identical-by-descent in sib-pairs with inflammatory bowel disease (n = 17)

Genes [a]	Allele-sharing (%) [b]			LOD score
	0	**1**	**2**	
IL-2	25	50	25	0
IL-2Rβ	25	50	25	0
IL-4R	17	33	50	0.95
IL-10	20	41	39	0.27
IL-10R	10	50	40	0.25
TNFα	22	45	33	0.1
TNFαR1	25	50	25	0
TNFαR2	25	50	25	0

[a] IL, interleukin; TNF, tumour necrosis factor; R, receptor.

[b] The probabilities of sharing zero, one or two alleles identical-by-descent were estimated by maximising the log likelihood function under the possible triangle constraints.

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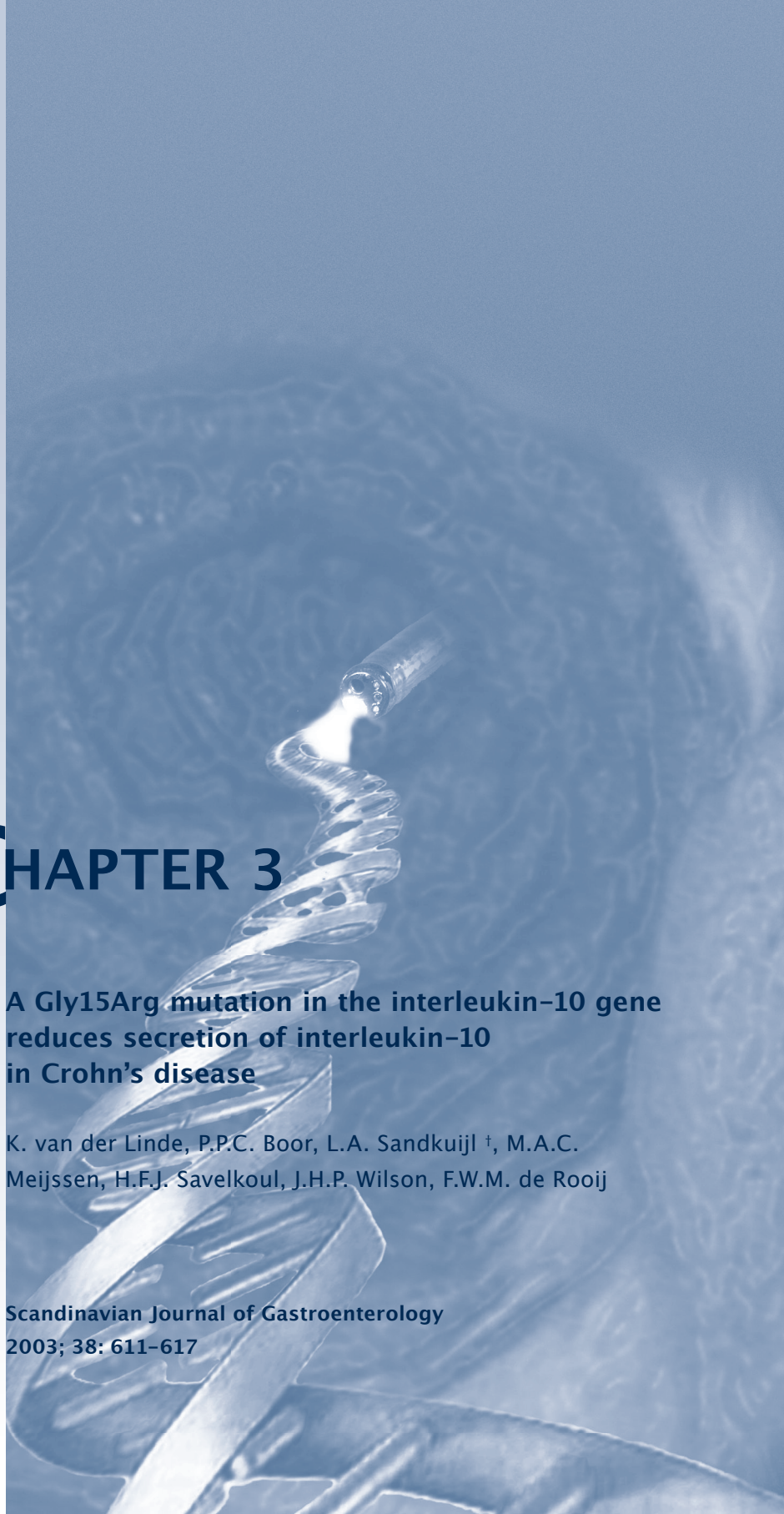

CHAPTER 3

A Gly15Arg mutation in the interleukin-10 gene reduces secretion of interleukin-10 in Crohn's disease

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ABSTRACT

Background

Genetic susceptibility, probably involving cytokines and their receptors, plays an important role in inflammatory bowel disease (IBD). In this study we examined the potential role of the interleukin-10 (IL-10) gene as a susceptibility gene in IBD.

Methods

We studied 17 sib-pairs with either Crohn's disease (CD) or ulcerative colitis. After microsatellite analysis for allele-sharing, the IL-10 gene of sib-pairs who shared alleles was screened for nucleotide alterations in and around exons and the promoter region. The IL-10 promoter polymorphism at position -1082 was also determined. Function was evaluated by measuring IL-10 secretion by peripheral blood mononuclear cells stimulated with lipopolysaccharide or phorbol ester. The activity of recombinant immature wild-type and mutated IL-10 was tested in a proliferation assay with a human monocytic leukaemia cell line (HL60 cells).

Results

DNA sequencing revealed a G → A point mutation in exon 1 at base position 43 in one sib-pair, both affected with CD. It was also found in two of their healthy siblings, but not in 75 unrelated healthy controls. This mutation results in a glycine to arginine substitution at amino acid position 15 of the leader sequence (Gly15Arg). The *in vitro* IL-10 secretion by mononuclear cells of the IL-10 Gly15Arg carriers was about 50% of healthy controls, matched for the -1082 polymorphism in the IL-10 promoter region. Incubation of HL60 cells with recombinant mutated IL-10 showed a markedly reduced cell proliferation compared with wild-type IL-10.

Conclusion

A Gly15Arg mutation in the leader sequence of IL-10 was found in a multiple CD-affected family. This altered leader sequence decreases IL-10 secretion, thereby reducing the anti-inflammatory effect.

INTRODUCTION

Genetic predisposition plays an important role in the pathogenesis of chronic inflammatory bowel disease (IBD). It seems increasingly likely that IBD is not a single disease but rather a group of aetiologically and genetically distinct diseases that have similar clinical presentations [1]. During past years, many attempts have been made to identify susceptibility loci for IBD, by using either a genome-wide linkage analysis with multiple highly polymorphic microsatellite markers, or association studies with candidate and positional genes [2]. The available data suggest that genetic susceptibility for IBD is heterogeneous, and probably not inherited in a simple monogenetic Mendelian fashion, although this type of inheritance might apply to subgroups [2, 3].

In IBD, the mucosal inflammation is associated with an exaggerated and prolonged immune response because of a dysregulated production and interaction of pro-inflammatory and anti-inflammatory cytokines and their receptors [4]. Interleukin-10 (IL-10) inhibits the synthesis of several pro-inflammatory cytokines derived from activated monocytes and macrophages such as tumour necrosis factor alpha and IL-1 [5]. The IL-10 gene knock-out mouse spontaneously develops a chronic enterocolitis [6]. The IL-10 gene is therefore of special interest for a candidate gene approach in IBD.

Linkage of the IL-10 gene with IBD was not demonstrated in a large group of sib-pairs [7]. However, recently three biallelic polymorphisms have been identified within the IL-10 gene promoter region. One polymorphism was associated with either a high (-1082*G) or low (-1082*A) IL-10 production of *in vitro* stimulated peripheral lymphocytes [8]. Tagore and co-workers found a decreased frequency of this high IL-10 producer allele in patients with IBD, and suggested that the lower IL-10 responses could contribute to the chronic intestinal inflammation [9]. These results were not confirmed by others 10. Treatment with recombinant IL-10 does not appear to be effective [11-13]. However, the possibility that mutations in the IL-10 gene may play an important role in a small percentage of patients is not excluded by the above studies.

In the present study, we examined the potential role of the IL-10 gene in 17 IBD-affected sib-pairs from 15 families. Sibs who share both alleles were considered to be the most likely candidates to harbour a mutation. After selecting suitable sib-pairs by IL-10 allele-sharing based on microsatellite analysis, we screened for exon nucleotide alterations and promoter polymorphisms. We identified a guanine (G) to adenine (A) substitution in exon 1 of the IL-10 gene. The functional consequences of this genetic alteration were tested *in vitro* using recombinant IL-10 (both wild-type and Gly15Arg) in a proliferation assay with LPS-stimulated HL60 cells which express IL-10 receptors. Secretion of mutated IL-10 was evaluated *in vitro* by stimulating monocytes and lymphocytes obtained from patients and controls.

MATERIALS AND METHODS

Patients and materials

Dutch Caucasian families with two or more siblings with either Crohn's disease (CD) or ulcerative colitis (UC) attending the Departments of Gastroenterology, Internal Medicine, and Surgery of the Erasmus MC University Medical Centre Rotterdam (UMCR) were recruited. Probands, affected siblings and non-affected first-degree relatives completed a questionnaire about IBD. Based on information supplied by their physician the diagnosis was verified according to standard criteria [14]. Seventy-five healthy Caucasian controls were also examined.

Genomic DNA was isolated from whole peripheral venous blood collected in ethylenediaminetetraacetic acid anticoagulated tubes using standard techniques. Blood collected in acid citrate dextrose solution containing tubes (Becton Dickinson, Leiden, The Netherlands) was used for DNA and RNA isolation from Epstein-Barr virus transformed lymphoblastoid cell lines [15]. All DNA sequence and polymerase chain reaction (PCR) fragment analyses were performed using an ABI 310 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) supported by Genescan (version 2.1.1) and Genotyper (version 2.1) software programs.

Peripheral blood mononuclear cells (PBMCs) were isolated from selected patients, their relatives and 22 healthy Caucasian controls for *in vitro* stimulation.

The study was approved by the Institutional Review Board of the Erasmus MC (UMCR). All participants gave written informed consent.

IL-10 genotyping

The microsatellite marker IL10.G was used for genotyping IL-10 alleles, as described by Eskdale and co-workers [16], with minor modifications in the PCR conditions, using a PCR thermocycler (PCT 200, Biozyme, Landgraaf, The Netherlands).

IL-10 gene sequence analysis

IL-10 exons were screened for nucleotide alterations by comparing its DNA sequence with the IL-10 gene sequence deposited in the Genbank of the National Centre for Biotechnology Information (NCBI) with accession number U16720 (unpublished data, internet http://www.ncbi.nlm.nih.gov). Primer pairs used to amplify the 5 exons, including the flanking intronic regions, are listed in Table 1. PCR fragments of the exons were sequenced with the forward primer, using a BigDye Terminator sequencing kit (Applied Biosystems). As discussed below, in one multiple affected family with IBD a point mutation was found in exon 1. Other participants were screened for this specific mutation, using the Mae I restriction enzyme. The PCR product of exon 1 was incubated with 1 unit of Mae I (Boehringer Mannheim, Almere, The Netherlands) at 45 ^{0}C for 4 hours (h), and then analysed on a 2% agarose electrophoresis gel. Bands were visualised with ethidium bromide staining.

IL-10 promoter analysis

IL-10 high and low secretion genotypes were determined by screening for promoter polymorphisms at position -1082 relative to the transcriptional start site. Positions -819 and -592 were also evaluated [8]. DNA amplification was performed by PCR, using the forward primer 5'-CCAAGACAACACTACTAAGGC-3', and the reverse primer 5'-GTCTCTGGGCCTTAGTTTCC-3'. This resulted in a PCR product of 629 base pairs, which was further sequenced with an internal reverse primer 5'-AAGCTTCTGTGGCTGGAGTC-3' to determine the -1082, and the 5'-GTCTCTGGGCCTTAGTTTCC-3' reverse primer to determine positions -819 and -592.

Excretion of IL-10 protein *in vitro* by stimulated PBMCs

Blood samples were taken between 09.00 and 11.00 h to minimise circadian variation [17]. PBMCs were prepared from heparinized blood within 3 h of venapuncture by Ficoll-Hypaque separation (Pharmacia, Uppsala, Sweden). PBMC's were cultured at 10^6 cells/mL in RPMI 1640 medium (Bio Whittaker Europe, Verviers, Belgium) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 50 µg/mL streptomycin, 1 mM pyruvate and 10% heat-inactivated foetal bovine serum (Gibco BRL Life Technologies, Breda, The Netherlands). Cells were stimulated in 24-well flat-bottom microtitre plates in triplicate separately with 1 µg/mL lipopolysaccharide (LPS) extracted from Escherichia (E.) coli O26:B6 bacteria (Difco, Detroit, Michigan, USA) and 10 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma, St. Louis, Missouri, USA) plus 1 µg/mL ionomycine (Sigma). After 24 h of incubating at 37 ^{0}C and 5% CO2, the supernatants were collected and stored at -70 ^{0}C for IL-10 protein enzyme-linked immunosorbent assay (ELISA, Biosource, Etten Leur, The Netherlands) or Western blotting [18].

LPS stimulates mainly monocytes [19], while PMA stimulation involves T cells [20]. The total IL-10 protein production of stimulated PBMCs depends on the relative numbers of monocytes and T cells. To identify the PBMCs, two triple stainings were performed for immunophenotyping. PBMCs were first stained with fluorescein isothiocyanate labelled monoclonal antibodies (MoAbs) against CD3 (UCHT1, Immunotech, Marseille, France) or CD14 (Leu-M3, Becton Dickinson) for 15 minutes (min) at room temperature, to determine T cells and monocytes, respectively. After washing, the samples were further stained with phycoerythrin-conjugated MoAbs against CD25 (3G10, Sanbio, Uden, The Netherlands) and phycoerythrin-cyanine 5-conjugated MoAbs against CD45 (T29/33, Dako, Denmark) for 15 min at room temperature, to identify IL-2 receptor carrying cells and total leukocytes, respectively. After a second wash, cells were analysed for specific fluorescence by flow cytometry (FACScan, Becton Dickinson) using CellQuest software (Becton Dickinson). Appropriate controls were included to rule out non-specific fluorescence.

Expression of IL-10 protein

Total RNA was isolated from lymphoblastoid cell lines using the Rneasy minikit (Qiagen, Leusden, The Netherlands). Complementary DNA (cDNA) was made using M-MLV reverse transcriptase (Gibco BRL Life Technologies) and hexamers (Promega, Leiden, The Netherlands). The full coding IL-10 sequence was amplified by reverse transcription PCR (RT-PCR) using the forward primer 5'-CGGGATCCATGCACAGCT CAGCACTGC-3', including a BamHI restriction site, and reverse primer 5'-GGAATT CTCAGTTTCGTATCTTCATTG-3', including an EcoRI restriction site. After digestion and purification, the RT-PCR fragments were ligated into the corresponding restriction sites of the expression vector pRSET A (Invitrogen, Groningen, The Netherlands), and transformed into TOP10 E. coli bacteria (Invitrogen). Inserts were sequenced, and vectors with the appropriate insert were transferred into BL21 E. coli bacteria (Invitrogen) for recombinant human IL-10 (rIL-10) protein production. The rIL-10 protein was demonstrated by ELISA (Biosource).

Effect of IL-10 protein *in vitro* on HL60 cells

HL60 cells (human monocytic leukaemia cell line) [21, 22] were cultured at 10^6 cells/mL in 24-well plates in RPMI 1640 medium (Bio Whittaker Europe) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 50 µg/mL streptomycin, 1 mM pyruvate, and 10% heat-inactivated foetal bovine serum (Gibco BRL Life Technologies). Cells were stimulated with 1 µg/mL LPS E. coli O26:B6 (Difco). After 16 h of incubation at 37 ^{0}C and 5% CO2, [3H]-thymidine 0.5 µCi/well (Amersham, Aylesbury, UK) was added, and plates were incubated for an additional 8 h before harvesting. Measurement of [3H]-thymidine was performed with a BetaPlate (Pharmacia LKB, Turku, Finland). In addition, similar HL60 quadriplate cultures were incubated in serum-free medium according Yssel and co-workers [23] in the presence of serial dilutions of equal amount of rIL-10 of mutated or wild-type origin obtained from transfected E. coli. The cloned immature wild-type rIL-10 and mutated rIL-10 proteins were tested as lysates of phosphate-buffered saline sonificated recombinant E. coli. These cultures were allowed to incubate for 24 h, after which culture supernatants were collected and assayed for the production of IL-10 by ELISA (Biosource).

Statistics

Statistical analysis of microsatellite markers to determine IL-10 allele-sharing in affected sib-pairs was performed with Mapmaker/sibs software. The data were evaluated by a non-parametric two-point linkage analysis [24]. The results of all IL-10 *in vitro* stimulation studies were expressed as mean values with standard errors of the mean (SEM). The data were analysed by the Mann-Whitney test using SPSS software version 8.0 (SPSS Inc., Chicago, Illinois, USA). Linear regression was evaluated using GraphPad Prism software version 3.0 (GraphPad Software, Inc., San Diego, California, USA). P values of 0.05 or less were considered to be statistically significant.

RESULTS

Affected sib-pairs

Fifteen families including 17 sib-pairs affected by IBD were examined in this study for genomic alterations of the IL-10 gene. In five pairs both siblings had CD, in seven pairs both had UC, and in 5 pairs one individual had CD and the other UC. One family included three UC-affected siblings of which two females were dizygous twins. Twenty-seven non-affected parents and three non-affected siblings were also studied.

IL-10 allele-sharing

In seven IBD-affected sib-pairs out of five families, siblings shared both parental IL-10 alleles. One sib-pair included two patients with CD. In four sib-pairs both family members had UC, of which two patients were dizygous twins. In two pairs one sibling had CD and the other UC. The LOD score for involvement of the IL-10 gene in all included sib-pairs (n = 17) was low (0.27). The diverse allele-sharing between the sib-pairs suggests that the IL-10 gene is not involved in pathogenesis in all families, but may play a role in a subgroup.

IL-10 gene sequence analysis

DNA sequence analysis of all IL-10 exons was performed in those probands of affected sib-pairs who shared or were statistically likely to share both parental IL-10 alleles, as the chance of finding gene alterations among these pairs is the highest. In one male proband with CD, a heterozygous point mutation (G → A) was found at position 43 of exon 1. This mutation was also present in his CD-affected sister and two unaffected sisters, but not in the father and another unaffected sister. The mother died several years ago, and could not be tested. On allele reconstruction, she most probably carried the mutated IL-10 allele. Unfortunately, no close relatives of the mother were available for testing. Two available sisters of the proband's father were tested for the mutation, but found to be negative. The pedigree is shown in Figure 1.

The heterozygous mutation at position 43 of exon 1 was confirmed in cDNA of the proband. According to NCBI U16720, the mutation results in a single amino acid change from glycine (Gly) to arginine (Arg) at position 15 of the 178 amino acids containing IL-10 protein. This position corresponds with the leader sequence of the immature IL-10 (position 1-18).

Subsequently, all other probands of affected sib-pairs who shared one or neither parental allele, and 75 unrelated controls were screened for this IL-10 exon 1 Gly15Arg mutation, using digestion of exon 1 PCR fragments by Mae I endonuclease or DNA sequence analysis. In none of them was the mutation found.

Clinical characteristics of two families with the IL-10 Gly15Arg mutation

Proband This 37-year-old non-smoking male patient presented with abdominal pain, diarrhoea and fever in 1979. CD of the terminal ileum was diagnosed and confirmed during an ileocecal resection in 1980. Additional resections of stenotic small bowel were necessary in 1987, 1988, 1995 and 1999. Histology of the resected specimens showed transmural granulomatous inflammation. In 1987, mild disease activity was seen in the rectum at colonoscopy. He was treated with salazopyrine and steroids from 1979, changing to oral budesonide in 1997. He has had mild anterior uveitis and sacroiliitis.

Affected sibling His 49-year-old smoking sister was diagnosed with CD in 1981, and underwent an ileocecal resection. Again in 1987 and 1993 small bowel resections followed because of recurrent stenotic inflammation around the anastomosis. Treatment included aminosalicylates from 1988 until 1997. She has not had extra-intestinal manifestations.

Unaffected siblings The proband has three sisters without IBD, born in 1953, 1954 and 1959. None of them has had other diseases or uses medication.

Unaffected parents Neither parent had a history of abdominal disorders. The father, born in 1924, died in 1999 of a myocardial infarction, some time after blood was taken for this study. The mother died at the age of 62 years due to metastatic cancer of unknown primary.

IL-10 gene promoter analysis

Promoter polymorphisms were examined in available individuals (n = 6) of the IBD family in which the IL-10 Gly15Arg substitution was found to determine whether they had a high or low IL-10 secretion genotype. The data are shown in the pedigree (see Figure 1). Only the -1082*A/A and -1082*G/A genotypes (low production) were found. IL-10 gene promoter polymorphisms were also analysed in 22 healthy unrelated Caucasian controls (11 males and 11 females). Seven controls had the -1082*A/A genotype, 11 the -1082*G/A genotype, and four the -1082*G/G genotype.

IL-10 secretion of *in vitro* LPS- and PMA-stimulated PBMCs

PBMCs of the CD-affected sib-pair with the IL-10 Gly15Arg mutation and their available family members (n = 3) were stimulated *in vitro* with LPS and PMA, after which IL-10 secretion was determined by ELISA. PBMCs of the father unfortunately could not be studied. The amount of IL-10 protein was expressed per 10^6 monocytes and T-cells.

As the number of individuals with the IL-10 mutation was low (n = 4), stimulation tests of these participants were performed twice with an interval of 6 weeks. These results were expressed as the mean of both tests and summarised in Table 2.

In the literature, both the -1082*G/A and -1082*A/A genotypes are reported to be associated with low IL-10 production. As shown in Figure 1, the mutation carri-

ers had either the -1082*G/A or -1082*A/A genotype. Therefore, IL-10 secretion was evaluated in healthy controls with comparable promoter polymorphisms. In the control group, IL-10 secretion was not significantly different between -1082*G/A and -1082*A/A carriers, so both genotypes were pooled to form a single control group. The IL-10 Gly15Arg mutation was associated with a significantly lower IL-10 protein production by PBMCs after stimulation with LPS or PMA (P = 0.002 and 0.033, respectively, see Table 2).

Western blot analysis of the IL-10 protein derived from LPS-stimulated PBMCs culture mediums of individuals with the IL-10 Gly15Arg mutation revealed only a single band of a molecular weight corresponding to the wild-type IL-10.

In vitro effect of wild-type rIL-10 and mutated rIL-10 on HL60 cells

Both the wild-type and mutated rIL-10 were recognised by the ELISA for IL-10. After incubation of LPS-stimulated HL60 cells with serial dilutions of rIL-10, we did not find a difference in the intrinsic release of IL-10 by HL60 cells comparing mutated rIL-10 with wild-type rIL-10. However, the mutated rIL-10 protein induced a significantly lower HL60 cell proliferation rate than the wild-type rIL-10 protein, as analysed by linear regression (P = 0.006, see Figure 2).

DISCUSSION

The IL-10 gene is an attractive candidate gene for susceptibility to IBD, as its product has important anti-inflammatory and immunomodulatory effects [4-6]. However, a recent linkage study using microsatellite analysis in 198 pairs of siblings with IBD failed to demonstrate an overall significant contribution of the IL-10 gene to the risk of developing IBD [7]. Assuming that IBD is a genetically heterogeneous disease, then failure to demonstrate a statistically significant linkage of the IL-10 gene with IBD in a large number of affected sib-pairs will not preclude a major importance of the IL-10 gene in a small portion of families. We therefore chose to look for functional mutations in the IL-10 gene in sib-pairs with IBD, selected on the base of allele-sharing.

The IL-10 gene was initially screened in sib-pairs who shared or were likely to share both parental alleles, as the chance of finding gene alterations among these pairs is the highest. In a sib-pair affected family with CD a point mutation was found in the IL-10 gene at position 43 of exon 1 (G → A). This mutation leads to the amino acid change Gly → Arg at position 15 of the leader sequence of the immature IL-10 protein. We showed that this mutation was associated with about a 50% reduction in IL-10 protein secretion by PBMCs following stimulation with either LPS or PMA. This effect of the mutation on IL-10 secretion is not surprising in view of the size and charge differences between Gly and Arg. To standardise the comparison with the mutation carriers, all healthy controls were matched for the IL-10 promoter poly-

morphism at position -1082 relative to the transcriptional start site, which has been found to be associated with the amount of IL-10 production [9].

Although it is likely that most IL-10 involved in the regulation of intestinal inflammation is the mature protein, we cloned both mutant and wild-type IL-10 including the leader sequence. Both recombinant cytokines were tested in a proliferation assay with LPS-stimulated HL60 cells, a human leukaemia cell line of monocytic lineage. HL60 cells were used as they are a model for monocytes, and express IL-10 receptors [22]. The Gly15Arg rIL-10 was less active than the wild-type rIL-10. We propose that in the CD-affected family the Gly15Arg mutation in the leader sequence of IL-10 predisposes to the development of IBD by causing a reduced IL-10 response to intestinal inflammation.

The IL-10 Gly15Arg mutation was also present in healthy siblings of the proband with CD. Although in many autosomal dominant hereditary disorders clinical expression is variable, we did consider the possibility that lack of symptoms might be due to differences in the promoter polymorphisms between the symptomatic and the non-symptomatic siblings. This was not the case, which suggests that in addition to the putative role of the IL-10 gene, other factors trigger the events to obtain clinically manifest IBD in this family.

Very recently, the IL-10 Gly15Arg mutation was reported as a very rare polymorphism in an extensive search for new IL-10 gene alterations in patients with myocardial infarction [25], and in patients with IBD [10]. None of these studies evaluated the biological effects of the mutated IL-10 protein. To what extent the IL-10 Gly15Arg mutation is associated with IBD or other chronic diseases, is at present unknown.

Our findings do have potentially important implications. So far, four multicentre clinical trials have been published about the therapeutic effect of subcutaneous administered recombinant IL-10 in CD. Although treatment with IL-10 is safe and well tolerated, the overall effect was disappointing [11-13, 26]. However, it is possible that IL-10 treatment could be beneficial in a subset of patients who have a genetic defect in IL-10 secretion or function.

The other implication of our findings is that IBD is a heterogeneous disease. Detailed examination of candidate genes in sib-pairs who share alleles of these genes in a relative small number of multiple affected families can provide further insight into the genetic aetiology of IBD.

Table 1. Primers used in polymerase chain reaction amplifications of interleukin-10 exons

Exon	Nucleotides [a]	Primer	Sequence
1	3946-3965	1a forward	5'-AGAGGCCTCCCTGAGCTTAC-3'
	4312-4331	1b reverse	5'-TGTTGGGGATGGAGGTGGAG-3'
2	4921-4942	2a forward	5'-TCCCATACTGTTGAATCCTCTG-3'
	5261-5282	2b reverse	5'-TGCTGAGTTAACATCTTCCCAC-3'
3	5326-5345	3a forward	5'-GGCTTCTGGTAAGGAGGATC-3'
	5658-5677	3b reverse	5'-TGTCTGTGGATGTGAGTGTC-3'
4	6452-6472	4a forward	5'-CATGGAAGCAGGGCTCAGTTC-3'
	6791-6810	4b reverse	5'-GCAGCAGCTCCTAATGGCTG-3'
5	7584-7603	5a forward	5'-CACAGCCTTTCCCAAGGCAG-3'
	7924-7946	5b reverse	5'-AGGTACAATAAGGTTTCTCAAGG-3'

[a] Nucleotide number according to accession number U16720 of the NCBI (http://www.ncbi.nlm.nih.gov).

Table 2. Interleukin-10 secretion by LPS- and PMA-stimulated PBMCs of interleukin-10 Gly15Arg mutation carriers and non-carriers

	LPS-stimulated PBMCs (ng/mL per 10^6 monocytes ± SEM)	PMA-stimulated PBMCs (pg/mL per 10^6 T-cells ± SEM)
Family with IL-10 Gly15Arg mutation [a]		
carriers (n = 4)	5.05 ± 0.59 [c]	175.40 ± 115.24 [d]
non-carrier (n = 1)	7.91	502.45
Healthy controls [b]		
-1082*A/A (n = 7)	9.18 ± 1.97	261.66 ± 67.77
-1082*G/A (n = 11)	10.44 ± 1.10	400.03 ± 60.14
-1082*A/A and -1082*G/A (n = 18)	9.95 ± 0.99 [c]	346.22 ± 46.89 [d]

[a] Multiple affected family with inflammatory bowel disease, including a Crohn's disease-affected sib-pair (brother and sister) and two healthy sisters carrying the interleukin-10 (IL-10) Gly15Arg mutation, and one healthy sister with the wild-type IL-10 gene. The father and mother could not be tested. All tested family members had the low IL-10 producing promoter genotype (-1082*G/A or -1082*A/A relative to the transcriptional start site).

[b] Selected for IL-10 promoter polymorphism at position -1082 relative to the transcriptional start site.

[c] IL-10 secretion by lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs) was significantly different between IL-10 Gly15Arg mutation carriers and carriers of the wild-type IL-10 gene (P = 0.002).

[d] IL-10 secretion by phorbol-12-myristate-13-acetate (PMA)-stimulated PBMCs was significantly different between IL-10 Gly15Arg mutation carriers and carriers of the wild-type IL-10 gene (P = 0.033).

SEM = standard error of the mean.

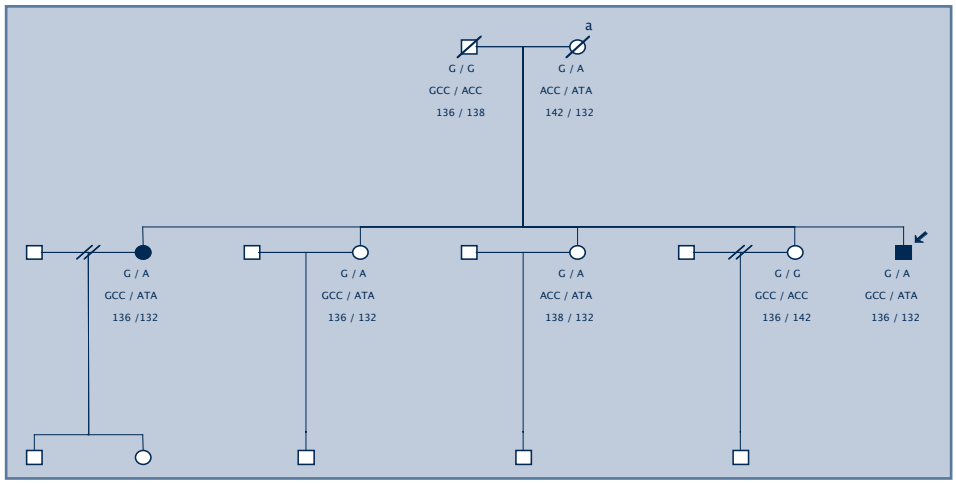

Figure 1. Pedigree of multiple affected family with Crohn's disease (CD) and the interleukin-10 (IL-10) Gly15Arg mutation. Tested individuals: ■, CD-affected male; ●, CD-affected female; □, non-CD-affected male; ○, non-CD-affected female; ◪, proband. Indicated data for each tested individual: *first row*, genotype of the IL-10 gene at position 43 of exon 1; *second row*, IL-10 promoter polymorphism at positions -1082, -819 and -592 relative to the transcriptional start site, respectively; *third row*, lengths of the IL-10.G microsatellite marker polymerase chain reaction fragments.

[a] Genotypes of the deceased mother were reconstructed.

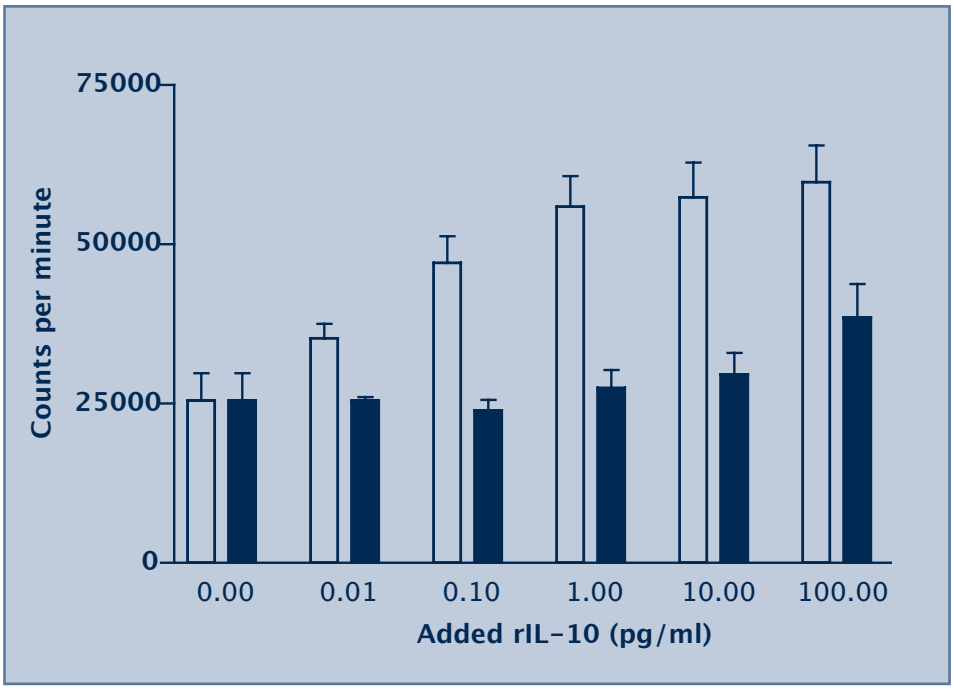

Figure 2. Proliferation rate of HL60 cells stimulated by lipopolysaccharide (LPS), and wild-type or mutated recombinant interleukin-10 (rIL-10). *Open bar*, added wild-type rIL-10; *black bar*, added rIL-10 with Gly15Arg mutation. A significant difference ($P = 0.006$) was found between the slopes of both correlation curves, meaning that stimulation of HL60 cells with LPS and rIL-10 Gly15Arg ($r = 0.95$) induced a lower proliferation than stimulation with LPS and wild-type rIL-10 ($r = 0.55$). Data are expressed as a mean value of a quadruple measurement ± standard error of the mean.

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CHAPTER 4

A functional interleukin-10 mutation in Dutch patients with Crohn's disease

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ABSTRACT

Background

Interleukin-10 (IL-10) is an anti-inflammatory and immunomodulatory cytokine. IL-10 deficient mice are prone to develop chronic colitis. Administration of recombinant human IL-10 has been proposed to have a beneficial effect in a subgroup of patients with Crohn's disease (CD). Recently, we found an IL-10 Gly15Arg mutation in a family with CD which is associated with reduced IL-10 secretion by *in vitro* stimulated monocytes and lymphocytes. We hypothesised that this IL-10 mutation plays a role in maintaining the inflammatory process in CD in some families.

Methods

We evaluated IL-10 Gly15Arg in 379 patients with CD, and 75 unrelated healthy controls. Also first-degree family members of IL-10 Gly15Arg carriers were evaluated. Additionally, mutation carriers and their relatives were evaluated for CARD15 R702W, G908R and L1007fs.

Results

Two patients with CD were heterozygous for the IL-10 Gly15Arg mutation. No homozygotes were found. The Gly15Arg mutation was not observed in the controls. In first-degree family members of the CD-affected IL-10 Gly15Arg carriers, the mutation was found in CD-affected as well as in their apparently healthy individuals. All family members carried one or two CARD15 mutation(s).

Conclusion

The IL-10 Gly15Arg mutation is rare in patients with CD, and is not associated with the disease in The Netherlands.

INTRODUCTION

Interleukin-10 (IL-10) is considered to be an important cytokine for immune modulation in the gut. It has anti-inflammatory and immunomodulatory effects, and is considered to play a role in inflammatory bowel disease (IBD). This hypothesis is supported by the observation that IL-10 gene knock-out mice develop chronic enterocolitis resembling Crohn's disease (CD) [1].

IL-10 influences the expression of a variety of cytokines, soluble mediators, and cell surface molecules produced by activated monocytes and macrophages. IL-10 can down-regulate the production of IL-1 and tumour necrosis factor alpha (TNFα), which are first-line cytokines in a Th1-driven inflammatory response [2]. Subcutaneously administered IL-10 has been evaluated in four clinical trials with CD-affected patients, with inconsistent results [3-6]. It has been suggested that a subgroup of CD patients might benefit from IL-10 administration [4].

Recently, we found an IL-10 single nucleotide gene alteration at position 43 in exon 1 (G → A) encoding for a Gly15Arg substitution in a family with multiple CD-affected members. The nucleotide substitution was associated with a reduced IL-10 protein secretion by *in vitro* stimulated monocytes and lymphocytes of IBD patients. Based on these findings we hypothesised that the IL-10 Gly15Arg mutation may play a role in the pathogenesis of IBD in this family, and that IL-10 administration could be useful in treating active CD in these patients [7].

CD is considered to be a polygenic disease with different genes contributing to susceptibility in different families and giving rise to differences in response to anti-inflammatory agents [4]. Recently, three coding polymorphisms R702W, G908R, and L1007fs in the CARD15 gene were shown to be independently associated with CD [8, 9]. As the IL-10 Gly15Arg also appeared to be a coding nucleotide variation and therefore may have potential therapeutic implications, we evaluated the prevalence of this mutation in a large group of CD-affected patients with or without a positive family history of IBD.

MATERIALS AND METHODS

Patients and materials

Patients with CD of Dutch Caucasian origin were recruited from three Dutch university referral centres. Patients were classified as familial CD if one or more first-, second- or third-degree relatives were known to have IBD. If the family history was negative or not clear, patients were considered sporadic CD or CD patients with an unknown family history. The diagnosis of CD was verified by each centre according to standard criteria [10]. The age of the CD-affected participants was calculated on the date of entry, i.e. July 1st 2002. Initially, only the proband from a family was studied. Family members of selected participants were also subsequently examined.

Seventy-five healthy Caucasian controls were included. These controls were recruited at the Erasmus MC University Medical Centre Rotterdam (UMCR), and the reports have been published previously [7].

Genomic DNA was isolated from whole peripheral venous blood using standard techniques.

The study was approved by the Institutional Review Board of the Erasmus MC UMCR. All participants provided written informed consent.

IL-10 Gly15Arg mutation analysis

Exon 1 of the IL-10 gene was amplified using the forward primer 5'-AGAGGCCTCCCTGAGCTTAC-3' and reverse primer 5'-TGTTGGGGATGGAGGTGGAG-3' resulting in a polymerase chain reaction (PCR) fragment of 386 base pairs (bp). The prevalence of the IL-10*G/A point mutation at base position 43 was evaluated with the restriction enzyme Mae I (Boehringer Mannheim, Almere, The Netherlands). After digestion with 1 unit of Mae I at 45 ^{0}C for 4 hours (h), the PCR fragment was analysed on a 2% agarose electrophoresis gel. Bands were visualised with ethidiumbromide staining. Wild-type PCR products resulted in fragments of 60 and 326 bp, while mutant PCR products gave fragments with a length of 60, 152 and 174 bp. Positive samples representing the IL-10 Gly15Arg mutation were confirmed by sequencing the 386 bp PCR fragments, using the forward primer 5'-AGAGGCCTCCCTGAGCTTAC-3' and a BigDye Terminator sequencing kit (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). All DNA sequences were performed using an ABI 310 Genetic Analyzer (Applied Biosystems) supported by Genescan (version 2.1.1) and Genotyper (version 2.1) software programs.

IL-10 haplotyping

Microsatellite markers IL-10.G and IL-10.R were used for haplotyping IL-10 alleles. Both microsatellites have been evaluated in healthy Caucasian individuals by Eskdale et al. [11]. The IL-10.G marker is highly polymorphic with a heterozygosity of around 72%. It is located in the promoter of the IL-10 gene [11]. The IL-10.R marker is less informative, having a heterozygosity of 50% 12. PCR conditions were used as published in the literature with minor changes. An ABI 310 Genetic Analyzer (Applied Biosystems) was used for DNA sequencing.

CARD15 mutation analysis

CARD15 R702W and G908R were determined by means of restriction enzyme analysis. First, the DNA regions of interest containing the alteration were amplified by PCR. For R702W (2104C → T in exon 4) the forward primer 5'-CAGCTGGGCAGCTTTGCTGC-3' and the reverse primer 5'-CATGGCATGCACGCTCTTGG-3' were used. The 448 bp was purified by phenol extraction followed by ethanol precipitation, after which the PCR fragment was digested with 8 units of Hpa II (New England BioLabs, Beverly, Massachusetts, USA) for 4 h at 37 ^{0}C. Bands were visualised after separation on a

2% agarose gel containing ethidium bromide. The wild-type exon 4 resulted in fragments of 29, 54, 76 and 289 bp, whereas the minor allele resulted in fragments of 29, 130, and 289 bp.

The G908R (2722G → C in exon 8) alteration was amplified using the forward primer 5'-CACTTTGCTGGGACCAGGAG-3' and reverse primer 5'-ACTCCATTGCCTAACATTGTGG-3'. The 363 bp PCR product was digested immediately with 4 units HinP1 I (New England BioLabs) for 3 h at 37 ^{0}C followed by separation on a 2% agarose gel containing ethidium bromide. It resulted in one fragment of 363 bp in case of the wild-type, and in 168 and 195 bp fragments in case of the rare allele of the G908R polymorphism.

CARD15 L1007fs (3020insC in exon 11) was determined by means of an allele-specific PCR reaction, followed by separation of PCR products on an agarose gel as described previously [9, 13].

When participants were positive for CARD15 R702W, G908R or L1007fs, sequencing of the corresponding PCR product was performed on an ABI 310 Genetic Analyzer (Applied Biosystems) to confirm the alteration.

Statistical analysis

Statistical analysis was performed by comparing the allele frequencies of the mutation in patients with CD and controls, using Fisher's exact test. The genotype frequencies in controls were tested for Hardy-Weinberg equilibrium (HWE) proportions. The analyses were performed with SPSS software version 9 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Patients

Initially, 79 probands from separate families with CD were studied. Subsequently, 300 CD patients with no or an unknown family history were investigated, giving a total of 379 patients with CD including 134 males and 245 females. Their mean age was 40.1 years (range: 18.7-81.6 years).

Prevalence of IL-10 Gly15Arg mutation

Two CD patients (0.5%) from two different multiple IBD-affected families were found to be heterozygous for the IL-10 Gly15Arg mutation (Figure 1). Both probands were from one centre (Erasmus MC UMCR). Homozygosity for the Gly15Arg mutation was not found. All other CD-affected patients and all 75 healthy controls carried the wild-type allele of the IL-10 gene.

Mutation analysis in family members of IL-10 Gly15Arg mutation carriers

First-degree family members of the two CD-affected IL-10 Gly15Arg mutation

carriers (probands) were evaluated for this mutation. In both families IL-10 haplotypes were evaluated as well. The results are summarised in Figure 1. The reports of one family have been published recently [7]. In the second family, in addition to the proband a niece of the probands' father had CD. She was evaluated as well, and did not have the Gly15Arg mutation.

One CD-affected sibling and several non-IBD-affected family members were carriers of the IL-10 Gly15Arg mutation. In both families, the mutation was present on the haplotype IL-10.43*A–IL-10.G–131bp–IL-10.R-110 bp. Therefore, it is possible that these families have a common ancestor.

Clinical characteristics of two families with the IL-10 Gly15Arg mutation

Family 1 This family has been described in detail recently [7]. In summary, the proband (born in 1963) has been known with CD since 1979. The disease is mainly located in the small bowel. He was treated with salazopyrine and steroids, and later budesonide. He underwent several resections for therapy resistance and stenotic lesions. The disease was complicated by uveitis and sacroiliitis. One female CD-affected sibling (born 1952) was diagnosed in 1981. She also underwent several small bowel resections, mainly because of stenotic inflammation. She was treated with aminosalicylates until 1997, and experienced no extra-intestinal manifestations. The proband has three female healthy siblings (born in 1953, 1954, and 1959). Both parents (father born in 1924, mother born in 1926) have died, but were not known to have IBD. All family members were evaluated for CARD15 L1007fs, as previously published in part [13]. The proband, the CD-affected sister, two non-IBD-affected sisters, and the father were homozygous carriers. One non-IBD-affected sister, and the mother were heterozygous carrier of mutant L1007fs. The proband and the non-IBD-affected sister which was heterozygous for L1007fs, were both negative for R702W and G908R. These mutations were not evaluated in the other family members.

Family 2 The proband is a non-smoking female patient born in 1977. She presented with weight loss, nausea, bloody diarrhoea, and abdominal pain in 1996. CD of the colon was diagnosed by endoscopy in 1996. Biopsies showed a chronic granulomatous inflammation with crypt abscesses. She was treated with prednison and subsequently budesonide until 1997, azathioprine until 2000 and mesalazine. Besides arthralgia, she had no serious extra-intestinal manifestations. Her sister, born in 1971, is healthy and has no abdominal complaints. Neither parent has a history of abdominal disorders. The father, born in 1949, is known to be affected with hypertension. The mother, born in 1951, is healthy and underwent a hysterectomy for a benign reason several years ago. A niece of her father, born in 1956, had been diagnosed as having CD of the colon in 1988. Her disease was complicated by perianal fistulas. The proband, sister and father had genotypes CARD15 R702W/G908R. The mother and niece of the father were heterozygous for R702W. The proband and niece were also evaluated for L1007fs, which was negative.

DISCUSSION

IL-10 has important anti-inflammatory and immunomodulatory effects making the IL-10 gene (chromosome 1q32.1) a good candidate for susceptibility to IBD [2, 14]. Several studies with IL-10 deficient mice have shown that exogenous IL-10 is effective in preventing and treating spontaneous enterocolitis [1, 15, 16]. In IBD patients, a lower frequency of the IL-10 -1082*G allele was found compared with controls [17]. As this allele is associated with higher production of IL-10, the presence of this allele might protect against IBD [18]. Genetic studies do not support a major role for IL-10 mutations in human IBD. In a candidate gene study, no significant association was found between IBD and IL-10 gene polymorphisms [19]. In a genome-wide screen significant linkage was found at chromosome 1q31-q32 [20]. This region is at least 15 cM apart from the IL-10 gene, and therefore linkage dysequilibrium between the IL-10 gene and 1q31-q32 is unlikely. This absence of significant linkage in large population studies does not exclude an essential role for mutations in the IL-10 gene in a limited number of CD patients.

We have recently described the IL-10 Gly15Arg mutation in a single multiple CD-affected family. This mutation is located in the leader sequence of IL-10, and was associated with about a 50% reduction in IL-10 protein secretion by peripheral blood mononuclear cells after stimulation with lipopolysaccharide or phorbol-12-myristate-13-acetate. This lead us to propose that the IL-10 Gly15Arg mutation might play an important role in the pathogenesis of CD in this specific family [7]. This mutation also has potential therapeutic implications, as these CD patients could theoretically benefit from recombinant human IL-10 administration.

In the present study, we evaluated the prevalence of the IL-10 Gly15Arg mutation in a large group of Dutch patients with CD. Only three patients in two families (Figure 1) were heterozygous carrier of this mutation (prevalence of 0.5%). The relative rarity of the IL-10 Gly15Arg mutation has been reported in two other studies. In a study of IL-10 gene polymorphisms, Klein et al. found the IL-10 Gly15Arg mutation in two out of 142 patients with CD and five out of 400 healthy controls [21]. Donger et al. evaluated the prevalence of IL-10 Gly15Arg in patients with cardiovascular disease and healthy controls [22]. The data are summarised in Table 1. Neither of these studies presented data evaluating the possible effect on functionality of this IL-10 mutation.

In this study, 75 unrelated healthy controls were analysed for the IL-10 Gly15Arg. No carriage was found. We concluded that the IL-10 Gly15Arg mutation is not associated with CD. Ideally, to evaluate the possibility of association, a much larger control group is necessary. However, as Klein et al. and Donger et al. [21, 22] showed a very low prevalence of IL-10 Gly15Arg in a large set of controls, enlarging our control group is unlikely to change the conclusion of this study.

As healthy carriers of the mutation were found in both families, the presence of the IL-10 Gly15Arg mutation is not by itself sufficient to cause CD in these specific

families. We speculate that in a subgroup of CD patients, the reduction in IL-10 production together with other yet unidentified predisposing genes involved in the immune response, may impair the capacity to appropriately overcome or down-regulate the inflammatory process in the gut. The finding that carriage of the mutation is not associated with CD, and the finding that within a family with two CD-affected relatives only one carries the mutation, does not exclude a pathogenetic role for this functional mutation. Similar disparities in association have for instance been reported for the CARD15 mutations in CD [8, 9, 13, 23]. Moreover, there might be an epistatic interaction between IL-10 and CARD15 as in both families CD-affected individuals were found with both the IL-10 Gly15Arg as well as one or two CARD15 mutations.

In conclusion, the prevalence of the IL-10 Gly15Arg mutation is very low (less than 1%) and is only found in a small number of Dutch patients with CD. We postulate that this mutation in the gene encoding the anti-inflammatory cytokine IL-10, which we have previously shown results in an impairment of anti-inflammatory function [7], may play a role in a small number of individuals with CD. However, additional gene polymorphisms and/or triggering events are necessary to result in clinical manifest CD.

Table 1. European studies on the prevalence of the interleukin-10 Gly15Arg mutation

Study	Crohn's disease (n)		Cardiovascular disease (n)		Healthy controls (n)	
	Gly15Arg +	Gly15Arg -	Gly15Arg +	Gly15Arg -	Gly15Arg +	Gly15Arg -
Klein et al. [21]	2	140	-	-	5	395
Donger et al. [22]	-	-	7	1001	5	987
This study	2	377	-	-	0	75
Total [a]	4	517	7	1001	10	1457
Prevalence *(%)* [a]	0.8	99.2	0.7	99.3	0.7	99.3

[a] Statistical analysis of the combined data using Fisher's exact test showed no association between interleukin-10 (IL-10) Gly15Arg and Crohn's disease.

All IL-10 Gly15Arg positive individuals are heterozygous.

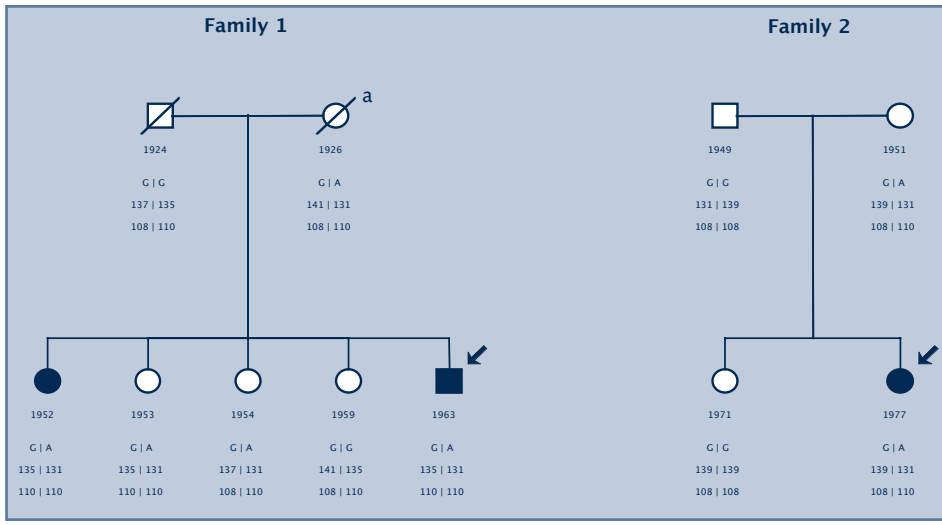

Figure 1. Pedigrees of families with carriers of the interleukin-10 (IL-10) Gly15Arg mutation Tested individuals: ■, Crohn's disease (CD)-affected male; ●, CD-affected female; □, non-CD-affected male; ○, non-CD-affected female; ↙, proband. Indicated data for each tested individual: *first row*, year of birth; *second row*, position 43 in exon 1; *third row*, length of the IL-10.G microsatellite marker PCR fragments; *fourth row*, lengths of the IL-10.R microsatellite marker PCR fragments.
[a] Haplotypes of the deceased mother in family 1 were reconstructed.

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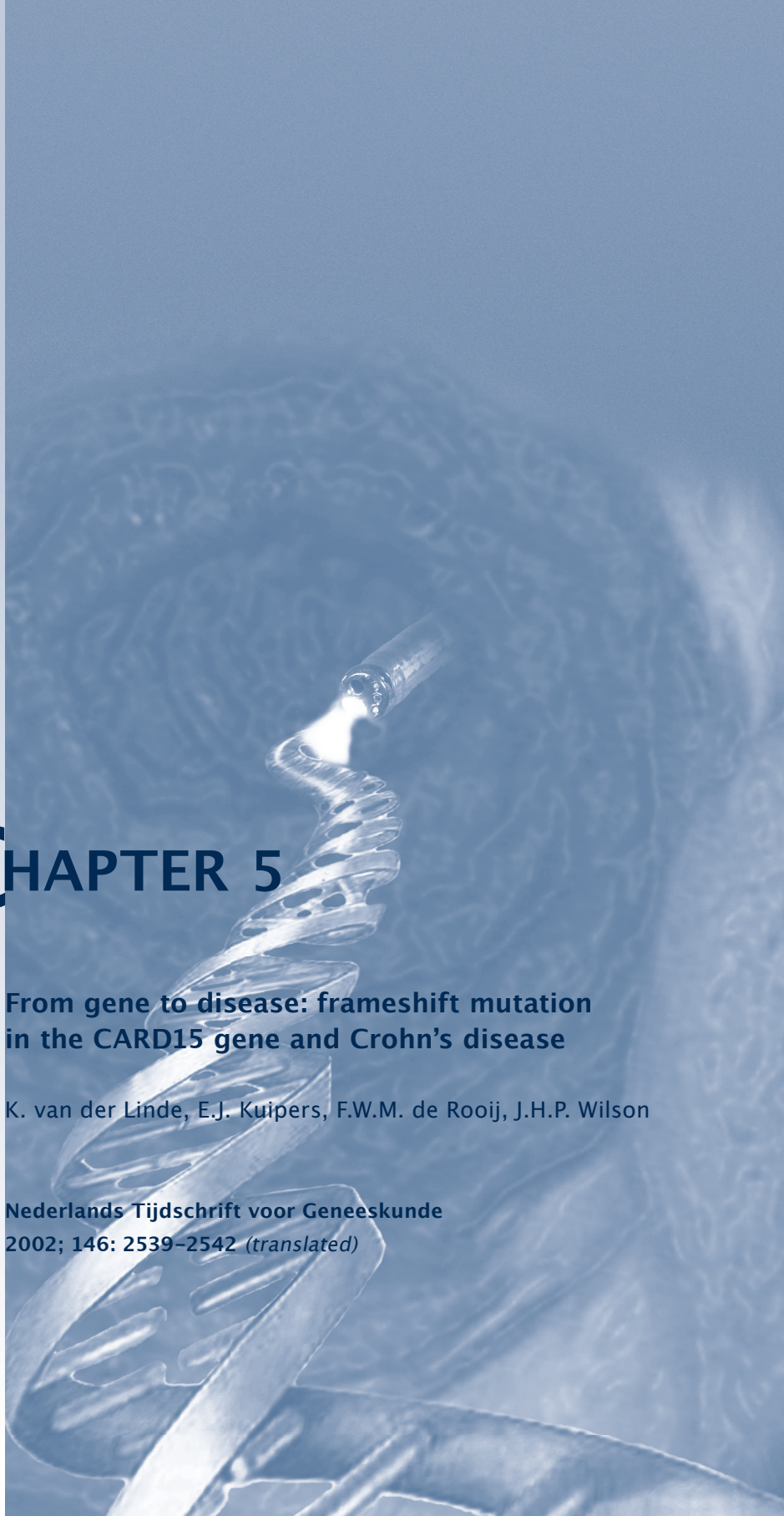

CHAPTER 5

From gene to disease: frameshift mutation in the CARD15 gene and Crohn's disease

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ABSTRACT

A pericentromeric region on chromosome 16 (IBD1 locus) has been linked with Crohn's disease (CD). Recently, three genetic variants in the CARD15 gene within the IBD1 locus have been identified which were highly associated with CD. Carriage increases the relative risk of developing CD. One specific mutation (3020insC) leads to a stop codon and truncation of the C-terminal tandem leucine-rich repeats (LRR) of the CARD15 protein. Of all patients with CD, 11-19% is heterozygous and 3-7% homozygous for this frameshift mutation. The CARD15 gene is expressed in monocytes. The LRR-domain is thought to be involved in the binding of bacterial lipopolysaccharide (LPS) and subsequent activation of nuclear factor kappa-B (NFκB). NFκB plays a central role in the regulation of the expression of other genes involved in the inflammatory response. *In vitro*, embryonic kidney cells transfected with the CARD15 3020insC mutant showed a reduced activity of NFκB after exposure to LPS compared with cells transfected with the wild-type CARD15 gene. How the reduced response to LPS contributes to CD is not clear yet.

THE DISEASE

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD). In The Netherlands the incidence is four to eight cases per 100,000 inhabitants per year. The prevalence is around 0.5% [1-3]. The disease is characterised by a chronic or intermittent transmural granulomatous inflammation of the gastrointestinal tract. It can affect any part of the gastrointestinal tract. The most common disease location is the ileocecal region (40%). Typical endoscopic features are presented in Figure 1. CD can be complicated by strictures and fistulas, such as perianal and enteroenteric fistulas. Furthermore, the disease can manifest outside the gastrointestinal tract, with signs including arthritis, erythema nodosum, pyoderma gangrenosum, and uveitis [4].

The disease cause is unknown, however, there is strong epidemiological evidence for genetic involvement in IBD. For example, Caucasians are more often affected than other ethnic groups. Especially, in American- and Europe-born Ashkenazi Jews a high prevalence of CD is reported [5]. Four to 18% of patients with CD have a first-degree relative with IBD [6]. In twins with IBD, the concordance for CD is 42-58% in monozygotic twin and 0-12% in dizygotic twins [7, 8]. High concordance rates for type of disease has also been found in families with two or more IBD-affected relatives other than twins [6, 9].

Non-genetic factors also play a role in the aetiology of CD. Several animal studies have demonstrated that an inflammatory bowel response occurs in the presence of bacterial flora, and not in a germ-free environment. In CD patients with severe perianal fistula, deviation of the faecal stream by means of an ileo- or colostomy can induce clinical improvement. Otherwise, antibiotic treatment can also reduce disease activity [10].

THE GENE

During the 1990s of the previous century, several research groups have searched for genes which might play a role in the pathogenesis of CD and ulcerative colitis (UC). The latter disease is also a major subtype of IBD [11]. Linkage analysis in multiple IBD-affected families have revealed several putative IBD susceptibility chromosomal loci (Table 1) [12-18]. The various results support the current hypothesis that IBD is a multigenetic disease [19].

The pericentromeric region of chromosome 16 (16q12) is the most documented locus. This so-called IBD1 locus showed linkage with CD [12, 20]. In 2001, two research groups independently demonstrated that linkage was based on specific nucleotide variations within the caspase recruitment domain (CARD)-15 gene (formally named nucleotide binding oligomerization domain (NOD)-2 gene) [21, 22]. So far, 67 nucleotide variations in the CARD15 gene have been identified [23]. Only three of these variations appeared independently highly associated with CD in Caucasian populations from

North America and Europe; 2104C → T in exon 4 (Arg702Trp), 2722G → C in exon 8 (Gly908Arg), and 3020insC in exon 11 (Leu1007fsinsC) [21, 23]. Although all three nucleotide variations are considered as mutations, only the 3020insC mutation has shown a functional defect *in vitro* [22]. This frameshift mutation includes a cytosine insertion at position 3020 in exon 11. It changes a leucine to a proline at position 1007, and is followed by a stop codon. It results in a truncated CARD15 protein. This frameshift mutation is located in the distal part of the gene, which codes for the tail of the protein, the so-called C-terminal leucine rich repeats (LRR) region [21, 22, 24].

THE PROTEIN

The CARD15 protein contains 1,040 amino acids. Its structure resembles the well-known CARD4 (NOD1) protein. CARD15 consists of two CARDs at the N-terminal site, a central nucleotide binding domain (NBD), and the LRR region at the C-terminal site [24]. A schematic illustration of the CARD15 protein with the three CD-associated amino acid changes is presented in Figure 2. The CARD domains can interact with other CARD containing cytoplasmatic proteins, like several pro-caspases [25]. The NBD domain can bind adenosine triphosphate and guanosine triphosphate, and probably comprises oligomerization of protein molecules [26, 27]. LRRs are found in proteins, which are involved in recognition of pathogens. Therefore, CARD15 might be a receptor for bacterial products, like lipopolysaccharide (LPS) which is a cell wall component of gram negative bacteria [24].

The function of CARD15 is not fully elucidated. *In vitro* studies with CARD15 cDNA transfected embryonal kidney cells (HEK293T) have shown that expression of the CARD15 protein leads to activation of the caspase cascade through the N-terminal CARD leading to apoptosis (programmed cell death). Apoptosis is necessary for normal development and homeostasis of organs and tissues. Binding with inactive pro-caspase-9 leads to conversion into active caspase-9 [24, 25]. Expression of CARD15 in HEK293T cells also leads to activation of nuclear factor kappa-B (NFκB). NFκB comprises a family of proteins which can induce transcription factors. It is an important regulator of immune- and inflammation-related genes of for example pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, cyclo-oxygenase-2 and inducible nitric oxide synthase. NFκB proteins are localised in the cytoplasm as inactive dimers. Activation of NFκB is mediated by RICK (RIP-like interacting CLARP kinase; RIP, receptor interacting protein; CLARP, caspase-like apoptosis regulatory protein), a CARD-containing kinase that associates with CARD of the CARD15 protein to a complex. The LRR domain is likely to be involved in the recognition of a wide array of pathogen components, and induction of a pathogen specific response. In case of CARD15, it is suggested that the LRR domain acts like an intracellular receptor for LPS. After binding (or interaction) of intracellular LPS with the LRR domain, cytoplasmatic NFκB becomes activated and subsequently translo-

cates into the nucleus where it activates expression of specific genes [24, 28].

The CARD15 frameshift mutation leads to truncation of the LRR domain lacking the 33 distal amino acids. CARD15 frameshift mutation transfected HEK293T cells showed reduced NFκB activity [22]. In contrast, patients with CD have increased expression of NFκB in the colon compared with healthy controls [29]. Therefore, the exact role of the CARD15 frameshift mutation in the aetiology of CD is unclear, and there exists a discrepancy between experimental and clinical findings. Otherwise, while NFκB is considered to be a pro-inflammatory factor, NFκB-deficient mice spontaneously develop colitis [30]. Together, the data have led to several hypotheses with respect to the relation between CARD15 mutations and the development of CD. As a consequence of altered (reduced) recognition of bacterial organisms the inflammatory response might become dysregulated. Otherwise, mutated CARD15 might lead to an inadequate anti-inflammatory response, for example through the anti-inflammatory cytokine interleukine-10 [22]. A CARD15 mutation might also result in a reduced apoptotic signal leading to an abnormal inflammatory response.

THE CELL

The CARD15 protein is expressed in monocytes [24] and possibly in enterocytes as well [31]. Monocytes are involved in non-specific cellular immunity and are precursors of tissue-bound cells, like intestinal macrophages and dendritic cells. These antigen presenting cells are important for initiation an immune response after binding with specific intestinal antigens. The inflammatory response in CD is based on a dysregulated T-helper-1 response characterised by release of pro-inflammatory cytokines, like tumour necrosis factor-alpha and interferon-gamma [32]. It is likely that CARD15 is also expressed in intestinal antigen presenting cells, however, this has not been proven.

THE POPULATION

The prevalence of the CARD15 frameshift mutation in patients with CD is 11-19% for heterozygous carriage, and three to seven percent for homozygous carriage. In patients with UC and in healthy controls only heterozygous carriage have been found in six to nine percent [21, 22]. The prevalence in CD is equal in sporadic patients and in patients with IBD-affected relatives [23, 33, 34].

Carriage means an increased risk of developing CD. While heterozygous carriage is associated with a two- to three-fold increased risk, homozygous carriers have a 17-42-fold increased risk for developing CD [21, 22, 33]. These calculations are based on the original publications in which no healthy homozygous carriers had been found. In our own populations we did find homozygous carriage in individuals with a relatively

high age and without signs of CD.

Besides the frameshift mutation, carriage of the two other common CARD15 mutations in exon 4 and 8 are also associated with an increased disease risk. None of the three mutations are located together on the same allele. In Table 2, the disease risks for developing CD are summarised for carriage of one or two of the three common CARD15 mutations [21]. The population attributable risk (PAR) of the three CARD15 mutations is around 15–20%. The PAR estimates the proportion of a disease attributable to a specific risk factor [35].

DIAGNOSTICS

The diagnosis CD is based on clinical, radiological and endoscopic criteria. So far, CARD15 DNA testing has no consequences concerning the diagnostic work-up and treatment of CD. It is also not accepted as a non-invasive screening test when considering the diagnosis CD in symptomatic patients. However, recent studies have shown that carriage of the CARD15 frameshift is associated with an early age of disease [36], disease localisation in the terminal ileum [34, 36–39], and a stenosing disease behaviour [36, 38, 39]. One study reported association with fistula [38], another did not [36]. These findings make it possible to subtype patients with CD. Maybe CARD15 genotyping will become a clinical tool to predict the course and prognosis of the disease.

CONCLUSION

The discovery of the CARD15 gene is a milestone in the history of IBD. It will give new insights on the pathogenesis of CD. At the same time, an important conclusion is that CD is a complex genetic disorder of which one single risk factor is neither necessary nor sufficient to develop the disease.

Table 1. Review of genome screens in inflammatory bowel disease

Authors (year) reference	Chromosome [a]	Disease [a]
Hugot et al. (1996) [12]	16 (IBD1 locus)	CD
Satsangi et al. (1996) [13]	12q (IBD2 locus), 7q, 3p	CD and UC
Cho et al. (1998) [14]	1p, 3q, 4q	CD and UC
	16	CD
Hampe et al. (1999) [15]	1q, 6p (IBD3 locus), 22	CD and UC
	10, 12, 16	CD
	4, X	UC
Ma et al. (1999) [16]	12, 14q	CD
Duerr et al. (2000) [17]	14q (IBD4 locus)	CD
Rioux et al. (2000) [18]	3p, 5q (IBD5 locus), 6p, 19q	CD and UC

[a] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

Table 2. Relative risk to develop Crohn's disease with respect to carriage of the three common CARD15 mutations [21]

Carriage of mutations	Relative risk	Absolute risk (%)
None	1	0.07
One, heterozygous carriage	3	0.2
One, homozygous carriage	38	3
Two, compound heterozygous carriage (on two different alleles)	44	3

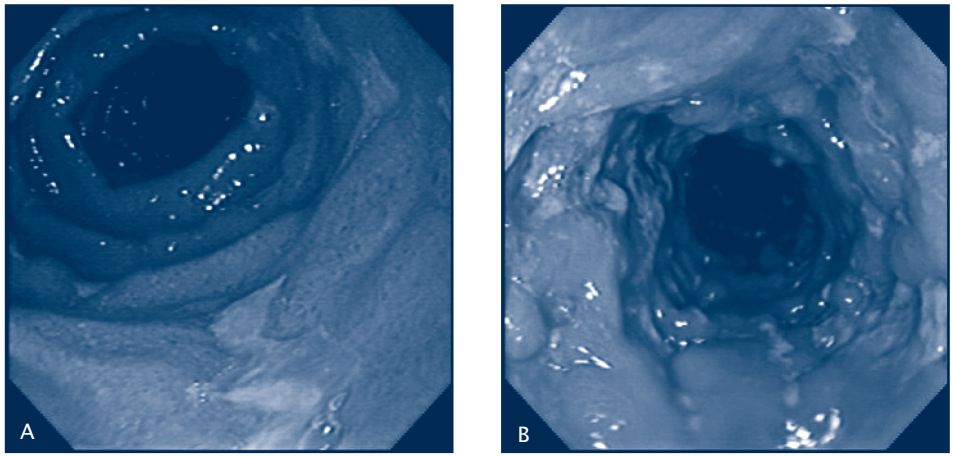

Figure 1. Endoscopic views of Crohn's disease. **A**, recurrent disease in the neo-terminal ileum after ileocecal resection. The mucosa is red and swollen with several irregular longitudinal ulcers. **B**, severe Crohn's disease of the colon with multiple irregular deep longitudinal ulcers and disappearance of haustration.

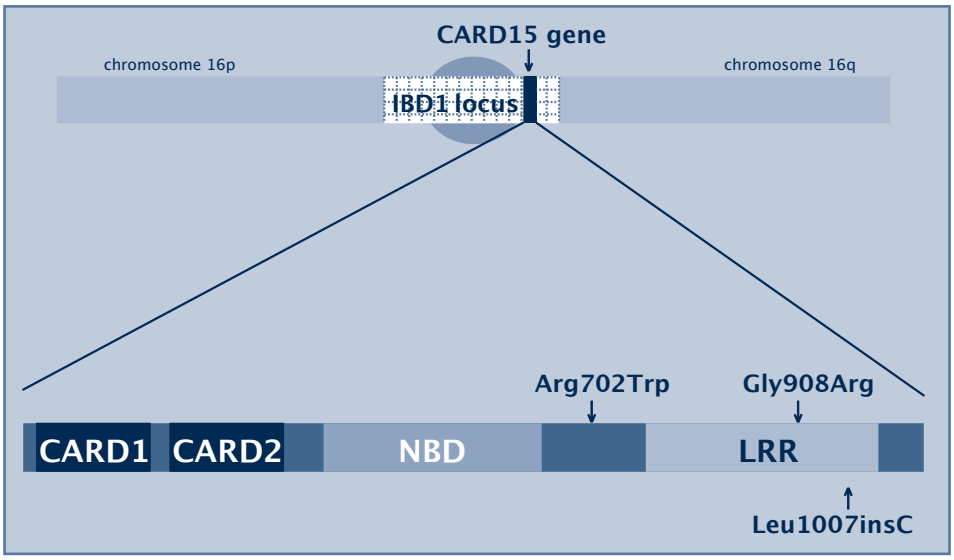

Figure 2. A schematic illustration of the inflammatory bowel disease (IBD)-1 locus, the caspase recruitment domain (CARD)-15 gene, and the CARD15 protein with the three common amino acid changes which are associated with Crohn's disease. Only 3020insC have demonstrated an *in vitro* functional defect. This frameshift mutation leads to truncation of the C-terminal protein by the change of leucine to proline at amino acid position 1007. NBD, nucleotide-binding domain; LRR, leucine-rich repeats.

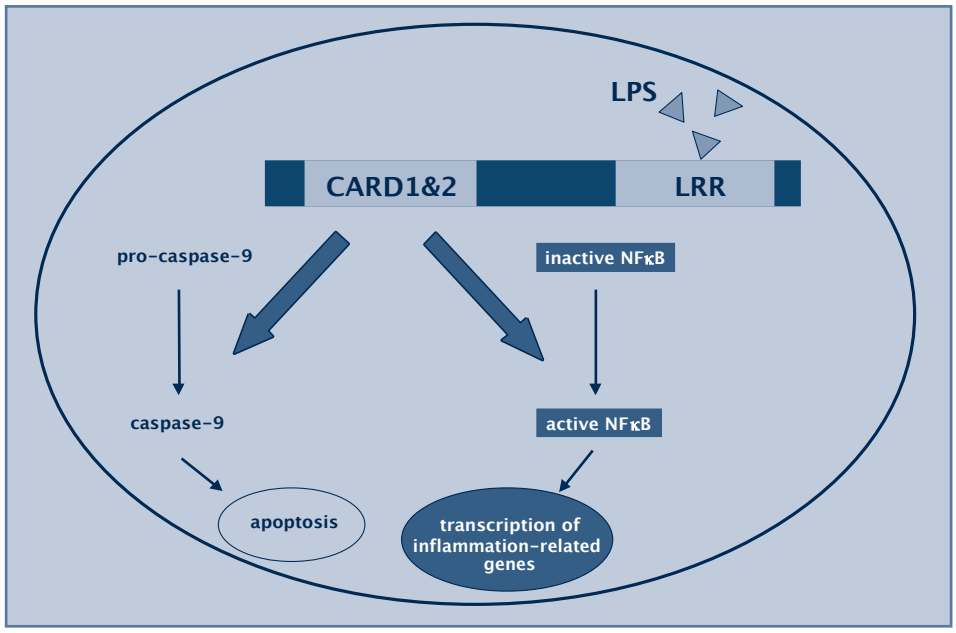

Figure 3. A schematic illustration of caspase recruitment domain (CARD)-15 dependant inflammatory and apoptotic response. CARD1 and CARD2 are important for the initiation of apoptosis and activation of nuclear factor kappa-B (NFκB). Binding (or interaction) of lipopolysaccharide (LPS) with the leucine-rich repeats (LRR) domain leads to NFκB activation. Whether this is also the first step towards apoptosis is yet not known.

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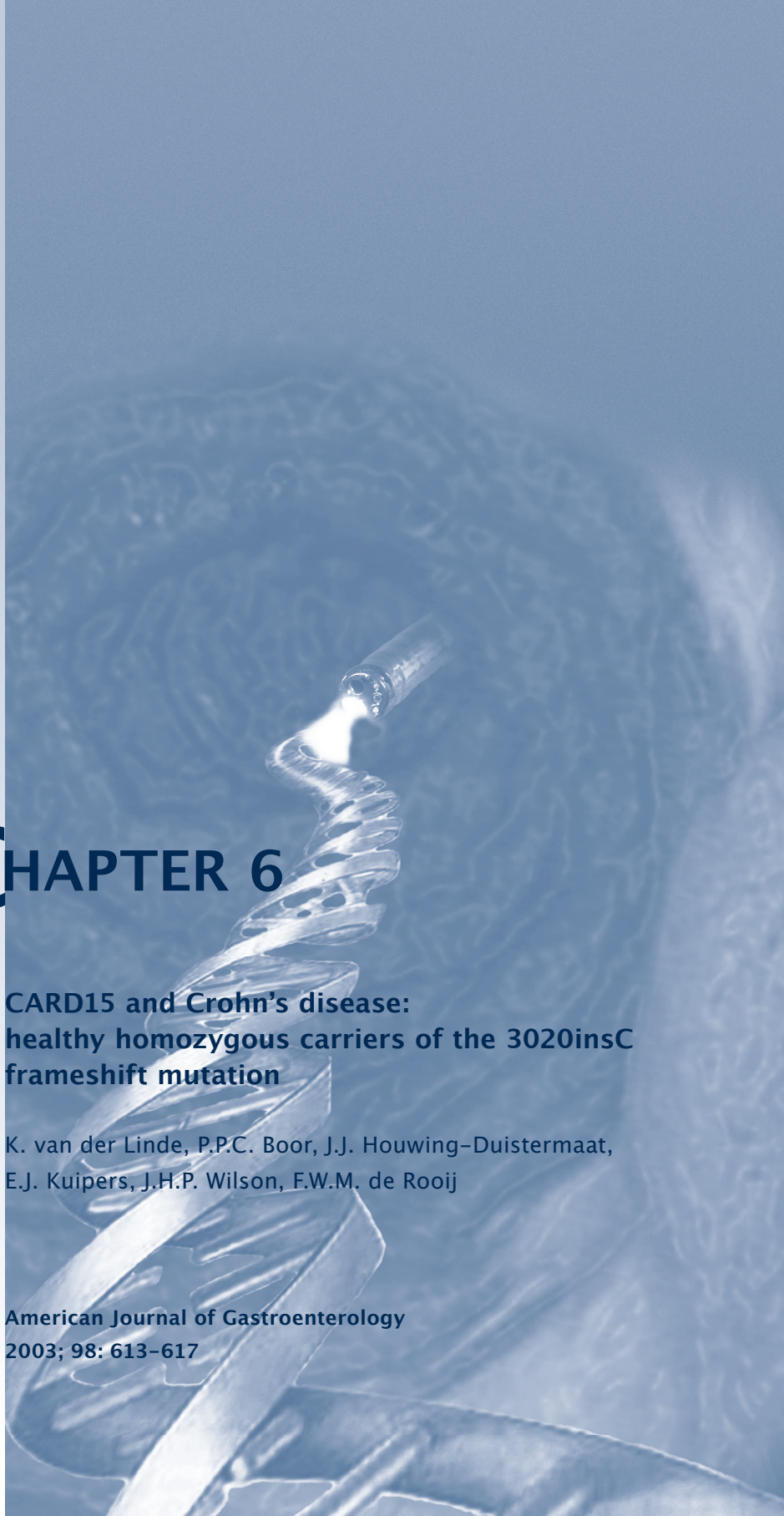

CHAPTER 6

CARD15 and Crohn's disease: healthy homozygous carriers of the 3020insC frameshift mutation

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2003; 98: 613-617

ABSTRACT

Background

Single nucleotide variations in the CARD15 gene have recently been shown to be associated with Crohn's disease (CD). Of special interest is a cytosine insertion at position 3020 of exon 11 (3020insC), which leads to a stop codon, truncation of the CARD15 protein, and an altered function of CARD15. The aim of the study was to evaluate this frameshift mutation in Dutch multiple affected families with inflammatory bowel disease (IBD).

Methods

Ninety-three Caucasian multiple affected families with IBD were recruited by interviewing patients attending our department. Sixty-one probands had CD, and 32 probands ulcerative colitis (UC). The diagnosis of probands and affected family members was verified according to standard criteria. In addition, 81 healthy unrelated controls were included. Genomic DNA was isolated from venous blood of all participants to determine the CARD15 3020insC mutation by using an allele-specific polymerase chain reaction, followed by agarose gel electrophoresis and DNA sequencing.

Results

Association with CARD15 3020insC was statistically significant for CD, but not for UC. In one of the multiple affected families, middle-aged and elderly homozygous carriers were identified without CD.

Conclusion

Although CARD15 3020insC appears to be aetiologically important in CD, homozygous carriage does not always lead to IBD.

INTRODUCTION

The aetiology of inflammatory bowel disease (IBD), a chronic recurrent gastrointestinal disease, is complex and largely unknown. Genetic susceptibility, however, plays an important role, as demonstrated by several epidemiological studies [1]. Additionally, during the 1990s several genome-wide linkage analyses with multiple highly polymorphic microsatellite markers in multiple affected families with IBD have revealed putative disease loci. The results of these studies are summarised in Table 1 [2-8].

A pericentromeric locus on chromosome 16 was the first genomic susceptibility region of interest, described by Hugot et al. in 1996. This so-called IBD1 locus appeared to be linked with Crohn's disease (CD) [2]. The findings were replicated by others [4, 5, 9-17]. Absence of linkage of CD with the IBD1 locus has also been reported [18, 19].

Linkage of CD with the IBD1 locus appears to be based on genetic alterations in the CARD15 gene. The CARD15 gene is located on chromosome 16q12 and was described by Ogura et al. in 2001 [20]. Three single nucleotide variants appeared to be highly associated with CD. One specific alteration involves a cytosine insertion at position 3020 of exon 11, leading to a stop codon and truncation of the CARD15 protein. It affects the C-terminal leucine rich repeats (LRR), shortening this protein domain by 33 amino acids. This 3020insC frameshift mutation showed preferential transmission in families with CD but not in families with ulcerative colitis (UC) [21, 22]. The spectacular findings have been subsequently confirmed by several other groups [23-30].

The goal of this study was to evaluate the prevalence of CARD15 3020insC in Dutch IBD-affected probands and their (non-)IBD-affected family members.

MATERIALS AND METHODS

Patients and families

Multiple affected families with IBD were identified by interviewing IBD patients attending the Departments of Gastroenterology, Internal Medicine, and Surgery of the Erasmus MC University Medical Centre Rotterdam (UMCR). Probands, affected relatives, and available non-affected relatives completed a questionnaire about IBD and other diseases. Based on information supplied by their physician, the diagnosis was verified according to standard criteria [31].

The study was approved by the Institutional Review Board of the Erasmus MC UMCR. All participants gave written informed consent.

DNA isolation

Genomic DNA was isolated from whole peripheral venous blood collected in ethylenediaminetetraacetic acid anticoagulated tubes (Becton Dickinson, Leiden, The

Netherlands) using standard techniques. In some instances, DNA was isolated from Epstein-Barr virus transformed lymphoblastoid cell lines [32], derived from peripheral venous blood collected in acid citrate dextrose solution containing tubes (Becton Dickinson).

Genotyping

The CARD15 3020insC mutation was determined by means of an allele-specific polymerase chain reaction (PCR), followed by separation on agarose gels, as described by Ogura et al. [22]. Briefly, a multiplex PCR with four primers was performed. Two control intronic primers were used, including forward 5'-CTGAGCCTTTGTTGATGAGC-3' and reverse 5'-TCTTCAACCACATCCCCATT-3' resulting in a 533 base pairs (bp) fragment including position 3020 of exon 11. Two additional exonic primers were used to discriminate between the wild-type and 3020insC allele, including reverse 5'-CGCGTGTCATTCCTTTCATGGGGC-3' and forward 5'-CAGAAGCCCTCCTGCAGGCCCT-3'. These primers result in a 319 bp containing wild-type fragment, or a 214 bp fragment in the case of a 3020insC allele. The PCR products were identified by electrophoresis on a 2% agarose gel and ethidium bromide staining. When participants appeared positive for CARD15 3020insC, the mutation was confirmed by DNA sequencing of a exon 11 PCR fragment amplified with the forward primers 5'-AGGATGTGTCTAAGGGACAG-3' and reverse primer 5'-CTGAATGTCAGAATCAGAAGG-3'. Sequencing was performed on an ABI 310 genetic analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands).

In one family, non-IBD homozygous CARD15 3020insC mutation carriers were found. To exclude these results to be caused by an unknown sequence variation in the binding site of the primers, a second confirmation test was used. Exon 11 was amplified and sequenced with the two control intronic primers mentioned earlier.

Statistics

Association was determined by comparing the frequencies of the CARD15 3020insC frameshift mutation in cases with CD and controls, using the Fisher exact test. It was expressed as relative risk (RR) with a 95% confidence interval (CI). The genotype relative risk (GRR) was evaluated by comparing the genotype frequencies in cases and controls. For cases, the observed frequencies were used, whereas for controls the genotype frequencies were estimated assuming the genotypes to be in Hardy-Weinberg equilibrium proportions (i.e., assuming no selection against the homozygotes) [22]. The analyses were performed with SPSS software version 8.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Ninety-three IBD-affected probands with either CD (n = 61) or UC (n = 32), and with a positive family history for IBD were screened for the CARD15 3020insC frameshift mutation. In addition, 81 unrelated healthy controls were examined.

Fourteen (23%) probands with CD, one (3%) proband with UC, and three (4%) healthy controls were carriers of the mutation. Three CD-affected probands were homozygous for the frameshift mutation. All other carriers, including UC-affected probands and controls were heterozygous. The allele frequency of the CARD15 3020insC among patients with CD was 14%, and approximately 2% in UC-affected patients and in controls. The frequency of the frameshift mutation was significantly higher in CD-affected patients than in controls ($P < 0.0001$, Fisher exact test). No association was found with UC.

In probands with CD and heterozygous for CARD15 3020insC, the GRR was 6.1 (95% CI = 1.6−23.7). As the CARD15 3020insC genotype frequencies in controls were assumed to be in Hardy-Weinberg equilibrium proportions ($P = 0.87$), we could estimate the expected frequency of homozygous frameshift carries in the control population. We used this to calculate the GRR for the homozygous carriers, which was 178 (95% CI = 0−∞).

Subsequently, available IBD-affected relatives of the 14 probands with CD who were positive for CARD15 3020insC were screened for the mutation. The data are summarised in Table 2. Most affected relatives were concordant for disease as well as for carriage of the mutation.

In addition, available non-IBD-affected relatives were screened for carriage of CARD15 3020insC when the proband had the frameshift mutation. In one family with a homozygous CD-affected proband, two sisters, the father and an aunt (sister of the father) were also homozygous carriers. Except for one sister, all these relatives had no symptoms or signs of IBD or other major gastrointestinal health problems. One relative (sister) was diagnosed with irritable bowel syndrome, based on a normal colonoscopy and blood tests. Follow-up of these non-IBD-affected family members was available until the beginning of 2002 (range of age: 42−83 years). The pedigree of the family is shown in Figure 1. Individual details are given in Table 3. In another family with a CARD15 3020insC homozygous CD-affected proband, the father was heterozygous for the mutation but appeared to have UC.

DISCUSSION

The CARD15 (formerly NOD2) gene is a recently described gene [20]. Functionally, the CARD15 gene product has been predicted to have similar activity to the apoptotic protease activating factor-1, which is a regulator of apoptosis [33]. The structure of the CARD15 protein is similar to that of CARD4 (formerly NOD1) [20]. CARD15 is located

on chromosome 16q12 within the IBD1 locus. It seems to play an important role in the aetiology of CD, as three single nucleotide variants are highly independently associated with the disease [21, 26].

CARD15 is a cytosolic protein expressed in monocytes. Its C-terminal region is presumed to be involved in recognition of bacterial lipopolysaccharide (LPS) through the LRR. Binding or interaction of LPS with the LRR domain triggers activation of the transcription factor nuclear factor kappa-B (NFκB) through two N-terminal caspase recruitment domains (CARD). NFκB is an important regulator of genes involved in a pro-inflammatory response [34].

One of the three CD-associated nucleotide variants is a 3020insC in exon 11, which results in a truncated CARD15 protein missing the final 33 amino acids of the LRR. It probably leaves the CARD motifs and the centrally located nucleotide binding domain functionally intact [21, 22]. Embryonic kidney cells (HEK293T) transfected with 3020insC-mutated CARD15 plasmids showed a reduced activity of NFκB after exposition to LPS from various bacteria, compared with wild-type CARD15 transfected cells [22]. Based on these experiments, the CARD15 frameshift mutation is thought to result in an altered monocytic response to bacterial components in CD. Whether this reduced NFκB response to LPS under artificial circumstances is relevant to the pathogenesis of CD is not clear, as colonic tissue from CD patients has been found to have increased rather than decreased NFκB activity compared with that of controls [35].

Carriage of the CARD15 3020insC mutation results in an increased RR of developing CD and appears to be a risk factor for early onset of disease [24], location of disease in the small bowel [24, 25, 28-30], and development of stenosis [24, 26, 28, 29]. Whether carriage is also associated with fistulas is not yet clear [24, 28]. In the initial studies on the prevalence of CARD15 3020insC mutation in IBD, no homozygous healthy controls have been detected. In these studies, the RR of homozygous CD-affected individuals was estimated to be as much as 18-42-fold, assuming that the genotype frequencies in controls were in Hardy-Weinberg equilibrium proportions [22, 23]. In this study also no unrelated homozygous controls were found. However, we did find homozygous carriers of CARD15 3020insC within a family of two CD-affected siblings. These homozygous relatives had never had important gastrointestinal complaints or features suggesting IBD. They remained asymptomatic 27–67 years beyond the age of diagnosis of the proband. Searching the literature for other reports on non-IBD-affected homozygous carriers, we found only one other study on the prevalence of CARD15 in psoriasis, which reported a 60-year-old healthy homozygous carrier in the control group [36]. These results demonstrate that homozygosity does not necessarily lead to manifest IBD, even at older age.

If the mutation by itself would be a major determinant of CD, one would expect, by analogy with other genetic diseases, homozygosity to result in a more severe phenotype. Other factors, both genetic and environmental, must be involved in the pathogenesis of CD. As CARD15 mutations have only been found in a minority of patients with CD patients [21-23], it is likely that IBD is not a single disease but rather

a group of aetiologically and genetically distinct diseases with similar clinical presentations. The various results of genome-wide linkage studies in IBD (Table 1) also support this multigenetic pathogenesis.

In conclusion, this study confirms the association of CARD15 3020insC with CD. Homozygous carriage, however, does not always lead to disease. Further studies on the function of both wild-type and truncated CARD15 protein will help unravel the role of this frameshift mutation in CD.

Table 1. Summary of genome screens in inflammatory bowel disease

Authors (year) [reference]	Significant results of linkage analysis [37]	
	Chromosome [a]	Disease [a]
Hugot et al. (1996) [2]	16 (IBD1 locus)	CD
Satsangi et al. (1996) [3]	12q (IBD2 locus), 7q, 3p	CD and UC
Cho et al. (1998) [4]	1p, 3q, 4q 16	CD and UC CD
Hampe et al. (1999) [5]	1q, 6p (IBD3 locus), 22 10, 12, 16 4, X	CD and UC CD UC
Ma et al. (1999) [6]	12, 14q	CD
Duerr et al. (2000) [7]	14q (IBD4 locus)	CD
Rioux et al. (2000) [8]	3p, 5q (IBD5 locus), 6p, 19q	CD and UC

[a] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

Table 2. CARD15 genotype of inflammatory bowel disease-affected relatives of probands with Crohn's disease who carried the CARD15 3020insC frame-shift mutation

	CD-affected relatives [a]		UC-affected relatives [a]	
Familial relation to proband with CD [a]	**3020insC positive**	**3020insC negative**	**3020insC positive**	**3020insC negative**
First-degree	4	1	1	0
Second-degree	1	1	0	1
Third-degree	4	2	0	0
> Third-degree	3	3	0	3
Not available	1 [b]		1 [c]	

[a] CD, Crohn's disease; UC, ulcerative colitis.
[b] A third-degree family member was not available for testing.
[c] A second-degree family member died before starting this study.

Table 3. Characteristics of an inflammatory bowel disease family with affected and non-affected homozygous CARD15 3020insC frameshift mutation carriers

	Year of birth (year of death)	CARD15 3020insC genotype	Age of diagnosis CD (yrs) [a]	Location of CD [a]	Other relevant diseases [a]
Proband	1963	+ / +	16 (1979)	small bowel, rectum	uveitis, sacroiliitis
Sister of proband	1952	+ / +	29 (1981)	small bowel	
Sister of proband	1953	+ / +			IBS, normal colonic barium study in 1995
Sister of proband	1954	+ / -			IBS, endoscopy at early adolescence was normal
Sister of proband	1959	+ / +			
Father of proband	1924 (1999)	+ / +			no history of abdominal disorders, died of an acute myocardial infarction
Mother of proband	1926 (1988)	+ / - [b]			no history of abdominal disorders, died due to metastatic cancer of unknown primary
Sister of father	1916 (2000)	+ / +			no history of abdominal disorders, known with cardiovascular disease, diabetes mellitus, and chronic obstructive pulmonary disease
Sister of father	1917 (2000)	- / -			IBS, normal colonoscopy in 1997, known with cerebral vascular accident

[a] CD, Crohn's disease; IBS, irritable bowel syndrome.
[b] Reconstructed.

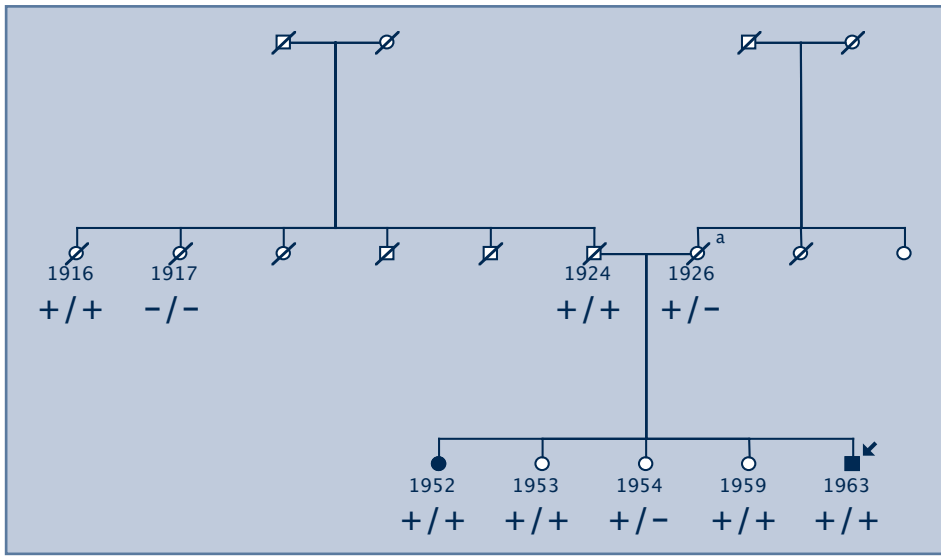

Figure 1. A multiple IBD-affected family with healthy homozygous CARD15 3020insC carriers. ■, CD-affected male; ●, CD-affected female; □, non-IBD-affected male; ○, non-IBD-affected female; ↙, proband; + / +, homozygous carriage of CARD15 3020insC; + / -, heterozygous carriage of CARD15 3020insC; - / -, homozygous carriage of wild-type CARD15. *Row below individuals*, year of birth. [a] Reconstructed genotype.

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CHAPTER 7

CARD15 mutations in Dutch familial and sporadic inflammatory bowel disease and an overview of European studies

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ABSTRACT

Background

The single nucleotide variations R702W, G908R and L1007fs in the CARD15 gene have been found to be independently associated with Crohn's disease (CD). The aim of this study was to evaluate the prevalence of these gene variations in Dutch multiple inflammatory bowel disease (IBD)-affected families, in sporadic IBD-affected patients and in healthy controls.

Methods

Dutch Caucasians from multiple IBD-affected families were recruited, including 78 probands with CD, 34 probands with ulcerative colitis (UC) and 71 IBD-affected and 100 non-IBD-affected family members. In addition, 45 sporadic IBD patients (36 CD and nine UC) and 77 unrelated healthy controls were included. Genomic DNA was isolated to determine CARD15 R702W, G908R and L1007fs. For these mutations, we evaluated disease susceptibility and correlation with IBD phenotypes.

Results

In all included unrelated IBD-affected probands, the R702W, G908R and L1007fs allele frequencies were 8.8, 6.1 and 11.0%, respectively, for CD, and 4.7, 0 and 2.3% for UC. In controls, the allele frequencies were 5.9, 0.7, and 1.9%, respectively. G908R and L1007fs were associated with CD ($P = 0.006$ and 0.001, respectively). Compound heterozygotes for any of the three mutations were 11 (9.2%) in CD patients, but none in UC patients or controls. Carriage of CARD15 mutations was not associated with familial disease ($P \geq 0.38$). IBD-affected family members of CD-affected probands carrying L1007fs, however, were significantly more often carrier of L1007fs than expected ($P < 0.001$). In CD-affected patients, a significant trend was found between carriage of at least one CARD15 mutation, and between carriage of L1007fs and behaviour of disease, including more carriers with stricturing and even more with penetrating disease ($P = 0.006$ and 0.017, respectively).

Conclusion

In the Dutch population, CARD15 G908R and L1007fs are associated with CD. Although no difference was found between sporadic and familial cases, in L1007fs positive multiple affected families the IBD-affected relatives are more likely than expected to carry this mutation. In CD, carriage of at least one CARD15 mutation is associated with a more complicated disease behaviour.

INTRODUCTION

Genetic susceptibility plays an important role in the aetiology of inflammatory bowel disease (IBD). Several epidemiological studies have in particular shown high concordance rates of Crohn's disease (CD) in monozygotic twins [1, 2]. Molecular genetic association studies including candidate gene and genome-wide screening studies have suggested that several immune and inflammation-related genes contribute to the pathogenesis of IBD [3, 4].

In 2001, two groups reported the first susceptibility gene in CD, originally called NOD2 and more recently CARD15. The gene is located on chromosome 16q12 and its discovery was based on the linkage analysis of genome screening in IBD [5, 6]. CARD15 is expressed in monocytes, macrophages and intestinal epithelial cells [7–10]. CARD15 is a member of the Ced4 superfamily that includes apoptotic protease activating factor-1 [11] and CARD4 [12, 13].

So far, more than 100 single nucleotide polymorphisms (SNPs) and mutations have been described in the CARD15 gene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp). Only three mutations are independently associated with CD in Caucasian populations, 2104C → T in exon 4 (SNP8, R702W, Arg702Trp), 2722G → C in exon 8 (SNP12, G908R, Gly908Arg), and 3020insC in exon 11 (SNP13, L1007fs, Leu1007fsinsC) [14]. Several studies from different countries have been published on the prevalence of these three mutations in IBD. The results from European countries are summarised in Table 1 [5, 15–55]. CD is most consistently associated with CARD15 L1007fs. One study also demonstrated a significantly increased prevalence of L1007fs in ulcerative colitis (UC) compared with controls [39].

While simple heterozygous carriage of R702W, G908R or L1007fs is associated with about a three-fold increased relative risk for CD, compound heterozygous carriage or homozygous carriage results in a CD genotype relative risk (GRR) of 23–44 [5, 55, 56]. All three nucleotide changes are considered to be disease-associated mutations. Especially L1007fs has been demonstrated *in vitro* to be associated with a reduced activation of transcription factor nuclear kappa-B (NFκB) in mononuclear cells [6, 57, 58]. Recent data in a L1007fs knock-in mouse model, however, showed that carriage of L1007fs is associated with an elevated NFκB activity [59]. This mouse model is more in line with the clinical observation of elevated NFκB activity in patients with active CD [60].

CARD15 is expressed in Paneth cells, which are specialised epithelial cells located in the mucosal crypts of Lieberkühn, and which are thought to play a protective role in the host against enteric flora [61]. While a high CARD15 expression has been demonstrated in CD-affected ileal Paneth cells, only weak expression was seen in normal ileal mucosa of CD and non-IBD-affected individuals [10]. An antimicrobial defence mechanism of Paneth cells is secretion of antimicrobial peptides defensin-5 and defensin-6, which is diminished in CD-affected ileal Paneth cells. CARD15 L1007fs carriage showed a further defensin deficiency [27, 62]. Intestinal barrier function can also

be harmed due to an increased intestinal permeability, which is found in patients with CD and in their healthy relatives. Carriage of CARD15 L1007fs appeared to be associated with an increased intestinal permeability in these healthy first-degree relatives [63, 64], except for one study [65].

In multiple IBD-affected families, there is a high concordance rate for type of disease [66, 67]. Not much is known about the concordance rate of CARD15 mutation carriage in familial IBD. Recently, we have published our data about CARD15 L1007fs in familial CD. CD-affected relatives were more often concordant for L1007fs carriage than UC-affected relatives [68]. Otherwise, a Finnish study demonstrated in CD probands an association between CARD15 L1007fs carriage and familial IBD [20].

The goal of this study was to determine the prevalence of the three CARD15 mutations R702W, G908R and L1007fs in Dutch patients, with either familial or sporadic IBD. In familial IBD, CARD15 mutations were examined in IBD-affected and non-IBD-affected relatives. Moreover, a correlation with clinical phenotypes in IBD probands was sought.

MATERIALS AND METHODS

Patients, families and controls

Caucasian patients with IBD, meeting standard criteria [69] and attending the Erasmus MC University Medical Centre Rotterdam (UMCR), were recruited for this study. Patients were classified as familial IBD if one or more relatives were known to have IBD. Probands, available affected relatives and non-affected relatives completed a questionnaire about IBD and other diseases. On the basis of the information supplied by their physician, the diagnosis was verified [69]. The disease phenotype of CD probands was classified according to the Vienna classification for CD [70]. UC-affected probands were phenotyped according to the age at onset (< 40 and ≥ 40 years) and the location of the disease (proctitis, left-sided colitis and extended or pancolitis). Some patients and their relatives have been included in other studies [68, 71]. Caucasian controls were recruited at the Erasmus MC UMCR and have been described before [68, 71]. The study was approved by the Institutional Review Board of the Erasmus MC UMCR. All participants provided written informed consent.

DNA isolation

Genomic DNA was isolated from whole peripheral venous blood collected in ethylenediaminetetraacetic acid anticoagulated tubes (Becton Dickinson, Leiden, The Netherlands) using standard techniques. In some cases, DNA was isolated from Epstein-Barr virus transformed lymphoblastoid cell lines, derived from peripheral venous blood collected in acid citrate dextrose solution containing tubes (Becton Dickinson) [72].

Genotyping

CARD15 R702W and G908R were determined by means of restriction enzyme analysis. First, the DNA regions of interest containing the mutation were amplified by polymerase chain reaction (PCR). For R702W (2104C → T in exon 4), the forward primer 5'-CAGCTGGGCAGCTTTGCTGC-3' and the reverse primer 5'-CATGGCATGCACGCTCTTGG-3' were used. The R702W PCR product (448 base pairs (bp)) was purified by phenol extraction followed by ethanol precipitation, after which the PCR fragment was digested with 8 units of Hpa II (New England BioLabs, Beverly, Massachusetts, USA) for 4 hours (h) at 37 ^{0}C. Bands were visualised after separation on a 2% agarose gel containing ethidium bromide. The wild-type exon 4 resulted in fragments of 29, 54, 76 and 289 bp, while the R702W polymorphism resulted in fragments of 29, 130, and 289 bp. The G908R (2722G → C in exon 8) mutation was amplified using the forward primer 5'-CACTTTGCTGGGACCAGGAG-3' and the reverse primer 5'-ACTCCATTGCCTAACATTGTGG-3'. The G908R PCR product (363 bp) was not further purified, and digested immediately with 4 units HinP1 I (New England BioLabs) for 3 h at 37 ^{0}C followed by separation on a 2% agarose gel containing ethidium bromide. It resulted in one fragment of 363 bp in case of the wild-type allele, and in fragments of 168 and 195 bp in case of the G908R mutation.

CARD15 L1007fs (3020insC in exon 11) was determined by means of an allele-specific PCR reaction, followed by separation of the amplicons on an agarose gel as described previously [6, 68].

When participants were found to be positive for CARD15 R702W, G908R or L1007fs, DNA sequencing of the corresponding PCR product was performed to confirm the mutation. Available relatives of these probands were screened for the same CARD15 mutation by DNA sequencing. Sequencing was performed on an ABI 310 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands).

Statistics

Allele and genotype frequencies in CD, UC cases and in controls were determined. Associations were tested by comparing the allele frequencies of the CARD15 R702W, G908R and L1007fs mutations in cases with IBD and controls, using Fisher's exact test. The genotype frequencies in controls were tested for Hardy-Weinberg equilibrium (HWE) proportions. In case of association, the GRR and 95% confidence interval (CI) in heterozygous and homozygous carriers were estimated by comparing the genotype frequencies in cases and controls, and expressed as an odds ratio. When homozygous carriers were observed among the cases but not among the controls, the GRR in homozygous carriers versus non-carriers was computed using the expected number of homozygous carriers in controls based on HWE. A comparison of genotype frequencies between different groups was evaluated using Fisher's exact test [6].

In familial IBD, the frequency of CARD15 mutations in affected family members

was evaluated. We computed the expected number of carriers among affected relatives of probands carrying the mutation under Mendelian transmissions and assuming a small allele frequency. Taking 'p' as the allele frequency in the population, then the probability of carrying the mutation for first-degree relatives is ½ + p, and for second-degree relatives this probability is ¼ + 1½p, etc. In this formula, the first term is the probability that relatives carry the same allele as the proband, and the second term is the probability that relatives carry another allele of the mutation. As an estimate of 'p', we used the observed frequency in the controls. We tested whether the observed number of carriers differed from the expected number of carriers. To this end, the variance of the observed number of carriers taking into account the correlation among affected relatives of one proband was computed. We used a normal distribution to derive a P value.

Associations between genotypes and IBD phenotypes were evaluated by χ^2 statistics. For ordinal variables, we also applied the χ^2 statistics for trend. In the case of small numbers, exact P values were computed.

Mean follow-up between groups was evaluated by Student's t test.

All analyses were performed with SPSS software version 9.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Patients, families and controls

In this retrospective study, 114 unrelated Caucasian patients with CD (46 men and 68 women, mean age at end of follow-up: 41.4 years, range: 21.8-78.8 years) and 43 with UC (21 men and 22 women, mean age at end of follow-up: 45.8 years, range: 28.0-71.5 years) were screened for CARD15 mutations. Their phenotypes are demonstrated in Tables 3 and 4. Seventy-eight (68%) CD- and 34 (79%) UC-affected patients had a positive family history for IBD. From these families, 45 CD-affected, 26 UC-affected, 100 non-IBD-affected blood-related and 19 non-IBD-affected non-blood-related family members were included. In addition, 77 unrelated healthy Caucasian adults were included as controls.

CARD15 mutations in patients with inflammatory bowel disease and controls

R702W Twenty (17.5% of 114) patients with CD, four (9.3% of 43) with UC and nine (11.8% of 76) healthy controls (one control not tested) were carriers of the CARD15 R702W mutation. All mutation carriers were heterozygous. In controls, the genotype frequencies were in HWE (P = 0.59). The allele frequency of CARD15 R702W among patients with CD was 8.8%, 4.7% in UC patients and 5.9% in controls. The frequency of this missense mutation was not statistically different (P = 0.33) between patients with CD and controls, nor between patients with UC and controls (P = 0.77).

G908R CARD15 G908R was found in 14 (12.3% of 114) patients with CD and in one (1.3% of 76) healthy individual (one control not tested). Only heterozygous carriers were found. In controls, the genotype frequencies were in HWE (P = 0.95). No UC-affected carriers were found. The allele frequency of the G908R mutation was 6.1% in CD-affected patients and 0.7% in controls, which was significantly different (P = 0.006). The GRR for CD in G908R carriers versus non-carriers was 10.5 (95% CI 1.4–81.6, P = 0.005).

L1007fs We found carriage of CARD15 L1007fs in 22 (19.3% of 114) patients with CD, in two (4.7% of 43) UC-affected patients, and in three (3.9% of 77) healthy controls. Three (2.6%) CD-affected patients were homozygous carriers. In controls, the genotype frequencies were in HWE (P = 0.86). The allele frequency was 11.0% in CD-affected patients, 2.3% in UC-affected patients and 1.9% in healthy controls. CARD15 L1007fs was associated with CD (P = 0.001), but not with UC (P = 1.0). The GRR for CD of heterozygous compared with wild-type carriers was 5.1 (95% CI 1.5–17.9, P = 0.005). On the basis of HWE, the estimate for the number of homozygous carriers in controls was 0.029, which yields a GRR for CD of homozygous versus wild-type carriers of 83.2 (95% CI = 0.31–∞, P = 0.26).

Combined carriage of CARD15 mutations in patients with inflammatory bowel disease and controls

The prevalence of CARD15 compound heterozygosity in CD-affected probands was 2.6% (n = 3) for R702W/G908R, 3.5% (n = 4) for R702W/L1007fs and 0.9% (n = 1) for G908R/L1007fs. In patients with UC and controls, no combined mutation carriage was found. The GRR for CD in patients with carriage of one mutation (R702W, G908R or L1007fs) versus wild-type carriers was 2.8 (95% CI = 1.3–5.7, P = 0.007). The GRR for CD of compound heterozygous or homozygous versus wild-type carriers was 17.8 (95% CI 1.23–258.0, P = 0.001).

Prevalence of CARD15 mutations in familial and non-familial inflammatory bowel disease-affected patients

Carriage of CARD15 R702W, G908R and L1007fs in CD-affected probands with a positive familial history of IBD was 15.4, 14.1 and 20.5%, respectively. In CD-affected probands with a negative familial history, the prevalences were 22.2, 8.3 and 16.7%, respectively. Although the G908R and L1007fs were more and the R720W was less prevalent in CD-affected probands with a positive familial history than in sporadic CD-affected probands, these differences were not statistically significant (P = 0.43, 0.54 and 0.80, respectively). Carriage of CARD15 R702W and L1007fs in UC-affected probands with a positive familial history of IBD was 11.8 and 2.9%, respectively. Prevalences in sporadic UC-affected probands were 0 and 11%, respectively. None of the prevalences between the two UC groups was significant different (P = 0.56 and 0.38, respectively).

As the group sizes of CD-affected probands with a negative family history (n = 36),

UC-affected probands with a negative family history (n = 9) and UC-affected probands with a positive family history (n = 34) are quite small, we also analysed the combined data. In familial IBD-affected probands (n = 112), carriage of CARD15 R702W, G908R and L1007fs was 14.3, 9.8 and 15.2%, respectively. In sporadic IBD-affected probands (n = 45), the prevalences were 17.8, 6.6 and 15.6%, respectively. These differences were not significant (P = 0.63, 0.54 and 1.0, respectively).

Prevalence of CARD15 mutations in patients and relatives with inflammatory bowel disease

Available IBD-affected and unaffected relatives (up to the sixth-degree) of probands positive for CARD15 R702W, G908R or L1007fs were screened for the mutation. Among first-degree IBD-affected relatives of CD-affected probands carrying R702W, G908R and L1007fs mutations, the number of carriers with the same mutation was three out of six (50%), one out of four (12%) and five out of six (80%), respectively. Among IBD-affected relatives of CD-affected probands carrying CARD15 L1007fs (n = 6), carriage of CARD15 L1007fs was significantly more frequent than expected (P < 0.001). Carriage of CARD15 R702W and G908R mutations in IBD-affected relatives was not different from what was expected. The pedigrees of the families with the L1007fs mutation are illustrated in Figure 1, and the statistical results are summarised in Table 2. The number of available families of UC-affected probands was too low to perform statistical analysis.

Genotype-phenotype correlation

Carriage of CARD15 mutations and clinical phenotypes of all CD- and UC-affected probands were established. The results are summarised in Tables 3 and 4. In CD, an association was found between carriage of at least one CARD15 mutation and disease behaviour (P = 0.023). Carriage of L1007fs was almost significant associated with disease behaviour (P = 0.052). In fact, in carriers of at least one CARD15 mutation and in carriers of L1007fs, a significant linear effect (trend) was found for inflammatory (B1), stricturing (B2) and penetrating (B3) disease (P = 0.006 and 0.017, respectively). For B1 disease eight (25.0% of 32), for B2 disease 10 (37.0% of 27) and for B3 disease 30 (54.5% of 55) patients were carrier of at least one mutation. Two (6.3%), five (18.5%) and 15 (27.3%) patients were carriers of L1007fs, respectively. It means that these carriers often show B2 disease, and even more often B3 disease. The mean follow-up of patients with B1 disease (12.4 years), however, was significantly shorter than patients with B3 disease (7.3 years, P = 0.016). Between B1 and B2 disease, and between B2 and B3 disease no significant differences were found for follow-up duration (P = 0.20 and 0.23, respectively). For UC, no association was found between CARD15 mutations and any phenotype (P ≥ 0.57).

DISCUSSION

The CARD15 gene has recently been described as the first susceptibility gene for CD [5, 6]. The CARD15 protein is mainly expressed in phagocytic cells. The CARD15 protein is able to induce NFκB after recognition of intracellular bacterial peptidoglycan [57, 73]. NFκB is an important regulator of genes involved in a pro-inflammatory response [74].

Three mutations in the CARD15 gene have been reported to be independently associated with CD, including R702W, G908R and L1007fs [5, 53]. In this study, the allele and genotype frequencies of these three common CARD15 mutations are comparable with those found in several other European studies (Table 1). We found associations with CD only for G908R and L1007fs. Two other Dutch studies have been published about the prevalence of CARD15 mutations in IBD [43, 44]. We observed higher allele frequencies of G908R and L1007fs in CD than that found by Murillo et al. [43] and Linskens et al. [44] (6.0 versus 4.2-4.3 and 10.8 versus 7.9-8.4, respectively). These authors also found an association between the L1007fs mutation and CD, but not between CD and the G908R mutation. Neither of them evaluated the R702W mutation.

Carriage of two CARD15 mutations, either two alike (homozygosity) or two different (compound heterozygosity), has been reported in IBD as well. We found frequencies of homozygous and compound heterozygous carriership in CD comparable to those mentioned in the European literature (see references in Table 1). No combined carriage was found in UC and healthy controls, which is also in accordance with the literature.

As CARD15 mutations have only been found in a minority of patients with CD, it is likely that IBD is not a single disease but rather a group of genetically distinct diseases with similar clinical presentations [14]. Moreover, we recently reported homozygous carriage of L1007fs mutations in unaffected first- and second-degree relatives of a CD-affected sib-pair, suggesting that in these individuals the CARD15 mutation does not play a pathogenetic role [68]. In Figure 2, families are presented in which closely related CD-affected relatives differ in CARD15 mutation carriership status. It is therefore unlikely that the CARD15 mutations detected in the IBD-affected probands contribute to the pathogenesis in the other IBD-affected family members. These individual families are clear examples of the complex multifactorial origin of IBD, in which no single genetic or environmental factor is necessary nor sufficient to cause disease [75]. Here, other (private) CARD15 mutations or mutations in other genes may be causative as recently reviewed. For example, nucleotide variations in the SLC22A4 and SLC22A5 genes (chromosome 5q31), and the DLG5 gene (chromosome 10q23) have also been found to be associated with CD [76].

A positive family history is reported in about 5-30% of IBD cases, especially in CD [1]. Reports regarding an association between CARD15 mutations and a positive family history for IBD are, however, not consistent. While some studies reported a

significant association [20, 40, 42, 55, 77], most studies did not [15, 25, 33, 38, 41, 43, 53, 78–82]. In this study, we also found no association between CARD15 carriage and a positive familial history, although the number of patients without a familial history was relatively low compared with the group of familial patients.

Associations have been reported between CARD15 mutations and CD phenotypes, including stricturing disease [19–21, 23, 37, 41, 47, 53, 80, 83], early onset disease [19, 26, 33, 34, 41, 53, 77, 80, 82, 84], ileal and ileocolonic disease [19–23, 25, 26, 33, 34, 37, 41, 47, 49, 55, 78–80, 82], penetrating disease [23, 34, 80] in some, but not in all studies [43, 44, 85]. In this study, an association was found only between the behaviour of disease in CD and carriage of at least one of the three common CARD15 mutations. CD behaviour was almost significant associated with carriage of the L1007fs mutation. Carriers of at least one CARD15 mutation and carriers of L1007fs also showed a significant trend for stricturing and even more for penetrating disease behaviour. This may be a true phenomenon, but we cannot exclude the possibility that the observed differences were partly related to difference in follow-up between the groups, as changing patterns of disease have been observed [86].

Not much is known about the carriage of CARD15 mutations in IBD-affected family members. By using an approach in which calculated allele frequencies were corrected for familial genetic dependency, we found that IBD-affected family members of CD-affected probands with CARD15 L1007fs were carriers of this mutation significantly more often than expected. The statistical analysis for unaffected relatives is much harder to interpret as these relatives may still develop IBD in the future. Some authors have reported a higher frequency of CARD15 mutations in unaffected first-degree relatives of familial CD-affected cases than in unrelated healthy controls [15, 38, 87]. These results can be criticised as multiple related healthy and CD-affected relatives were included, which may yield a deflated P value.

We found statistically significant higher concordance rates for carriage of CARD15 L1007fs in CD-affected probands and their IBD-affected family members. On the other hand, the prevalence of L1007fs in probands with a positive family history was similar to probands with a negative family history. This may be due to small sample sizes or to the used definition of a positive family history. Larger families and families with more relatives of age near 30 years (median age of diagnosis of IBD in The Netherlands is 28–29 years [66]) are more likely to be positive a priori.

In conclusion, we evaluated the CARD15 R702W, G908R and L1007fs mutations in Dutch IBD patients, and we confirmed the association of G908R and L1007fs with the susceptibility of CD. The prevalence of the three common mutations did not differ between familial and sporadic IBD cases. Homozygosity and compound heterozygous carriage of the mutations was only found in CD (and not in UC). IBD-affected family members of L1007fs positive CD-affected probands were positive for L1007fs more often than expected. In CD, carriers of at least one CARD15 mutation and carriers of L1007fs were more likely to have stricturing and penetrating disease, which however might be the result of differences in follow-up duration.

Table 1. Allele frequencies of CARD15 R702W, G908R and L1007fs in European adult patients with inflammatory bowel disease

		R702W			G908R			L1007fs			At least one mutation		
European population	**Authors (year)** [reference]	**CD**	**UC**	**C**	**CD**	**UC**	**C**	**CD**	**UC**	**C**	**CD**	**UC**	**C**
Belgium	Esters et al. (2004) [15]	12.9	7.8	5.8	6.0	3.2	1.8	8.6	1.4	3.0	46.3	22.0	20.6
	Vermeire et al. (2004) [16] [a]	16.7	–	6.4	3.8	–	1.9	12.7	–	2.3	51.4	–	19.7
	Vander Cruyssen et al. (2005) [17]	15.1	–	–	5.5	–	–	8.7	–	–	49.3	–	21.8
Denmark	Vind et al. (2005) [18]	0	–	1.5	2.6	–	1.0	16.4	–	2.1	22.4	–	9.3
England	Ahmad et al. (2002) [19]	12.5	–	5.2	3.3	–	1.4	9.4	–	1.6	38.5	–	15.8
Finland	Heliö et al. (2003) [20]	3.3	1.5	1.8	0.6	0	0	4.8	3.0	1.7	15.5	9.1	6.7
France	Heresbach et al. (2004) [21]	11.5	–	4.7	3.7	–	1.6	9.0	–	4.2	38.0	–	20.0
Germany	Hampe et al. (2002) [22]	10.5	–	4.8	5.2	–	0.7	14.5	–	4.1	–	–	–
	Radlmayr et al. (2002) [23]	–	–	–	–	–	–	13.9	2.1	1.7	–	–	–
	Mascheretti et al. (2002) [24] [b]	6.7	–	–	1.7	–	–	13.9	–	–	36.1	–	–
	Sun et al. (2003) [25] [c]	14.0	–	–	5.0	–	–	26.0	–	2.0	56.4	–	4.0
	Büning et al. (2004) [26]	7.2	2.1	3.6	4.2	2.1	2.1	12.2	4.3	2.1	35.6	14.3	15.5
	Wehkamp et al. (2004) [27]	4.4	–	–	2.9	–	–	13.2	–	–	27.9	–	–
Greece	Roussomoustakaki et al. (2003) [28]	–	–	–	–	–	–	2.7	0	1.5	–	–	–
	Gazouli et al. (2005) [29]	10.0	7.1	1.0	14.2	13.5	3.5	17.9	3.5	6.0	81.7	47.0	21.0
Hungary	Büning et al. (2005) [30]	7.1	3.1	2.6	3.0	1.6	1.2	10.8	2.3	2.2	32.4	13.2	11.5
	Lakatos et al. (2005) [31]	7.3	–	3.0	6.3	–	1.8	10.9	–	2.5	35.1	–	16.5
Iceland	Thjodleifsson et al. (2003) [32]	0	–	–	0	–	–	0	–	–	–	–	–
Ireland	Bairead et al. (2003) [33]	7.0	–	4.0	3.0	–	1.0	4.0	–	1.0	27.4	–	–
	Arnott et al. (2004) [34]	9.7	–	5.1	2.2	–	0.9	3.0	–	0.9	27.4	–	11.4
Italy	Palmieri et al. (2003) [35]	11.0	3.0	6.0	9.0	3.0	2.0	10.0	3.0	1.0	–	–	–
	Annese et al. (2003) [36]	15.0	7.0	6.0	9.0	2.0	2.0	11.0	2.0	1.0	45.3	22.6	18.5
	Giachino et al. (2004) [37]	9.0	10.9	5.9	4.3	2.7	1.4	6.3	0.5	2.3	33.1	25.0	18.7
	Annese et al. (2004) [38] [d]	9.0	–	5.0	5.5	–	2.0	7.7	–	1.3	22.0	–	6.9
	Andruilli et al. (2004) [39]	–	2.9	4.0	–	1.6	2.0	–	2.5	0.7	–	8.8	6.9
	Vavassori et al. (2004) [40]	1.2	–	0.8	5.2	–	2.0	11.2	–	1.2	27.9	–	8.0
	Annese et al. (2005) [41]	8.7	–	4.1	7.3	–	2.7	9.3	2.7	0.7	38.2	13.7	15.1
	Laghi et al. (2005) [42]	10.0	–	–	4.6	–	–	7.7	–	–	33.0	–	–

		R702W			G908R			L1007fs			At least one mutation		
European population	**Authors (year)** [reference]	**CD**	**UC**	**C**	**CD**	**UC**	**C**	**CD**	**UC**	**C**	**CD**	**UC**	**C**
Netherlands	Murillo et al. (2002) [43] [e]	–	–	–	4.3	–	3.0	8.5	–	1.0	23.1	–	7.9
	Linskens et al. (2004) [44]	–	–	–	4.2	–	–	7.9	–	–	21.3	–	–
	This study	8.8	4.7	5.9	6.1	0	0.7	11.0	2.3	1.9	42.1	14.0	16.9
Norway	Hampe et al. (2002) [22]	4.3	–	2.8	0.9		1.2	2.6	–	1.2	–	–	–
Portugal	Ferreira et al. (2005) [45]	12.2	–	4.0	2.8	–	1.3	6.8	–	1.6	34.6	–	12.9
	Vind et al. (2005) [18]	12.1	–	6.3	1.7	–	0.5	0	–	2.9	20.7	–	15.5
Scotland	Arnott et al. (2004) [34]	7.2	2.6	5.5	1.8	2.0	0.2	4.6	3.0	1.4	22.8	–	13.1
	Russell et al. (2004) [46]	7.1	–	–	1.8	–	–	4.7	–	–	–	–	–
Spain	Mendoza et al. (2003) [47]	6.9	–	2.9	4.2	–	1.4	8.6	–	2.9	32.8	–	10.7
	Núñez et al. (2004) [48]	6.7	–	5.8	4.5	–	1.0	4.5	–	1.0	27.9	–	15.2
	Alvarez-Lobos et al. (2005) [49]	–	–	–	–	–	–	–	–	–	30.5	–	–
	Barreiro et al. (2005) [50]	–	–	–	–	–	–	–	–	–	27.9	–	–
Switzerland	Ruegg et al. (2004) [51]	5.7	–	–	2.8	–	–	4.7	–	–	22.8	–	–
Belgium, Denmark, France, Ireland, Spain, Sweden	Hugot et al. (2001) [5] [f]	11.0	3.0	4.0	6.0	0	1.0	12.0	1.0	2.0	42.9	8.7	14.6
England, Germany, Netherlands	Hampe et al. (2001) [52] [f]	–	–	–	–	–	–	16.0	3.1	4.4	–	–	–
Belgium, Denmark, France, Germany, Ireland, Italy, Spain, Sweden	Lesage et al. (2002) [53] [f]	10.8	3.1	4.4	6.1	0.3	1.0	10.6	1.3	1.9	37.7	8.8	14.6
Belgium, France, Luxembourg	Vermeire et al. (2002) [54] [b,f]	10.0	–	2.1	4.7	–	0.5	5.3	–	1.1	32.6	–	15.0
England, Germany, Netherlands	Cuthbert et al. (2002) [55] [f]	9.1	3.7	3.5	3.4	1.6	0.6	6.6	1.5	2.1	35.8	14.3	13.6

[a] Unclear whether CD-affected relatives were included.
[b] Patients treated with infliximab.
[c] Data of controls only for L1007fs.
[d] Combined data of several centres.
[e] Data only for G908R and L1007fs.
[f] Combined data of several countries.
CD, Crohn's disease; UC, ulcerative colitis; C, controls.

Table 2. Frequency of CARD15 mutations in inflammatory bowel disease–affected relatives

Mutation	Proband	Observed IBD-affected patients with mutation	Expected IBD-affected patients with mutation	P [a]
R702W	CD	5	4.8	0.9
	UC	4	1.7	–
G908R	CD	4	2.9	0.4
	UC	0	–	–
L1007fs	CD	13	6.6	< 0.001
	UC	0	–	–

[a] Statistical analysis for UC was not possible because of too low numbers.
IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis.

Table 3. Crohn's disease phenotypes and CARD15 mutations

		Carriage frequency (%)				
	Probands (n)	R702W	G908R	L1007fs	At least one mutation	Two mutations
Age at diagnosis						
A1 (< 40 years)	102	17.6	12.7	17.6	41.2	9.8
A2 (≥ 40 years)	12	16.7	8.3	33.3	50.0	8.3
Location of disease						
L1 (terminal ileum)	32	15.6	15.6	31.3	56.3	12.5
L2 (colon)	27	18.5	11.1	7.4	33.3	7.4
L3 (ileocolon)	47	21.3	10.6	21.3	42.6	10.6
L4 (upper gastrointestinal)	8	0	12.5	0	12.6	0
Behaviour of disease [a]						
B1 (inflammatory)	32	12.5	9.4	6.3	25.0	3.1
B2 (stricturing)	27	22.2	7.4	18.5	37.0	11.1
B3 (penetrating)	55	18.2	16.4	27.3	54.5	12.7

[a] An association was found between carriage of at least one CARD15 mutation and disease behaviour (P = 0.023). Carriage of L1007fs was almost significant associated with disease behaviour (P = 0.052). Carriers of at least one mutation and carriers of L1007fs showed a significant trend for behaviour of disease (P = 0.006 and P = 0.017, respectively), including often stricturing and even more often penetrating disease.

Table 4. Ulcerative colitis phenotypes and CARD15 mutations

		Carriage frequency (%)			
	Probands (n)	R702W	G908R	L1007fs	At least one mutation
Age at diagnosis					
A1 (< 40 years)	30	10.0	0	6.7	16.7
A2 (≥ 40 years)	13	7.7	0	0	7.7
Location of disease					
Proctitis	3	0	0	0	0
Left-sided colitis	14	7.1	0	0	7.1
Extended or pancolitis	26	11.5	0	7.7	19.2

No association was found between any phenotype and carriage of CARD15 mutations.

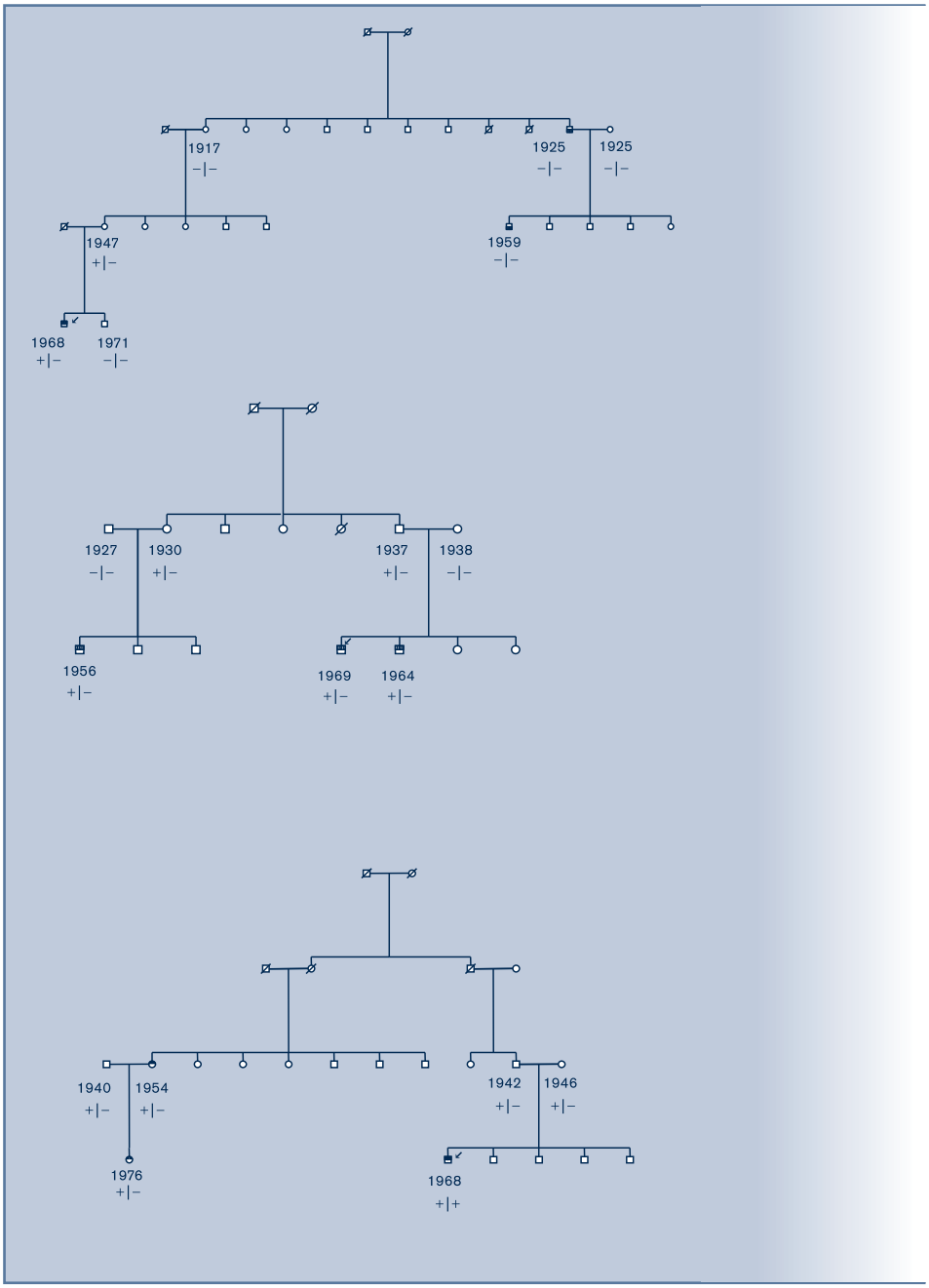
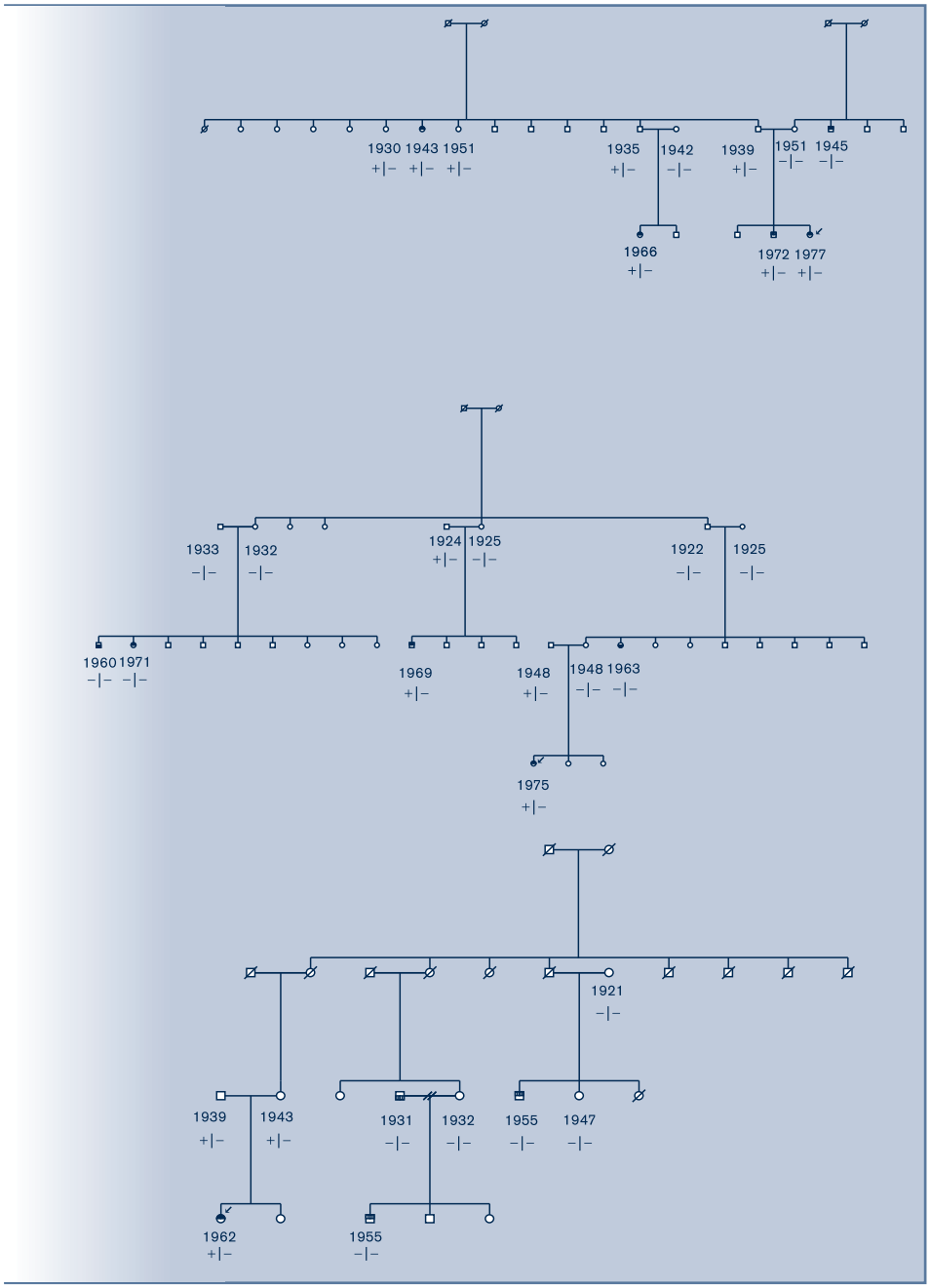


Figure 1. In these six multiple inflammatory bowel disease (IBD)–affected families, in which the proband was positive for CARD15 L1007fs, carriage of CARD15 L1007fs in the IBD-affected relatives was significantly more frequent than expected ($P < 0.001$). − | −, Homozygous carriage of wild-type allele; + | −, heterozygous car-



riage of mutation; + | +, homozygous carriage of mutation; ⬒, male with Crohn's disease (CD); ◓, female with CD; ⬓, male with ulcerative colitis (UC); ◒, female with UC; □, non-IBD-affected male; ○, non-IBD-affected female; ◪, proband.

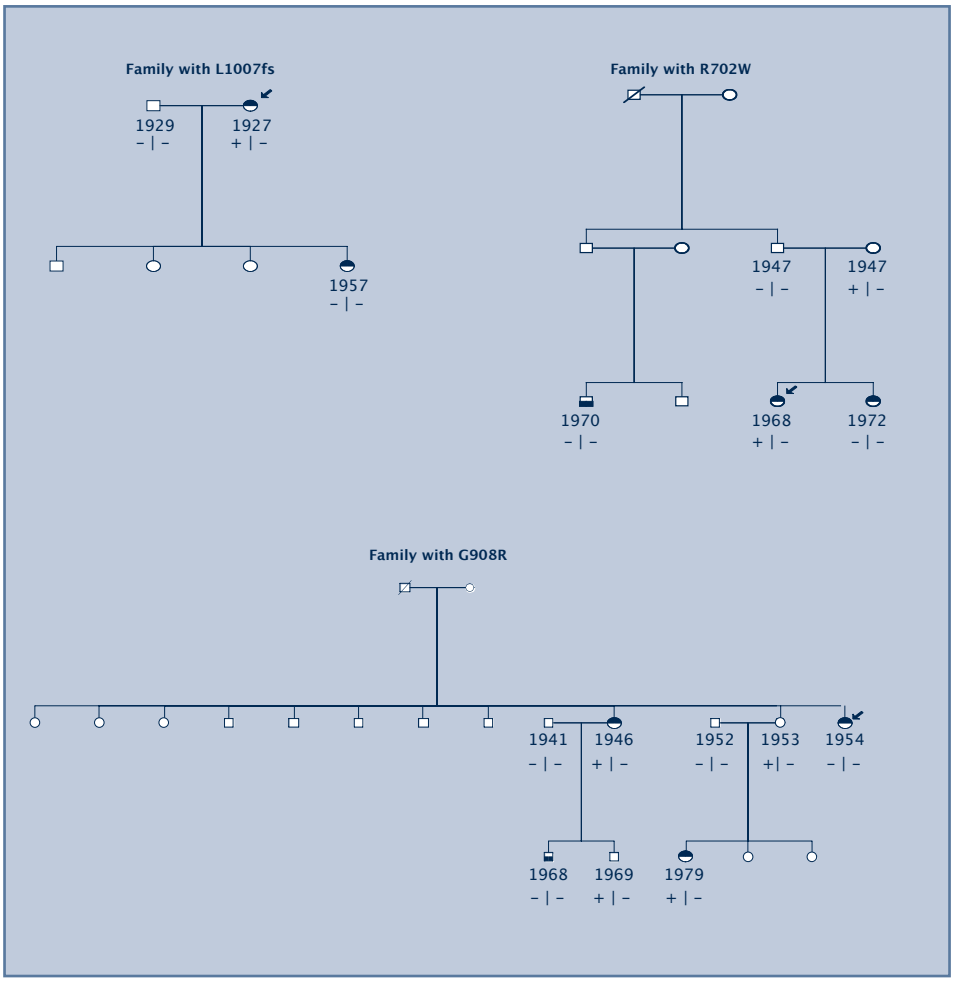


Figure 2. In these three families, it is demonstrated that common CARD15 mutations do not segregate with the disease. It is therefore likely that in these families other genes than CARD15 contribute to the pathogenesis. - | - , Homozygous carriage of wild-type; + | - , heterozygous carriage of mutation; + | + , homozygous carriage of mutation; ⬒, male with Crohn's disease (CD); ◓, female with CD; ⬓, male with ulcerative colitis (UC); ◒, female with UC; □, non-IBD-affected male; ○, non-IBD-affected female; ↙, proband.

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CHAPTER 8

No evidence for genetic anticipation in Dutch familial inflammatory bowel disease

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ABSTRACT

Background

Genetic predisposition plays an important role in inflammatory bowel disease (IBD). Some non-controlled studies suggested genetic anticipation in IBD, including disease onset in children at an earlier age than in parents. This study was planned to evaluate the existence of genetic anticipation in Dutch familial IBD-affected patients.

Methods

Forty familial IBD-affected parent-child pairs and 40 control IBD-affected parent-child pairs were studied. The control pairs included sporadic patients matched for age, sex and type of IBD, either Crohn's disease (CD), ulcerative colitis (UC) or indeterminate colitis (IC). Age at onset of first symptoms, age at diagnosis and disease severity, including the extent of inflammation, use of steroids, number of bowel resections and number of reported symptoms at onset of disease, were evaluated within and between the familial and control parent-child pairs.

Results

In IBD-IBD, CD-CD and UC-UC parent-child pairs, children presented symptoms and were diagnosed at a significant earlier age than their parents ($P = 0.01$ and 0.003, respectively). The same significant findings were present in control parent-child pairs. Moreover, in familial and control pairs the age at presenting first symptoms and the diagnosis was significantly correlated with the age at start of follow-up ($P < 0.0001$). In CD-CD and UC-UC pairs, no differences were found for number of symptoms, number of bowel resections, steroid use and extent of inflammation between familial and control pairs.

Conclusion

Genetic anticipation in IBD is very unlikely as age differences between affected parents and children for onset of symptoms and diagnosis was similar in familial and matched control pairs. Also no differences were found for disease severity. The most probable cause of these results is ascertainment bias.

INTRODUCTION

Inflammatory bowel disease (IBD), including Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis (IC), has a complex multifactorial pathogenesis. Genetic predisposition plays an important role in the disease susceptibility. Epidemiological evidence for genetic involvement includes ethnic differences in the frequency of disease and familial clustering up to 20% of the patients [1]. Convincing evidence for a genetic role in IBD also comes from the observed higher concordance rate of disease in monozygotic twins than in dizygotic twins, especially in CD [2]. Also high concordance rates for type, location and behaviour of disease have been described in multiple IBD-affected families other than twins [3]. More recently, the CARD15 gene has been identified as the first susceptibility gene in CD [4,5]. The pathogenetic role of the CARD15 in IBD has now extensively been evaluated, especially its role in the activation of transcription factor nuclear kappa-B [6].

Early non-controlled clinical studies, demonstrated that CD-affected children have a lower age at diagnosis than their CD-affected parents, suggesting the existence of genetic anticipation [7,8]. This age difference was also found in UC-affected parent-child pairs [9], and can in both conditions even reach 15 to 17 years. Genetic anticipation is defined as disease manifestation in offspring at an earlier age and/or with a more severe course than their affected parents [10]. It is thought that expansion of unstable trinucleotide repeats in the disease-causing gene in subsequent generations can be the biological basis for genetic anticipation [11]. Expansion of such DNA triplet repeats has been described in some classic Mendelian monogenetic neurological diseases, such as Huntington's disease, Friedreich's ataxia and Fragile X-syndrome [12]. There is some preliminary evidence that CAG repeats are involved in a subset of CD-affected families [13]. Whether genetic anticipation is present in IBD is doubtful, as IBD is a polygenic disease with a non-Mendelian inheritance pattern [14]. Moreover, studies about genetic anticipation are often systematically biased in that disease-affected parent-child pairs are by definition characterised by a young onset in affected children. However, as IBD is a heterogenetic disease simple Mendelian susceptibility might be true for a subset of IBD-affected patients [14]. This may imply that anticipation perhaps is only present in such a subgroup.

The aim of this study was to evaluate clinical data of Dutch IBD-affected parent-child pairs in order to evaluate the existence of genetic anticipation.

PATIENTS AND METHODS

Patients and controls

Between July 1998 and October 2002, patients with IBD attending the Erasmus MC University Medical Centre Rotterdam (UMCR) were registered. The diagnosis of IBD was based on standard criteria [15]. At the end of the inclusion period, the registry

included 694 patients with CD, 406 with UC and 67 with IC. A positive family history for IBD, meaning one or more relatives affected with IBD, was reported in 291 cases. IBD-affected relatives were offered to participate in this study. Completely documented IBD-affected parent-child pairs (familial pairs) were included for this study. In those cases in which one of the affects did not attend the Erasmus MC UMCR but another Dutch (non-academic) hospital, medical and surgical information of these 'unknown' relatives were supplied by their treating physician. Based on this information the diagnosis was verified to the same criteria [15].

To evaluate genetic anticipation, familial parent-child pairs were compared with control IBD-affected parent-child pairs, including sporadic patients matched for age, sex and type of IBD. All matched controls were retrieved from the IBD registry of the Erasmus MC UMCR.

The study was approved by the Institutional Review Board of the Erasmus MC UMCR. All participants provided written informed consent.

Clinical data

All clinical data were retrieved from medical hospital records and from a questionnaire, which was completed by most patients. A few patients were interviewed by phone instead. We evaluated age at onset of first symptoms, age at diagnosis and disease severity. Disease severity was based on the extent of inflammation, use of steroids, number of bowel resections, and number of reported symptoms at the onset of disease. These symptoms included weight loss, nausea, vomiting, diarrhoea, rectal blood loss, rectal mucus loss, abdominal pain, arthralgia, eye symptoms and skin problems. The extent of bowel inflammation in UC was classified as proctitis (involving rectum only), left-sided colitis (involving rectum, sigmoid and descending colon) or pancolitis (involving inflammation beyond the splenic flexure). In CD, more severe extended inflammation was classified as disease involving the colon, small intestine or both, respectively. When the exact day or month was uncertain, day 15th and July 1st was chosen, respectively.

Statistics

Clinical characteristics between parents and children, and between parent-child pairs were evaluated by using Student's t test or χ^2 statistics. A P value of 0.05 or less was considered statistically significant. When data were significant while multiple tests were performed, the analysis was corrected using the formula $P_{corr} = 1 - (1 - P)^N$ (N, number of performed tests; P, the significant P value to be corrected) [16]. All analyses were performed with SPSS software version 9.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Characteristics of parent-child pairs with inflammatory bowel disease

Forty familial IBD-affected parent-child pairs were included, retrieved from thirty-nine families. One family included a CD-affected parent and two CD-affected children, resulting in two parent-child pairs. Parents and children were concordant for CD in 19 pairs, and for UC in 13 pairs. In eight pairs the parent and child were discordant for type of disease (mixed pairs). The familial patients' characteristics are summarised in Table 1.

Seventy-nine sporadic matched control IBD-affected patients were included. The mean age of the control parents and children was 58.9 years (range: 39.0-86.0 years) and 33.6 years (range: 9.6-68.9 years), respectively. In five control parents and five control children, the difference of age between the matched familial patients was more than one year (range: 1.0-9.1 years). However, the mean age of the familial parents and children was not different from controls ($P = 0.86$ for parents and $P = 0.94$ for children).

Age at onset of symptoms and diagnosis

In the familial parent-child pairs, 80% (n = 32) of the children presented with symptoms of IBD at an earlier age than their affected parents (range: 1.1-37.4 years). In 85% (n = 34) children are diagnosed with IBD earlier than their parents (range: 1.3-52.4 years). In the matched control parent-child pairs, children presented in 93% (n = 37) with symptoms and in 98% (n=39) with the diagnosis at an earlier age than their affected parents (range: 91 days-35.8 years and 2.1-38.5 years, respectively). With respect to age at diagnosis, more children from control pairs than children from familial pairs were younger than their parents, however, this was borderline significant ($P = 0.05$).

In familial pairs, the mean age at onset of symptoms and diagnosis was significant lower in children than in their affected parents ($P \leq 0.01$ and ≤ 0.01, respectively). These data are summarised in Table 2. Similar significant data were found for control pairs (Table 2). The age differences between familial and sporadic parent-child pairs for onset of symptoms and diagnosis are summarised in Table 3. No significant differences were found between the two groups ($P = 0.6$ and 0.2, respectively).

In familial patients, a significant linear correlation was found between start of follow-up (age at inclusion) and age at onset of symptoms ($P < 0.0001$), and between age at diagnosis ($P < 0.0001$, Figure 1). The same significant correlations were found for control patients ($P < 0.0001$).

Disease severity

In familial parent-child pairs, the number of reported symptoms at onset of disease was significantly higher in parents than in their children ($P = 0.04$, Table 4). It became not significant ($P = 0.39$) after correcting for multiple testing

(n = 12 tests). No significant differences were found for number of resections (Table 4). In the IBD-affected and CD-affected control parent-child pairs, a significant higher use of steroids was found in children (P = 0.004 and 0.006, respectively, Table 4). After correcting this findings for multiple testing (n = 12 tests), the difference is steroid use between CD-affected control parents and children lost significance (P = 0.07). In the IBD-affected control pairs, a higher steroid use in children remained borderline significant (P = 0.05). The parent-child differences for number of symptoms at onset and steroid use between familial and sporadic pairs was not significantly different (P = 0.1 and 0.7, respectively).

The extent of disease as a parameter of disease severity was evaluated in the CD-CD (n = 19) and UC-UC parent-child pairs (n = 13). The data are summarised in Tables 5 and 6. In CD-CD pairs, concordance for extent of disease between parents and children was found in 47% (n = 9) familial pairs and in 37% (n = 7) control pairs, which was not significantly different (P = 0.5, not shown in Table 5). In UC-UC parent-child pairs, concordance rates were 39% and 23%, respectively (P = 0.4, not shown in Table 6).

DISCUSSION

Studies on genetic anticipation in IBD were initiated based on positive findings in some monogenetic neurological diseases, like Huntington's disease, Friedreich's ataxia and fragile X-syndrome [12]. Several studies in familial IBD have shown a lower age of disease onset in children compared with their parents. This was in particular explained as genetic anticipation in two studies evaluating CD-affected parent-child pairs [7, 8]. One of these studies also showed more extensive disease in offspring compared with their parents [7]. However, the results were based on the comparison of mean ages between patients from two different generations, and without including a control group.

In this study, we therefore compared clinical data of IBD-affected parent-child pairs with control IBD-affected parent-child pairs. These control pairs included age, sex and type of IBD matched patients with a negative family history. Similar to other studies [7-9, 16-22], we found in the familial pairs that children presented at a significantly lower age with their first symptoms of IBD, and also were diagnosed at a significantly younger age than their IBD-affected parents. However, the same results were found in the control IBD-affected parent-child pairs.

The initial studies suggesting anticipation in IBD [7, 8], have been criticised as the observations were probably caused by ascertainment bias. Ascertainment bias means that in case of retrospective cross-sectional disease-affected parent-child studies both generations are included at different ages, which leads to preferential inclusion. Therefore, the parent and child have a different completed risk period for their disease. This type of study does not include unaffected children who may still develop

their disease after the inclusion date, which will lead to a false lower age of disease in children compared with their affected parents [23-25].

To study the role of ascertainment bias several methods have been used. Some authors studied the age of diagnosis in relation to the age of birth in IBD-affected parent-child pairs and sporadic IBD-affected patients. They found a significant linear correlation, in which the age at diagnosis decreased with later birth cohorts [16, 20, 26]. The same analysis was done in this study and presented in Figure 1. Instead of date of birth, we chose the age at start of follow-up (age at the time of inclusion). Evaluating the parental age at diagnosis and the number of 'anticipation years' (age at diagnosis of the parent minus age of diagnosis of the child) also showed a significant linear correlation [18]. Otherwise, the age at onset of symptoms in (first-degree) parent-child pairs has been compared with the age of onset in uncle/aunt-nephew/niece pairs, which showed comparable significant age differences [19, 22]. In grandparent-grandchild pairs, even a higher age difference was found [22].

In all these analyses, the time of follow-up from birth to date of inclusion in IBD-affected children is lower than in their affected parents. This will lead to an artificial lower age of diagnosis in the children. To overcome his inadequate follow-up time, the age at diagnosis in children has been compared with age matched affected parents and sporadic affected patients. This also showed a significant linear correlation, in which the age at diagnosis decreased in younger birth cohorts [16]. A similar analysis but based on age stratification (child ≥ or < 45-years-old at time of survey) showed the same results [22]. Another study evaluated the age of diagnosis in a subgroup of CD-affected patients, who had lived for at least 40 years and had received their diagnosis by 40 years of age. In this subgroup in which the observation time was equal for each patient, a minimal decreasing lower age at diagnosis per 10 year birth cohort was found [26]. The method we used in this study has not been used before in genetic anticipation IBD studies, although it is somewhat similar to the method described by Hampe et al., as described above [16]. None of the genetic anticipation studies in IBD have included healthy but future IBD-affected individuals. This requires a prospective study and probably will increase the mean age of diagnosis, especially in children.

In this study, we also evaluated disease severity in familial and control parent-child pairs based on the number of patients' reported symptoms at onset of disease, the number of resections, the use of steroids and the extent of disease. No significant differences were found, except for steroid use in IBD-affected control parent-child pairs. Steroid use in CD- and UC-affected pairs was not different. We found high concordance rates for extent of disease, which is in accordance with the literature [8, 9]. Concordance rates for surgery need are variable [8, 9].

All together, there are no strong arguments to assume that genetic anticipation plays a role in IBD. The age differences between parents and children are most probably caused by ascertainment bias. Moreover, other forms of bias can play a role, such as recall bias and truncation bias [25, 26]. A long-term prospective study is prob-

ably the best way to evaluate whether anticipation is present in IBD or not. However, even the results of such a study can be influenced by confounding factors, like age effect, cohort effect and period effect [25, 26].

In conclusion, in this study we demonstrated that IBD-affected children have a significant lower age at onset of symptoms and diagnosis than their IBD-affected parents. Similar results were found in matched control IBD-affected parent-child pairs. Also no major differences were found between familial and control parent-child pairs for disease severity. These observations argue against genetic anticipation and are most likely to be caused by ascertainment bias.

Table 1. Familial parent-child pairs with inflammatory bowel disease

	Familial pairs	
	Parents n = 39	**Children n = 40**
Sex *(n)*		
Males	14	20
Female	25	20
Age *(mean years, range)*	59.4 (39.3 – 95.1)	33.4 (11.1 – 68.6)
Type of IBD *(n)* [a]		
CD	19	25
UC	19	15
IC	1	0

[a] CD, Crohn's disease; UC, ulcerative colitis; IC, indeterminate colitis.

Table 2. Age differences in familial and control parent-child pairs with inflammatory bowel disease

	Familial pairs			Control pairs		
	Parents	Children	P	Parents	Children	P
Age at onset of symptoms *(mean years, SD)* [a]						
IBD-IBD pairs (n = 40)	32.5 (13.1)	20.3 (7.3)	< 0.001	36.2 (14.5)	22.3 (10.8)	< 0.001
CD-CD pairs (n = 19)	28.3 (12.4)	19.8 (5.9)	0.01	30.3 (9.3)	20.4 (3.8)	< 0.001
UC-UC pairs (n = 13)	37.5 (12.7)	22.3 (8.5)	0.001	43.4 (16.3)	25.6 (16.8)	0.01
Mixed pairs (n = 8)	33.9 (13.6)	18.3 (8.5)	0.02	37.7 (17.2)	21.1 (9.5)	0.3
Age at diagnosis *(mean years, SD)* [a]						
IBD-IBD pairs (n = 40)	36.4 (14.1)	22.8 (6.8)	< 0.001	40.7 (14.0)	23.2 (10.6)	< 0.001
CD-CD pairs (n = 19)	32.8 (12.5)	22.4 (6.1)	0.003	36.4 (10.1)	21.5 (3.4)	< 0.001
UC-UC pairs (n = 13)	39.9 (11.4)	24.2 (7.3)	< 0.001	47.6 (14.0)	26.2 (16.7)	0.002
Mixed pairs (n = 8)	39.0 (20.1)	21.3 (8.2)	0.04	39.3 (18.5)	22.1 (9.6)	0.4

[a] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IC, indeterminate colitis; mixed pairs, parent and child discordant for type of IBD; SD, standard deviation.

Table 3. Age differences between familial and control parent-child pairs with inflammatory bowel disease

	Differences between parents and children		
	Familial pairs	**Control pairs**	**P**
Age at onset *(mean years, SD)* [a]			
IBD-IBD pairs (n = 40)	12.2 (13.2)	13.5 (11.2)	0.6
CD-CD pairs (n = 19)	8.6 (10.8)	9.3 (10.6)	0.8
UC-UC pairs (n = 13)	15.2 (14.6)	17.7 (9.7)	0.6
Mixed pairs (n = 8)	15.7 (15.5)	16.6 (12.6)	0.9
Age at diagnosis *(mean years, SD)* [a]			
IBD-IBD pairs (n = 40)	13.5 (13.3)	17.4 (10.7)	0.2
CD-CD pairs (n = 19)	10.2 (11.1)	14.7 (9.4)	0.2
UC-UC pairs (n = 13)	15.6 (12.5)	21.3 (10.5)	0.2
Mixed pairs (n = 8)	17.7 (18.7)	17.2 (13.2)	1.0

[a] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IC, indeterminate colitis; mixed pairs, parent and child discordant for type of IBD; SD, standard deviation.

Table 4. Disease severity in familial and control parent-child pairs with inflammatory bowel disease

	Familial pairs			Control pairs		
	Parents	Children	P	Parents	Children	P
Mean number of symptoms *(mean n, SD)* [a]						
IBD-IBD pairs (n = 40)	5.7 (1.9)	4.8 (1.9)	0.04	4.9 (2.2)	4.7 (1.8)	0.7
CD-CD pairs (n = 19)	6.1 (1.9)	5.2 (2.1)	0.2	5.3 (2.5)	4.6 (1.9)	0.4
UC-UC pairs (n = 13)	4.7 (1.4)	4.5 (1.8)	0.7	4.7 (1.9)	4.5 (1.0)	0.7
Mixed pairs (n = 8)	6.4 (2.1)	4.4 (1.7)	0.5	4.5 (1.9)	5.4 (2.5)	0.4
Number of bowel resections *(mean n, SD)* [a]						
IBD-IBD pairs (n = 40)	1.0 (1.4)	0.6 (0.9)	0.1	1.0 (1.3)	0.8 (1.3)	0.5
CD-CD pairs (n = 19)	1.7 (1.7)	0.7 (1.2)	0.06	1.3 (1.5)	1.1 (1.3)	0.5
UC-UC pairs (n = 13)	0.4 (0.7)	0.4 (0.8)	1.0	0.5 (0.8)	0.4 (0.8)	0.8
Mixed pairs (n = 8)	0.4 (0.7)	0.5 (0.5)	0.7	0.9 (1.1)	0.8 (1.8)	0.9
Use of steroids *(%)* [a]						
IBD-IBD pairs (n = 40)	77	68	0.4	67	93	0.004
CD-CD pairs (n = 19)	83	68	0.3	67	100	0.006
UC-UC pairs (n = 13)	69	69	1.0	62	85	0.2
Mixed pairs (n = 8)	75	63	0.6	75	88	0.5

[a] IBD, inflammatory bowel disease; CD, Crohn's disease; UC, ulcerative colitis; IC, indeterminate colitis; mixed pairs, parent and child discordant for type of disease; SD, standard deviation.

Table 5. Extent of disease in parent-child pairs with Crohn's disease

	Familial pairs		Control pairs	
	Parents (n=18)	Children (n=19)	Parents (n=18)	Children (n=19)
Colon *(n)*	5	6	4	3
Small intestine *(n)*	6	3	8	5
Both *(n)*	7	10	6	11

Table 6. Extent of disease in parent-child pairs with ulcerative colitis

	Familial pairs		Control pairs	
	Parents (n=13)	Children (n=13)	Parents (n=13)	Children (n=13)
Proctitis *(n)*	2	6	4	2
Left-sided colitis *(n)*	6	5	6	4
Pancolitis *(n)*	5	2	3	7

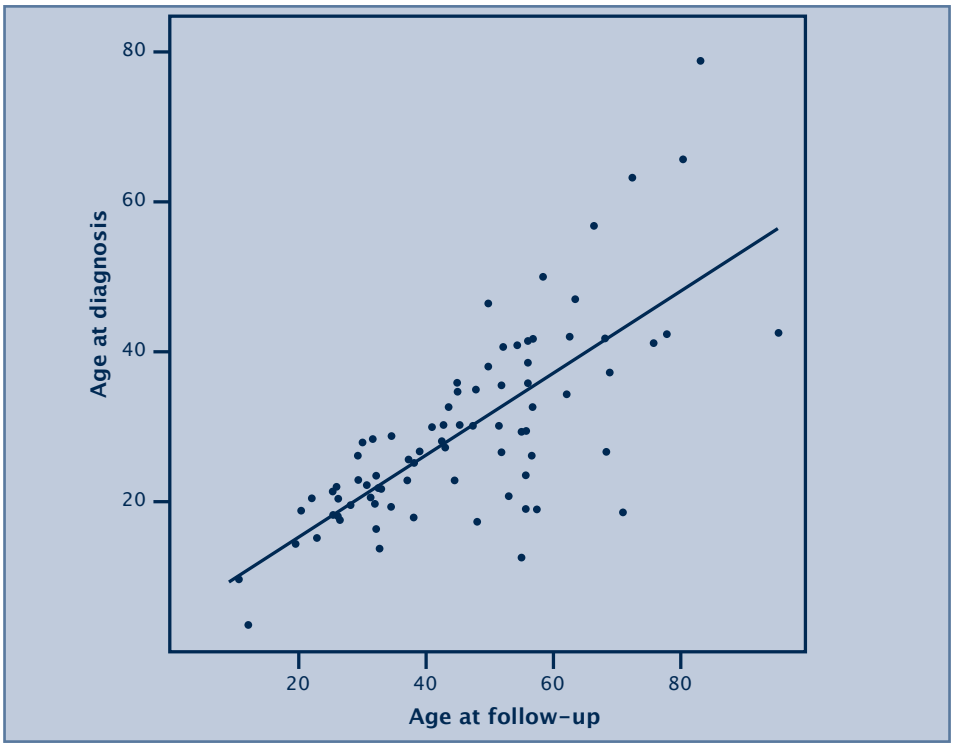

Figure 1. Correlation between the age at follow-up and the age at diagnosis of parents and their children both affected with inflammatory bowel disease ($P < 0.001$, $r = 0.5$).

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CHAPTER 9

Summary, general discussion and future perspectives

SUMMARY AND GENERAL DISCUSSION

The goal of this thesis is to explore aspects of the genetic susceptibility in inflammatory bowel disease (IBD), which is considered to be a major factor in the aetiology of IBD. This thesis started with creating a clinical and DNA database including multiple IBD-affected families and sporadic affected patients. The subsequent genetic studies mainly focused on the caspase recruitment domain 15 (CARD15) and interleukin-10 (IL-10) genes.

Evidence for a genetic role in the aetiology of IBD comes from epidemiological [1, 2] and basic molecular studies [3]. The current hypothesis includes a complex multigenic disease, in which several gene mutations combined with environmental factors can lead to IBD, either Crohn's disease (CD) or ulcerative colitis (UC) [4]. These two disease entities are the major subtypes of IBD [5]. Probably some disease-causing genes are related to both CD and UC, while others are disease-specific [3].

The genome scans in IBD, based on linkage analysis using the allele-sharing method in multiple affected families, have revealed several IBD-linked loci (IBD1-9), which are likely to contain susceptibility genes [6]. Some of these genes have been discovered by either fine mapping or using a candidate gene strategy [7]. The CARD15 gene is the first discovered IBD gene. This gene is located on chromosome 16q12 within the IBD1 locus [7].

The IL-10 gene is not part of any IBD linkage locus. Interest in this gene in relation to IBD is based on its anti-inflammatory function [8]. Therefore, the IL-10 gene is a candidate gene in the search for susceptibility genes in a complex multigenic disorder like IBD.

In **chapters 1 and 5**, a review of the epidemiology, pathogenesis and genetics of IBD is presented.

Genetic heterogeneity can be part of complex multigenic disorders. It is thought that multiple (mutated) genes with a low penetrance lead to similar phenotypes [4]. Genetic heterogeneity in IBD is demonstrated in **chapter 2**. In this chapter a linkage study is described in a small group of 17 IBD-affected sib-pairs. Several inflammatory and anti-inflammatory cytokine and cytokine receptor candidate genes were evaluated for allele-sharing. As expected, the results were quite different per individual sib-pair. A significant LOD score (outcome of linkage studies) was only found for the IL-4 receptor (IL-4R), which is located within the IBD1 locus. However, it is unlikely that this gene is an important disease-causing gene as in other studies no association with IBD was found [9-12]. Linkage is also not caused by carriage of CARD15 mutations. While nine (out of 17) sib-pairs (four CD-CD pairs and five UC-UC pairs) showed allele-sharing for the IL-4R gene, only one CD-affected proband carried CARD15 mutations (compound heterozygous R702W/L1007fs). One female UC-affected proband was not tested, however, her twin sister and brother both affected

with UC carried no CARD15 mutations (these data have not been published). These findings are in accordance with another Dutch study [12].

Although linkage analysis and candidate gene association studies can exclude involvement of a specific gene in a large group of IBD patients, (low level) involvement in some individual affected families is not excluded. Even simple Mendelian inheritance, based on a major single gene mutation among several other involved genes might be the case [13]. This hypothesis was worked out in **chapters 3 and 4** for the IL-10 gene. Sib-pairs who shared both IL-10 alleles were considered to be most likely to harbour a (major or minor) mutation. The IL-10 gene of these sib-pairs was screened for exon and promoter nucleotide alterations. We identified a point mutation Gly15Arg in exon 1 in one CD-affected sib-pair. The mutation was also found in their non-IBD-affected first-degree relatives. Additionally, the prevalence of IL-10 Gly15Arg was evaluated in 379 CD-affected patients recruited from our own Erasmus MC IBD database and from two other Dutch academic hospitals. One additional patient with CD and a related healthy parent were found to be heterozygous for Gly15Arg. No carriage among 75 healthy controls was found. On the basis of these data, IL-10 Gly15Arg appeared to be not associated with CD. However, IL-10 secretion is likely to be reduced in carriers of IL-10 Gly15Arg, which was demonstrated by an *in vitro* stimulation test with human peripheral blood mononuclear cells. Moreover, the secreted IL-10 protein also showed functional impairment, as was shown by an experiment using an IL-10 dependent HL60 cell line (human leukaemia cell line of monocytic lineage). We have concluded that in IL-10 Gly15Arg positive CD-affected families, the Gly15Arg may predispose to the development of IBD by causing a reduced IL-10 response to intestinal inflammation.

A major genetic breakthrough was the identification of the CARD15 gene in 2001. Three common mutations in this gene, i.e. R702W, G908R and L1007fs are independently associated with CD, meaning that carriage increases the risk of developing this disease [14-16]. Meanwhile, the (dys)function of CARD15 and its mutations are widely explored. At the moment, there are two opposite hypotheses about the function of CARD15. Carriage of CARD15 mutations shows a reduced transcription factor nuclear kappa-B (NFκB) activity in human studies ('loss-of-function'). In contrast, in animal studies mutated CARD15 leads to a defective down-regulation of NFκB ('gain-of-function') and subsequently a pro-inflammatory cytokine response [17]. The latter is more in line with the clinical observation of elevated NFκB activity in patients with active CD [18].

In **chapters 6 and 7**, the presence of the three common CARD15 mutations are evaluated in Dutch Caucasian familial and sporadic IBD patients, and in healthy controls. We found allele and genotype frequencies comparable with other European studies. Around 40% of patients with CD appeared to be carrier of at least one of the three mutations. In UC-affected patients and healthy controls carriage was

found in about 15%. Association with CD was found for G908R and L1007fs, but not for R702W. Association was also found between carriage of at least one of the three CARD15 mutations and disease behaviour, especially for carriage of L1007fs. Patients carrying L1007fs were more likely to have intestinal strictures and fistula formation. These findings, however, are not completely in line with the literature. A meta-analysis and a recent pooled analysis showed that carriage of at least one CARD15 variant is in particular associated with small bowel disease and to a lesser extent with stricturing disease [19, 20].

We did not find an association between carriage of a common CARD15 mutation and familial IBD. However, carriage of CARD15 L1007fs in IBD-affected relatives of L1007fs-positive CD-affected probands occurred significantly more often than expected. In other studies, increased carriage of L1007fs was also found in unaffected first-degree relatives of CD-affected probands compared with healthy unrelated controls [21-23]. Although healthy relatives can still develop IBD it is unlikely that carriage of the L1007fs mutation leads per se to IBD. In **chapter 6**, we have demonstrated this by a multiple IBD-affected family in which several first- and second-degree relatives of a homozygous L1007fs CD-affected sib-pair also showed homozygous carriage of this mutation but yet had not developed CD even at very high age. In this specific family, the CARD15 mutation probably does not play an (important) role in the disease pathogenesis.

In the literature, CD-affected sib-pair families have been described which show linkage with the IBD1 locus, but without segregation of one of the three CARD15 mutations [14, 24-26]. There is evidence for the existence of a second IBD gene on the proximal region of chromosome 16p [24], as the IBD1 locus contains at least a second linkage peak not corresponding with the CARD15 gene [24, 27]. Otherwise, in families with more than two affected sibs a decreased linkage with the IBD1 locus [27] and a lower frequency of CARD15 mutations [22] was found. As discussed before, in **chapter 2** linkage between the IL-4R gene and IBD has been found in 17 affected sib-pairs. The IL-4R gene is located on chromosome 16p12.1-p11.2 (MIM 147781), which is close to the second linkage peak 16p13.1-p13.3 within the IBD1 locus [6]. Although not tested, this might explain the linkage result between IL-4R and IBD.

CD is a T-helper-1 cell driven disease, characterised by increased secretion of pro-inflammatory cytokines [28]. In contrast, IL-10 is an anti-inflammatory cytokine, however, it may play a significant role in the pathogenesis of CARD15 mutation positive CD-affected patients. IL-10 secretion by human mononuclear cells of CD-affected patients homozygous for L1007fs is significantly reduced after stimulation with a toll-like receptor 2 (TLR-2) agonist compared with CD-affected patients and healthy controls homozygous for wild-type CARD15. In contrast, no difference was seen in IL-10 secretion after stimulation with a TLR-4 agonist like lipopolysaccharide (LPS) [29].

The family members carrying the IL-10 Gly15Arg mutation, as described in **chap-**

ter 3, were also carrier of CARD15 L1007fs (three homozygotes and one heterozygote, **chapter 6**). In these family members, it is unlikely that the observed reduced IL-10 secretion is caused by L1007fs carriage as we have performed the stimulation tests with LPS. We also performed cell stimulation with phorbol-12-myristate-13-acetate, which is an agonist of intracellular protein kinase C that does not follow the TLR-signalling pathway [30]. Stimulation with phorbol-12-myristate-13-acetate also showed a reduced IL-10 secretion of mononuclear cells from carriers of IL-10 Gly15Arg. Therefore, our hypothesis that the nucleotide variation Gly15Arg in the IL-10 gene is functional and may predispose to the development of CD in patients carrying this mutation remains valid.

In **chapter 8**, a study is presented on genetic anticipation. This phenomenon is defined as disease manifestation in offspring at an earlier age and/or with a more severe course than in their affected parents [31]. Genetic anticipation in IBD was suggested by some uncontrolled studies. In our study, we compared clinical data of familial parent-child pairs with those from control parent-child pairs, including sporadic IBD-affected patients matched for age, sex and type of disease. We confirmed that children presented with symptoms of IBD and are diagnosed significantly earlier than their IBD-affected parents. As this finding was also present in the control pairs, we concluded that genetic anticipation in IBD is very unlikely. Moreover, also no major differences were found for several clinical parameters involving the severity of the disease. The results regarding differences in age at onset of symptoms and age at diagnosis are probably caused by ascertainment bias. Our conclusion is in accordance with the literature [32]. In fact, studies about genetic anticipation based on parent-offspring pairs are by definition characterised by a relative young onset in affected children as future IBD-affected individuals are not included. A long-term prospective study including a large cohort of IBD-affected patients may give a definitive answer whether genetic anticipation in IBD exists.

FUTURE PERSPECTIVES

Due to its genetic complexity and heterogeneity it will probably take many years to discover all the IBD genes. However, new genetic technology involving a very large amount of single nucleotide polymorphisms (SNPs) to perform genome screens (linkage analysis) in multiple affected families or genome-wide association studies in multiple case-control pairs, might increase the progress of gene finding [33]. Very recently, the first genome-wide association study in IBD was published, showing a highly significant association between CD and the IL-23R gene [34].

The discovery of new IBD genes will initiate additional research on their biological (dys)function, hopefully leading to a better understanding of the pathogenesis. This has nicely been demonstrated for the CARD15 gene, which provided insight into

mechanisms of bacteria recognition in CD. However, the paradox whether CARD15 mutations lead to 'gain-of-function' or 'loss-of-function' with respect to cytokine production is still unresolved [17].

Based on the results of genetic research, new treatment strategies can be developed. It may also lead to the development of clinical genetic tests, for example to confirm the diagnosis in IBD-affected patients [35], or it may provide a screening test in symptomatic individuals. Otherwise, genotyping in patients diagnosed with IBD may be useful as a prognostic test concerning the disease course and response to therapy [36, 37].

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CHAPTER 10

Samenvatting, algemene discussie en toekomstperspectief

SAMENVATTING EN ALGEMENE DISCUSSIE

Dit promotieonderzoek richt zich op genetische factoren die van invloed zijn op het ontstaan van inflammatoire darmziekten (IBD), zoals de ziekte van Crohn en colitis ulcerosa [1]. Gestart is met de opbouw van een dataset bestaande uit klinische gegevens en DNA materiaal van patiënten met IBD, met en zonder een positieve familieanamnese voor dit complex van aandoeningen. Vervolgens heeft het onderzoek zich toegespitst op de caspase recruitment domain 15 (CARD15) en interleukine-10 (IL-10) genen.

Epidemiologisch [2, 3] en moleculair biologisch onderzoek [4] heeft aangetoond dat erfelijke factoren een belangrijke rol spelen bij het ontstaan van IBD. Waarschijnlijk leiden bepaalde genen (in combinatie met omgevingsfactoren zoals roken) tot zowel Crohn als colitis ulcerosa, terwijl andere genen juist specifiek zijn voor het ontstaan van een van beide aandoeningen [4, 5]. Genoomscans hebben door middel van koppelingsonderzoek (linkage analyse) bij families met IBD aangetoond dat er tenminste negen chromosoomgebieden (IBD1 tot en met IBD9 locus) zijn waarop IBD-genen kunnen liggen [6]. Met behulp van 'fine mapping' en de kandidaat-gen methode zijn deze IBD-gebieden verder onderzocht en is op chromosoom 16 in het IBD1 locus het CARD15 gen ontdekt. Dit is het eerste gen waarvan is aangetoond dat het een belangrijke rol speelt bij het ontstaan van de ziekte van Crohn [7].

In tegenstelling tot het CARD15 gen maakt het IL-10 gen geen deel uit van een IBD locus. Het IL-10 gen heeft een ontstekingsremmende (anti-inflammatoire) werking en kan daardoor ook van invloed zijn op het ontstaan van IBD [8]. Het IL-10 gen is dus als kandidaat-gen geschikt voor verdere genetische studies in de zoektocht naar genen die van invloed zijn op het ontstaan van complexe multigen ziekten zoals IBD.

In de **hoofdstukken 1 en 5** wordt een overzicht gegeven van de epidemiologie, pathogenese en genetica van IBD.

Genetische heterogeniteit kan een rol spelen bij complexe ziektebeelden. Dit betekent dat meerdere (gemuteerde) genen kunnen leiden tot het ontstaan van eenzelfde (uitingsvorm van een) ziekte [5]. Genetische heterogeniteit bij IBD komt aan de orde in **hoofdstuk 2**. In dit hoofdstuk wordt een koppelingsonderzoek beschreven bij een relatief kleine groep sib-paren (broer/zus relatie) met IBD, waarin verschillende inflammatoire en anti-inflammatoire cytokine en cytokinereceptor kandidaatgenen zijn geëvalueerd. Daarbij is specifiek gekeken naar overeenkomstige allelen per sib-paar. Zoals verwacht is de uitkomst van deze zogenaamde 'allele-sharing' methode heel verschillend per sib-paar. Voor de hele groep is alleen voor het IL-4 receptor (IL-4R) gen een significante LOD score (uitkomst van koppelingsonderzoek) gevonden. Het IL-4R gen maakt deel uit van het IBD1 locus, echter het is niet waarschijnlijk dat dit gen een belangrijke rol speelt bij het ontstaan van IBD. In andere

studies is namelijk geen associatie aangetoond tussen dit kandidaat-gen en IBD [9-12]. De significante LOD score, zoals hiervoor beschreven, is waarschijnlijk ook niet veroorzaakt door dragerschap van CARD15 mutaties in de onderzochte sib-paren. Namelijk van de zeventien onderzochte sib-paren zijn er negen sib-paren (vier paren met de ziekte van Crohn en vijf paren met colitis ulcerosa) die IL-4R allelen delen. Daarvan blijkt slechts één patiënt (proband met de ziekte van Crohn) drager te zijn van CARD15 mutaties (dubbel heterozygoot R702W/L1007fs). Een vrouwelijke proband met colitis ulcerosa is niet getest, doch haar tweelingzus en broer met colitis ulcerosa zijn geen drager van CARD15 mutaties (niet gepubliceerde data). Deze bevindingen zijn in overeenstemming met een andere Nederlandse studie [12].

Binnen een grote onderzoeksgroep kan met koppelingsonderzoek en de kandidaat-gen methode worden aangetoond dat een bepaald gen geen rol speelt bij het ontstaan van de onderzochte ziekte. Met deze onderzoeksmethoden is echter niet uitgesloten dat het onderzochte gen toch betrokken is bij het ontstaan van de ziekte in individuele gevallen en/of families. Het is zelfs niet uitgesloten dat in bepaalde families IBD wordt veroorzaakt door één belangrijk gen wat overerft volgens de wetten van Mendel [13]. In **hoofdstuk 3 en 4** is van deze hypothese uitgegaan in het onderzoek naar het IL-10 gen. Sib-paren met volledig overeenkomstige IL-10 allelen werden geacht de hoogste kans te hebben op een IL-10 mutatie. Het IL-10 gen van deze sib-paren is gescreend op nucleotide veranderingen van de exonen en de promotorgebieden. Door middel van DNA-sequencing is de puntmutatie Gly15Arg gevonden in één sib-paar met de ziekte van Crohn. De mutatie werd ook aangetroffen bij eerstegraads familieleden zonder IBD. Daarnaast is het voorkomen van deze mutatie bestudeerd in 379 patiënten met de ziekte van Crohn, afkomstig uit de Rotterdamse IBD database en de databases van twee andere Nederlandse academische ziekenhuizen. In dit onderzoek is bij nog één patiënt en haar gezonde ouder de IL-10 Gly15Arg mutatie gevonden. Deze mutatie komt niet voor in de controlegroep van 75 personen. Geconcludeerd is dat de IL-10 Gly15Arg mutatie niet geassocieerd is met de ziekte van Crohn. De mutatie is overigens wel functioneel; bij dragers blijkt de secretie van IL-10 verlaagd. Dit is aangetoond in een *in vitro* stimulatietest met humane perifere mononucleaire cellen. In een tweede experiment waarbij gebruik is gemaakt van een IL-10 afhankelijke HL60 cellijn (humane leukemie cellijn), blijkt de werking van het gemuteerde IL-10 eiwit ook veranderd. Geconcludeerd is dat in families met de ziekte van Crohn de IL-10 Gly15Arg mutatie kan bijdragen aan het ontstaan van de ziekte vanwege een verminderde IL-10 respons bij een ontsteking van het maag-darmkanaal.

Een belangrijke doorbraak in het genonderzoek bij IBD was de ontdekking van het CARD15 gen in 2001. Drie belangrijke mutaties in dit gen - te weten R702W, G908R en L1007fs - zijn onafhankelijk van elkaar geassocieerd met de ziekte van Crohn. Dit betekent dat dragerschap van één of meerdere van deze mutaties leidt

tot een verhoogd risico op het krijgen van de ziekte [14-16]. Intussen is er veel onderzoek gedaan naar het biologische effect van CARD15 mutaties. Dit heeft geleid tot twee tegengestelde hypothesen. In onderzoek op menselijk materiaal leidt de aanwezigheid van CARD15 mutaties tot een verlaagde activiteit van het transcription factor nuclear kappa-B (NFκB). Dit betekent functieverlies van het gen ('loss-of-function') waardoor er geen ontstekingsproces in gang wordt gezet. Daarentegen heeft onderzoek op dierlijk materiaal aangetoond dat gemuteerd CARD15 juist leidt tot een verminderde down-regulatie van NFκB. Deze 'gain-of-function' zorgt voor een verhoogde NFκB activiteit. Dit leidt tot toename van ontstekingsbevorderende cytokinen en daarmee tot het juist wel in gang zetten van een ontstekingsproces [17]. De laatste hypothese is meer in overeenstemming met klinische bevindingen bij patiënten met de ziekte van Crohn. Patiënten met Crohn hebben namelijk een verhoogde NFκB activiteit in de darm [18].

In de **hoofdstukken 6 en 7** zijn onderzoeken beschreven naar het voorkomen van de drie hiervoor genoemde CARD15 mutaties in een groep Nederlandse patiënten met IBD en een gezonde controlegroep. De gevonden allel- en genotypefrequenties komen overeen met de resultaten van andere Europese studies. Ongeveer 40% van de patiënten met de ziekte van Crohn blijkt drager te zijn van tenminste één van de drie mutaties. Bij patiënten met colitis ulcerosa en in de gezonde controlegroep blijkt dit 15% te zijn. De mutaties G908R en L1007fs zijn geassocieerd met de ziekte van Crohn. Daarnaast is associatie gevonden tussen dragerschap van tenminste één van de drie mutaties en het ziektegedrag van Crohn. Dit verband is het sterkst voor de L1007fs mutatie. Zo hebben patiënten met L1007fs mutatie een groter risico op darmstricturen en fistels. Deze bevindingen zijn niet volledig in overeenstemming met de literatuur. Twee grote studies hebben namelijk aangetoond dat dragerschap van tenminste één van de drie CARD15 mutaties in patiënten met de ziekte van Crohn is geassocieerd met lokalisatie van de ziekte in de dunne darm en daarnaast (in mindere mate) met darmstricturen [19, 20].

Bij patiënten met de ziekte van Crohn is er geen associatie tussen dragerschap van één van de drie CARD15 mutaties en het hebben van een positieve familieanamnese voor IBD. Daarentegen blijkt wel dat bij patiënten met de ziekte van Crohn én de CARD15 L1007fs mutatie, familieleden met IBD significant vaker dan verwacht ook drager zijn van deze mutatie. Ander studies vonden ook een hoger percentage dragerschap van de L1007fs mutatie in gezonde eerstegraads familieleden van patiënten met Crohn in vergelijking met gezonde (niet-bloedverwante) controles [21-23]. Hoewel gezonde familieleden nog IBD kunnen ontwikkelen, blijkt uit dit onderzoek (**hoofdstuk 6**) dat het niet waarschijnlijk is dat dragerschap van L1007fs per definitie leidt tot het krijgen van IBD. Bij familieleden van een sib-paar met de ziekte van Crohn én homozygoot drager van CARD15 L1007fs, blijken meerdere eerste- en tweedegraads familieleden ook homozygoot drager van deze mutatie. Echter zij hebben tot op hoge leeftijd geen kenmerken van de ziekte van Crohn vertoond. In deze specifieke familie speelt het CARD15 gen waarschijnlijk geen (belangrijke) rol

bij het ontstaan van de ziekte.

In de literatuur zijn sib-paren met de ziekte van Crohn beschreven waarbij koppeling met het IBD1 locus niet blijkt te berusten op dragerschap van één van de drie CARD15 mutaties [14, 24-26]. Het koppelingsonderzoek wijst uit dat er waarschijnlijk naast het CARD15 gen nog een tweede IBD gen op het IBD1 locus ligt [24], aangezien er twee koppelingspieken zijn gevonden binnen dit locus [24, 27]. In families met meer dan twee aangedane sibs (broer/zus relatie) is een minder sterke koppeling gevonden met het IBD1 locus [27]. In dergelijke families komt dragerschap van CARD15 mutaties ook minder voor [22]. In **hoofdstuk 2** en zoals hiervoor beschreven, wordt in dit onderzoek bij zeventien sib-paren koppeling aangetoond tussen het IL-4R gen en IBD. Het IL-4R gen ligt op chromosoom 16p12.1-p11.2 wat dicht in de buurt ligt van de tweede koppelingspiek 16p13.1-p13.3 binnen het IBD1 locus [6]. Hoewel dit niet verder is uitgezocht, kan dit verklaren waarom er koppeling is gevonden tussen het IL-4R gen en IBD bij de zeventien sib-paren.

Bij de ziekte van Crohn speelt de T-helper-1 cel een belangrijke rol. Deze cel produceert ontstekingsbevorderende cytokines [28]. Daarentegen is IL-10 een ontstekingsremmende cytokine. Laatstgenoemde cytokine kan ook een belangrijke rol spelen bij patiënten met de ziekte van Crohn en drager zijn CARD15 mutaties. Stimulatie van humane mononucleaire cellen via de toll-like receptor 2 (TLR-2) van patiënten met de ziekte van Crohn en homozygoot drager van de CARD15 L1007fs mutatie, leidt tot een verlaagde IL-10 secretie ten opzichte van patiënten met de ziekte van Crohn en gezonde controles die drager zijn van het niet gemuteerde CARD15 gen. Daarentegen is geen verschil gezien in IL-10 secretie na celstimulatie via de TLR-4 met lipopolysaccharide (LPS) [29].

De familieleden met de IL-10 Gly15Arg mutatie (**hoofdstuk 3**) blijken in dit onderzoek ook drager te zijn van de CARD15 L1007fs mutatie. Drie personen zijn homozygoot en één persoon is heterozygoot drager (**hoofdstuk 6**). De celstimulatietest zoals beschreven in hoofdstuk 3 is uitgevoerd met LPS. Daarom is het onwaarschijnlijk dat de verlaagde IL-10 secretie bij de bovenstaande familieleden het gevolg is van de CARD15 L1007fs mutatie. In het onderzoek is ook celstimulatie verricht met phorbol-12-myristate-13-acetaat (**hoofdstuk 3**), wat ook een verlaagde IL-10 secretie toont. Stimulatie met phorbol-12-myristate-13-acetaat loopt via een andere route dan de toll-like receptoren [30]. De hiervoor genoemde hypothese blijft daarom gehandhaafd; de nucleotideverandering Gly15Arg in het IL-10 gen betreft een functionele mutatie en kan bijdragen aan het ontstaan van de ziekte van Crohn.

In **hoofdstuk 8** wordt een studie beschreven naar genetische anticipatie bij IBD. Dit betekent dat de ziekte zich in opeenvolgende generaties op steeds jongere leeftijd en/of met een steeds ernstiger beloop manifesteert [31]. Genetisch anticipatie bij IBD is gesuggereerd in enkele studies zonder een controlegroep. In dit onderzoek zijn klinische kenmerken vergeleken tussen ouders en hun kinderen met IBD en een

controlegroep. De controlegroep bestond uit volwassen en kinderen met IBD van dezelfde leeftijd en hetzelfde geslacht als de onderzoeksgroep, maar zonder een positieve familieanamnese voor IBD. Geconcludeerd is dat kinderen met een positieve familieanamnese op significant jongere leeftijd symptomen ontwikkelen en ook op jongere leeftijd worden gediagnosticeerd met IBD dan hun aangedane ouders. Echter dezelfde bevindingen zijn gevonden in de controlegroep. Op grond hiervan is geconcludeerd dat genetische anticipatie bij IBD waarschijnlijk niet bestaat. Verder zijn er ook geen relevante verschillen gevonden tussen de onderzoeksgroep en controlegroep wat betreft klinische parameters die de ernst van de ziekte bepalen. Waarschijnlijk wordt het verschil in leeftijd waarop symptomen optreden en de diagnose kan worden gesteld, veroorzaakt door ascertainment bias (inclusie-bias). Deze conclusie komt overeen met de bevindingen in andere studies [32]. Onderzoek naar genetische anticipatie waarbij gebruik wordt gemaakt van aangedane ouder-kind paren wordt per definitie gekenmerkt door een relatief jonge leeftijd waarop de ziekte zich manifesteert bij de kinderen. In dergelijke onderzoeken worden namelijk geen familieleden onderzocht die mogelijk erfelijk belast zijn en dus in de toekomst de ziekte nog kunnen ontwikkelen. Een langdurig lopend prospectief onderzoek bij een grote groep patiënten met IBD kan mogelijk uitwijzen of genetische anticipatie bij IBD werkelijk voorkomt.

TOEKOMSTPERSPECTIEF

De genetische achtergrond van IBD is complex en heterogeen. Het zal waarschijnlijk nog vele jaren duren voordat alle IBD genen zijn ontdekt. Nieuwe genetische onderzoekstechnologieën die gebruik maken van zeer grote aantallen enkelvoudige nucleotideveranderingen (SNP, single nucleotide polymorphism) voor de uitvoering van koppelings- en associatieonderzoek van het gehele menselijke genoom, kunnen de zoektocht naar IBD genen een impuls geven [33]. Op deze manier is heel recent het IL-23 receptor (IL-23R) gen aangetoond. Het IL-23R gen blijkt sterk geassocieerd te zijn met de ziekte van Crohn [34].

De ontdekking van (nieuwe) IBD genen maakt het mogelijk om meer inzicht te krijgen in het ontstaan van de ziekte. Een goed voorbeeld is het CARD15 gen. Uitgebreid onderzoek naar de functie van CARD15 heeft meer inzicht gegeven in herkenningsmechanismen van bacteriën bij de ziekte van Crohn. Echter er zijn nu twee plausibele, doch tegenstrijdige hypothesen ('gain-of-function' versus 'loss-of-function') over de rol van het gemuteerde CARD15 gen bij een darmontsteking [17].

Verder genetisch onderzoek kan de behandelmogelijkheden van patiënten met IBD in de toekomst verbeteren. Tevens kan onderzoek de ontwikkeling van klinische gentesten mogelijk maken. Deze testen kunnen een waardevolle aanvulling zijn bij het stellen van de diagnose [35] en bepalen van de prognose [36, 37] van IBD. Een dergelijke test zou ook ingezet kunnen worden om mensen met klachten verdacht voor IBD te diagnosticeren.

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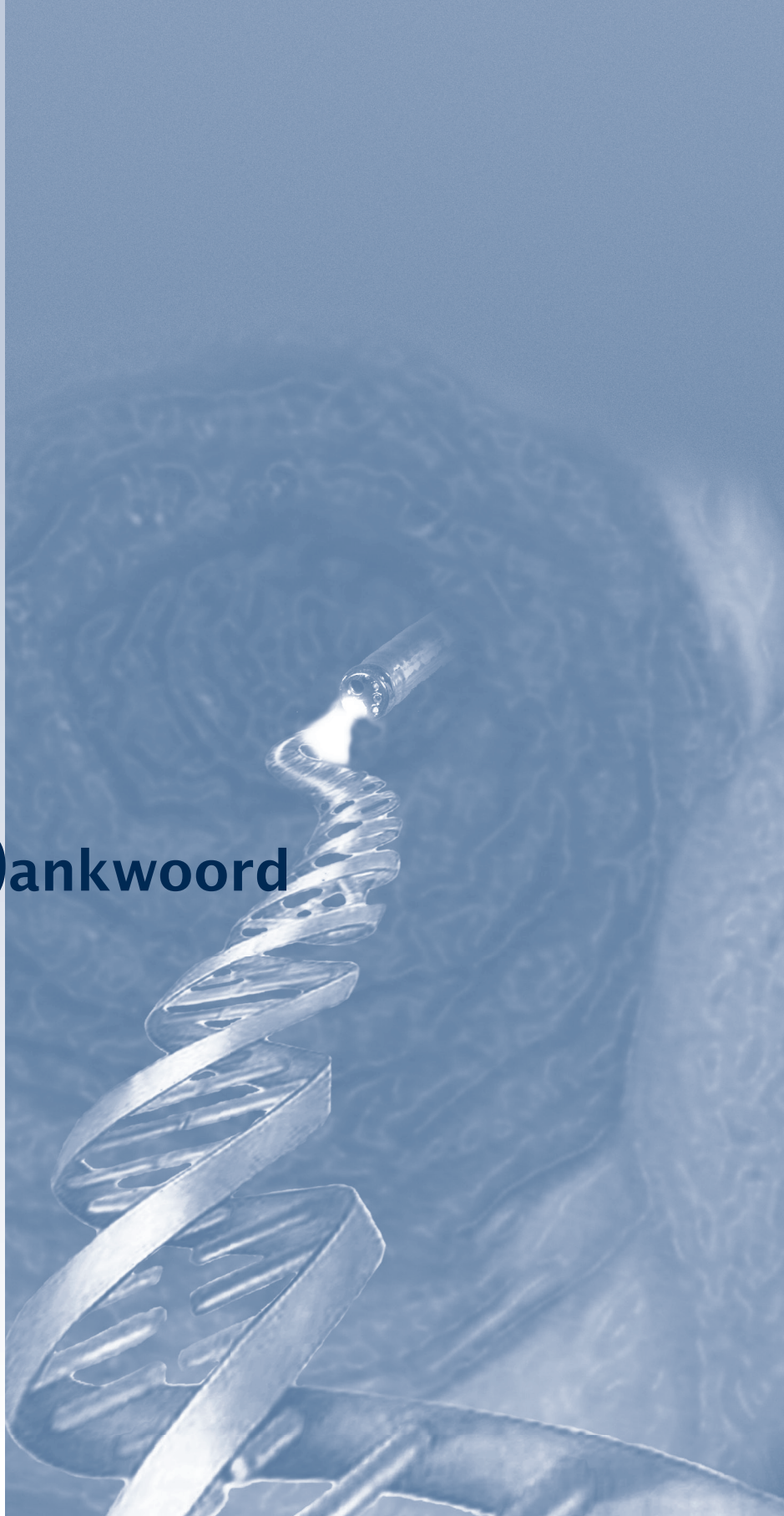
Dankwoord



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It is mear as ynoarder.

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Curriculum vitae

Klaas van der Linde is geboren op eerste kerstdag 1965 in Emmeloord. Na het behalen van het eindexamen atheneum B aan de Christelijke Scholengemeenschap (thans Emelwerda College) in Emmeloord, start hij in 1984 met de studie Geneeskunde aan de Rijksuniversiteit Groningen. In zijn studententijd is hij actief binnen diverse commissies en fotografeert voor studentenbladen. Ook richt hij met studiegenoten de Stichting Medisch Interfacultair Congres en de Stichting Medische Congres Organisatie op.

Tijdens de studie Geneeskunde wordt zijn speciale interesse gewekt voor de maag-, darm- en leverziekten. Om zich verder in dit vakgebied te verdiepen, volgt hij wetenschappelijke stages aan de University of Pittsburgh (USA) en Calgary (Canada). Later volgen keuzecoschappen in de Mayo Clinic te Rochester (USA) en de University of Southern California te Los Angeles (USA).

In 1994 neemt Van der Linde zijn artsenbul in ontvangst en begint als arts-assistent Interne Geneeskunde in het Drechtsteden ziekenhuis te Dordrecht (thans Albert Schweitzer ziekenhuis). Nog datzelfde jaar wordt hij daar toegelaten tot de zesjarige opleiding tot internist (opleider Dr. B. Hazenberg, later Dr. A.J.M. van Vliet). De laatste twee opleidingsjaren werkt Van der Linde in het Erasmus MC Universitair Medisch Centrum Rotterdam (opleider Prof. J.H.P. Wilson). Aansluitend volgt hij in datzelfde ziekenhuis de driejarige opleiding maag-, darm- en leverziekten (opleider Prof.dr. S.W. Schalm, later Prof.dr. E.J. Kuipers).

Naast de opleiding start hij in 1998 zijn promotieonderzoek naar genetische factoren die van invloed zijn op het ontstaan van inflammatoire darmziekten.

Januari 2003 begint Van der Linde als maag-darm-leverarts in het Medisch Centrum Leeuwarden. Daar heeft hij de afgelopen jaren de gastrointestinale endo-echografie geïntroduceerd.

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