The role of genetic alterations
in the gut-joint axis of
Crohn’s disease and Spondyloarthropathies

Harald PEETERS

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Co-promoter: Prof. Dr. Filip De Keyser

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for the Degree of PhD in Medical Sciences
2006
"Ask a question and you're a fool for three minutes; do not ask a question and you're a fool for the rest of your life."

- Chinese Proverb -

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* equal contribution
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*equal contribution*  

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Submitted

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Submitted
CHAPTER 6
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* equal contribution

CHAPTER 7
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Altered gut transcriptome in Spondyloarthropathy.


Ann Rheum Dis 2006 Feb 13; [Epub ahead of print]

CHAPTER 8
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<th>Full Form</th>
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<tbody>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylates</td>
</tr>
<tr>
<td>AIEC</td>
<td>adherent-invasive <em>Escherichia coli</em></td>
</tr>
<tr>
<td>ANCA</td>
<td>anti-neutrophil cytoplasmic antibodies</td>
</tr>
<tr>
<td>AS</td>
<td>ankylosing spondylitis</td>
</tr>
<tr>
<td>ASCA</td>
<td>anti-<em>Saccharomyces cerevisiae</em> antibodies</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CARDIAK</td>
<td>CARD-containing interleukin-1β converting enzyme-associated kinase</td>
</tr>
<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB kinase</td>
</tr>
<tr>
<td>IRAK</td>
<td>interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucin-rich repeat</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDP</td>
<td>muramyl dipeptide</td>
</tr>
<tr>
<td>MIHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MT</td>
<td>metallothionein</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB-inducing kinase</td>
</tr>
<tr>
<td>NOD</td>
<td>nuclear oligomerization domain</td>
</tr>
<tr>
<td>OmpC</td>
<td>outer membrane porin C</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PGN</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>PsA</td>
<td>psoriatic arthritis</td>
</tr>
<tr>
<td>PSC</td>
<td>primary sclerosing cholangitis</td>
</tr>
<tr>
<td>ReA</td>
<td>reactive arthritis</td>
</tr>
<tr>
<td>RICK</td>
<td>RIP-like CARD-containing domain kinase</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor-interacting protein</td>
</tr>
<tr>
<td>SI</td>
<td>sacroiliitis</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SpA</td>
<td>spondyloarthropathy</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/IL-1 receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF-receptor-associated factor</td>
</tr>
<tr>
<td>UC</td>
<td>ulcerative colitis</td>
</tr>
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</table>
CHAPTER 1

General introduction
1. Crohn’s Disease

The concept of inflammatory bowel diseases (IBD) comprises Crohn’s disease (CD) and ulcerative colitis (UC). Both disorders are characterized by chronic, relapsing intestinal inflammation (1). UC is limited to the colon whereas CD can affect the whole gastrointestinal tract with preference for the terminal ileum. Inflammation in CD is transmural, asymmetrical and segmental.

Although the exact pathogenesis is still unclear, it is supposed that an inadequate immunologic response towards certain environmental (microbial) factors in genetic susceptible individuals leads to a dysregulated cytokine balance and subsequently to uncontrolled inflammation (2).

1.1. Clinical Features

1.1.1. Epidemiology

Important geographic differences have been noted considering prevalence and incidence of CD. The highest incidence and prevalence rates can be found in northern Europe, the United Kingdom and North America, whereas clearly lower rates are seen in southern and central Europe, Asia (except Israel), Latin America and Africa (except South Africa), indicating a “North-South gradient” (table 1) (3, 4). Incidence rates seem to stabilize in many high-incidence areas whereas they increase in developing countries becoming “Westernized” or industrialized. These findings reflect the importance of environmental factors in the pathogenesis of CD (e.g. diet, lifestyle, bacterial or parasitic infections, etc) although ethnicity also plays a significant role (e.g. Ashkenazi Jews).

There is a slight female predominance in CD and an incidence peak is found in the second or third decade in life. Whether this is followed by a second, smaller peak between the fifth and eighth decades has become uncertain. Recent studies rather suggest a gradual decrease in incidence following the first peak (5).

Numerous epidemiological studies have demonstrated cigarette smoking as a risk factor for the development of CD (6). Whether dietary antigens, appendectomy, oral contraceptives, vaccination or infections (e.g. measles, mycobacteria) play a role, is still uncertain.
Table 1. Incidence and Prevalence rates for CD

<table>
<thead>
<tr>
<th>Geographic area</th>
<th>Incidence*</th>
<th>Prevalence**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Europe</td>
<td>3.6 – 8.3</td>
<td>54 - 213</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>5.6 - 9.8</td>
<td>144 - 214</td>
</tr>
<tr>
<td>North America</td>
<td>3.6 - 15.6</td>
<td>26 - 198.5</td>
</tr>
<tr>
<td>Southern/Central Europe</td>
<td>0.7 - 3.4</td>
<td>8.3 - 40</td>
</tr>
<tr>
<td>Africa</td>
<td>0.3 - 1.8</td>
<td>NA</td>
</tr>
<tr>
<td>Asia</td>
<td>0.5</td>
<td>3.6 - 5.8</td>
</tr>
<tr>
<td>Latin America</td>
<td>0.03</td>
<td>NA</td>
</tr>
</tbody>
</table>

* cases per 100,000 person-years; ** cases per 100,000 persons

1.1.2. Clinical Findings & Diagnosis

The most common presenting symptoms are increased stool frequency and decreased stool consistency with or without loss of mucus or blood, abdominal pain, weight loss and fatigue. Possible complications represent (sub)obstruction due to intestinal fibrostenosis, intraabdominal or perianal fistulas and abscesses, toxic megacolon and extraintestinal manifestations. Diagnosis of CD is based on clinical, endoscopical, histopathological and radiological findings. Nevertheless, a clear differentiation from UC is not always possible (indeterminate colitis). Irritable bowel syndrome, intestinal infections, microscopic colitis, appendicitis and non-steroidal anti-inflammatory drug induced mucosal lesions represent other important differential diagnostic syndromes.

1.1.3. Extraintestinal Manifestations

Extraintestinal manifestations (EIM) occur in 21-36% of IBD patients (7). Joint involvement and cutaneous lesions are most frequent, but also ophthalmologic and hepatobiliary manifestations are not uncommon. Furthermore, a list of possible and often rare complications “at distance” of the gut is quite extensive (8).

➤ Articular

Articular involvement represents the most frequent extraintestinal manifestation of IBD and can be divided into peripheral and axial arthropathy (7-9). According to the European
Spondyloarthritis Study group (ESSG) criteria, the presence of inflammatory low back pain and/or peripheral arthritis in IBD patients is classified as spondyloarthritis (SpA) (10).

Peripheral arthritis occurs in 15-20% of IBD patients and is mostly pauciarticular and asymmetrical, involving predominantly the large and small joints of the lower limbs (fig 1A). It is frequently transient, migratory and usually non-deforming (11). It has been associated with colonic involvement and usually coincides with intestinal activity. Often, other extraintestinal manifestations like erythema nodosum or uveitis occur simultaneously. This type of arthropathy has been classified as type 1 by Orchard et al (12). Type 2 arthropathy was defined as polyarticular and symmetrical with persistence of symptoms and not associated with IBD activity. Besides a different clinical phenotype and course, both types of peripheral arthropathies could be distinguished immunogenetically according to different associated HLA genotypes (13). The prevalence of type 2 arthropathy found by this group however could not be confirmed (14, 15).

Peripheral enthesopathies have also been associated with IBD and usually affect the Achilles tendon or the insertion of the fascia plantaris (11).

Axial involvement consists of spondylitis or sacroiliitis (fig 1B) (16). Up to 10% of IBD patients fulfil the criteria for ankylosing spondylitis (AS) (17, 18). Symptoms of spondylitis tend to be unrelated to the bowel activity. Usually a chronic progressive course is seen. Clinical features comprise morning stiffness, inflammatory low back pain or alternating buttock pain, limited Schober index and reduced chest expansion. However, asymptomatic sacroiliitis has been described in up to 32% of patients (15, 19, 20). In contrast to idiopathic AS, the association of axial involvement with HLA-B27 is much weaker in IBD. Whereas more than 90% of patients with idiopathic AS carry HLA-B27, the prevalence decreases to 25-75% in IBD associated AS and to even normal prevalences in asymptomatic sacroiliitis (15, 20). These findings point at a different genetic background.

Fig 1. A. synovitis of the left knee      B. sacroiliitis at the right sacroiliac joint.
Non-articular

Erythema nodosum has been reported in up to 20% of IBD patients (8). These skin lesions are usually found on the extensor surface of the lower extremities and appear as tender, red and painful nodules. A good correlation with bowel disease activity is known and occurrence is often in concordance with peripheral arthritis (21).

Pyoderma gangrenosum is a chronic, ulcerating skin disorder. These sometimes severe and debilitating lesions have been described in 1 to 10% of IBD patients (8). Its occurrence is usually independent of IBD disease activity.

Most common ocular complications are episcleritis and uveitis (3.5 – 6.3% in CD) (8). The occurrence of episcleritis, presenting as a red eye with irritation or burning, usually correlates with IBD activity. Uveitis, which is painful and can be associated with visual blurring, photophobia and headache, often coincides with joint and skin manifestations of IBD (22).

Primary sclerosing cholangitis (PSC) is characterized by progressive inflammation, fibrosis and destruction of intra- and extrahepatic bile ducts, usually resulting in an evolution towards cirrhosis and portal hypertension. PSC is associated with colonic involvement in IBD. Similar rates of hepatobiliary involvement (2.4 to 7.5%) are found in UC and colonic CD patients (23).

1.1.4. Serological markers

Several antibodies have been detected in the serum of IBD patients (24). Serological markers could have a potential role in differentiating CD from UC in patients with indeterminate colitis. Theoretically, they might also be useful in a screening test for patients with compatible gastrointestinal symptoms or relatives of IBD patients or serve as a predictor of disease course or treatment response. However, due to the low and variable sensitivity, the individual use of these described serological markers for screening purposes in clinical practice is limited (25).

Anti-Saccharomyces cerevisiae antibody (ASCA)

ASCA have been associated with CD and are directed against the cell wall mannan of the baker’s or brewer’s yeast Saccharomyces cerevisiae (26, 27). These IgA and/or IgG antibodies can be found in 40 to 65% of CD patients (25). Reported specificities for CD are higher and vary between 85 and 95%, although ASCA have also been detected in celiac disease, spondyloarthopathies, autoimmune liver disorders and cystic fibrosis (28-
An important range in sensitivity and specificity has been described comparing different available ASCA assays (35). Consequently, standardization between assays concerning the cut-off value is mandatory. The exact pathophysiological role of these anti-yeast antibodies is still unclear and even cross-reactivity with self-antigens in human gut is still not fully excluded (36). Nevertheless, the presence of ASCA has been associated with specific clinical phenotypes: ileal involvement, penetrating or stricturing disease, younger age at onset and the need for resective bowel surgery (37-41). Twin and family studies have suggested genetic influences on the formation of ASCA (39, 42-46). Higher ASCA titers have been demonstrated in healthy monozygotic twins and healthy first-degree relatives of CD patients.

> **Anti-neutrophil cytoplasmic antibodies (ANCA)**

A distinctive subset of ANCA has been related to UC (47, 48). Prevalence of ANCA in UC ranges from 45 to 82% compared to 2 to 28% in CD (25). This wide variation is most probably due to differences in laboratory techniques and reporting standards between different laboratories. In contrast to cANCA with a cytoplasmic staining pattern (as found in Wegener’s granulomatosis), the antibodies in IBD are often defined as pANCA due to their perinuclear staining pattern. However, the name “atypical ANCA” would be more appropriate since the staining pattern in IBD differs from the classical pANCA seen in microscopic angiitis or small-vessel vasculitides (25). Various antigens, recognized by IBD-related ANCA, have been put forward varying from nuclear (histone H1) over cytoplasmic antigens (e.g. cathepsin G, lactoferrin, lysozyme, elastase and others) to epitopes on colonic bacteria (OmpC) (25, 49, 50).

> **Others**

Anti-OmpC antibodies are directed against the outer membrane porin C of *Escherichia coli* (*E. coli*). IgG antibodies have been found in UC, in particular in patients with pANCA (50). In contrast, IgA antibodies can be detected in 55% of CD patients (51).

A fragment of bacterial DNA (I2), more specifically from *Pseudomonas fluorescens*, was isolated from mononuclear cells of active CD patients (52, 53). IgA antibodies against this I2 sequence can be found in 50% of CD patients (51).

Antibodies to pancreatic antigens (PAB) have also been reported in patients with IBD, in particular with CD (54). PAB can be found in 27 to 39% of CD sera versus less than 5% in UC (55, 56).
Immunoreactivity to flagellin, the primary structural component of bacterial flagella, has been observed with the serum of CD, but not UC patients (57, 58). Anti-flagellin IgA and IgG were detected in 52% of CD patients. Flagellin can induce an acute inflammatory response by recruiting polymorphonuclear cells via epithelial, TLR5-driven chemokine secretion (mainly IL-8) (59). It might however also trigger the adaptive immune system via dendritic cell activation and CD4 T-cell driven B-cell response.

The individual usefulness of these described markers is limited in clinical practice. However, recent studies suggest that combining ASCA with pANCA, anti-OmpC and anti-I2 antibodies may still help in differentiating CD from UC or in predicting the course of the disease (38, 60). Since the level of immune response seems stable over time and with change in disease activity, these antibodies can however not be used for the monitoring of disease activity or treatment response (51, 61).

1.1.5. Disease Classification

The Vienna classification, proposed at the 1998 World Gastroenterology Congress, represents the most widely used clinical classification system of CD (62). Three variables are used to allocate CD patients to 24 different phenotypic subgroups (table 2): age at diagnosis, disease location and behaviour. Location points at the maximum extent of the lesions at any time before the first surgical resection. Stricturing (or fibrostenosing) disease is characterized by the presence of radiologically, endoscopically or surgically proven strictures. A history of inflammatory masses, abscesses, fistulas or perianal ulcers is defined as penetrating (or fistulizing) disease. A hierarchy was defined for stricturing and penetrating disease (B1<B2<B3). Overall, a good intra- and interobserver agreement is found using this classification system (62, 63). Location of disease, as defined by this system, seems relatively stable over time, however an important change of disease behaviour can be noticed (64). Nevertheless, due to the lack of more suitable classification systems, this model is still often used in genotype-phenotype studies.
Table 2. Vienna classification for Crohn’s Disease

<table>
<thead>
<tr>
<th>Age at diagnosis</th>
<th>A1</th>
<th>&lt;40 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>&gt;40 years</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>L1</td>
<td>Terminal ileum</td>
</tr>
<tr>
<td>L2</td>
<td>Colon</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>Ileocolon</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>Upper gastrointestinal</td>
<td></td>
</tr>
<tr>
<td>Behaviour</td>
<td>B1</td>
<td>Non-stricturing non-penetrating</td>
</tr>
<tr>
<td>B2</td>
<td>Strictures</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>Penetrating</td>
<td></td>
</tr>
</tbody>
</table>

1.1.6. Therapy

**Medical treatment**

- **Aminosalicylates**
  5-ASA formulations like mesalamine or sulfasalazine are commonly used for the treatment of Crohn’s disease. However, evidence for their efficacy in CD is not fully convincing and the indications are limited to the management of mild to moderate active disease (65, 66). There is no place for 5-ASA drugs in the maintenance of medical induced remission (67).

- **Antibiotics**
  Although clear data are relatively scarce, metronidazole and ciprofloxacin seem to have a limited efficacy in the treatment of active disease (68, 69). These antibiotics can also be used for perianal disease where they can induce a decrease in fistula drainage (70). Responses are however mostly incomplete and transient.

- **Corticosteroids**
  Prednisone or prednisolone are quite successful in inducing remission but many patients are or become steroid-resistant, -refractory or -dependent (71). Moreover, long-term steroid treatment has significant systemic side effects. The use of the topical drug budesonide overcomes this partially but does not change long-term outcomes.


- **Immunosuppressives**

Immunosuppressive therapy, including azathioprine, 6-mercaptopurine or methotrexate, is used in refractory disease to maintain remission (72-74). Due to their toxicity profile, close monitoring is mandatory. These agents are slowly acting and hence not useful for induction of remission.

- **Biologicals**

The introduction of infliximab, a chimeric mouse/human monoclonal antibody against tumor necrosis factor (TNF)-α, ushered in a new era in the treatment of CD (75). This biologic agent appeared to be very efficient in both induction and maintenance of remission and in the management of perianal disease, providing hope for patients having left few non-surgical options. Nevertheless, some patients do not respond to infliximab and several other biologicals are currently under investigation including humanized and pegylated anti-TNFα antibodies and antibodies directed against interleukin (IL)-12/IL-23 p40, integrins, interferon (IFN)-γ and CD25 (76).

**Surgical therapy**

Despite significant progress in the medical treatment of active CD, up to two thirds of the patients will need surgery during the course of their disease (65, 77). Strictures, perianal involvement (fistulas and/or abscesses), unresponsive fulminant disease and perforation are the most frequent indications for surgical intervention.

**1.2. Pathogenesis**

**1.2.1. Normal mucosal homeostasis**

The gut mucosa represents the largest area of our body in contact with environmental (microbial, dietary and other) antigens (ca. 400m²) (78). Only a single layer of epithelium separates the luminal content from the internal milieu. Integrity of the surface epithelium with its tight junctions is important for an adequate intestinal barrier. Furthermore, specialized epithelial cells (goblet cells) produce mucus containing mucin glycoproteins and trefoil
peptides. This protective mucus layer also contains antimicrobial peptides or defensins (produced by Paneth cells) and secretory IgA. The underlying mucosal immune system, containing elements of both innate and acquired immunity, also makes part of the intestinal barrier. Interactions between the host and intraluminal flora are mutually beneficial. Hence the host needs to avoid an overly aggressive response to this microbial population that would lead to the elimination of beneficial organisms and could result in inflammation and extensive tissue damage. Nevertheless, the capacity to limit the spread of bacteria from the lumen into underlying tissues and to respond effectively to episodic challenges with pathogens needs to be maintained. Cross-talk between immune and epithelial cells is mandatory to obtain an adequate immunological tolerance.

Due to continuous host-flora interactions, a “controlled” or “physiologic” mucosal inflammation is present in normal circumstances. This is characterized by a perfect and tightly regulated balance between pro- and anti-inflammatory cytokines produced by antigen-presenting cells and T lymphocytes (T-helper (Th) and regulatory T (Treg) cells).

1.2.2. Sensing the microenvironment

Several cell types are involved in sensing the microenvironment. M-cells overlying lymphoid follicles actively transport microbial antigens and deliver them to antigen-presenting cells (dendritic cells, macrophages and B-cells) within the follicle but also surface enterocytes can transport soluble antigens. Dendritic cells can extend their dendrites into the lumen between the enterocytes without disrupting the tight junctions and sample luminal antigens themselves. The antigen-presenting cells process and present the antigens to lymphocytes, initiating an acquired immune response. Innate immune cells use different pattern-recognition receptors to recognize highly conserved structures of pathogenic microorganisms, called PAMPs (pathogen-associated molecular patterns).

Toll-like receptors

Toll-like receptors (TLRs) are transmembrane receptors for PAMPs, expressed in monocytes/macrophages, dendritic cells, mast cells, polymorphonuclear cells, epithelial and
some endothelial cells (82, 83). The TLR family consists of at least 10 different receptors with specific bacterial components as ligands (84, 85). TLR2 recognizes lipoproteins/lipopeptides and peptidoglycan (PGN). TLR4 senses lipopolysaccharide (LPS). TLR5 binds flagellin while CpG DNA is the ligand for TLR9 (fig 2).

All TLRs contain an extracellular leucin-rich repeat (LRR) domain (that interacts with the PAMPs) and an intracellular portion designated TIR (Toll/IL-1 receptor) that activates intracellular signalling pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (86). Binding of the TLR with its ligand gives rise to NF-κB activation via myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase (IRAK), TNF-receptor-associated factor (TRAF)-6, NF-κB-inducing kinase (NIK) and inhibitor of κB kinase (IKK) complex (fig 3) (83). IKK phosphorylates inhibitor of κB (IκB), resulting in ubiquination and degradation of IκB and subsequently in the release of the active dimer NF-κB (87). NF-κB, a nuclear transcription factor, will translocate towards the nucleus where it activates multiple genes resulting in the production of inflammatory cytokines. A Myd88-independent pathway has been described as well for TLR4 (88).

Fig 2. Toll-like receptors and their ligands

NOD receptors

Both NOD1 (CARD4) and NOD2 (CARD15) are intracellular sensors of bacterial products (89). NOD proteins have been found in monocytes/macrophages, dendritic cells, granulocytes
and epithelial cells. They have a C-terminal LRR domain, N-terminal CARDs (caspase activation and recruitment domains) and a central nucleotide-binding site and detect components of peptidoglycan (a component of the bacterial cell wall) in the cytosol with specificity for distinct muropeptides (90). NOD1 has been shown to detect a muropeptide found mostly in Gram-negative bacteria whereas NOD2 senses another muropeptide found in most bacteria (91-93). Similar to TLRs, binding of NOD with its ligand muramyl dipeptide (MDP) gives rise to activation of caspases (linking NOD with apoptotic pathways) and NF-κB although via a signalling cascade different from TLRs (fig 3). Activation of NOD induces recruitment of receptor-interacting protein (RIP)-2 (also known as RIP-like CARD-containing domain kinase [RICK] or CARD-containing interleukin-1β converting enzyme-associated kinase [CARDIAK]) through homotypic CARD-CARD interactions followed by activation of the IKK complex and subsequently NF-κB (89, 94, 95). The final result is again production of inflammatory cytokines. Some cytokines like TNFα and IFNγ can in turn induce an overexpression of the NOD protein (96, 97).

**Fig 3. Nod2 and TLR4 signalling pathways**

**NOD/TLR synergy**

Cross-talk between NOD and TLR signalling pathways has been demonstrated in several studies (98-101). TLR mediated responses are enhanced by simultaneous MDP-costimulation.
1.2.3. Intestinal inflammation in Crohn’s disease

In CD, the normal mucosal homeostasis is disturbed, resulting in a cytokine imbalance in favour of pro-inflammatory mediators (fig 4) (1, 2). Dendritic cells, primed by antigens that have permeated the epithelial barrier or that are obtained by direct luminal sampling, interact with naïve Th cells through CD40/CD40L, resulting in IL-12 release and polarization towards the Th1 phenotype (1, 2, 102, 103). These Th1 lymphocytes will in turn produce IL-2 and IFNγ (among others), activating macrophages. Several inflammatory mediators will then be secreted by stimulated macrophages: IL-12 and IL-18 will further stimulate Th1 cells, creating a self-sustaining cycle, whereas IL-1, IL-6 and TNFα will have a broader effect, causing inflammatory damage and amplification of a variety of inflammatory cascades by stimulating other, diverse cell types. During the process of active intestinal inflammation, an infiltration by a large number of lymphocytes, monocytes/macrophages and polymorphonuclear cells is seen in the affected mucosa.

Fig 4. Induction of pro-inflammatory cytokines in CD
By inducing a Th1 response, IL-12 related cytokines like IL-12, IL-23 and IL-27 play a pivotal role in the mucosal cytokine imbalance found in CD (103, 104). Moreover, the activated Th1 cells in CD are resistant to apoptosis, leading to accumulation of T cells in the mucosa and perpetuation of the inflammatory response (2, 105, 106). In addition, an inadequate activation and/or expansion of regulatory T (Treg) cells has been demonstrated in CD (1, 107, 108). Treg cells, producing anti-inflammatory cytokines like IL-10 and TGFβ, are generally considered to be the “peace keepers” in the intestinal mucosa and have an important role in controlling local immune responses.

The exact underlying mechanisms of the immune dysregulation, observed in CD, are still not fully clarified but both environmental triggers and genetic susceptibility seem to play an important role.

1.2.4. Environmental factors

The striking increase in the incidence of IBD, and CD in particular, during the last half century points to changes in the environment as major causes for this evolution, since genetic variations are negligible in such a short period of time (109).

Industrialized countries represent high-incidence areas and subsequently the rising emergence of IBD was linked to the “westernization” process (109). Almost every new compound introduced in the population during the last century has been proposed to play a role in the aetiology of IBD including toothpaste, chewing gum, fast food, margarine and cornflakes (110). The “hygiene hypothesis” of allergic and auto-immune diseases has also been invoked to explain the world-wide spread of IBD (109, 111). Another theory is pointing at the possible contribution of domestic refrigeration and subsequently increased exposure to psychotropic bacteria (“cold chain hypothesis”) (112).

Nevertheless, numerous epidemiological studies made it clear that some environmental factors indeed contribute to the physiopathogenesis of CD.

**Smoking**

As opposed to UC, where smoking seems to have a protective effect, several studies have identified cigarette smoking as a risk factor for CD (113-115). Smokers are more than twice as likely to develop CD. Even second-hand exposure to cigarette smoke increases the risk
Furthermore, smoking also influences the course of the disease. Patients who smoke are more likely to have ileal involvement and develop more frequently fistulizing or stenosing disease (116, 117). Continued smoking also increases the risk of disease recurrence, the need for surgery and the requirement of immunosuppressive drugs, whereas cessation of smoking has a beneficial effect (118, 119). The underlying mechanism is unclear but smoking seems to influence both systemic and mucosal immunity, altering a wide range of innate and adaptive immune functions (120).

**Diet**

Since dietary antigens are, in addition to bacterial antigens, the most common type of luminal antigen, numerous studies have searched for dietary factors influencing the pathogenesis of CD, particularly since elemental diets seem to have some beneficiary effect as primary or adjuvant therapy (121). Nevertheless, the studies conducted on dietary factors (including sugar and fat intake) could not reach consensus (122-125).

**Socio-economic status**

High social, economical, educational and occupational status seems to increase the risk for developing CD (110). However, these findings might stand for differences in diet, crowding and hygiene (126).

**Stress**

Although most probably not an initiating factor, stress might modulate the course of disease. This belief was provided by clinical observations, animal models of colitis and neuro-immune interactions in laboratory animals (127). A complex interplay of nervous, endocrine and immune factors is likely to be implicated in the induction of exacerbations by stress (128).

**Appendectomy**

Whereas appendectomy would protect against UC, it has been associated with a higher future risk for CD (129). Development of CD with a history of perforated appendicitis is
characterized by an even worse clinical outcome with increased risk for intestinal resections (130). The underlying mechanisms are not clear.

**Microbial factors**

- **Specific infectious agents**
  Numerous studies have been performed to identify a causative micro-organism for CD. *Listeria monocytogenes, Chlamydia trachomatis, Escherichia coli, Cytomegalovirus, Saccharomyces cerevisiae, Mycobacterium paratuberculosis* and (vaccination against) *Paramixovirus* (measles virus) have among others been proposed as potential etiologic agents (109, 131, 132). However, results were often conflicting and no convincing data are available until now.

- **Intestinal commensal flora**
  During the last decade, a shift from infectious to commensal agents was noticed in the search for potential microbial triggers of IBD (133). The magnitude and complexity of the alimentary ecosystem is striking. It represents a living mass accounting for 1 to 2 kg of body weight, a cellular content that outnumbers 10-fold the number of eukaryotic cells of the host, a genetic microbiome exceeding the human genome with a 100-fold and an enormous metabolic activity (134, 135). There is now increasing evidence that the antigens that drive the tissue-damaging response in CD lie within this bacterial flora.

- The most convincing arguments arose from animal models of IBD. Intestinal inflammation fails to develop when animals are kept in germ-free conditions (128, 136-138). The presence of a normal flora is necessary. This was seen in different species (mouse, rat and guinea pig) and occurs in knockout or transgenic animals but also in induced models of IBD. Moreover, in some models, disease can be transferred with effector T cells against enteric bacteria (139, 140).

- Loss of immune tolerance against enteric flora has also been reported in human studies (141). T cells isolated from the lamina propria of these patients responded in vitro to the antigens of their own flora. This is reflected by a serologic reactivity to enteric bacteria and (cross-)reactivity of serum antibodies (such as ASCA, ANCA, anti-I2, -OmpC and –flagellin antibodies) to enteric bacterial antigens (52, 57-59, 142, 143).
- IBD lesions predominate in the parts of the gastrointestinal tract where the intestinal microflora is most abundant (terminal ileum and colon) and differences have been found in the bacterial composition of the faecal microflora between CD patients and healthy subjects after ileocaecal resection (144, 145, 146). Furthermore, much larger numbers and concentrations of (adherent) bacteria have been found in the biofilms covering the epithelium of IBD patients compared to healthy subjects (146).

- Diversion of the faecal stream from inflamed bowel loops induces improvement in CD whereas re-anastomosis results in relapse (147, 148). Moreover, direct infusion of luminal contents into excluded ileum also causes inflammation (149, 150).

- Pouchitis, inflammation of a surgically constructed ileo-anal pouch, is associated with a dysbiosis caused by the contact of the once near sterile small bowel mucosa with a rich colon-like flora repopulating the pouch soon after surgery (151). A beneficiary effect of antibiotics and probiotics has been demonstrated in the treatment of this pouchitis (152, 153).

- **Adherent-invasive Escherichia coli**

  *Escherichia coli* strains with particular adhesion and invasion characteristics have been associated with CD (154, 155). These adherent-invasive *E. coli* (AIEC) colonize predominantly the ileal mucosa of CD patients. They can be found in the ileum of 30% of patients with chronic lesions, in 34% of patients with early lesions (in the neoterminal ileum) and in 22% of CD patients with healthy mucosa (156). In contrast, in only 6% of healthy controls AIEC can be detected in ileal specimens.

  Adherence to the epithelium by AIEC strain LF82 is mediated by type 1 pili and this active phenomenon is a key step in the establishment of its invasive process (157). Indeed, AIEC strain LF82 is able to invade cells of different epithelial cell lines in vitro (158). After invasion, the bacteria can lyse the vacuole membrane and become free in the host cell cytoplasm. Moreover, they can survive and even replicate intracellularly in both epithelial cells and (murine and human) macrophages without inducing host cell death (158-160).
1.2.5. Genetic factors

1.2.5.1. Genetic epidemiology

Multiple lines of evidence derived from epidemiological data have suggested a genetic contribution to the pathogenesis of IBD, including racial and ethnic differences, familial aggregation and twin studies and association with other genetic disorders (161).

- Incidences and prevalences of IBD vary significantly depending on geographic location and racial or ethnic background, although this may be mainly due to differences in environmental factors (162). Incidences increase in populations emigrating from low-risk geographic areas to those with higher risk (163, 164). A study from the United States reported higher prevalence rates for CD (per 100,000) in Caucasians (43.6) compared to African Americans (29.8), Hispanics (4.1) and Asians (5.6) (165). However, the incidence in African Americans increases and this might be consistent with changing environmental exposures (166). Nevertheless, ethnicity seems to play a role since Jews (particularly Ashkenazi Jews) in the United States have clearly the highest risk to develop CD (167, 168).

- A large number of studies have reported clustering of IBD within families. Between 6 and 32% of IBD patients have an affected first- or second-degree relative (161). Risk factors for familial aggregation include Jewish ancestry and early-onset disease (169, 170). About 75% of multiple affected families with IBD are concordant for disease type with the remaining 25% being mixed (CD and UC in one family) (171). Relative risks for developing CD in case one has a family member with the disease vary significantly between studies. For siblings, the relative risk for CD has been estimated between 13 and 36 (172). High concordance rates for pattern of disease (location, behaviour and occurrence of EIM) are found in affected pedigrees (173).

- Twin studies have provided the strongest evidence for a genetic susceptibility. The concordance rate for CD in monozygotic twins varies between 42 and 58%, whereas it is not significantly different in dizygotic twins from that for all siblings (174-177). The incomplete
penetrance in monozygotic twins is most probably due to non-genetic factors, such as environmental triggers.

- Associations have been made between susceptibility to IBD and a number of genetically determined syndromes, such as ankylosing spondylitis, psoriasis and eczema (178-180). Furthermore, intestinal inflammation resembling IBD has also been reported in several other (rare) genetic syndromes (181-185).

1.2.5.2. Genome-wide scans

Significant progress was made during the last decade in the understanding of the genetic basis of CD. Genetic linkage studies used genome-wide scans to type genetic markers in families containing more than one affected member (186). Typically, about 400 microsatellites spaced evenly across a chromosome are genotyped. If the actual disease-susceptibility gene is located close enough to the marker, it will be co-inherited. Subchromosomal regions where the degree of allele sharing between affected individuals exceeds the statistical expectation will then identify regions of interest, implying that a disease gene resides in that particular chromosomal region.

Genome-wide scans have identified putative loci on almost every chromosome, reaffirming the concept that inflammatory bowel diseases are complex genetic disorders with several predisposing genes (figure 5) (161, 187). However, owing to this genetic heterogeneity, linkage results have often not been consistently replicated between studies. Looking at more homogeneous populations or subsets of the data (common ethnicity and/or geographic characteristics) is a way to reduce this heterogeneity. At least 9 susceptibility loci, designated IBD1-9, met the strict criteria for linkage and have been replicated in independent studies (188, 189).
1.2.5.3. CARD15/NOD2

CARD15 polymorphisms in Crohn’s disease

Narrowing down these susceptibility loci represents a major challenge and is complicated by clustering of multiple candidate genes within one locus. Genetic association studies are used to identify the specific disease genes. This candidate gene approach led in 2001 to the detection of the first CD susceptibility gene CARD15 or NOD2, within the IBD1 locus (190-192). This gene on chromosome 16q encodes the intracellular PAMP-receptor NOD2/CARD15.

Three single nucleotide polymorphisms (SNPs) including 2 missense and 1 frameshift mutation have been independently associated with Crohn’s disease susceptibility: Arg702Trp (R702W, SNP8), Gly908Arg (G908R, SNP12) and Leu1007finsC (1007fs, SNP13) (figure 6). These CARD15 gene variants have been found in 30 to 45% of CD patients compared to only 8 to 16% in the healthy population (190, 192, 193). About 30% of CD patients carry one risk allele whereas 8 to 17% carry 2 copies (178). The relative risk of developing CD has been estimated as 3-fold in single heterozygotes whereas it increases to 40-fold in compound heterozygous and homozygous patients (187).

Important ethnic differences have been noted. Compared to European, North American, Israeli and Australian cohorts, much lower prevalences of CARD15 polymorphisms have been
found in Finnish, Irish and Scottish CD populations (194-196). Even striking is the almost total absence of these variants in Japanese, Korean and Chinese populations (197-199).

**Fig 6: Structure of the CARD15 gene and location of the CD-associated polymorphisms**

**Genotype-phenotype associations**
An extensive clinical heterogeneity is observed in CD, most likely representing a heterogeneous subset of disorders, with differing pathogenic mechanisms. Numerous genotype-phenotype association studies have been performed to link the presence of *CARD15* polymorphisms with clinical disease characteristics (194, 200-210).

Most of these studies have associated the presence of *CARD15* polymorphisms with ileal involvement, younger age at onset and stricturing or fibrostenosing disease. It is however unclear whether the latter is not just related to the presence of ileal disease. Using multivariate analysis, an independent association with stricturing disease could not be confirmed (201, 211). Associations with familial CD, fistulizing disease and a history of surgical resection, as reported in some of these studies, have not been consistently confirmed and it is again unclear whether the latter is simply explained by the association with ileal involvement.

**Functional studies**
The intracellular pattern recognition receptor CARD15 or NOD2 binds specifically MDP, the minimal naturally occurring motif of PGN (present in the cell wall of all bacteria). Binding of CARD15 with its ligand gives rise to activation of caspases (involved in apoptotic pathways) and NF-κB, a nuclear transcription factor inducing the transcription of pro-inflammatory genes and the subsequent production of several cytokines (212). The mechanism by which
MDP enters the cell is uncertain. Possibilities include invasion of pathogens, active endocytosis of bacteria or their components and a specialized transporter molecule. The protein hPepT1 has been proposed as a transporter molecule for MDP in enterocytes and recently its expression was also confirmed in mononuclear cells (213, 214).

- Several in vitro studies have been performed to identify the functional role of the CARD15 polymorphisms. No NF-κB activation could be measured in HEK293T cells transfected with the frameshift mutation 1007fs after stimulation with MDP or PGN (91, 92, 215). R702W and G908R transfectants also showed a decreased NF-κB activation compared to NOD2 wild-type cells but less pronounced than the 1007fs variant (92, 215).

- Functional studies have also been performed on isolated mononuclear cells from (mostly limited numbers) of CD patients (92, 216, 217). These ex vivo studies confirmed the reduced NF-κB activation in response to MDP associated with CARD15 polymorphisms. A decreased production of pro-inflammatory cytokines (IL-8 and IL-1β) was demonstrated as well. Interestingly, a study from Netea et al also showed a reduced production of anti-inflammatory cytokines like IL-10 and transforming growth factor (TGF)-β whereas TNFα secretion was similar in cells from patients with CARD15 variants stimulated with PGN or heat-killed Bacteroides or Salmonella species (218). This actually results in a reduced and thus pro-inflammatory IL-10/TNFα ratio. All the effects in these studies were however only detected in patients carrying 2 copies of the mutant alleles (compound heterozygotes or homozygotes) with the most pronounced effect in 1007fs homozygotes. Until now no real functional deficits could be detected in cells from (the more common) patients being single heterozygous for CARD15 variants.

- Two recent studies also described a loss of NOD2/TLR synergy in CD patients carrying CARD15 variants (217, 219). Peripheral blood mononuclear cells from CD patients carrying CARD15 polymorphisms respond normally to TLR ligands. However, when co-stimulation with MDP is performed, the synergistic effect between MDP and TLR1/2, TLR4, TLR5, TLR2/6, TLR7/8 and TLR9 ligands is profoundly impaired.

- Common structural and functional features between human and mouse NOD2 have been identified (220). This finding opened the door to the use of animal models, in particular CARD15- or NOD2- deficient (CARD−/− or NOD−/−) mice, to evaluate the role of NOD2 in
chronic inflammatory disorders. Interestingly, NOD2^{−/−} mice are indistinguishable from controls and do not develop spontaneous intestinal inflammation (221). Results from animal studies are however not consistent.

- A study of Kobayashi et al reported a strongly reduced NF-κB activation in macrophages from NOD2^{−/−} mice stimulated with MDP (221). The synergistic effect of TLR2 ligand and MDP costimulation was abrogated. These animals were also more susceptible to bacterial infection (with *Listeria monocytogenes*) via the oral route but not through intravenous or peritoneal delivery. This may be due in part to the deficient production of certain antimicrobial peptides, called cryptdins (the homologue of human α-defensins), in these mice. *In vitro* infection of NOD^{−/−} macrophages did not result in an altered intracellular bacterial growth or killing.

- Pauleau et al showed that NOD2^{−/−} macrophages were indeed refractory to MDP stimulation but responded normally to multiple TLR agonists (222). However, these mice were protected in intraperitoneal endotoxin challenge assays.

- Watanabe et al reported an increased TLR2-mediated NF-κB activation and IL-12 production (Th1 response) in splenocytes from NOD2^{−/−} mice compared to wild-type mice (223). Similar results were obtained in isolated macrophages from NOD2^{−/−} mice repleted with the mouse construct L908fs, corresponding to the human 1007fs *CARD15* mutation. These findings suggest a NOD2-mediated inhibition of TLR2-driven response in normal circumstances and an abrogation of this inhibition in the presence of *CARD15* polymorphisms, which is in contrast with all studies on NOD2/TLR synergy previously reported.

- Macrophages from NOD2^{2939iC} mice, another construct corresponding to the human 1007fs mutation, even exhibited elevated NF-κB activation and increased IL-1β secretion in response to MDP (224). TLR2-driven responses were normal. Furthermore, mortality of these mice was higher in a dextran sodium sulphate (DSS) colitis model compared to wild-type mice. This difference in mortality was restored with oral antibiotic treatment, reflecting the role of enteric bacteria.

The “NF-κB paradox”

NF-κB is a key intracellular signalling molecule in a variety of inflammatory pathways and has been shown to be elevated in IBD tissues (225). However, most of the functional studies on the role *CARD15* polymorphisms point at a decreased NF-κB activation. How these
conflicting results can be explained is not yet understood. Nevertheless, certain observations can be used to formulate some hypotheses.

Expression of CARD15 has been demonstrated in mononuclear cells but also in intestinal epithelial cells and particularly in Paneth cells and is upregulated in active Crohn’s disease (226-228). Paneth cells are specialized intestinal epithelial cells in the base of the crypts of Lieberkühn in the ileum. Although they are generally absent in the colon and rectum, some metaplastic Paneth cells can be found in IBD (226, 229). They are critically involved in host defence against enteric infection and respond to bacterial products by secreting antimicrobial peptides including lysozyme, secretory phospholipase A2 (sPLA$_2$) and $\alpha$- and $\beta$-defensins (230, 231). Cytologic study has localized NOD2 in the cytosol in close proximity to the granules containing antimicrobial peptides (227). Recently, it was demonstrated that the presence of CARD15 mutations in CD patients is associated with a diminished mucosal expression of $\alpha$-defensins and this might induce a loss of control over bacterial growth (early immune pathogen clearance) at the epithelial surface (232). In combination with an increased intestinal permeability as reported in IBD patients, it may lead to an enhanced influx of bacteria into the mucosa and subsequently to the activation of CARD15-independent inflammatory pathways (233-235). Consistent with these findings, mice deficient in TLR signalling, resulting in decreased NF-$\kappa$B activation, exhibit an increased susceptibility to pathogen invasion and NF-$\kappa$B knock-out mice seem to develop colitis (236, 237). Moreover, defective eradication of intracellular pathogens (*Salmonella typhimurium*) has been demonstrated in CARD15-negative epithelial cell lines transfected with the CD-associated frameshift mutation (238). An altered sensing and handling of intracellular bacteria might lead to a prolonged bacterial survival and/or intracellular persistence of antigens, serving as a chronic stimulus for other immune cells.

An alternative hypothesis was postulated by the group of Strober and colleagues (104, 223). They showed in mouse splenocytes that stimulation of NOD2 with MDP downregulates the production of IL-12, IL-18 and IFN$\gamma$ in response to TLR2 ligands in wild-type cells but not in CARD15$^{-/-}$ cells. The absence of a negative regulation of this TLR2 response (towards PGN) by MDP-NOD2 signalling in CARD15$^{-/-}$ mice, could indicate that the “immunostat” for innate immune responses to PGN in these mice is set to a lower level, resulting in an excessive Th1 response with higher basal levels of IL-12 (and other Th1 mediators). However, this hypothesis still needs to be proven in humans. Moreover, the results of the Strober group are in contradiction with findings of other groups, demonstrating NOD2-TLR synergy and the loss of it in the presence of CARD15 polymorphisms (217, 219, 221).
1.2.5.4. TLR4

A polymorphism in another PAMP receptor gene, TLR4, has been associated with CD and UC (239). The prevalences of the Asp299Gly variant in the separate CD and UC populations were about 20% compared to less than 10% in the control population. Carriage of both CARD15 and TLR4 polymorphisms would imply a higher relative risk to develop CD compared with CARD15 or TLR4 variants alone.

Studies on transfected cell lines and isolated mononuclear cells associated homozygous carriage of this variant with hyporesponsiveness to the TLR4 ligand LPS (240-243). However, Asp299Gly homozygotes are rare and until now no functional defects could be linked with heterozygous carriage.

The association of this TLR4 variant with CD and UC were however not fully confirmed by other groups. Török et al found a higher prevalence of this polymorphism in German UC patients but not in CD whether a recent Greek study demonstrated the opposite (244-246). In a Scottish CD population no associations could be found (247).

Genetic heterogeneity may be an explanation for the lack of confirmation of these associations (247). However, it is also noteworthy that the associations found between TLR4 and CD and UC result from a primary candidate gene approach. The gene encoding TLR4 is located on chromosome 9q where no susceptibility loci have been described. A case-control association design in which the allele frequencies of candidate genes are compared in patients and matched healthy controls has been subject to considerable criticism because of a lack of reproducibility (248-250). This is reflected by a meta-analysis of 370 studies addressing 36 genetic associations with a number of diseases (251).

1.2.5.5. Other genetic associations

Numerous and often non-confirmed genetic associations with CD have been reported during the last years. There is however a growing interest in SLC22A4/5 and DLG5, since they have been identified as possible susceptibility genes by fine mapping of previously described IBD-loci.
The \textit{SLC22A4} and \textit{SLC22A5} genes are located within the IBD5 locus (on chromosome 5q31) and respectively encode the organic cation transporters OCTN1 and OCTN2 (229). These membrane proteins are widely expressed and mediate multispecific and bidirectional transmembrane transport of carnitine and organic cations. Variants in these genes would increase the risk for CD by 2.5-fold when present as a single copy and by 4-fold when present as 2 copies (252-254). The relevant susceptibility alleles may however differ among ethnic groups (253, 255-257). Several hypotheses on the functional role of these variants are currently under investigation. Impaired carnitine transport could inhibit fatty acid oxidation which could lead to intestinal inflammation whereas on the other hand, enhanced cation transporter activity may engender an aberrant uptake of toxic substrates (188, 258, 259). Limited genotype-phenotype studies have been performed and showed associations with ileal and perianal disease (253, 260).

The \textit{DLG5} (Drosophila Discs Large Homolog 5) gene is located within an IBD-linked locus on chromosome 10q (261). Dlg5 has a putative role as scaffolding protein involved in maintenance of epithelial cell structure and regulation of cell growth (262). Associations of \textit{DLG5} variants with IBD have been reported although not yet replicated (254, 261, 263).
2. The Spondyloarthropathy concept

2.1. Clinical features

Spondyloarthropathies constitute a cluster of interrelated and overlapping chronic inflammatory rheumatic diseases characterized by pauciarticular, peripheral, asymmetrical arthritis and/or axial involvement with ankylosing spondylitis as prototype (table 3) (264). A clear differentiation among these forms is not always possible. The European Spondyloarthropathy Study Group (ESSG) classification criterion has been validated in various groups and is currently most widely used to diagnose SpA (265-267).

Table 3. Clinical entities belonging to the Spondyloarthropathy concept

<table>
<thead>
<tr>
<th>Ankylosing spondylitis</th>
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<tr>
<td>Reactive arthritis</td>
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<tr>
<td>Psoriatic arthritis</td>
</tr>
<tr>
<td>IBD-associated arthritis</td>
</tr>
<tr>
<td>Juvenile spondyloarthropathy</td>
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<tr>
<td>Undifferentiated spondyloarthropathy</td>
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Prevalences of SpA between 0.2 and 1.9% have been reported (268, 269). Ethnic differences are important, since Eskimo and Inuit persons have a much higher prevalence whereas SpA is relatively uncommon in the Japanese population (270, 271). Although both sexes can be affected, there is overall a male preponderance. Onset of disease is mostly seen in the late teens and the early 20s and familial aggregation may occur (272).

The primary pathologic sites include the entheses, which are the sites of bony insertion of ligaments and tendon (particularly the plantar fascia, Achilles and patellar tendon), the sacroiliac joints and axial skeleton, the limb joints and some non-articular structures such as the gut, the skin, the eye or aortic valve (figure 7) (272).
Arthritis (dactylitis)  spondylitis (bamboo spine)  enthesitis

Figure 7. Some clinical manifestations of SpA

Ankylosing spondylitis is the most common and most typical form of SpA. The diagnosis is based on the modified New York criteria for AS, combining (unspecific) clinical criteria with radiologic evidence of sacroililitis (SI) (table 4) (18). SI is still considered to be the radiographic hallmark of AS.

Table 4. Modified New York criteria for the diagnosis of AS

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Details</th>
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<tbody>
<tr>
<td>1.</td>
<td>Low back pain of at least 3 month’s duration, improving by exercise and not relieved by rest</td>
</tr>
<tr>
<td>2.</td>
<td>Limited lumbar spinal motion in sagittal and frontal (Schober index) planes</td>
</tr>
<tr>
<td>3.</td>
<td>Chest expansion decreased relative to normal values for sex and age</td>
</tr>
<tr>
<td>4.</td>
<td>Bilateral sacroiliitis grade 2-4 or unilateral sacroiliitis grade 3 or 4 *</td>
</tr>
</tbody>
</table>

* Grades are 0 - normal; 1 - suspicious; 2 - localized sclerosis, erosion, joint widening; 3 - diffuse sclerosis, erosion, widening; 4 - ankylosis

The diagnosis of AS is made whenever criterion 4 with any of the other criteria is fulfilled.

Reactive arthritis (ReA) is diagnosed when an episode of aseptic peripheral arthritis occurs, preceded within 1 month by a genitourinary infection with *Chlamydia trachomatis* or an enteritis with gram-negative enterobacteria (such as *Shigella, Salmonella, Yersinia* or *Campylobacter*) (273). It is typically an acute, asymmetric and oligoarticular arthritis, associated with one or several characteristic extraarticular features, such as ocular inflammation (conjunctivitis or acute iritis), enthesitis, mucocutaneous lesions or rarely carditis. The rare triad of arthritis, conjunctivitis and urethritis is called Reiter syndrome (274).
Psoriatic arthritis (PsA) is defined by the presence of inflammatory arthritis associated with psoriasis, although no internationally agreed-on criteria for this diagnosis are available (275). Oligoarticular and polyarticular forms of PsA can be distinguished. PsA has been reported in up to one third of patients with psoriasis.

Arthritis associated with IBD, also called enteric arthritis, has been discussed previously. Some patients fulfil the ESSG criteria but can not be classified into one of the clear SpA subentities. This subgroup is categorized as undifferentiated SpA (276).

2.2. Genetic susceptibility

There is an undisputed influence of HLA-B27 carriership on the development of SpA (277). About 90% of AS patients are HLA-B27 positive whereas the prevalence of HLA-B27 varies between 25 and 80% in the other subgroups of SpA patients (15, 272). In contrast, only 8% of healthy Caucasian persons carry this genetic marker (278). The exact functional role of HLA-B27 in the pathogenesis of SpA is however not yet understood. Altered cytokine production from monocytes and T cells associated with HLA-B27 positivity has been reported (279). The latest data suggest that misfolding of the HLA-B27 heavy chain and dimerization (through disulfide linkage) at the cell surface might somehow affect cell function (280).

However, family and twin studies suggested that also other genes might play a role in the genetic predisposition for SpA, determining it as a complex genetic disorder (281, 282). This was confirmed by genome-wide scans (282-284). Next to the major histocompatibility complex (MHC) locus, these genome-wide studies also provided evidence for non-MHC susceptibility loci. Laval et al identified linkage regions in families with AS on chromosomes 1q, 2q, 6p, 9q, 10q, 16q and 19q (283). Recently, the non-MHC susceptibility locus on chromosome 9q was confirmed in SpA patients (284).

Since one of the linkage regions was located on chromosome 16q, the CD-associated CARD15 gene was investigated as putative susceptibility gene. No associations with CARD15 polymorphisms were found in SpA and AS populations (285-290). One study reported an association with PsA but this could not be confirmed by other groups (291-293).
3. The Gut-Joint axis in IBD and SpA

Similar to IBD, SpA are currently considered as multifactorial diseases occurring in genetically predisposed persons and triggered by environmental factors. Different combinations of genes and environmental factors may predispose to variation in disease expression (294).

The association of ReA with enteric infections initiated the search for an infectious aetiology of SpA. Bacterial antigenic components and DNA have been found in synovial fluid of patients with ReA, but also in other SpA subgroups (295-299). However, although carriage of Klebsiella pneumoniae in the gut was proposed as a trigger for AS, no definite evidence exists for an infectious trigger for AS or SpA (300).

Nevertheless, several lines of evidence point at a close relationship between gut and joint and more specifically between SpA and CD (301).

3.1. Clinical evidence

As already stated, rheumatic manifestations in IBD are frequent and can often be classified as SpA. On the other hand, colonoscopies with ileal and colonic biopsies, performed in SpA patients, have demonstrated that up to 60% of these patients had histological signs of (subclinical) acute or chronic gut inflammation (302-304). Acute inflammatory lesions were similar to those seen in acute self-limiting bacterial enterocolitis whereas the features of chronic lesions resembled mucosal alterations found in CD (305). The presence of gut inflammation was most frequently seen in AS patients and represented a risk factor for non-AS SpA patients to develop AS (306, 307). Furthermore, gut inflammation was also associated with active rheumatic disease (308). There was no influence of NSAID intake on the occurrence of these lesions (302).

The high prevalence of intestinal inflammation in SpA was confirmed by several other groups (309-313). Interestingly, about 13% of SpA patients having chronic gut lesions (representing about half of the patients with intestinal inflammation) will evolve over time towards a clinically overt Crohn’s disease (314).
Also animal models have provided evidence for the simultaneous occurrence of gut and joint inflammation. Both HLA-B27-transgenic rats and TNF$_{AARE}$ mice spontaneously develop Crohn’s-like gut inflammation and chronic inflammatory arthritis (315, 316).

### 3.2. Therapeutic evidence

Sulfasalazine has a beneficial effect in both CD and peripheral manifestations of SpA but more important was the development of anti-TNF$_{a}$ monoclonal antibodies. The introduction of these antibodies in CD meant an enormous breakthrough in the management of both intestinal and articular symptoms. Later on, a similar effect was demonstrated in SpA with a rapid and dramatic improvement of arthritis, enthesitis and axial symptoms (317, 318). Whether this drug also has a disease-modifying effect in CD or SpA needs to be proven.

### 3.3. Immunological evidence

Both types of tissue inflammation (gut and joint) in SpA and CD are characterized by increased numbers of T cells and macrophages and a greater expression of pro-inflammatory cytokines (like IL-1$\beta$, TNF$_{a}$ and IFN$\gamma$) at the site of inflammation (102, 319). In the intestine of both CD and SpA patients, an enrichment of gut-mucosal T cell lines with $\alpha$E$\beta$7-integrins and an increased expression of its ligand, E-cadherin, were observed (320-322). An altered expression of $\beta$7-integrins was also found on synovial T cells from patients with SpA but not with rheumatoid arthritis (RA) (323). This might reflect a recirculation between the gut and the joint of T cells, primed in the gut.

In CD and SpA patients, an augmented infiltration of CD163 positive macrophages (producing IL-1$\beta$ and TNF$_{a}$) has been found in both the gut and synovium (324, 325). This finding could reflect a trafficking of antigen presenting cells between the gut and the joint (326).

These findings led to the concept that the group of SpA patients with gut inflammation could be considered as a model for early immune alterations in CD.
3.4. Serological evidence

Antibodies, directed against the cell wall mannan of *Saccharomyces cerevisiae*, also called ASCA, are known to be specific serological markers for CD. Significant higher levels of ASCA IgA were also found in SpA patients, particularly in AS patients, compared to healthy controls or RA patients (30, 31).

3.5. Genetic evidence

A study in 1st degree relatives of AS patients provided evidence for a common, not HLA-B27 related (and yet unknown) genetic risk factor for the development of subclinical intestinal inflammation (327). Moreover, there was a close relationship between the presence of this subclinical intestinal inflammation (assessed by measuring fecal calprotectin concentrations) and sacroiliac changes, suggestive of early AS.

Overall, there is clear evidence for the close relationship between Crohn’s disease and SpA. However, the etiopathogenic mechanisms underlying this link are still unknown and studies are needed to elucidate the common genetic and/or environmental triggers, predisposing for both disease entities.
4. Research objectives

The objective of this work was to investigate the role of genetic alterations in the gut-joint axis of CD and SpA.

4.1. The role of CD associated CARD15 polymorphisms in the relationship between gut and joint.

4.1.1. CARD15 polymorphisms in SpA

The CD-associated CARD15 gene is located on chromosome 16q which was also found to have a linked region in a genome-wide scan of AS patients. We wanted to investigate the possible role of CARD15 polymorphisms in SpA patients and in particular in SpA patients with chronic gut inflammation, since exactly these patients are at risk to develop clinical CD. A genetic association study was conducted in a population of SpA patients who underwent an ileocolonoscopy with biopsies between 1983 and 2004.

4.1.2. Relation between genetic markers and rheumatic phenotypes in patients with CD

4.1.2.1. CARD15 polymorphisms and rheumatic manifestations in CD

Despite the numerous genotype-phenotype studies on the role of CARD15 polymorphisms in CD, no clear data were available concerning the association with the distinct rheumatic manifestations of CD. In a cohort of CD patients, we investigated the relation of these polymorphisms with the history or presence of peripheral arthritis, enthesopathies and sacroiliitis (with or without AS).

4.1.2.2. Axial involvement in CD: a clinical and genetic association study

Overall, data on the association of axial disease in CD (including asymptomatic sacroiliitis and AS) with other clinical phenotypes and genotypes (except for HLA-B27) are scarce. Therefore, we initiated a multicentre study, in cooperation with Leuven and Liège, to investigate the association of radiological sacroiliitis and AS in CD with the most common
clinical phenotypes of the disease, including other extraintestinal manifestations, and CARD15 polymorphisms.

4.1.3. Influence of CARD15 polymorphisms on cellular and humoral response

4.1.3.1. The functional impact of CARD15 polymorphisms on peripheral blood monocytes in CD

CARD15 and TLR4 polymorphisms have been linked with an altered innate immune response to bacterial components but the exact functional roles (in particular in single heterozygotes) are actually unknown. Therefore we investigated the functional role of these genetic variants on isolated blood monocytes, stimulated with MDP or LPS or infected with AIEC, an enteroinvasive E. coli strain specifically associated with CD. Since one of the hypotheses of simultaneous gut and joint inflammation includes circulation of microbial antigens between both sites, we also looked at intracellular survival and replication of these bacterial strains within these monocytes.

4.1.3.2. CARD15 polymorphisms and ASCA formation

ASCA can be found in both CD and SpA and a genetic influence on the generation of these antibodies has been suggested. Since CARD15 polymorphisms and ASCA share associations with similar clinical phenotypes (ileal and structuring disease and early age at onset), we wanted to investigate the direct link between these polymorphisms and this aberrant serological response (ASCA formation) in CD patients.

4.2. The search for new molecular targets in CD and SpA

4.2.1. Genetic expression assays on colonic biopsies of CD and SpA patients

Important immunological similarities have already been found in the gut of both CD and SpA patients. We performed macro- and microarrays on non-inflamed colonic biopsies of healthy controls, CD patients and SpA patients (with or without subclinical gut inflammation) to investigate whether similarities could also be found at the transcriptome level.
4.2.2. Identification of new candidate genes for CD by linking gene expression variation with known susceptibility genes

Differentially expressed genes, revealed by transcriptome analysis of colonic biopsies, were linked with known disease susceptibility loci for CD in order to identify new putative disease-modifying genes. We wanted to confirm the altered expression of potentially interesting candidate genes at both the RNA and protein level and investigated the functional significance. Mutation screening was performed to search for significant polymorphisms within the involved genes.

References


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CHAPTER 2

Study of the association of *CARD15* polymorphisms with gut inflammation in spondyloarthropathies

*CARD15* gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to Crohn’s disease.


* equal contribution

**EXTENDED REPORT**

**CARD15 gene polymorphisms in patients with spondyloarthropathies identify a specific phenotype previously related to Crohn’s disease**

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*These authors made an equal contribution to the work

**Background:** The association between spondyloarthropathy and Crohn’s disease is well known. A risk for evolution to Crohn’s disease has already been shown in the subgroup of patients with spondyloarthropathy associated with chronic gut inflammation.

**Objective:** To investigate whether the reported polymorphisms in the CARD15 gene, a susceptibility gene for Crohn’s disease, are associated with the presence of preclinical intestinal inflammation observed in spondyloarthropathies.

**Methods:** 104 patients with spondyloarthropathies were studied. All underwent ileocolonoscopy with biopsies between 1983 and 2004. The prevalence of three single nucleotide polymorphisms in the CARD15 gene (R702W, G908R, and 1007fs) was assessed using restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR); the patients were compared with an ethnically matched Crohn’s disease population and a control population.

**Results:** The carrier frequency of R702W, G908R, or 1007fs variants in the spondyloarthropathy populations (20%) was similar to the control population (17%), but increased to 38% in the spondyloarthropathy subgroup with chronic gut inflammation. This frequency was significantly higher than in the other spondyloarthropathy subgroups (p = 0.001) or the control group (p = 0.006), but not different from the Crohn’s disease group (49%) (NS). This indicates that CARD15 polymorphisms are associated with a higher risk for development of chronic gut inflammation.

**Conclusions:** CARD15 gene polymorphisms clearly identify a subgroup of patients with spondyloarthropathies associated with chronic intestinal inflammation.

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The spondyloarthropathies are a group of interrelated inflammatory diseases characterised by a pauciarticular, peripheral, asymmetrical arthritis with or without axial involvement, with ankylosing spondylitis as the prototype.\(^1\) Reported prevalences of spondyloarthropathy vary between 0.2% and 1.9%.\(^2\) Although an association with HLA-B27 is strong, recent genetic studies suggest a polygenic model of susceptibility.\(^3-5\)

In up to 60% of spondyloarthropathy patients, articular involvement is associated with subclinical histological evidence of chronic or acute gut inflammation in the ileum or colon.\(^6\) We described a long term evolution to overt Crohn’s disease in 13% of patients with initial chronic gut inflammation.\(^11-13\) The presence of chronic intestinal inflammation was not related to HLA-B27, but a weak association with HLA-B62 was found.\(^14\)

The observed immunological similarities between spondyloarthropathy with gut inflammation and Crohn’s disease support the concept that this subgroup of spondyloarthropathy patients can be considered a model for early immune alterations related to Crohn’s disease. An enrichment of gut mucosal T cell lines with \(\alpha E\beta 7\) integrin and an increased expression of its ligand, E-cadherin, is found in the intestine in Crohn’s disease as well as in spondyloarthropathy patients.\(^15-17\) Recirculation of gut primed T cells to synovial tissue is one potential mechanism whereby gut and synovial inflammation could be linked. This hypothesis is supported by an altered expression of \(\beta 7\) integrins, which are highly expressed within the gut, on synovial T cells from patients with spondyloarthropathies compared with rheumatoid arthritis.\(^18\) Another potential mechanism includes trafficking of antigen presenting cells between gut and joints. Consistent with this was the augmented infiltration of gut mucosa and synovium with CD163 positive macrophages (producing interleukin 1 (IL-1) and tumour necrosis factor \(\alpha\) (TNF\(\alpha\))) in both Crohn’s disease and spondyloarthropathy.\(^19-22\) Finally, a comparable beneficial clinical effect of infliximab, a monoclonal antibody to TNF\(\alpha\), suggests a key role for this cytokine in both diseases.\(^23-26\)

In 2001, a correlation was reported between polymorphisms in the CARD15 gene and an increased susceptibility to Crohn’s disease.\(^21-23\) Three independent single nucleotide polymorphisms (SNPs) in CARD15 are associated with Crohn’s disease in around 30–46% of patients (one frame shift mutation (1007fs (SNP13)) and two missense mutations (R702W (SNP8) and G908R (SNP12))).\(^22\) These variants increase the risk for Crohn’s disease by a factor of 3 for heterozygotes and by a factor of 38 or 44 for, respectively, homozygous or compound heterozygous individuals.\(^22\) Lower prevalences have been described in Crohn’s disease patients in Scotland, Ireland, and northern Europe, whereas no association could be found in Japan.\(^25-28\)

The CARD15 gene encodes for an intracellular protein, which is expressed in monocytes, granulocytes, and dendritic, epithelial, and Paneth cells, and has binding affinity for

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**Abbreviations:** CARD, caspase recruitment domain; ESSG, European Spondyloarthropathy Study Group; SNP, single nucleotide polymorphism
bacterial cell wall components such as muramyl dipeptides. The CARD15 protein is involved in NFκB activation and in apoptosis by two N-terminal caspase recruitment domains (hence the term CARD), although its precise pathogenic role in Crohn's disease remains to be determined.19–21

CARD15 gene polymorphisms have also been linked to another related disorder, Blau's syndrome, characterised by granulomatous inflammation of the uvea, skin, and joints.22–25

Several studies have been carried out to investigate the role of CARD15 polymorphisms in spondyloarthropathies. These studies did not show an association with spondyloarthropathies or ankylosing spondylitis in particular.11–17 An increased prevalence of CARD15 polymorphisms was found in psoriatic arthritis though not in psoriatic skin disease.18–20 However, a recent Italian study could not confirm this association.21 Nevertheless, this finding could emphasise the importance of investigating the possible role of these genetic variants in specific clinical subpopulations of patients. In Crohn's disease, CARD15 polymorphisms also seem to be related to certain clinical phenotypes.22–26

In view of the apparent correlation between gut inflammation in spondyloarthropathies and their clinical evolution to Crohn's disease, we investigated whether the presence of polymorphisms in this susceptibility gene for Crohn's disease is associated with gut inflammation in spondyloarthropathy.

### METHODS

#### Study population

This study included 104 white patients with spondyloarthropathies (according to the ESSG criteria), who underwent an ileocolonoscopy with concomitant ileal and colonic biopsies between 1983 and 2004. This population consisted of 74 male and 30 female patients with a mean age of 46 years (range 21 to 77). Spondyloarthropathy patients were systematically referred by the rheumatologist for an ileocolonoscopy with biopsies, independent of the presence of gastrointestinal grounds was also included. This cohort included 57 male and 99 female patients with a mean age of 38 years (range 18 to 80). Prevalences were also compared with those in a control population of 140 individuals.

The study was approved by the local ethics committee. All patients signed their informed consent.

#### Histological classification

A classification of histological lesions was used as reported in previous studies.9–12, 49–50 Three subgroups were distinguished: patients with normal gut histology, and those with acute and chronic inflammation.51

In acute inflammatory lesions normal architecture was well preserved. A mucosal and epithelial infiltrate of neutrophils and eosinophils was present, without a significant increase in lymphocytes. Small superficial ulcers covered with fibrin and neutrophils overlying hyperplastic lymphoid follicles were occasionally observed. The lamina propria was oedematous and haemorrhagic and contained mainly polymorphonuclear cells. The pattern of inflammation was similar to that seen in acute self-limiting bacterial enterocolitis.

The principal features of chronic inflammatory lesions were mucosal architectural alterations with crypt distortion and atrophy in the colon, and villous blunting and fusion in ileal mucosa. In both ileum and colon there was an increased mixed cellularity and formation of basal lymphoid aggregates in the lamina propria. Whenever one of several biopsies featured chronic lesions, regardless of acute or active inflammation in other fragments, a diagnosis of chronic inflammation was made.

Although non-steroidal anti-inflammatory drugs may induce intestinal disorders, we and others excluded these drugs as factors in the aetiology of reported chronic inflammation.44–47

### Table 1 Prevalence of CARD15 variants in the populations according to subtypes defined at baseline

<table>
<thead>
<tr>
<th>Classification</th>
<th>n</th>
<th>Carriers of CARD15 variant(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>140</td>
<td>24 (17%)</td>
</tr>
<tr>
<td>Crohn's disease</td>
<td>156</td>
<td>77 (49%)†</td>
</tr>
<tr>
<td>Spondyloarthropathy</td>
<td>104</td>
<td>21 (20%)††</td>
</tr>
<tr>
<td>Ankylosing spondylitis</td>
<td>75</td>
<td>18 (24%)</td>
</tr>
<tr>
<td>Undifferentiated spondyloarthropathies</td>
<td>29</td>
<td>5 (17%)</td>
</tr>
</tbody>
</table>

**Gut histology in the spondyloarthropathy population**

- **Acute inflammation**: 24 (23%) 0 (0%)
- **Normal histology**: 29 (38%) 6 (15%)

* p = 0.001 (carrier frequency in Crohn's disease v control population).
†† p = 0.5 (carrier frequency in general spondyloarthropathies v control population).
† p = 0.001 (chronic inflammation in patients with CARD15 variant v chronic inflammation in patients without CARD15 polymorphism).

### CARD15 genotyping (R702W, G908R, and 1007fs), and HLA-B27 and HLA-B62 typing

Genomic DNA was extracted from whole blood using the Qiagen blood and cell culture DNA kit (Westburg BV, Leusden, Netherlands). All patients were genotyped for R702W, G908R, and 1007fs using restriction fragment length polymorphism–polymerase chain reaction (RFLP-PCR), followed by separation of the DNA fragments on a 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site forMspI, resulting in an intact 130 base pair (bp) band for mutant

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Cytotoxicity test, according to Terasaki and McClelland. Typing of these markers was done using the microlymphocytotoxicity test, according to Terasaki and McClelland.  

Statistical analysis
Statistical significance was determined by the $\chi^2$ test and odds ratios using SPSS (SPSS Inc, Chicago, Illinois, USA). Multivariate analysis (logistic regression) was carried out to investigate whether an association found in univariate analysis was independent of other genetic markers. Probability (p) values of less than 0.05 were considered significant.

RESULTS
We subdivided our cohort into three groups according to gut histology. Forty patients (38%) had normal histology, 24 (23%) had acute gut inflammation, and 40 (38%) had chronic gut inflammation (table 1).

Univariate analysis
Prevalence of CARD15 polymorphisms in the various populations
The prevalences of CARD15 polymorphisms in the total spondyloarthropathy (20%), specific ankylosing spondylitis (21%), and undifferentiated spondyloarthropathy (17%) populations did not differ significantly (table 1). All except one (homozygous for the 1007fs allelic variant) were heterozygous for at least one mutation. The prevalences of R702W, G908R, and 1007fs allelic variants in these spondyloarthropathy populations were 12%, 4%, and 5% respectively (table 2). No compound heterozygosity was found. All carriers of CARD15 polymorphisms in the spondyloarthropathy group had (a history of) peripheral disease (table 3). There were no significant differences in disease duration or the duration of the follow up period between the spondyloarthropathy patients carrying CARD15 polymorphisms and the patients without these polymorphisms (data not shown).

In the Crohn’s disease population, a carrier frequency of 49% (77 of 156 patients) was observed (table 1). Forty three Crohn’s disease patients carried at least one R702W polymorphism, 14 carried at least one G908R polymorphism, and 27 carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms, of which seven were homozygous and seven were compound heterozygous (table 2).

In the control group, 24 individuals (17%) carried CARD15 polymorphisms (table 1). All except one (compound heterozygous for the R702W and 1007fs variant) were single heterozygotes (table 2).

The prevalence of polymorphisms in the spondyloarthropathy cohort (20%) was not different from that in the control group (17%) (p = 0.5; odds ratio (OR) = 1.22 (95% confidence interval (CI), 0.64 to 2.34)) and significantly lower than in our Crohn’s disease population (49%) (<0.001; OR = 3.85 (95% CI, 2.17 to 6.83)).

Association between CARD15 polymorphisms and intestinal inflammation in spondyloarthropathy patients
The carrier frequency in the subgroup of spondyloarthropathy patients with chronic gut inflammation was 38% (15 of 40 patients) which was significantly higher than in the control population (p = 0.006; OR = 2.9 (95% CI, 1.33 to 6.30)) or the other spondyloarthropathy populations (p = 0.001; OR = 5.80 (2.02 to 16.68)) and not statistically different from that in our Crohn’s disease population (49%) (p = 0.2; OR = 1.62 (0.80 to 3.31)) (table 1).

Of all spondyloarthropathy patients carrying CARD15 polymorphisms, 71% (15 of 21 patients) had chronic gut inflammation, none had acute inflammation, and 29% had normal histology (table 1). The only spondyloarthropathy patient carrying two CARD15 variants also had chronic gut inflammation. In contrast, only 25 of 83 patients with a wild type genotype (30%) had chronic gut inflammation, 29% had acute inflammation, and 41% had normal histology. Consequently, the presence of CARD15 polymorphisms was associated with a higher risk of the development of chronic gut inflammation.

There were no significant differences between the ankylosing spondylitis and the undifferentiated spondyloarthropathy groups for the prevalence of CARD15 polymorphisms in patients with normal gut (3/29 v 3/11, respectively; p = 0.3; OR = 3.3 (95% CI, 0.5 to 19.41), acute gut inflammation (0/13 v 0/11), or chronic gut inflammation (13/33 v 2/7; p = 0.7; OR = 1.6 (95% CI, 0.3 to 9.7)).
In the subgroup of 54 patients who were clinically reassessed, four had evolved from histological chronic gut inflammation towards clinically overt Crohn’s disease. Two of these four patients carried CARD15 polymorphisms. The other 22 patients with chronic gut inflammation in this group did not develop clinical Crohn’s disease.

Association between CARD15 polymorphisms and HLA-B27 in spondyloarthropathy patients

There was no significant association between the presence of these two genetic markers. Six of 34 HLA-B27 positive patients carried CARD15 polymorphisms (p = 0.3; OR = 1.8 (95% CI, 0.6 to 5.3)).

Multivariate analysis

In the subgroup of 53 spondyloarthropathy patients in whom both HLA-B27 and HLA-B62 status was known, logistic regression was undertaken (with the presence of chronic gut inflammation as the dependent variable). This showed that the association between chronic gut inflammation and CARD15 polymorphisms (p = 0.01; OR = 17.3 (95% CI, 2.0 to 152.3)) was independent of HLA-B27 (p = 0.42; OR = 1.7 (0.5 to 6.0)) and HLA-B62 (p = 0.28; OR = 2.5 (0.5 to 13.0)).

DISCUSSION

In this study we describe a novel and remarkably strong association between variants in a host defence gene located on chromosome 16 (CARD15) and a chronic form of gut inflammation in patients with spondyloarthropathies. The prevalence of CARD15 polymorphisms in this subgroup of spondyloarthropathy patients was not significantly different from that seen in patients with Crohn’s disease.

Three single nucleotide polymorphisms have been associated with Crohn’s disease.2–12 One variant (10071G) encodes a truncated protein which results in altered activation of NFκB in response to bacterial stimuli.13,14 The two other single nucleotide polymorphisms (R702W and G908R) result in an amino acid substitution.

More recently, several groups assessed the linkage of CARD15 variants in Crohn’s disease to particular clinical phenotypes, but the results of these retrospective studies are disparate. The presence of two mutations has been shown at younger age at onset and preferential involvement of small bowel.1,13 Preference for ileal involvement was also reported by Cuthbert et al14 and by Ahmad et al.15 Fibrostenosing disease was the dominant type in a study by Abreu et al.16 In these studies, no association of CARD15 variants with extraintestinal involvement could be shown.

Our study shows a new association between these three Crohn’s disease associated variants in the leucine-rich region of the CARD15 gene and a distinct subpopulation of patients with spondyloarthropathies. As in previous reports, the overall prevalence of mutations in spondyloarthropathy patients was not statistically different from the prevalence in our control population.10–12 However, unlike the previous studies, we identified a distinct clinical subgroup—characterised by the presence of chronic inflammatory gut lesions—with a high prevalence of CARD15 polymorphisms similar to the Crohn’s disease population and significantly higher than in the control population or the other spondyloarthropathy patients. Previous studies from our group showed that these patients with chronic gut inflammation were at particular risk for progression to Crohn’s disease.11,12

Strikingly, in the present study none of the spondyloarthropathy patients with only axial disease carried CARD15 polymorphisms. Carriers of these polymorphisms all had (a history of) peripheral disease. This is in accord with previous studies of patients with ankylosing spondylitis where more chronic gut inflammation could be found in those with peripheral disease than in those with strict axial disease.

One previous study investigated CARD15 polymorphisms in patients with ankylosing spondylitis, Crohn’s disease, and ulcerative colitis.13 It did not show a higher prevalence of CARD15 variants in patients with ankylosing spondylitis plus Crohn’s disease compared with idiopathic ankylosing spondylitis, ankylosing spondylitis plus ulcerative colitis, or healthy controls. However, the low prevalence of CARD15 variants in the Crohn’s disease population with ankylosing spondylitis was not compared with the prevalence in a general Crohn’s disease population and it unexpectedly revealed a possible association between the G908R CARD15 variant and ankylosing spondylitis plus ulcerative colitis.

Moreover, in a recent study we found an association between CARD15 polymorphisms and the presence of radiological sacroiliitis in Crohn’s disease patients, unrelated to the HLA-B27 status of these subjects. These data already suggested a role for the CARD15 gene in the link between gut and joint inflammation.

Our findings confirm the previous reported clinical, therapeutic, and immunological links between spondyloarthropathies and Crohn’s disease and provide genetic proof for the association between these two disorders. As chronic gut inflammation in the majority of spondyloarthropathy patients remains asymptomatic, this might suggest that CARD15 polymorphisms could be linked to the development of (subclinical) chronic gut inflammation rather than to Crohn’s disease as such.

The underlying pathogenic mechanisms that could explain the phenotypic expression of CARD15 mutations in spondyloarthropathies need to be investigated. CARD15 encodes a cytosolic protein that could play a role in spondyloarthropathies by interference with transport of antigens by macrophages from mucosal surfaces to the joints.13 CARD15 seems to function as an intracellular receptor for bacterial components, where the C-terminal leucine-rich repeat domain (LRR domain) is crucial for responsiveness. The cellular response to bacterial products has been shown to be altered in HEK293T cells transfected with expression plasmids containing any of the three SNPs.12,14 Moreover, expression of CARD15 in myeloblastic and epithelial cells is enhanced by proinflammatory cytokines and bacterial components, through NFκB.15,16 This response is likely to mediate cytokine production including TNFs, suggesting that upregulation of CARD15 may be part of a positive regulatory loop and facilitate the response of the host to pathogens. A genetically determined disturbance of handling of bacterial products in the intestinal tract, leading to altered transport of antigens by macrophages to synovial tissue, is an interesting hypothesis that should be investigated in spondyloarthropathy. A further identification and characterisation of inflammatory cells involved in gut and joint inflammation may also lead to new therapeutic targets.

Conclusions

A distinct phenotype associated with the three main Crohn’s disease associated CARD15 variants is reported in patients with spondyloarthropathies. Our data show that the presence of CARD15 variants in spondyloarthropathy patients strongly predisposes to chronic intestinal inflammation, defining a population at risk for evolution to Crohn’s disease. However, the persistence of the subclinical character of the inflammation in a large proportion of patients may reflect the fact that Crohn’s disease is a multigenic disease or alternatively that the heterogenous carriage of CARD15 polymorphisms predisposes only to subclinical inflammation.
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CARD15 gene polymorphisms in spondyloarthropathy


 CHAPTER 3

Study of the association of *CARD15* polymorphisms with different rheumatic manifestations of CD

Radiological sacroiliitis, a hallmark of spondylitis, is linked with *CARD15* gene polymorphisms in patients with Crohn’s disease.


*equal contribution

Radiological sacroiliitis, a hallmark of spondylitis, is linked with CARD15 gene polymorphisms in patients with Crohn’s disease

H Peeters, B Vander Cruyssen, D Laukens, P Coucke, D Marichal, M Van Den Bergh, C Cuvelier, E Remaut, H Mielants, F De Keyser, M De Vos

Background: Sacroiliitis is a common extraintestinal manifestation of Crohn’s disease but its association with the HLA-B27 phenotype is less evident. Polymorphisms in the CARD15 gene have been linked to higher susceptibility for Crohn’s disease. In particular, associations have been found with ileal and fibrostenosing disease, young age at onset of disease, and familial cases.

Objectives: To investigate whether the presence of sacroiliitis in patients with Crohn’s disease is linked to the carriage of CARD15 polymorphisms.

Methods: 102 consecutive patients with Crohn’s disease were clinically evaluated by a rheumatologist. Radiographs of the sacroiliac joints were taken and assessed blindly by two investigators. The RFLP-PCR technique was used to genotype all patients for three single nucleotide polymorphisms (SNP) in the CARD15 gene. Every SNP was verified by direct sequencing. The HLA-B27 phenotype was determined.

Results: Radiological evidence of sacroiliitis with or without ankylosing spondylitis was found in 23 patients (23%), of whom only three were HLA-B27 positive. In contrast, 78% of patients with sacroiliitis carried a CARD15 variant v 48% of those without sacroiliitis (p = 0.01; odds ratio 3.8 (95% confidence interval, 1.3 to 11.5)). Multivariate analysis (logistic regression) showed that the association between sacroiliitis and CARD15 polymorphisms was independent of other CARD15 related phenotypes (ileal and fibrostenosing disease, young age at onset of disease, familial Crohn’s disease) (p = 0.039).

Conclusions: CARD15 variants were identified as genetic predictors of Crohn’s disease related sacroiliitis. An association was demonstrated between these polymorphisms and an extraintestinal manifestation of Crohn’s disease.

Methods

Study population

One hundred and two white patients with Crohn’s disease, attending the gastroenterology department of the Ghent University Hospital, agreed to participate in this cross sectional study and were included consecutively over a period of 13 months. The diagnosis of Crohn’s disease was based on clinical, endoscopic, histological, and radiological findings. All patients were seen by both a gastroenterologist and a rheumatologist. The localisation of the disease was assessed as ileal, ileocolonic, or colonic. Types and amount of surgical interventions were recorded. The need for resective small bowel surgery was used as an index of fibrostenosing disease. A positive family history was defined as the presence of at least one first, second, or third degree relative with Crohn’s disease and AS.

Our aim in this cross sectional clinical and radiological study was to evaluate, in a cohort of Crohn’s disease patients, the possible association between CARD15 polymorphisms and the presence of radiological sacroiliitis, a hallmark of spondylitis and the most frequent objective rheumatic manifestation of Crohn’s disease.

Abbreviations: AS, ankylosing spondylitis; CARD, caspase activation and recruitment domain; IBD, inflammatory bowel disease; RFLP-PCR, restriction fragment length polymorphism polymerase chain reaction; SNP, single nucleotide polymorphism
proven Crohn’s disease. The age at diagnosis of the disease was also recorded.

For controls, we used a group of 54 patients without any symptoms or clinical evidence of Crohn’s disease, spondyloarthopathies, or sclerosing cholangitis, who were attending the department of hepatology.

The study was approved by the regional ethics committee and all patients gave their signed informed consent.

Assessment of articular involvement

All patients were assessed for the presence of inflammatory low back pain, had measurements of the Schönber index and chest expansion, and were examined for peripheral arthritis and enthesopathy (fasciitis plantaris or Achilles tendon tendinitis).

A history of peripheral arthritis or enthesopathy was recorded if observed, confirmed by a physician, and noted in the patient’s medical record. Inflammatory low back pain was defined by the history or presence of spinal pain in the neck, dorsal, or cervical region with at least four of the five following criteria: onset before the age of 45 years, insidious onset, improvement with exercise, association with morning stiffness, and duration of at least three months. The modified New York criteria were used to determine the diagnosis of AS.

Radiological classification

Radiographs of the sacroiliac joints were made and assessed blindly by two rheumatologists. They were scored using the New York grading system: 0, normal; 1, suspicious; 2, localised sclerosis, erosion, joint widening; 3, diffuse sclerosis, erosion, widening; 4, ankylosis. Radiological sacroilitis was diagnosed by the presence of at least unilateral sacroilitis grade 2, and only when agreed on by both radiological assessors.

CARD15 and HLA-B27 typing

Genomic DNA was extracted from whole blood using a Qiagen blood and cell culture DNA kit (Qiagen, Valencia, California, USA).

All patients were genotyped for R702W (SNP8), G908R (SNP12), and 1007fs (SNP13) using a restriction fragment length polymorphism polymerase chain reaction technique (RF-LPCR), followed by separation on 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for MspI (5′-CAGCCC TGATGACATTTCTCTT-3′ and 5′-AGCCGCTCCTCCTGCATCTC-3′), resulting in an intact 130 base pair (bp) band for mutant alleles, compared with two bands of 54 and 76 bp for wild type alleles. The missense mutation G908R (GenBank accession number G67951) creates a restriction site for HinfII. The frameshift mutation 1007fs (GenBank accession number G67953) creates a restriction site for NlaIV (5′-CTGAGCCTTTGGATGAGC-3′ and 5′-CGACATTTCTCTGCATCTC-3′). The presence of a mutant allele results in two bands of 219 and 41 bp, while the wild type allele produces a single 260 bp product. We verified every SNP by direct sequencing of the PCR product in an ABI 377 analyser (Applied Biosystems, Foster City, California, USA).

HLA-B27 typing was undertaken using Dynal Classic SSP (Dynal Biotech, Bromborough, Cheshire, UK).

Statistical analysis

Odds ratios were calculated with Pearson’s χ² test or Fisher’s exact test when the expected count was less than 5 in at least one cell. A model for multivariate analysis (logistic regression) was constructed, with sacroilitis as the dependent variable. As covariates we included the CARD15 genotype, HLA-B27, and CARD15 related phenotypes. A probability (p) value of less than 0.05 was considered to indicate statistical significance. Statistical analyses was carried out using SPSS software (SPSS Inc, Chicago, Illinois, USA).

RESULTS

Patient characteristics

In all, 102 patients with Crohn’s disease were included (34 men, 68 women), mean age 41.6 years (range 18 to 71). The mean age at diagnosis was 28.5 years (range 9 to 57). Twenty-two of the patients (21.6%) had ileal disease, 26 (25.5%) had colonic disease, and 54 (52.9%) had ileocolonic involvement. Fifty-two patients (51.0%) had needed resection of a small bowel segment. Fifteen patients (14.7%) had a family history of Crohn’s disease.

Radiological sacroilitis and other rheumatic manifestations

Radiological sacroilitis was found in 23 patients: eight had unilateral sacroilitis grade 2, 14 had bilateral sacroilitis grade 2, and one had unilateral sacroilitis grade 3. Nine patients fulfilled the criteria for AS. Among the 14 remaining patients, three had inflammatory low back pain without fulfilling the AS criteria, and 11 were clinically asymptomatic.

The history or presence of peripheral arthritis was recorded in 17 patients, of whom five actually had arthritis at the time of study assessment. Three patients had monoarticular arthritis and 14 had oligoarticular involvement. A history of enthesopathy was present in 11 patients, of whom four had tendinitis at the time of the study.

CARD15 status

The overall prevalence of CARD15 polymorphisms in the Crohn’s disease group was significantly higher than in the control group, at 56/102 (54.9%) versus 54/150 (15%), p<0.001; odds ratio (OR) 7.00 (95% confidence interval (CI), 3.00 to 16.31)). Eighteen of 23 patients with radiological sacroilitis (78%) carried at least one CARD15 variant, compared with 38 of 79 patients (48%) without sacroilitis (p = 0.01; OR 3.88 (95% CI, 1.31 to 11.49)) (table 1). There were two compound heterozygotes and no homozygotes in the group of patients with sacroilitis (table 1). Seven of nine patients with AS carried a CARD15 variant (table 2).

In another perspective, among all carriers of CARD15 variants, 32% of the patients had sacroilitis compared with 11% in patients without variants.

<table>
<thead>
<tr>
<th>Table 1 Carrier frequency of CARD15 variants in patients with Crohn’s disease</th>
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</thead>
<tbody>
<tr>
<td>CARD15 variants</td>
</tr>
<tr>
<td>CARD15−/−</td>
</tr>
<tr>
<td>CARD15+/−</td>
</tr>
</tbody>
</table>

Crohn’s disease with no S (n = 79)

CARD15−/− | 21 | 6 | 13 |
CARD15+/− | 2 | 1 | 0 |

Crohn’s disease with S (n = 23)

CARD15−/− | 12 | 0 | 8 |
CARD15+/− | 0 | 0 | 0 |

Numbers of patients carrying R702W, G908R, or 1007fs variants.

*Total number of patients in the group carrying at least one variant. The sum of all allelic CARD15 variants is greater than the overall number of patients carrying at least one variant, as some patients carry two different single nucleotide polymorphisms (compound heterozygotes). CARD15−/−: the heterozygous for CARD15 gene; CARD15+/−: homozygous for CARD15 gene, S, sacroilitis.
There was no significant association between the presence of CARD15 polymorphisms and peripheral arthritis (p = 0.37; OR 1.6 (95% CI 0.55 to 4.81)) or enthesopathy (p = 0.34; OR 0.5 (0.14 to 1.94)).

**HLA-B27 status**
Overall, six of the 102 patients with Crohn’s disease (6%) carried HLA-B27. HLA-B27 positivity was present in three of the 23 patients (13%) with sacroiliitis. Those three patients were also carriers of CARD15 variants and fulfilled the modified New York criteria for AS (table 2).

**Multivariate analysis**
Logistic regression showed the presence of CARD15 variants as the only significant predictor of sacroiliitis (p = 0.039), independent of HLA-B27, ileal involvement, need for resective small bowel surgery, familial Crohn’s disease, or age at onset (table 3).

**DISCUSSION**
Our data identify CARD15 variants as a possible genetic predictor of sacroiliitis in Crohn’s disease. Sacroiliitis is a distinct extraintestinal manifestation of Crohn’s disease. In contrast to the clinically evident peripheral arthritis, the prevalence of axial involvement in Crohn’s disease is probably underestimated.14,15 Previous studies showed that 10–32% of patients with inflammatory bowel disease (IBD) have evidence of sacroiliitis, a hallmark of spondylitis, on conventional radiographs.16,17,18,19,20,21,22,23,24 With technetium scintigraphy, tracer uptake in the sacroiliac joints can be seen in up to 32% of patients.20 Many patients with sacroiliitis remain asymptomatic.21,22,23,24 Only about one quarter of the patients with radiological sacroiliitis also fulfill the criteria for AS.24 In contrast to idiopathic AS, involvement of the sacroiliac joints is asymmetrical in about 25% of the patients with Crohn’s disease.25,26,27,28

In our cohort, 23% of patients had evidence of sacroiliitis of at least grade 2 unilaterally on conventional radiographs. Diagnosis of AS was retained in 39% of these patients with sacroiliitis.

In contrast to other forms of spondyloarthropathy, the association between axial involvement and HLA-B27 is weak in IBD patients. Whereas HLA-B27 is present in more than 90% of patients with idiopathic AS, the prevalence decreases to 25–75% in patients with AS associated with IBD, and to low or even normal prevalences in patients with asymptomatic IBD-associated radiological sacroiliitis.15,16,17,18

Recently, the possibility of an as yet undefined common genetic link between intestinal inflammation and sacroiliac changes was again suggested in a study assessing first degree relatives of patients with AS.29 This study showed an association between asymptomatic intestinal inflammation, recorded in 41% of these relatives, and sacroiliac changes suggestive of early AS. The presence of intestinal inflammation and sacroiliac changes did not relate to the HLA-B27 status of these subjects.

Our data suggest that CARD15 polymorphisms may predispose to sacroiliac involvement in Crohn’s disease: 78% of Crohn’s patients with sacroiliitis carried at least one variant, versus 48% of patients without sacroiliitis. Multivariate analysis showed that the presence of CARD15 variants was the only significant predictor of sacroiliitis, independent of other known CARD15 related phenotypes such as ileal involvement, fibrostenosing disease, family history, and age of onset, and independent of HLA-B27.

Interestingly, the three patients with sacroiliitis who carried HLA-B27 also carried a CARD15 variant, and all had clinical AS. In contrast none of the patients with isolated sacroiliitis carried HLA-B27. Whether or not the presence of both genetic markers in Crohn’s patients with sacroiliitis predisposes to evolution to AS cannot be concluded from this study. It seems interesting, however, to explore this question with larger studies.

The percentage of Crohn’s disease patients with CARD15 variants was higher in our study (54.9%) than generally reported. A variation in the methodology was excluded as the prevalence in our control group was similar to values in previous reports. Moreover, the prevalence of CARD15 variants in our group was not significantly greater than in another Flemish population studied by Vermeire et al (46.1%).29 To the best of our knowledge, no studies are available about a possible relation between the CARD15 genotype and sacroiliitis in Crohn’s disease. Until now, studies in idiopathic AS have not been able to demonstrate a possible association with CARD15 variants.29,30 However, these studies provided no information on the possible presence and relative number of patients with Crohn’s disease who were included. Only one study included cases of AS with Crohn’s disease and ulcerative colitis.29 The investigators could not demonstrate a higher prevalence of CARD15 variants in patients with AS plus Crohn’s disease compared with idiopathic AS, AS plus ulcerative colitis, or healthy controls. The low prevalence of CARD15 variants in their Crohn’s population with AS was not compared with the prevalence in a general Crohn’s disease population. Moreover, they unexpectedly showed a possible association between the G908R CARD15 variant and AS with ulcerative colitis.

In contrast to that study, we found CARD15 variants in seven of nine Crohn’s patients with AS. Although it only represents a small group of patients in our study, a difference in prevalence of CARD15 variants between Crohn’s disease related AS and idiopathic AS (and also for HLA-B27) could reflect a different aetiopathogenetic mechanism.

The CARD15 gene encodes for an intracellular protein that is found in monocytes, macrophages, epithelial cells, granulocytes, and dendritic cells. The gene product acts as a
receptor for bacterial cell wall components like muramyl-di-peptides derived from peptidoglycan and lipopolysaccharides, resulting in the activation of NF-κB. The polymorphisms described could cause a disturbed cellular response to bacterial components, leading to intracellular persistence of pathogens. Previous studies have suggested that recirculation of inflammatory cells between the gut and peripheral joints might contribute to inflammation at a distance.

Conclusions
In Crohn’s disease, CARD15 variants could play a role in the development of sacroiliitis, as an intraintestinal manifestation of the disease. However, the exact mechanism of the link between gut and axial joints remains to be elucidated.

Acknowledgements
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REFERENCES
CHAPTER 4

Axial involvement in CD: a clinical and genetic
multicentre association study

Clinical and genetic factors associated with Sacroiliitis in Crohn’s disease

H Peeters, B Vander Cruyssen, H Mielants, K de Vlam, S Vermeire, E Louis, P
Rutgeerts, J Belaiche, M De Vos

Submitted
CHAPTER 4

CLINICAL AND GENETIC FACTORS ASSOCIATED WITH SACROILIITIS IN CROHN’S DISEASE

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Abbreviations

SI: sacroiliitis; IBD: inflammatory bowel disease; CD: Crohn’s disease; UC: ulcerative colitis;
EIM: extraintestinal manifestation; PSC: primary sclerosing cholangitis; CT: computed
tomography; MRI: magnetic resonance imaging;
ABSTRACT

Radiographic sacroiliitis (SI), often asymptomatic, is considered to be the most frequent extra-intestinal manifestation (EIM) of Crohn’s disease (CD). Data on the association of SI with other clinical features of CD are limited. Association of SI with CARD15 polymorphisms has recently been suggested. In a cross-sectional, multicentre study, we investigated the association of SI in CD patients with clinical phenotypes, other EIM and CARD15 polymorphisms.

Radiographs of the sacroiliac joints were taken in 251 CD patients from 3 Belgian university hospitals and blindly scored by 2 rheumatologists. Clinical features were obtained from medical records. Forty-three percent of patients carried at least one CARD15 polymorphism. Sacroiliitis, defined as the presence of at least grade 2 unilateral changes, was diagnosed in 65 of the 244 scorable radiographs (27%). Only 16 of these patients were previously diagnosed with ankylosing spondylitis (AS). HLA-B27 positivity was observed in 53% of patients with AS and 7% of patients with radiographic SI. In univariate and multivariate analysis, associations between the presence of SI and peripheral arthritis (P=0.005) and between AS and uveitis (P=0.005) were found. No associations with other recorded clinical features or with CARD15 polymorphisms were observed.

In conclusion, we confirm the high prevalence of radiographic sacroiliitis in a multicentre CD cohort. Uveitis is only associated with AS whereas all patients with SI are more prone to develop peripheral arthritis during their disease course, suggesting similar pathogenetic mechanisms in the development of these EIM. The previously reported association between SI and CARD15 polymorphisms was not confirmed.

KEY WORDS

Sacroiliitis, Crohn’s disease, extraintestinal manifestation, association, CARD15
INTRODUCTION

Extraintestinal manifestations (EIM) are quite common in inflammatory bowel disease (IBD) and prevalence rates vary between 21 and 36% (1). Rheumatic (peripheral and/or axial) articular involvement and cutaneous lesions (erythema nodosum and pyoderma gangrenosum) are commonly seen but ocular and hepatobiliary complications can also occur (2).

According to the European Spondyloarthropathy Study Group (ESSG) criteria, the presence of peripheral arthritis and/or axial inflammatory complaints in IBD patients is classified as spondyloarthropathy (3). Peripheral arthritis is usually pauciarticular and asymmetrical and occurs in 5-20% of IBD patients (4, 5). It is mostly transient, migratory and non-deforming and often coincides with intestinal activity. Associations have been described with colonic involvement, uveitis and erythema nodosum (5-10).

Axial involvement consists of inflammatory low back pain, spondylitis and sacroiliitis (SI) (6). The criteria for ankylosing spondylitis (AS) are fulfilled in 1.1 to 11% of IBD patients (6, 11-14). The clinical symptoms, including morning stiffness, inflammatory low back pain or alternating buttock pain, are often unrelated to the bowel disease activity. Usually, a chronic and progressive disease course is observed. Radiographic SI, often asymptomatic, has been described in 10 to 32% of patients and can be considered the most frequent extraintestinal manifestation of IBD (11, 13, 14). Unilateral involvement of the sacroiliac joints is not uncommon (11, 13). The prevalence rate of radiographic SI is probably underestimated as reflected by higher prevalence rates found in studies using more sensitive imaging techniques than plain radiographs, such as quantitative SI scintigraphy, computed tomography (CT) and magnetic resonance imaging (MRI) (15-19). However, these newer techniques are not useful for screening purposes and plain radiographs remain the first line of imaging investigation.

Despite the high prevalence of SI in IBD, data on the association with different clinical phenotypes are scarce.
Although IBD associated AS is clinically and radiologically indistinguishable from idiopathic AS, the association with HLA-B27 is much weaker. Whereas more than 90% of patients with idiopathic AS carry HLA-B27, it decreases to 25-75% in IBD associated AS and to normal prevalence rates in asymptomatic SI (11, 18, 20, 21). An association between radiographic SI in Crohn’s disease (CD) and polymorphisms in \textit{CARD15}, a well-known susceptibility gene for CD, was observed by our group in a first cohort of patients (22).

The aim of the present study was to further investigate in a Belgian multicentre cohort of Crohn’s disease patients the association of SI with different articular and non-articular clinical phenotypes of the disease and the \textit{CARD15} genotype.
METHODS

Study population
A total of 251 CD patients from 3 Belgian university hospitals (Gent, Leuven and Liège) were
randomly included in this study, independent of the presence of articular symptoms.
Clinical features were obtained from medical records including sex, age, age at onset of CD,
disease duration, family history of CD (1st or 2nd degree relative), maximal location of the
disease (ileal, ileocolonic or colonic), upper GI involvement, history of ileal resections,
fistulas (abdominal or perianal), peripheral arthritis, AS, uveitis, erythema nodosum,
pyoderma gangrenosum, primary sclerosing cholangitis (PSC) and smoking habit. In 5
patients, smoking history was unknown. Peripheral arthritis was distinguished from
arthralgias (articular pain without evidence of joint swelling or effusion).
Two hundred thirty-three patients were genotyped (by RFLP-PCR) for the presence of the
three common CD associated CARD15 polymorphisms (R702W, G908R and 1007fs). There
were 136 wild-type patients, 77 heterozygotes (38 R702W, 15 G908R, 24 1007fs), 12
compound heterozygotes (4 R702W/G908R, 8 R702W/1007fs) and 8 homozygotes (4
R702W, 2 G908R, 2 1007fs). DNA was not available in the remaining 18 patients.
Table 1 shows the patient characteristics from the cohorts of the 3 different centres. The
disease duration in Leuven and Liège was longer compared to Gent and the cohort from Liège
had more (ex-)smokers compared to the other 2 cohorts. There was also a higher prevalence
of uveitis in Leuven compared to Liège. No other significant differences in clinical
phenotypes or CARD15 genotype were observed between the 3 different centres.
Diagnosis of sacroiliitis

Radiographs of the sacroiliac joints were taken in all 251 patients and blindly and independently scored by 2 senior rheumatologists from 2 different centres using the New York grading system: 0, normal; 1, suspicious changes (no specific abnormalities); 2, localised sclerosis, minimal erosions and joint narrowing; 3, diffuse, definite sclerosis on both sides of the joint, blurring and indistinct margins and erosive changes with loss of joint space; 4, complete fusion or ankylosis (23). Radiographic SI was diagnosed by the presence of at least unilateral SI grade 2 and only when agreed on by both radiological assessors (11, 13). For discordant scores, a consensus score was obtained after rereading the radiographs. HLA-B27 typing was performed by PCR using Dynal AllSet SSP HLA-B27 (Dynal Biotech, Invitrogen, Merelbeke, Belgium) in patients with pathological sacroiliac joints.

Statistical analysis

For univariate analysis, $X^2$ test, Fisher’s exact test (when at least 1 cell had an expected count less than 5) and Mann-Whitney U test (for continuous variables) were used where needed. Multivariate analysis was performed using logistic regression with SI, non-AS SI and AS as dependent variables (compared to non-SI patients). Clinical phenotypes and $CARD15$ genotype were included as covariates. A probability value ($P$) of less than 0.05 was considered to indicate statistical significance. Statistical analysis was performed using SPSS software (SPPS Inc, Chicago, Illinois, USA).

Based upon the previous study on the association between sacroiliitis in CD and the presence of $CARD15$ polymorphisms, a sample size calculation was performed. The number of patients needed to obtain a power of 95% was calculated as 228.
Ethics Approval

The local ethical committees approved the collection of all clinical data and the genotyping for *CARD15* polymorphisms and all patients gave their informed consent.
CHAPTER 4

RESULTS

Prevalence of radiographic sacroiliitis

The sacroiliac joints of seven patients were not scorable due to insufficient quality of the plain radiographs. Sixty-five (27%) of the remaining 244 patients had radiographic SI. The radiographs of all 16 patients, previously classified as having concomitant ankylosing spondylitis, were recognized as being pathological. Twenty-five of the 65 patients (42%) with positive sacroiliac radiographs had unilateral SI grade 2 whereas 40 patients (58%) had a score more than unilateral grade 2 or bilateral sacroiliitis.

Association of SI with clinical phenotypes and \textit{CARD15} genotype

Age, age at onset of CD, duration of CD, familial CD, ileal or colonic involvement, upper GI involvement, history of ileal resection, fistulas, smoking history, erythema nodosum and \textit{CARD15} genotype were not associated with the presence of SI (Table 2).

In relation to other extraintestinal manifestations, there was a significantly higher prevalence of peripheral arthritis and uveitis in patients with SI. No significant difference was found for erythema nodosum. One of the 2 patients with a history of pyoderma gangrenosum had radiologic SI, whereas the 2 patients with PSC had normal sacroiliac joints.

In both subgroups of patients with non-AS SI and AS, the association with peripheral arthritis was confirmed, whereas uveitis only seemed to be related to AS.

Association of AS, uveitis and peripheral arthritis with HLA-B27

Conclusive results on the HLA-B27 phenotype could be obtained in 60 of 65 patients with SI. Eight of 15 patients (53%) with AS, of whom DNA was available, were positive for HLA-
B27, whereas this was only the case in 3 of 45 patients (7%) with pure radiographic SI (P=0.0003, OR 16, 95% CI 3.4-75.3).

Within the group of patients with SI, no relationship with HLA-B27 could be found for uveitis (P=0.37, OR 2.3, 95% CI 0.5-10.6) and peripheral arthritis (P=0.75, OR 0.8, 95% CI 0.2-2.9) (data not shown).

**Multivariate analysis**

Logistic regression was performed to investigate whether peripheral arthritis and uveitis could be identified as independent predictors for SI or AS. Three models were fit, using three different dependent variables: the presence of radiographic SI (n=65), non-AS SI (n=49) or AS (n=16) (Table 3). As covariates we included sex, disease duration (more or less than 10 years), familial CD, ileal involvement, history of ileal resection, fistulas, peripheral arthritis, uveitis, smoking (ever versus never) and CARD15 genotype (wild-type versus polymorphism carrier).

The multivariate analyses confirmed that the presence of radiographic SI is associated with the occurrence of peripheral arthritis (independent of the presence of AS). Furthermore, CD patients with AS also have a higher risk of developing uveitis.
DISCUSSION

The present study confirms the high prevalence of radiographic SI in CD (6, 11, 13, 14, 22). Overall, we found radiographic signs of SI in 27% of patients. The prevalence of AS in our population was 6%, which is consistent with previously reported prevalence rates (11-14).

In our study, patients with CD associated AS have a higher risk of developing peripheral arthritis and uveitis compared to CD patients without axial involvement, confirming previous findings (5, 6, 12, 18, 24). Peripheral arthritis and uveitis are also known to be mutually associated (5-7, 25). However, multivariate analysis confirmed that both EIM were independently associated with AS. This finding might point to similar pathogenetic mechanisms in the development of AS, peripheral arthritis and uveitis.

Patients with non-AS SI also had a higher prevalence of peripheral arthritis in their medical history. Previous data on this association have been reported in only 2 studies and are somewhat conflicting. No association was found between non-AS SI and peripheral arthritis in a study by de Vlam et al (in a population of 78 CD and 25 UC patients) (11). In a study by Queiro et al, a higher incidence of peripheral arthritis (40%) was found in a population of 15 CD patients with subclinical SI compared to 47 patients without SI (27%), although statistical significance was not reached (probably due to the low number of patients) (13). As in the latter study, we found no association between non-AS SI and the incidence of uveitis (as opposed to AS).

Similar to a study by McEniff et al, we found no association between SI and disease duration. This is in contrast to the study by de Vlam et al (where the mean disease duration was slightly longer, 10.6 years versus 8 years in our study). We found no associations with other clinical characteristics such as age, sex, disease location or behaviour.

An association with CARD15 polymorphisms, as suggested in a recent paper from one of the centres in this study, could not be confirmed in this larger, multicentre cohort (22). This may
reflect issues of poor reproducibility in genetic association studies. In complex multigenic diseases, many potential confounding factors, including lack of power, population stratification, genetic heterogeneity, environmental interactions, genotype and phenotype quality control and the choice of statistical models, can lead to inconsistent results between different genetic association studies (26-29). In the numerous genotype-phenotype studies concerning CARD15 polymorphisms, several conflicting data have been reported considering associations with fibrostenosing and fistulizing disease, familial occurrence of CD and early onset of disease, even in ethnically similar populations (30, 31).

Although SI is generally seen as the hallmark of ankylosing spondylitis, isolated radiographic SI and AS are considered separate entities in IBD. HLA-B27 positivity has been described in 25-75% of IBD patients with AS (11, 18, 20). On the contrary, isolated radiographic SI is not related to HLA-B27. In our study, 53% of patients with AS were positive for HLA-B27. HLA-B27 was found in only 7% of patients with non-AS SI, which corresponds to the prevalence rates reported in the normal population (12, 13). Furthermore, the clinical evolution and importance of radiographic SI is still unclear. To our knowledge, only one follow-up study has been performed addressing this question (13). Sixty-two IBD patients were followed up for 4 years and no significant change in radiological score was observed. In the present study there was no association between disease duration and the presence of AS or SI. To determine whether radiographic SI is clinically insignificant or whether it represents a fruste form of AS or a partially resolved SI in response to treatment, larger and longer follow-up studies are necessary.

Recognition of SI on radiographs of the sacroiliac joints is difficult and requires experience. Many studies have addressed the inter- and intra-observer variability of this technique and CT and MRI scans have proven to be more sensitive and specific (3, 15-17, 32). Nevertheless, due to the limited availability, high radiation exposure and/or relative high cost of these
techniques, plain sacroiliac radiographs remain the initial diagnostic tool (11, 28). Moreover, no validated classification criteria for SI based on these newer techniques are available (13). CT or MRI scan may be particularly helpful in patients with a high probability of SI in whom conventional radiographs are negative or inconclusive.

In conclusion, we confirm the high prevalence of radiographic SI as an EIM of CD. Furthermore, the presence of (both non-AS and AS) SI is associated with a higher incidence of peripheral arthritis, suggesting common pathogenetic factors in the occurrence of arthritic manifestations of CD. Uveitis is associated with AS but not with isolated radiographic SI. The clinical importance of radiographic SI is still uncertain and future studies are needed to identify those patients at risk for development of AS.

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References


Table 1. Patient characteristics according to the 3 participating centres.

<table>
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<tr>
<th>Centre</th>
<th>Gent</th>
<th>Leuven</th>
<th>Liège</th>
<th>Total</th>
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<td>5 (9)</td>
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<td>location</td>
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<tr>
<td>ileal (%)</td>
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<td>25 (26)</td>
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<td>colonic (%)</td>
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<td>44 (17.5)</td>
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<td>upper GI involvement (%)</td>
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<td>10 (10)</td>
<td>3 (6)</td>
<td>17 (7)</td>
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<td>ileal resection (%)</td>
<td>33 (33)</td>
<td>44 (45)</td>
<td>24 (44)</td>
<td>99 (39)</td>
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<td>48 (50)</td>
<td>22 (41)</td>
<td>125 (50)</td>
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<td>abdominal (%)</td>
<td>13 (13)</td>
<td>13 (13)</td>
<td>8 (15)</td>
<td>34 (14)</td>
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<td>perianal (%)</td>
<td>48 (48)</td>
<td>40 (41)</td>
<td>18 (33)</td>
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<td>extraintestinal manifestations</td>
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<td>peripheral arthritis (%)</td>
<td>29 (29)</td>
<td>31 (32)</td>
<td>15 (24)</td>
<td>73 (29)</td>
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<td>AS (%)</td>
<td>5 (5)</td>
<td>7 (7)</td>
<td>4 (7)</td>
<td>16 (6)</td>
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<td>uveitis (%) **</td>
<td>7 (7)</td>
<td>13 (13)</td>
<td>1 (2)</td>
<td>21 (8)</td>
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<td>erythema nodosum (%)</td>
<td>9 (9)</td>
<td>11 (11)</td>
<td>3 (6)</td>
<td>23 (9)</td>
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<td>pyoderma gangrenosum (%)</td>
<td>1 (1)</td>
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<td>PSC (%)</td>
<td>2 (2)</td>
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<tr>
<td>Smoking ***</td>
<td></td>
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<tr>
<td>never (%)</td>
<td>54 (54)</td>
<td>48 (52)</td>
<td>17 (31)</td>
<td>119 (48)</td>
</tr>
<tr>
<td>ever (%)</td>
<td>46 (46)</td>
<td>44 (48)</td>
<td>37 (69)</td>
<td>127 (52)</td>
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<td>CARD15 genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>wild-type (%)</td>
<td>55 (55)</td>
<td>56 (61)</td>
<td>25 (46)</td>
<td>136 (58)</td>
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<tr>
<td>heterozygous (%)</td>
<td>36 (36)</td>
<td>26 (28)</td>
<td>15 (33)</td>
<td>77 (33)</td>
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<tr>
<td>compound heterozygous (%)</td>
<td>4 (4)</td>
<td>7 (8)</td>
<td>1 (2)</td>
<td>12 (5)</td>
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<td>homozygous (%)</td>
<td>5 (5)</td>
<td>3 (3)</td>
<td>0 (0)</td>
<td>8 (4)</td>
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</table>

N: total number
AS: ankylosing spondylitis; PSC: primary sclerosing cholangitis
* P=0.001 between Gent and Leuven; P=0.023 between Gent and Liège
** P=0.019 between Leuven and Liège
*** P=0.007 between Liège and Gent; P=0.015 between Liège and Leuven
Table 2. Prevalence of the different clinical phenotypes according to the presence of sacroiliitis

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<tr>
<th></th>
<th>No SI N=179</th>
<th>SI N=45</th>
<th>non-AS SI N=49</th>
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<tr>
<td></td>
<td>years (range)</td>
<td>years (range)</td>
<td>years (range)</td>
<td>years (range)</td>
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<tr>
<td>median age</td>
<td>35 (17-80)</td>
<td>36 (16-67)</td>
<td>36 (16-67)</td>
<td>33.5 (25-50)</td>
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<tr>
<td>median age at onset of CD</td>
<td>24 (9-76)</td>
<td>24 (10-68)</td>
<td>24 (10-68)</td>
<td>26 (18-38)</td>
</tr>
<tr>
<td>median disease duration</td>
<td>7 (0-41)</td>
<td>9 (0-30)</td>
<td>9 (0-30)</td>
<td>8.5 (0-26)</td>
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<tr>
<td></td>
<td>N (%) P* OR 95% CI</td>
<td>N (%) P* OR 95% CI</td>
<td>N (%) P* OR 95% CI</td>
<td>N (%) P* OR 95% CI</td>
</tr>
<tr>
<td>disease duration</td>
<td>&lt; 5y 64 (36%)</td>
<td>23 (33%)</td>
<td>18 (33%)</td>
<td>5 (31%)</td>
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<tr>
<td></td>
<td>30 (17) P* 0.96</td>
<td>6 (12) P* 0.44</td>
<td>43 (88) P* 0.90</td>
<td>13 (81) P* 0.74</td>
</tr>
<tr>
<td></td>
<td>≥ 5y 115 (64)</td>
<td>42 (65)</td>
<td>61 (63)</td>
<td>11 (69)</td>
</tr>
<tr>
<td></td>
<td>146 (82) P* 0.58</td>
<td>55 (85)</td>
<td>41 (64)</td>
<td>14 (87.5)</td>
</tr>
<tr>
<td>sex</td>
<td>M 73 (41)</td>
<td>27 (42)</td>
<td>19 (35)</td>
<td>8 (50)</td>
</tr>
<tr>
<td></td>
<td>F 106 (59) P* 0.92</td>
<td>38 (58)</td>
<td>30 (61)</td>
<td>8 (50)</td>
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<td>P* 1.0 OR 0.6-1.8</td>
<td>P* 1.2 OR 0.5-1.8</td>
<td>P* 1.3 OR 0.5-1.8</td>
<td>P* 1.2-4.3</td>
</tr>
<tr>
<td>familial CD</td>
<td>No 149 (83)</td>
<td>36 (86)</td>
<td>13 (81)</td>
<td>13 (81)</td>
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<td>Yes 30 (17) P* 0.96</td>
<td>9 (14)</td>
<td>3 (19)</td>
<td>3 (19)</td>
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<tr>
<td></td>
<td>P* 1.0 OR 0.5-1.8</td>
<td>P* 1.2 OR 0.5-1.8</td>
<td>P* 1.3 OR 0.5-1.8</td>
<td>P* 1.2-4.3</td>
</tr>
<tr>
<td>ileal involvement</td>
<td>No 33 (18)</td>
<td>10 (15)</td>
<td>10 (20)</td>
<td>6 (37.5)</td>
</tr>
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<td></td>
<td>Yes 146 (82)</td>
<td>55 (85)</td>
<td>41 (64)</td>
<td>14 (87.5)</td>
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<tr>
<td></td>
<td>P* 0.8 OR 0.4-1.8</td>
<td>P* 1.2 OR 0.5-1.8</td>
<td>P* 1.3 OR 0.5-1.8</td>
<td>P* 1.2-4.3</td>
</tr>
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<td>colonic involvement</td>
<td>No 45 (25)</td>
<td>16 (25)</td>
<td>10 (20)</td>
<td>6 (37.5)</td>
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<tr>
<td></td>
<td>Yes 134 (75)</td>
<td>49 (75)</td>
<td>39 (60)</td>
<td>10 (62.5)</td>
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<td>P* 1.0 OR 0.5-1.8</td>
<td>P* 1.2 OR 0.5-1.8</td>
<td>P* 1.3 OR 0.5-1.8</td>
<td>P* 1.2-4.3</td>
</tr>
<tr>
<td>upper GI involvement</td>
<td>No 164 (92)</td>
<td>63 (97)</td>
<td>4 (2)</td>
<td>16 (100)</td>
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<td>Yes 30 (17) P* 0.25</td>
<td>2 (3)</td>
<td>2 (4)</td>
<td>0 (0)</td>
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<td>P* 0.3 OR 0.1-1.6</td>
<td>P* 0.5 OR 0.1-1.6</td>
<td>P* 0.5 OR 0.1-1.6</td>
<td>P* 0.6</td>
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<tr>
<td>ileal resection</td>
<td>No 100 (59)</td>
<td>41 (63)</td>
<td>32 (63)</td>
<td>9 (56)</td>
</tr>
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<td>24 (37)</td>
<td>17 (35)</td>
<td>7 (44)</td>
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<td>P* 0.5 OR 0.3-1.6</td>
<td>P* 0.8 OR 0.4-1.5</td>
<td>P* 0.8 OR 0.4-1.5</td>
<td>P* 0.8</td>
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<tr>
<td>fistulas</td>
<td>No 83 (47)</td>
<td>36 (55)</td>
<td>27 (55)</td>
<td>9 (56)</td>
</tr>
<tr>
<td></td>
<td>Yes 94 (53)</td>
<td>29 (45)</td>
<td>22 (45)</td>
<td>7 (44)</td>
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<td>P* 0.28 OR 0.1-0.8</td>
<td>P* 0.35 OR 0.2-0.8</td>
<td>P* 0.35 OR 0.2-0.8</td>
<td>P* 0.5</td>
</tr>
<tr>
<td>abdominal fistulas</td>
<td>No 155 (87)</td>
<td>56 (86)</td>
<td>42 (86)</td>
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<td></td>
<td>Yes 24 (13)</td>
<td>9 (14)</td>
<td>7 (14)</td>
<td>2 (12.5)</td>
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<td>P* 1.0 OR 0.5-2.4</td>
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<td>31 (63)</td>
<td>10 (62.5)</td>
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<td>Yes 81 (45)</td>
<td>24 (37)</td>
<td>18 (37)</td>
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<td>P* 0.25 OR 0.1-0.7</td>
<td>P* 0.29 OR 0.1-0.7</td>
<td>P* 0.4 OR 0.3-1.3</td>
<td>P* 0.7</td>
</tr>
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<td>peripheral arthritis</td>
<td>No 135 (75)</td>
<td>37 (53)</td>
<td>29 (59)</td>
<td>8 (50)</td>
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<td></td>
<td>Yes 44 (25)</td>
<td>28 (43)</td>
<td>20 (42)</td>
<td>8 (50)</td>
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<td>P* 0.085 OR 2.3</td>
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<td>P* 0.1 OR 1.1-4.1</td>
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<td>uveitis</td>
<td>No 168 (94)</td>
<td>35 (83)</td>
<td>44 (90)</td>
<td>11 (69)</td>
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<td></td>
<td>Yes 11 (6)</td>
<td>10 (15)</td>
<td>5 (10)</td>
<td>5 (31)</td>
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<td>P* 0.023 OR 2.8</td>
<td>P* 0.32 OR 1.7</td>
<td>P* 0.3 OR 0.6-5.3</td>
<td>P* 0.005</td>
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<td>erythema nodosum</td>
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<td>56 (86)</td>
<td>82 (106)</td>
<td>14 (87.5)</td>
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<td></td>
<td>Yes 14 (8)</td>
<td>9 (14)</td>
<td>7 (14)</td>
<td>2 (12.5)</td>
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<tr>
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<td>P* 0.15 OR 1.9</td>
<td>P* 0.17 OR 2.0</td>
<td>P* 0.7 OR 0.5-2.2</td>
<td>P* 0.63</td>
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<tr>
<td></td>
<td>OR 0.6-4.6</td>
<td>OR 0.7-5.2</td>
<td>OR 1.1-8.7</td>
<td>OR 0.3-8.2</td>
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<tr>
<td>Smoking</td>
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<td>non-AS SI (N=46)</td>
<td>AS (N=16)</td>
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<td>---------</td>
<td>---------------</td>
<td>-----------</td>
<td>------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>N (%)</td>
<td>N (%)</td>
<td>P*</td>
<td>OR 95% CI</td>
<td>N (%)</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>N (%)</td>
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<td>88 (50)</td>
<td>27 (44)</td>
<td>22 (40)</td>
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<td>N (%)</td>
<td>35 (36)</td>
<td>35 (36)</td>
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<tr>
<td>P*</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
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<tr>
<td>OR</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
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<tr>
<td>95% CI</td>
<td>0.7-2.3</td>
<td>0.7-2.3</td>
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</table>

<table>
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<th>AS (N=14)</th>
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<tr>
<td>wt</td>
<td>mut</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (%)</td>
<td>93 (56)</td>
<td>73 (44)</td>
<td>38 (63)</td>
<td>29 (63)</td>
</tr>
<tr>
<td>N (%)</td>
<td>73 (44)</td>
<td>73 (37)</td>
<td>22 (37)</td>
<td>17 (17)</td>
</tr>
<tr>
<td>P*</td>
<td>0.33</td>
<td>0.33</td>
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<td>0.39</td>
</tr>
<tr>
<td>OR</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.4-1.4</td>
<td>0.4-1.4</td>
<td>0.4-1.4</td>
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</tr>
</tbody>
</table>

N: number
SI: sacroiliitis; OR: Odds ratio; CI: confidence interval; wt: wild-type; mut: mutant
* versus “No SI”
Table 3. Logistic Regression with the presence of SI, non-AS SI and AS as dependent variable

<table>
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<th>Covariate</th>
<th>SI versus non-SI</th>
<th>non-AS SI versus non-SI</th>
<th>AS versus non-SI</th>
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<tr>
<td></td>
<td>P</td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Sex</td>
<td>0.69</td>
<td>1.1</td>
<td>0.6-2.3</td>
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<tr>
<td>Disease duration</td>
<td>0.54</td>
<td>0.8</td>
<td>0.4-1.7</td>
</tr>
<tr>
<td>Familial CD</td>
<td>0.33</td>
<td>0.6</td>
<td>0.2-1.6</td>
</tr>
<tr>
<td>Ileal involvement</td>
<td>0.10</td>
<td>2.2</td>
<td>0.9-5.9</td>
</tr>
<tr>
<td>Ileal resection</td>
<td>0.57</td>
<td>0.8</td>
<td>0.4-1.7</td>
</tr>
<tr>
<td>Fistulas</td>
<td>0.42</td>
<td>0.8</td>
<td>0.4-1.5</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.31</td>
<td>1.4</td>
<td>0.7-2.8</td>
</tr>
<tr>
<td>Peripheral arthritis</td>
<td>0.007</td>
<td>2.7</td>
<td>1.3-5.6</td>
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<tr>
<td>Uveitis</td>
<td>0.11</td>
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<td>0.8-6.7</td>
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<tr>
<td>CARD15 genotype</td>
<td>0.19</td>
<td>0.6</td>
<td>0.3-1.3</td>
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</table>

SI: sacroiliitis; AS: ankylosing spondylitis
OR: Odds ratio; CI: confidence interval
Study of the functional impact of *CARD15* polymorphisms on peripheral blood monocytes in CD

*CARD15* variants determine a disturbed early response of monocytes to adherent-invasive *Escherichia coli* strain LF82 in Crohn’s disease.

H Peeters, S Bogaert, D Laukens, P Rottiers, F De Keyser, A Darfeuille-Michaud, AL Glasser, D Elewaut, M De Vos

Submitted
CARD15 Variants Determine a Disturbed Early Response of Monocytes to Adherent-invasive Escherichia coli Strain LF82 in Crohn’s Disease

Harald Peeters¹, Sara Bogaert¹, Debby Laukens², Pieter Rottiers², Filip De Keyser³, Arlette Darfeuille-Michaud⁴, Anne-Lise Glasser⁴, Dirk Elewaut³, Martine De Vos¹

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Key Words

CARD15, TLR4, Crohn’s disease, monocytes, AIEC

Abbreviations

IBD: inflammatory bowel disease; CD: Crohn’s disease; UC: ulcerative colitis; AIEC: adherent-invasive Escherichia coli; CARD: caspase activation and recruitment domain; NOD: nucleotide-binding oligomerization domain; TLR: toll-like receptor; SNP: single nucleotide polymorphism; MDP: muramyl dipeptide; LPS: lipopolysaccharide; PBMC: peripheral blood mononuclear cells; LDH: lactate dehydrogenase; CDAI: Crohn’s disease activity index; MOI: multiplicity of infection.
ABSTRACT

CARD15 and TLR4 are respectively intracellular and membrane-bound receptors for bacterial cell wall components (respectively muramyl dipeptide (MDP) and lipopolysaccharide (LPS)). Polymorphisms in \textit{CARD15} and \textit{TLR4} have been linked with Crohn's disease (CD). Adherent-invasive \textit{Escherichia coli} (AIEC), strains with particular adhesion and invasion characteristics, have been specifically associated with CD ileal mucosa. The aim of this study was to investigate the functional impact of these polymorphisms on monocytes from CD patients, in response to MDP, LPS and AIEC strain LF82. Monocytes were isolated from 40 CD patients using magnetic cell sorting, stimulated with LPS or MDP or infected with AIEC. IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα induction was assessed using qRT-PCR, Cytometric Bead Array and ELISA. Bacterial intracellular survival and replication was assessed using a gentamicin protection assay. Results were linked with the presence of \textit{CARD15} and \textit{TLR4} polymorphisms. Monocytes of patients with \textit{CARD15} polymorphisms showed an early reduced cytokine response (IL-1β, IL-6 and IL-10) to infection with AIEC, which was restored after 20 hours. A gene-dose effect was seen, comparing wild-types, heterozygotes and homozygotes. We found no differences in intracellular survival and replication of AIEC. Heterozygous carriage of \textit{TLR4} polymorphisms did not influence monocyte response. In conclusion, CD patients carrying \textit{CARD15} polymorphisms show a disturbed early inflammatory monocyte response after infection with AIEC strain LF82. For the first time, a functional defect was detected in single heterozygous carriers. These findings reflect the potential role of a genetically altered host response to disease-related bacteria in the pathogenesis of CD.
INTRODUCTION

Crohn’s disease (CD) represents a complex multigenic disorder. It is generally thought that in genetically susceptible individuals, an inappropriate innate immune response towards environmental (bacterial) triggers contributes to the development of chronic intestinal inflammation.[1] Polymorphisms in two pathogen associated molecular pattern (PAMP) receptors, involved in host/pathogen interactions, have been associated with inflammatory bowel disease (IBD).[2][3][4]

The CARD15 gene on chromosome 16 encodes an intracellular protein (CARD15 or NOD2) expressed in monocytes, macrophages, dendritic, polymorphonuclear and epithelial cells and acting as a receptor for muramyl dipeptide (MDP).[5][6] MDP is a component of peptidoglycan and can be found in the cell wall of most bacteria. Three polymorphisms (2 missense mutations (R702W, G908R) and 1 frameshift mutation (1007fs)) have been independently associated with a higher susceptibility for CD.[2][3] The CARD15 protein is involved in NF-κB activation (via Rick/Rip2) and apoptosis by two N-terminal Caspase Activation and Recruitment Domains.[7] However, its exact role in CD is still unclear. Most functional data arose from transfection studies in vitro and pointed to a decreased NFκB activation (most pronounced for the frameshift mutation) upon stimulation with MDP.[5][6][8] A number of ex vivo studies have been performed and confirmed a reduced pro-inflammatory activity, however predominantly focussing upon the rare 1007fs homozygous patients.[5][9][10]

Besides, it is still unclear how a decreased NF-κB activation could match with an inflammatory condition, characterised by enhanced NF-κB activity. Several hypotheses have been postulated including compensatory activation of the adaptive immune system, yet unknown anti-inflammatory properties of the NF-κB pathway, a defect in apoptosis of
activated immune cells, differences in the epithelial barrier function (e.g. defensins) and impaired killing of intracellular/invasive microorganisms.[11]

A Toll-like receptor 4 (TLR4) gene polymorphism (Asp299Gly) has recently been associated with IBD.[4] TLR4 is a membrane-bound PAMP receptor, belonging to the LPS (lipopolysaccharide)-signaling complex.[12][13] LPS can predominantly be found in the cell wall of Gram negative bacteria. Binding of TLR4 with its ligand also gives rise to NF-κB activation, albeit via another pathway involving MyD88, IRAK, TRAF6. Transfection studies showed again a disturbed NF-κB activation.[14] However, in vivo this LPS hyporesponsiveness could only be confirmed in isolated monocytes of the extremely rare homozygous carriers of the TLR4 variant.[15][16][17]

The discovery of these CD-associated polymorphisms stressed the importance of enteric flora in the pathogenesis of IBD. In animals raised under germ-free conditions, no intestinal inflammation can be seen in experimental IBD models.[18] In human IBD, most lesions are found in parts of the gastrointestinal tract with the highest bacterial counts.[19] In addition, faecal stream diversion has a beneficiary effect on intestinal inflammation whereas re-exposure with faecal material leads to relapse.[20][21] The use of antibiotics and probiotics in the management of IBD also points to a pivotal role for bacterial agents.[22] Moreover, differences in the bacterial composition of the faecal microflora between CD patients and healthy subjects have been shown.[23] Adherent-invasive Escherichia coli (AIEC), strains with particular adhesion and invasion characteristics, have been specifically associated with Crohn’s disease.[24][25] These strains are mainly associated with early ileal lesions in CD patients, with a prevalence of 36.4%.[24] AIEC are able to adhere in vitro to differentiated Caco-2 cells, a property that could enable them to colonize the intestinal mucosa.[25] Moreover, the AIEC LF82 strain has been proven to invade intestinal epithelial cells.[26]
Intracellular survival and even replication of this LF82 strain was also demonstrated in human macrophages without inducing host cell death.[27]

The purpose of this study was to assess the relationship between the presence of *CARD15* and/or *TLR4* gene polymorphisms and the functional characteristics of peripheral blood monocytes in CD patients in response to stimulation with MDP, LPS and infection with AIEC.
MATERIALS AND METHODS

CARD15 AND TLR4 GENOTYPING

Genomic DNA was extracted from whole blood from 250 CD patients using Qiagen blood and cell culture DNA kit (Westburg BV, Leusden, The Netherlands). Genotyping for the 3 CD-associated CARD15 polymorphisms R702W (SNP8), G908R (SNP12) and 1007fs (SNP13) was performed using RFLP-PCR, followed by separation of the DNA fragments on a 2.5% agarose gel. Table 1 shows the different CARD15 genotypes in this population. Remarkably, none of these 250 patients was homozygous for the 1007fs frameshift mutation. The TLR4 Asp299Gly polymorphism was assessed in 159 CD patients by direct sequencing of the PCR product in an ABI 3100 analyser (Applied Biosystems, Foster City, CA, USA). (forward primer: 5'-CCATTGAAGAATTCCGATTAGC-3', reverse primer: 5'-ATGGTAATAACACCATTGAAGC-3'). Twenty-seven patients (17%) were heterozygous carriers of this TLR4 polymorphism. No homozygous carriers could be identified.

MONOCYTE RESPONSE TO MDP, LPS AND INFECTION WITH AIEC

Study population

From this genotyped cohort of CD patients, a total of 40 patients (11 males, 29 females) with a mean age of 35 years (range 24-69y) were included for this part of the study. Mean age at diagnosis was 26 years (range 9-54y). Ten patients had ileal disease and 16 patients had ileocolonic disease, whereas 14 patients only had colonic involvement. Twenty-one patients never smoked whereas 7 patients were active smokers and 12 patients were ex-smokers. Mean Crohn’s disease activity index (CDAI) was 73 (range 0-253) with 7 patients having a CDAI above 150. Nine patients did not take any medication, 8 patients were on 5-ASA or sulfasalazine, 17 patients took azathioprine and 1 patient methotrexate whereas 5 patients...
were on a combination of 5-ASA and azathioprine or methotrexate. The distribution of the different *CARD15* and *TLR4* genotypes in this cohort is shown in table 2.

Isolation and stimulation of peripheral blood monocytes

Peripheral blood monocytes were isolated using magnetic cell sorting (MACS) with the Human Monocyte Isolation Kit II (Miltenyi Biotec, Amsterdam, The Netherlands), according to the manufacturer's instructions. In summary, the mononuclear cell (PBMC) fraction was isolated from 40 ml of venous blood using density gradient centrifugation with Ficoll-Pacque (Amersham Biosciences, Roosendaal, The Netherlands). PBMC were incubated with a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and Glycophorin A (directed against T cells, NK cells, B cells, dendritic cells and basophils) for 10 minutes at 4-8 °C, followed by an indirect magnetic labeling using Anti-Biotin Microbeads for 15 minutes at 4-8 °C. Applying this labeled cell suspension onto a MACS LS separation column resulted in the negative selection of a highly purified untouched monocyte population. The isolated monocytes were suspended at 10⁶ cells/ml in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) supplemented with 0.5% L-glutamine and 10% autologous serum and incubated 8 and 20 hours with culture medium (negative control), MDP 100 ng/ml (N-acetylmuramyl-L-alanyl-D-isoglutamine, Calbiochem, Merck Biosciences Ltd, Nottingham, UK) or LPS 10 ng/ml (Ultra Pure *E. coli* K12 LPS, Invivogen, San Diego, USA). Since 100 ng/ml MDP and 10 ng/ml LPS induced the highest TNFα mRNA induction after 2 hours of stimulation in a dose-response study, these concentrations were chosen in the present study. Using an E-TOXATE® LAL-test (Sigma-Aldrich, Bornem, Belgium), no detectable endotoxin contamination could be measured in the used MDP diluted to the working concentration. Infection with AIEC LF82 was performed with 10⁷ bacteria/ml (MOI 10) as described previously.[26] As this strain is not resistant to the complement found in the incubation
medium by the addition of autologous serum, no antibiotic was added during the infection period.[25] Cells were collected in RLT buffer (Qiagen Westburg, Leusden, The Netherlands) with 1% β-mercapto-ethanol and stored at –80 °C until assayed. Supernatants were harvested and stored at –20 °C prior to analysis.

Cytometric Bead Array immunoassay
Concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNFα in the culture supernatants were measured simultaneously using Cytometric Bead Array (CBA) (Human Inflammation Kit, Becton Dickinson (BD), Erembodegem, Belgium) according to the manufacturer’s instructions. The BD CBA technique uses amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immuno-assay. Flow cytometric analysis was performed using a BD FACScan. Data acquisition and analysis was performed using BD CBA software. Cytokine induction is calculated as the concentration of the cytokine in the culture medium after stimulation – the concentration in the culture medium of unstimulated monocytes.

IL-8 ELISA
IL-8 concentrations were assessed in duplo with a sandwich ELISA technique using the R&D DuoSet human IL-8 ELISA kit (R&D Systems, Abingdon, UK). The assay was run according to the manufacturer’s instructions.

Quantitative Real Time – Polymerase Chain Reaction
Total RNA from monocytes was isolated using RNeasy mini kit (Qiagen Westburg, Leusden, The Netherlands) according to the manufacturer’s instructions. Quantitative Real Time – Polymerase Chain Reaction (qRT-PCR) was used to measure mRNA induction of IL-1β, IL-6
and IL-10 using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization. The quantitative PCR reaction was performed in duplo using the SYBR Green I qPCR Core Kit (Eurogentec, Seraing, Belgium). All reactions were run in the Biorad iCycler. Melt curve analysis confirmed primer specificities.

MONOCYTE VIABILITY
To assess the influences of infection with AIEC and CARD15 genotype on monocyte viability, lactate dehydrogenase (LDH) concentrations were measured in the supernatant of cultured monocytes of 4 CARD15 wild-type and 3 homozygous (2 snp8 and 1 snp12) CD patients. Quantification of the release of LDH is a widely accepted method to estimate the number of non-viable cells.[27] LDH was measured photometrically using the LDH Optimized kit (Roche Diagnostics, Mannheim, Germany) on an automated clinical chemistry analyzer following the manufacturer’s instructions.

BACTERIAL SURVIVAL AND REPLICATION IN MONOCYTES
Survival and replication of AIEC within monocytes were measured using an adapted protocol of the previously described gentamicin protection assay.[27] Monocytes of 2 CARD15 wild-type, 6 heterozygous (3 snp8, 3 snp13) and 2 homozygous (snp8) CD patients were isolated as described above and cultured at a concentration of 4 x 10^5 cells/ml in RPMI 1640 medium supplemented with 0.5% L-glutamin and 10% heat-inactived autologous human serum. Heat-inactivation was obtained by incubating the serum for 30 minutes at 55°C. The monocytes were infected and incubated with AIEC for 2 hours at 37°C with 5% CO₂. The cells were then washed twice with RPMI and fresh culture medium containing 100μg/ml gentamicin was added for an additional hour to kill extracellular bacteria. Subsequently the medium was removed and replaced by fresh RPMI medium containing 50μg/ml gentamicin for longer
postinfection periods. At several time points (0, 1, 4 and 24 hours) after gentamicin treatment, monocytes were washed and 0.5ml of Triton-X100 (Sigma-Aldrich, Bornem, Belgium) 1% in PBS was placed in each well for 10 minutes to lyse the eukaryotic cells. Samples were removed, diluted and plated onto Luria Bertani (LB) medium plates to determine the number of CFU recovered from the lysed monolayers. Survival was expressed as the percentage of the number of intracellular bacteria recovered after 1 hour postinfection (time point 0), defined as 100%.

STATISTICAL ANALYSIS
Comparison of cytokine induction and LDH concentration between wild-type patients and polymorphism carriers was performed using Mann-Whitney $U$ test. Gene-dose effects (correlation of cytokine induction with the number of mutant alleles) were assessed using a Spearman’s rho test. Statistical significance was tested two-tailed. A $P$-value less than 0.05 was considered to indicate statistical significance.

Statistical analysis was performed using SPSS software (SPSS inc., Chicago, Illinois, USA).

ETHICS APPROVAL
The study was approved by the local Ethics Committee of the Ghent University Hospital. All patients signed an informed consent.
RESULTS

Monocyte responses to MDP, LPS and AIEC stimulation in the global CD population

Very low to almost absent cytokine induction was seen in supernatant of unstimulated monocytes after 8 hours of culture, whereas gradually higher concentrations were measured after respectively MDP stimulation, LPS stimulation and infection with AIEC (Fig 1). The highest cytokine levels were found for IL-8, IL-1β, and IL-6, followed by TNFα and IL-10. IL-8 concentrations surmounted the upper detection limit of the CBA kit in the chosen dilution and were reassessed in a higher dilution using ELISA. IL-12p70 was almost undetectable after 8 hours, only a slight induction could be noticed after 20 hours of culture after AIEC infection.

Cytokine induction according to patient characteristics

There were no significant differences in cytokine production according to age, age at diagnosis, sex, disease location, smoking habit, disease activity (CDAI) and medication intake between wild-type patients and polymorphism carriers (data not shown).

Cytokine induction according to CARD15/TLR4 genotype

CARD15 polymorphisms

Cytokine secretion

In culture supernatants of unstimulated monocytes no differences in cytokine concentrations were found between CARD15 wild-types (n=18) and polymorphism carriers (n=22) (data not shown). An aberrant monocyte response was seen after 8 hours infection with AIEC (Fig 2). Monocytes of patients with CARD15 polymorphisms produced significantly less IL-1β (P=0.01) and IL-6 (P=0.04). Also lower levels of IL-10 were found in these patients (P=0.05).
No significant differences were found for IL-8 and TNFα. After 20 hours of stimulation this aberrant cytokine induction in response to AIEC infection was no longer notable.

Differences in cytokine induction between both groups after 8 hours of stimulation with MDP were not statistically significant (Fig 3).

No differences could be detected between the 3 different single nucleotide polymorphisms (SNPs) (data not shown). The detected differences in monocyte response could not be explained by differences in disease activity, medication intake or smoking habit (data not shown).

Cytokine mRNA induction
Quantitative RT-PCR confirmed the lower production of IL-1β by monocytes of patients with CARD15 polymorphisms 8 hours after infection with AIEC (P=0.01), with a similar trend for IL-6 although not reaching statistical significance (P=0.1) (Fig 4). No significant differences were found for IL-10 at the mRNA level at 8 hours.

Gene-dose effect
IL-1β and IL-6 induction was significantly inversely correlated with the number of CARD15 mutant alleles carried (Spearman’s rho IL-1β P<0.01, IL-6 P=0.02) (Fig 5). This gene-dose effect 8 hours after infection with AIEC in wild-types, heterozygotes and homozygotes was not observed for IL-10, IL-8 and TNFα. After MDP stimulation, no significant correlations could be found between the number of copies of CARD15 mutant alleles and cytokine induction (data not shown).
**TLR4 polymorphisms**

Similar cytokine production was detected in unstimulated monocytes from patients with the *TLR4* wild-type genotype (n=22) compared to heterozygous carriers (n=18) of the Asp299Gly variant. Also after stimulation with LPS or infection with AIEC, no significant differences could be found between both groups (Table 3).

There was also no difference in response to AIEC between patients carrying both a *CARD15* and *TLR4* polymorphisms as compared to patients with *CARD15* polymorphisms alone (data not shown).

**Monocyte viability**

Infection with AIEC did not affect monocyte viability after 8 and 20 hours (figure 6). The mean LDH concentration in the supernatants of unstimulated monocytes did not differ significantly from those of monocytes infected with AIEC (after 8 hours of culture respectively 66.1 vs 62.5 U/l, P=0.9; after 24 hours of culture respectively 61.2 vs 56.6 U/l, P=0.7).

There were also no significant differences in LDH concentration after infection with AIEC between *CARD15* wild-types and homozygotes after 8 hours (P=0.3) and 24 hours (P=0.6) of culture.

**Survival and replication of AIEC within monocytes**

The gentamicin protection assay showed that AIEC were able to survive and replicate within the isolated human monocytes (figure 7). After phagocytosis, there is a short period of bacterial replication within the cell, followed by a progressive clearance of the AIEC by the monocytes. We found however no differences in bacterial survival and replication between *CARD15* wild-type patients, heterozygous or homozygous polymorphism carriers.
DISCUSSION

This study demonstrates an altered early inflammatory response of monocytes, isolated from CD patients with CARD15 polymorphisms, after infection with CD-associated adherent-invasive E. coli. Infection with the LF82 AIEC strain resulted after 8 hours of infection in a decreased induction of IL-1β, IL-6 and IL-10. TNFα and IL-8 secretions in response to AIEC infection were not influenced by carriage of CARD15 variants. Only minimal secretion of IL-12 was detected, merely measurable after 20 hours of infection, most probably due to the absence of IFNγ in these purified monocyte cultures. IFNγ normally provides a positive loop for IL-12 induction.[28][29][30] There were no differences between the 3 different SNPs but interestingly, a gene-dose effect was noted for IL-1β and IL-6.

The reduced cytokine response could not be explained by differences in cellular viability since the levels of LDH measured in the supernatant of monocytes from wild-type patients and polymorphism carriers were similar. Moreover, this aberrant innate immune response towards AIEC seems to be an early phenomenon, since cytokine production is restored after 20 hours.

Association of CARD15 polymorphisms with Crohn’s disease is well known and the prevalence of these mutations in our CD population is similar to previous findings in the Flemish population.[31] Obvious is the very low prevalence of homozygosity (3.6%) with even a complete absence of homozygosity for the frameshift mutation in our tested population. The exact role of these polymorphisms in CD pathogenesis is still not fully understood. A defective MDP-mediated NFκB activation, suggestive for an altered bacterial sensing, has been shown in HEK293T cell transfectants.[5][6][8] This reduced NFκB activation was confirmed twice in macrophages of NOD2−/− mice, although one study of Maeda et al demonstrated the opposite in mice homozygous for a CARD15 insertion mutation similar to 1007fs.[32][33][34] In man, a similar impaired inflammatory response to MDP was
shown in isolated PBMC from patients homozygous for the 1007fs variant.\cite{5,9,10} No functional implications of \textit{CARD15} polymorphisms have been described in single heterozygous carriers.

In this study, AIEC strain LF82 was used since this strain was previously associated with CD and can be found in high prevalences in the ileum of patients with Crohn’s disease.\cite{24} AIEC strains are adherent to the mucosa and able to invade intestinal epithelial cells. Moreover, the LF82 strains can survive and replicate within host macrophages without inducing cell death.\cite{26,27} By measuring LDH levels in the supernatant of cultured monocytes we also found a similar cell viability in non-infected monocytes and monocytes infected with AIEC.

We found no significant differences in cytokine induction after MDP stimulation between \textit{CARD15} wild-types and polymorphism carriers. Previous studies demonstrating an aberrant inflammatory response towards MDP have been predominantly described in 1007fs homozygotes and no such patients were included in our study.\cite{5,9} Due to the limited number of purified monocytes, we chose a single concentration of MDP (100 ng/ml) in this proof of principle study, based upon the highest TNF\(\alpha\) response in a dose-response study. However, recent findings suggest that a lower dose of MDP (10 ng/ml) might be more suitable to detect differences between \textit{CARD15} wild-types and polymorphism carriers.\cite{35,36}

Our findings reflect the importance of synergy between NOD2 and TLR immune responses.\cite{37,38} Although a study of Watanabe et al pointed at NOD2 as a negative regulator of TLR2-mediated cytokine response, the theory of NOD2-TLR synergy was again confirmed by two recent studies from Van Heel et al showing a loss of synergistic effect in \textit{CARD15} homozygotes as compared to wild-types.\cite{35,36,39} PBMC of homozygotes had a markedly lower cytokine production when stimulated with a combination of MDP with different TLR ligands. The altered immune response after infection with AIEC, reported in
our study, could not be detected after MDP stimulation which may support the importance of synergy between NOD2 and TLRs. In contrast to MDP alone, AIEC could indeed, being an enteroinvasive pathogen, stimulate immune cells through NOD2 and TLRs simultaneously.

Our study might also help to explain the paradox of a decreased NFκB activation in CARD15 polymorphism carriers in CD. A lower induction of IL-10 but not TNFα, as found in our CARD15 polymorphism carriers, leads to a lower (and more inflammatory) IL-10:TNFα ratio. Netea et al. found a similar change in IL-10:TNFα ratio after stimulation of PBMCs with heat-killed Bacteroides in patients homozygous for the 1007fs variant.[9] Furthermore, due to a defective IL-1β and IL-6 release after phagocytosis of pathogens, a disturbed neutrophil activation and chemotaxis can, in combination with a defective secretion of antimicrobial defensins by Paneth cells, facilitate further entrance of bacteria into the mucosa.[40] It has been demonstrated that Nod2−/− mice are more susceptible to invasion by Listeria monocytogenes after intragastric challenge.[32] A prolonged survival of these invading bacteria in phagocytes in combination with a possible loss of apoptotic activity of these cells can also lead to the persistence of intracellular antigens and subsequently to a chronic inflammatory stimulus for other immune cells. Different bacterial antigens have been found in mucosa of Crohn’s disease patients using immunohistochemistry in a study by Liu et al.[41] Moreover, Hisamatsu et al. demonstrated a higher survival of S. typhimurium in epithelial cell lines transfected with the 1007fs variant.[42] However, in our study we could not detect a difference in intracellular survival and replication of AIEC between CARD15 wild-types and polymorphism carriers. Whether the (early) altered immune response towards these bacteria, as found in the present pilot study, really has significant functional implications needs to be further investigated with studies including other cell types (e.g. macrophages, dendritic cells and epithelial cells). Further studies are also needed to clarify whether infection with other living (invasive) bacteria leads to a similar defective cytokine response.
Our study also shows that heterozygous carriersonhip of the TLR4 polymorphism Asp299Gly does not influence monocyte response to LPS or AIEC, confirming previous ex-vivo studies.[15][16][17] Besides, there is still controversy around the association of this variant with IBD. In 2004, this TLR4 gene polymorphism was associated with CD and ulcerative colitis (UC).[4] However, three other European studies could not fully confirm these associations. In a Scottish CD population no association could be found.[43] Török et al found a higher prevalence of this polymorphism in German UC patients but not in CD whether a recent Greek study demonstrated the opposite.[44][45]

In conclusion, monocytes of CD patients carrying CARD15 polymorphisms show a disturbed (early) inflammatory response after infection with adherent-invasive E coli. For the first time a functional defect seems to be detected in the most common CARD15 genotypes presently described in CD. These findings reflect the importance of an altered innate immune response to intracellular bacteria in the pathogenesis of CD.
REFERENCES


FIGURE LEGENDS

Fig 1. Cytokine secretion in the supernatant of monocytes of all CD patients (n=40) cultured for 8 hours without stimulus or in the presence of MDP, LPS or AIEC. IL1β, IL6, IL10, TNFα and IL12p70 were assessed using CBA, IL8 using ELISA.

Fig 2. Comparison of cytokine induction (mean ± SEM) between CARD15 wild-types (n=18) and polymorphism carriers (n=22) according to the duration of stimulation (8 or 20 hours infection with AIEC). Cytokine induction (Δ) is defined as the concentration of the cytokine in the culture medium after stimulation with substraction of the concentration in the culture medium of unstimulated monocytes. Mann-Whitney U test: at 8 hours P=0.01 (IL1β), P=0.04 (IL6) and P=0.05 (IL10).
wt: wild-type; mut: mutant

Fig 3. Comparison of cytokine induction (mean ± SEM) in the supernatant of monocytes cultured for 8 hours in the presence of MDP between CARD15 wild-types (n=18) and polymorphism carriers (n=22). Cytokine induction (Δ) is defined as the concentration of the cytokine in the culture medium after stimulation with substraction of the concentration in the culture medium of unstimulated monocytes.
wt: wild-type; mut: mutant

Fig 4. IL1β, IL6 and IL10 mRNA induction (mean ± SEM) 8 hours after infection with AIEC. Mann-Whitney U test: *P=0.01, ns* P=0.1.

Fig 5. Cytokine induction (mean ± SEM) in CARD15 wild-types (wt, n=18), heterozygotes (het, n=16) and homozygotes (homo, n=6) (8 hours after infection with AIEC). Cytokine induction (Δ) is defined as the concentration of the cytokine in the culture medium after stimulation with substraction of the concentration in the culture medium of unstimulated monocytes. Spearman’s rho *P<0.01, **P=0.02.

Fig 6. Concentration of LDH in the supernatant of cultured monocytes from CARD15 wild-type (n=4) and homozygous (n=3) patients at 8 (a) and 20 (b) hours after infection with AIEC. unstim: unstimulated monocytes; AIEC: monocytes infected with AIEC

Fig 7. AIEC survival and replication within monocytes. Results at 1, 4 and 24 hours after 1h of gentamicin treatment are expressed as the number of intracellular bacteria relative to that obtained at 1h of gentamicin treatment (time point 0) taken as 100%.
Table 1. Different *CARD15* genotypes in a population of 250 CD patients

<table>
<thead>
<tr>
<th><em>CARD15</em> genotype</th>
<th>wt</th>
<th>single heterozygous</th>
<th>comp heterozygous</th>
<th>homozygous</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>snp8</td>
<td>snp12</td>
<td>snp13</td>
<td>snp8/12</td>
<td>snp8/13</td>
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<tr>
<td></td>
<td>135</td>
<td>46</td>
<td>13</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>54%</td>
<td>36.8%</td>
<td>5.6%</td>
<td>3.6%</td>
<td>100%</td>
</tr>
</tbody>
</table>

wt: wild-type; comp: compound; snp: single nucleotide polymorphism
Table 2. Distribution of the different CARD15 and TLR4 genotypes in the study population (n=40) (number of patients).

<table>
<thead>
<tr>
<th>TLR4</th>
<th>wild-type</th>
<th>TLR4 wt</th>
<th>CARD15</th>
<th>snp8</th>
<th>snp12</th>
<th>snp13</th>
<th>TLR4 -/+</th>
<th>CARD15</th>
<th>snp8</th>
<th>snp12</th>
<th>snp13</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>snp12</td>
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<td>TLR4 wt</td>
<td>10</td>
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<td>5</td>
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<td>2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>1</td>
<td>0</td>
<td></td>
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</tbody>
</table>

snp8: R702W; snp12: G908R; snp13: 1007fs
TLR4 wt: TLR4 wild-type
TLR4 -/+: heterozygous TLR4 variant carrier
Table 3. Cytokine induction after 8 hours stimulation with LPS or AIEC strain LF82 according to the presence of TLR4 polymorphisms

<table>
<thead>
<tr>
<th></th>
<th>LPS</th>
<th></th>
<th>AIEC</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLR4 wt</td>
<td>TLR4 mut</td>
<td>P-value</td>
<td>TLR4 wt</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4805 ± 862</td>
<td>4210 ± 753</td>
<td>0.5</td>
<td>26504 ± 3538</td>
</tr>
<tr>
<td>IL-6</td>
<td>26553 ± 1894</td>
<td>26953 ± 1690</td>
<td>0.8</td>
<td>21937 ± 2824</td>
</tr>
<tr>
<td>IL-8</td>
<td>51050 ± 6215</td>
<td>51677 ± 5815</td>
<td>0.9</td>
<td>39391 ± 7438</td>
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<tr>
<td>IL-10</td>
<td>152 ± 21</td>
<td>132 ± 32</td>
<td>0.3</td>
<td>437 ± 78</td>
</tr>
<tr>
<td>TNFα</td>
<td>873 ± 248</td>
<td>1448 ± 387</td>
<td>0.2</td>
<td>5029 ± 1325</td>
</tr>
</tbody>
</table>

Cytokine induction after 8 hours of culture in pg/ml (mean conc ± SEM)

Cytokine induction is calculated as the concentration of the cytokine in the culture medium after stimulation – the concentration in the culture medium of unstimulated monocytes.

TLR4 wt: TLR4 wild-type patients; TLR4 mut: TLR4 polymorphism carriers
FIGURES

Figure 1

- **IL1β**
- **IL6**
- **IL10**
- **IL8**
- **TNFα**
- **IL12p70**

Different panels show the levels of cytokines IL1β, IL6, IL10, IL8, TNFα, and IL12p70 in response to various stimuli (unstim, MDP, LPS, AIEC). The y-axis represents the concentration in pg/ml.
Figure 2

hours after infection with AIEC

CARD15 wt
CARD15 mut

* P=0.05

* P=0.01

* P=0.04

* P=0.05

CARD15 wt
CARD15 mut
Figure 3
Figure 4

- **Δ IL1β**: 
  - Wild-type: 250,000
  - Mutant: 100,000
  - * P=0.01

- **Δ IL6**: 
  - Wild-type: 150,000
  - Mutant: 75,000
  - ns P=0.1

- **Δ IL10**: 
  - Wild-type: 75
  - Mutant: 25
  - ns
Figure 5

**Δ IL1β**

* Spearman’s rho P<0.01

**Δ IL6**

** Spearman’s rho P>0.02

**Δ IL10**

**Δ IL8**

**Δ TNFα**
Figure 6

a) 8h

- CARD15 homozygote
- CARD15 wildtype

b) 20h

- CARD15 homozygote
- CARD15 wild-type
Figure 7

% of bacteria

hours after gentamicin treatment
Study of the association of \textit{CARD15} polymorphisms with ASCA in CD

\textit{CARD15} polymorphisms are associated with anti-Saccharomyces Cerevisiae antibodies in Caucasian Crohn’s disease patients.

*equal contribution

CARD15 polymorphisms are associated with anti-Saccharomyces cerevisiae antibodies in caucasian Crohn's disease patients

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Summary
Carriage of CARD15 gene polymorphisms and the serological marker anti-Saccharomyces cerevisiae antibodies (ASCA) are two markers for Crohn's disease (CD). Similar phenotypes have been associated with both markers. In the present study we analysed whether both markers were associated with each other and, if so, whether this association could be explained by a direct link or by an indirect association with those phenotypes. Therefore, we included 156 consecutive Caucasian CD patients and assessed the prevalence of the three common single nucleotide polymorphisms in the CARD15 gene. Serum samples were analysed for IgA and IgG ASCA by ELISA. CD patients with CARD15 polymorphisms were more frequently ASCA positive (OR 2·7 (1.4–5.2); \(P=0.002\)) and had higher titres for ASCA IgA (\(P=0.005\)) and ASCA IgG (\(P<0.001\)) compared to patients carrying the wild type polymorphisms. Multivariate analysis demonstrated that this association was independent from ileal disease, penetrating disease and stricturing disease, the need for resective bowel surgery, familial cases, smoking habits and early age at onset. Homozygotes or compound heterozygotes for CARD15 polymorphisms had significantly more frequent ASCA positivity compared to single heterozygotes (OR 9·1 (1.1–74.2), \(P\) (corrected \(P\)-value) = 0.030). These data indicate that there is a significant association between the carriage of CARD15 polymorphisms and ASCA, independent of the described phenotypes. Moreover, ASCA positivity is more frequent in CD patients carrying 2 CARD15 polymorphisms compared to single heterozygotes.

Keywords: ASCA, CARD15, Crohn’s disease, caucasian, antibodies

Introduction
Although the exact pathogenesis of Crohn’s disease (CD) remains unclear, it is well accepted that an impaired microbial immune response, triggered by environmental and genetic factors, is important [1–4]. Recently, 3 polymorphisms in the CARD15 gene (two missense mutations (R702W and G908R) and one frame shift mutation (1007fs)), were independently associated with CD [5,6]. It has been estimated that heterozygotes have a 3-fold risk to develop CD and homozygotes or compound heterozygotes a 40-fold risk to develop the disease [5,6]. Genotype-phenotype studies showed different possible associations of these CARD15 polymorphisms with ileal and stricturing disease [2,7–10], familial cases [11] and early onset of disease [12].

Anti-Saccharomyces cerevisiae antibodies (ASCA) are directed against the cell wall mannann of Saccharomyces cerevisiae, commonly known as baker’s or brewer’s yeast [13]. ASCA are considered as a serological marker for CD. However, their pathophysiological role is not yet clear [14]. Sensitivity and specificity of ASCA for CD range from 39% to 65% and 80% to 97.5% [15–18], respectively. Combinations of ASCA with other serological markers as pANCA, I2 and OmpC antibodies are under investigation to obtain a better
CARD15 polymorphisms and ASCA are associated in Crohn’s disease

serological diagnostic tool for IBD [19]. In CD patients, ASCA are linked with earlier onset of disease [16], ileal involvement, penetrating and stricturing disease and need for resective bowel surgery [19–22]. One study also pointed at a possible negative association with smoking behaviour [23]. Previous family and twin studies already suggested a genetic influence on ASCA formation. Indeed, family and twin studies revealed that unaffected twins and unaffected relatives have higher ASCA titres compared to healthy controls [20,24–27]. The aim of the present study was to analyse whether CARD15 and ASCA are related and if so, whether this association can be explained by a direct link or an association by phenotypes.

Materials and methods

Study population and assessment of clinical characteristics

The study population consisted of 156 consecutive, Caucasian CD patients (57 male, 99 female). The diagnosis of CD was based on clinical, endoscopical, histological and/or radiological findings. All patients were seen by a gastroenterologist. Family and personal medical history, onset of disease, localizations of inflammation, amount and types of surgical interventions, history or current presence of fistulae, ease, localizations of inflammation, amount and types of fistulae secondary to surgery were excluded. Stricturing disease was considered in those patients without fistulae, who had radiological or surgical involvement, penetrating and stricturing disease and need for resective bowel surgery [19–22]. One study also pointed at a possible negative association with smoking behaviour [23]. Previous family and twin studies already suggested a genetic influence on ASCA formation. Indeed, family and twin studies revealed that unaffected twins and unaffected relatives have higher ASCA titres compared to healthy controls [20,24–27]. The aim of the present study was to analyse whether CARD15 and ASCA are related and if so, whether this association can be explained by a direct link or an association by phenotypes.

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Genotyping of R702W, G908R and 1007fs and sequencing

Genomic DNA was extracted from whole blood using Qia gen blood and cell culture DNA kit (Qiagen, Germany). All patients were genotyped for R702W, G908R and 1007fs using a RFLP-PCR technique, followed by separation on 2.5% agarose gel. The missense mutation R702W (GenBank accession number G67950) abolishes the restriction site for MspI (5'-CAGCCCTGTAGACATTCTTCTT-3' and 5'-AGC CGTTCCTCTGCATCTCGTA-3'), resulting in an intact 130-bp band for mutant alleles compared to two bands of 54- and 76-bp for wild type alleles. The missense mutation G908R (GenBank accession number G67951) creates a restriction site for HinP1l. The frame shift mutation 1007fs (GenBank accession number G67955) creates a restriction site for NlaIV (5'-CTGAGGCTTTTGATGAACGC-3' and 5'- CTTTCAACCCATCCCATCCATT-3'). The presence of a mutant allele results in two bands of 219 and 41 base pairs, while the wild type allele produces a single 260-bp product.

Detection of ASCA by ELISA

The Medizym ASCA kits (Medipan Diagnostica, Germany) were used. Tests were performed according to instructions from the manufacturer, including the use of cut-offs that were determined at 20 U/ml for both IgA and IgG ASCA. Briefly, serum was diluted 1:50 and applied to the microtitre plates (100 μl/well), coated with cell wall mannan from a mixture of different Saccharomyces strains. The plates were incubated for one hour at 37°C. To remove unbound serum components, plates were washed five times. Consequently 100 μl of conjugate, specific for either IgG of IgA, coupled with horseradish peroxidase was added, followed by an incubation period of 30 min at 37°C. The plates were washed again five times, after which substrate was added (3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide). Plates were incubated in the dark at room temperature for 10 min. The reaction was stopped using a stop solution containing sulphuric acid, turning the colour of the solution from blue to yellow. Plates were read at a wavelength of 450 nm.

ASCA IgG and IgA levels were determined using a standard curve, for which the manufacturer supplied calibrators. Study personnel were blinded for diagnosis during these assays. Each sample was tested in duplo and 2 positive control samples were run on each plate. The mean values for the IgG-samples were 60 U/ml and 29 U/ml with a coefficient of variation (CV%) of 2% and 4.5%, respectively. The mean values for the ASCA IgA samples were 18 U/ml and 28 U/ml with a CV% of 6.9% and 5.6%, respectively. The mean CV% between duplo's of all samples was 3.15% for ASCA IgA and 4.23% for ASCA IgG. Unless otherwise specified, ASCA were considered positive when either ASCA IgA or IgG was positive.

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS inc., Chicago, Illinois, USA). Groups were compared with Mann–Whitney U-test because normality was not achieved. Dichotomous data were analysed using Pearson’s χ² test or with Fisher’s exact test when the expected count was less than five in at least one cell. Odds ratios were calculated with their corresponding 95% confidence interval (CI). When indicated, a corrected P-value (Pcorr) was calculated using Bonferroni’s correction. We also calculated conditional odds ratios.
and their corresponding 95% CI by binary logistic regression.

**Ethics**

The study was approved by the local ethics committee. All patients gave written informed consent.

**Results**

**Patients characteristics**

Thirty-eight patients had ileal disease, 79 patients had ileocolonic disease and 39 patients had only colonic involvement. Seventy-three patients needed resective small bowel surgery, 92 patients had penetrating disease and 33 patients had stricturing disease. Thirty patients had at least one affected relative. Forty patients were ex-smokers and 44 patients smoked at the time of evaluation. Mean age was 38 years (range 18–80 years) and mean age at diagnosis was 27 years (range 9–66 years). In 48 patients, onset of disease was before the age of 20.

**Prevalence of CARD15 mutations**

Seventy-seven (49·3%) of 156 CD patients carried CARD15 polymorphisms. Forty-three CD patients carried at least one R702W polymorphism, 14 patients carried at least one G908R polymorphism and 27 patients carried at least one 1007fs polymorphism. Fourteen patients carried two polymorphisms of which 7 patients were homozygous and 7 patients compound heterozygous (Table 1). In a local control population, 19/87 (21·8%) controls carried at least one CARD15 variant.

**Prevalence of ASCA positivity**

Eighty-two (52·6%) CD patients were ASCA positive, 71 (45·5%) patients had ASCA IgA and 57 (36·5%) patients ASCA IgG. In a control population, 5/188 (2·6%) tested positive for ASCA (3/24 OA patients, 0/56 RA patients and 2/108 blood donors), confirming the high specificity of the test. Only 1/87 controls who were typed for CARD15 polymorphisms had ASCA. This control carried the 1007fs CARD15 polymorphism.

**Univariate analysis of association between CARD15 genotype and ASCA positivity**

Fifty of 77 (64·9%) carriers of CARD15 polymorphisms had positive ASCA IgA or IgG, in contrast to 32 (40·5%) of 79 wild type patients (OR 2·7; 95% CI (1·4–5·2), \( P = 0·002 \)).

Forty-four (57·1%) of 77 carriers of CARD15 polymorphisms were positive for ASCA IgA versus 27 of (34·2%) 79 wild type patients (OR 2·87; 95% CI (1·49–5·50), \( P = 0·004 \)) and 37 (48·1%) of 77 carriers tested positive for ASCA IgG versus 20 (25·3%) of 79 wild type patients (OR 2·7; 95% CI (1·4–5·4), \( P = 0·003 \)) (Table 2).

**Carriage of 1 versus 2 CARD15 polymorphisms and ASCA positivity**

Carriers of 2 CARD15 polymorphisms were more frequently ASCA positive compared to patients who carried only 1 polymorphism: 13 (93%) of 14 patients who carried 2 CARD15 variants were ASCA positive compared to 37 (58·7%) of 63 carriers of 1 polymorphism (OR 9·135, 95% CI (1·1–74·2), \( P = 0·015, P_c = 0·030 \)). When looking at ASCA IgA and ASCA IgG separately, we could again find higher prevalences of ASCA IgA or ASCA IgG in those patients who

| Table 1. Number of patients carrying R702W, G908R or 1007fs polymorphisms. |
|---------------------------------|--------------------|--------------------|--------------------|
|                                  | CD patients (\( n = 156 \)) |
| R702W heterozygotes              | 33 (21·3%)          |
| G908R heterozygotes              | 9 (5·8%)            |
| 1007fs heterozygotes             | 21 (13·5%)          |
| R702W homozygotes                | 4 (2·6%)            |
| G908R homozygotes                | 3 (1·9%)            |
| R702W + G908R compound heterozygotes | 1 (0·6%)      |
| G908R + 1007fs compound heterozygotes | 1 (0·6%)      |
| R702W + 1007fs compound heterozygotes | 5 (3·2%)      |

| Number of CD patients carrying the different CARD15 polymorphisms. There were no patients homozygous for 1007fs. |

| Table 2. ASCA status in relation to the carriage of CARD15 polymorphisms. |
|---------------------------------|-----------------|-----------------|-----------------|
| Carriage of CARD15 polymorphisms | ASCA positivity (IgA or IgG) | ASCA IgA positive | ASCA IgG positive |
| No                               | 32 (40·5%)      | 27 (34·2%)      | 20 (25·3%)      |
|                                  | \( P = 0·002 \)  | \( P = 0·004 \)  | \( P = 0·003 \)  |
| Yes                              | 50 (64·9%)      | 44 (57·1%)      | 37 (48·1%)      |
| Total                            | 82 (52·6%)      | 71 (45·5%)      | 57 (36·5%)      |

ASCA positivity for ASCA IgA or IgG, ASCA positivity for ASCA IgA and ASCA positivity for ASCA IgG in relation to the carriage of CARD15 polymorphisms.
ASCAs. The fact that data on a possible association between carriage of \textit{CARD15} polymorphisms and ASCA are so disparate might be explained by differences in environmental factors and different genetic backgrounds. Associations between genetic markers and serological markers have previously been described for several diseases. In insulin dependent diabetes, disease associated autoantibodies are associated with susceptibility HLA class II alleles [30]. Coeliac associated antibodies can identify healthy first-degree relatives who express coeliac associated HLA haplotypes [31]. In rheumatoid arthritis, rheumatoid factor is associated with the HLA shared epitope and the \(2849\) IL-10 promoter polymorphism [32,33]. Carriage of different HLA class II alleles is associated with a specific antibody response to nuclear antigens, in particular anti-SSA/Ro52, both in primary Sjögren’s syndrome and lupus [34,35] and may be more strongly associated with the antibody subsets than with the disease status itself [36]. Other nuclear antibodies (anti-SSB/La), associated with HLA class II genes, have also recently been linked with carriage of polymorphisms in the genes for transforming growth factor \(\beta\) and tumour necrosis factor \(\alpha\) in patients with primary Sjögren’s syndrome [37]. Different hypotheses can be generated to explain how the carriage of different HLA types or different gene polymorphisms on the promoters of cytokines, might influence antibody responses [33,35]. How the different polymorphisms on the \textit{CARD15} gene are involved in the antibody response against mannan from \textit{Saccharomyces cerevisiae} is still unclear. The \textit{CARD15} gene encodes for the Nod2/CARD15 protein, a member of the Apaf-1/Ced-4 family of apoptosis regulators and an intracellular protein expressed in monocytes, macrophages, epithelial cells, granulocytes and dendritic cells. Nod2 activates the NF-\(\kappa\)B pathway after stimulation by bacterial products. Initial reports suggested lipopolysaccharide as a possible ligand for Nod2, but recently, 2 independent groups highlighted muramyl dipeptide as the major activator of the Nod2-receptor [6,38–41]. The activation of the NF-\(\kappa\)B–pathway is followed by an enhanced production and secretion of proinflammatory cytokines [42].

It has been suggested that the described polymorphisms in the \textit{CARD15} gene give rise to an impaired NF-\(\kappa\)B activation by a deficient recognition of microbial antigens. This might result in an impaired killing of intracellular microbes, which leads to inflammation [38,40]. Whether ASCA is just a side-effect of this inflammation or whether ASCA have themselves a pathological role by cross-reactivity with self-antigens in human gut or other tissues is still unknown [43].

In conclusion, our data suggest that there is an association between the presence of \textit{CARD15} gene polymorphisms and the serological marker ASCA in a West-European CD population. This could be interpreted in a double way. \textit{CARD15}, as an immune response gene, may modulate in some way humoral immunity predisposing to the generation of ASCA. Or, carriage of \textit{CARD15} polymorphisms and ASCA may be both associated with a particular, as yet undefined, phenotypical subset of CD. However, we proved in the present study that the association between carriage of \textit{CARD15} polymorphisms and ASCA could not be explained by an indirect link with the common \textit{CARD15} and ASCA related phenotypes.

\section*{Acknowledgements}
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References

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CHAPTER 7

Genetic expression assays on colonic biopsies of CD and SpA patients

Altered gut transcriptome in Spondyloarthropathy


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Altered gut transcriptome in spondyloarthritis

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Keywords: spondyloarthritis, Crohn’s disease, microarray, baseline expression

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Objectives Intestinal inflammation is a common feature of spondyloarthropathy (SpA) and Crohn's disease (CD). Inflammation is manifested clinically in CD, and subclinical in SpA. However, a fraction of SpA patients develop overt CD. The aim of this study was to investigate whether subclinical gut lesions in SpA patients are associated with transcriptome changes comparable to those seen in CD. We examined global gene expression in non-inflamed colon biopsies, and screened for differentially expressed genes.

Methods Macroarray analysis was used as an initial genome-wide screen for selecting a comprehensive set of genes relevant to CD and SpA. This led to the identification of 2,625 expressed sequence tags (ESTs) that are differentially expressed in the colon of CD and/or SpA patients. These clones, together with appropriate controls (6,779 in total) were used to construct a glass-based microarray, which was then used to analyze colon biopsies from 15 SpA patients, 11 CD patients, and 10 controls.

Results Ninety-five genes were identified as differentially expressed in SpA patients with a history of subclinical chronic gut inflammation as well as in CD patients. Principal component analysis of this filtered set of genes successfully distinguished colon biopsies from the three groups studied. SpA patients with subclinical chronic gut inflammation cluster together, and are more related to CD.

Conclusion The transcriptome in the intestine of SpA patients differs from that of controls. Moreover, these gene alterations are comparable to those seen in CD, confirming initial clinical observations. Based on these findings, new (genetic) markers for detection of early CD in SpA patients can be considered.
The clinical association between Spondyloarthritis (SpA) and Crohn’s disease (CD) is illustrated by the concurrence of similar arthropathy and intestinal inflammation in the two diseases, indicating a shared etiology and pathogenesis. Depending on the imaging technique used, up to one third of CD patients have peripheral and/or sacroiliac joint abnormalities similar to those seen in various SpA subgroups.\textsuperscript{1,2} In addition, 60% of SpA patients who have no evidence of CD exhibit endoscopic and/or histological signs of subclinical gut inflammation.\textsuperscript{3} In general, two types of inflammation are observed: acute inflammation as seen in infectious colitis, and chronic inflammation resembling that in CD.\textsuperscript{3} A striking parallel exists between the activity of inflammation at the joints and the intestine. Moreover, long-term evolution to CD was observed in 13% of SpA patients with initial chronic gut inflammation, supporting the concept of preclinical CD in those patients.\textsuperscript{4} Since these clinical observations, several studies provided additional evidence for a joint-gut axis on the molecular as well as the genetic level. The early immune alterations observed are up-regulation of αEβ7 integrin on T-cell lines from SpA patients, and an increase in lymphoid follicles and lamina propria mononuclear cells in intestinal biopsies.\textsuperscript{5,7} Increased expression of αEβ7 and the E-cadherin/catenin complex was found in gut mucosa from CD and SpA patients.\textsuperscript{5,8} A specific subset of CD163\textsuperscript{+} macrophages is augmented in both groups of patients, supporting the hypothesis of a recirculation of similar clones in the intestinal mucosa and synovium.\textsuperscript{9}

Both CD and SpA are termed complex genetic traits, because many genes are probably involved in pathogenesis, and environmental factors have a substantial influence on the outcome of the disease. Evidence exists for a common genetic risk factor in the development of subclinical intestinal inflammation in first-degree relatives of patients with ankylosing spondilitis, which is the prototype of SpA.\textsuperscript{10} Furthermore, we found that \textit{CARD15}, which was the first CD susceptibility gene
identified, is associated with chronic subclinical inflammation in patients with SpA.\textsuperscript{11} In this regard, SpA patients can serve as a unique model for early CD.

In order to determine whether the association between the two disorders occurs not only at the clinical but also at the transcriptome level, we compared global gene expression in non-inflamed colon biopsies from SpA and CD patients. We propose that it is possible to identify a set of genes that distinguish CD patients and SpA patients with a history of chronic gut inflammation from SpA patients without chronic gut inflammation and from controls.

METHODS

Patients, tissue collection and histological classification

Colon biopsies from CD and SpA patients and healthy controls were obtained during colonoscopy. All biopsies were taken from non-inflamed sigmoid at 30 cm. Biopsy specimens were immediately placed in RNALater (Ambion, Cambridgeshire, UK) and frozen at -80°C until sample processing. Three biopsies were obtained from each of 34 patients diagnosed with CD according to clinical, endoscopic and histological criteria, and 20 patients diagnosed with SpA according to ESSG criteria.\textsuperscript{12} Sixteen patients without clinical manifestations of CD or SpA, who were undergoing colonoscopy for colon cancer screening, were included as a control population.

Histological classification of the SpA ileum and colon was performed as in our previous studies.\textsuperscript{3, 4, 13-15} We distinguished three classes: patients with normal histology, patients with acute inflammatory lesions, and those with chronic inflammatory lesions.\textsuperscript{16} In acute lesions, normal architecture was well preserved. There was infiltration by neutrophils and eosinophils without a considerable increase in lymphocytes. Small superficial ulcers covered with fibrin and neutrophils overlying hyperplastic lymphoid follicles were occasionally observed. The lamina propria was
edematous and hemorrhagic, and contained mainly polymorphonuclear cells. The pattern of inflammation was similar to that seen in acute self-limiting bacterial enterocolitis. The principal features of chronic lesions were crypt distortion, atrophy of the villous surface of the mucosa, villous blunting and fusion, increased mixed cellularity, and basal lymphoid aggregates in the lamina propria. Though several biopsies were obtained from each patient, a diagnosis of chronic inflammation was made even if only one biopsy showed chronic lesions, regardless of acute or active inflammation in the other biopsies.

SpA patients who had chronic inflammation in colon and/or ileum in previous examinations were termed SpA patients with chronic gut inflammation.

RNA extraction
Total RNA was extracted from biopsies using the Qiagen Rneasy Mini Kit (Westburg BV, Leusden, The Netherlands) with on-column DNase treatment (Qiagen). Needle homogenization was performed. RNA quality and concentration were checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

Macroarray hybridization and analysis
Colony filters containing 74,828 expressed sequence tag (EST) clones (Human UniGene collection 2, RZPD, Germany) were used as initial screen. Radioactively labeled probes were produced by incorporation of $[^3P]dCTP$ during reverse transcription of 50 μg total RNA (MMLV, Promega, Leiden, The Netherlands), using oligoT as primer. $^{35}P$-cDNA probes were purified on G-50 spin columns (Amersham Biosciences, Roosendaal, The Netherlands). Hybridization was performed at $10^5$ cpm/ml at 65°C for 20 hours. Images were acquired after 6, 18 and 24 hours of exposure, using a Phosphorimager system (Amersham Biosciences). Spot definition
and intensity measurement was done using Visualgrid (GPC Biotech AG, Munich, Germany). The raw expression data were processed with an in-house algorithm based on MS Access. Spot intensities were corrected for the local background, followed by a quality control of spots to exclude those influenced by intense signals of adjacent spots. The detection limit for expression values above background was calculated based on the variation of the local background intensity. Constitutive genes (those that show the lowest coefficient of variation over all arrays) were used for normalization. Subsequently, quantitative measures of each clone (gene) were calculated by log₂ transformation of the ratio of the mean spot intensity of CD or SpA patients to the mean spot intensity of controls.

Microarray hybridization, scanning and analysis

Construction of a focus microarray chip, probe labeling, hybridization, washing and scanning were carried out at the MicroArray Facility of the Flanders Interuniversity Institute for Biotechnology (MAF, Leuven, Belgium). Clones selected from the macroarray screen were PCR-amplified from RZPD clones using universal M13 primers. PCR fragments were purified on MultiScreen PCR plates (Millipore, Brussels, Belgium) and resuspended in 50% DMSO at an average concentration of 100 ng/μl. The PCR products were arrayed in duplicate on Type VII silane-coated slides using a Molecular Dynamics Generation III printer (Amersham Biosciences, Buckinghamshire, UK). Total RNA (5 μg) was amplified using a modified protocol of in vitro transcription as described.¹² Five μg of the amplified RNA were Cy3- or Cy5-labeled as described at http://www.microarrays.be/service.htm. Arrays were scanned at 532 and 635 nm using a Generation III scanner (Amersham BioSciences). Images were analyzed with ArrayVision (Imaging Research Inc., Ontario, Canada). Each hybridization was repeated in a dye swap experiment. Spot intensities were measured,
corrected for local background, and those that exceeded the background by more than two standard deviations (SD) were included. For each gene, ratios of red (Cy-5) to green (Cy-3) intensities (I) were calculated and normalized via a Lowess Fit of the \( \log_2 \) ratios \( \log_2(\text{ICy-5} / \text{ICy-3}) \) over the log2 total intensity \( \log_2(\text{ICy-5} \times \text{ICy-3}) \).

For comparing the microarray datasets, a mixture of RNA from 5 CD patients, 5 SpA patients and 5 controls was used as reference RNA. This reference sample provides a positive hybridization signal at each probe element on the microarray, which is essential when calculating and comparing fluorescence ratios. The data were imported into GeneMaths® XT (Applied Maths, St-Martens-Latem, Belgium). Weighted mean ratios and their corresponding error (pixel SD) were calculated from the dye swap.

Data were normalized over all arrays, and missing values were imputed using k-nearest neighbor algorithm (20 neighbors). GeneMaths® XT was used to perform all subsequent supervised and unsupervised analyses.

Statistics

All p-values chosen for cut-off are subjective.

Ethics

The study was approved by the regional ethics committee (project 2004/242). All patients signed an informed consent form.

RESULTS

Design of the custom microarray

In order to provide a practical and cost effective tool for conducting a large number of hybridizations, a self-designed focus microarray chip was constructed specifically for studying colonic gene expression in SpA and CD. To accomplish this, a genome-wide
survey of gene expression in colon biopsies of 4 CD patients, 4 SpA patients and 6 controls was conducted using high-density nylon arrays containing 74,828 cDNA sequences (table 1, macroarrays).

Spots that showed aberrant morphology, encompassed variation in replicates or were impaired because of over shining (characteristic of radioactive signals) were filtered out and considered as clones lost through experimental error. To select for clones that are differentially expressed in CD or SpA patients, we arbitrarily selected for those that have a log2 transformed mean ratio of less than -0.6 or more than +0.6 (1.5 fold down- or up-regulated). Genes that might be differentially expressed between groups (control versus CD or control versus SpA) were identified using a simple algorithm based on the t-test (p<0.05) and F-values (p<0.05) as selection criteria, providing that at least three consistent intensity values were present in each group. F-values were chosen for selection because we assumed that differences in variances within groups can be important. A total of 2,652 clones were identified as “potentially differentially expressed”. These genes, together with 4,127 ESTs lost through experimental error and which might include, beside control ESTs, additional differentially expressed genes, were used to produce a glass-based microarray platform. This allowed us to screen more patients in a more accurate and sensitive manner.

Clustering of unfiltered data

We hybridized an independent cohort of 15 SpA, 11 CD and 10 control patients to the focus microarray (table 1, microarrays). Unsupervised clustering (without prior knowledge of groups) using all genes revealed no clustering with respect to disease or phenotype (e.g. type of intestinal inflammation). The inability to find discriminatory genes using unfiltered data is perhaps not too surprising, as we are analyzing the steady state transcriptome in non-inflamed tissue samples of complex inflammatory diseases. Subtle differences in only a few genes are lost in the vast number of random
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*UspA = undifferentiated SpA; AS ex =ankylosing spondylitis with only axial involvement; AS perip = ankylosing spondylitis with peripheral involvement; histology of SpA patients is a historical classification; IC = ileocolonic; 1 = ileal involvement only; 5-ASA = 5-aminosalicylates; AZA = azathioprine; NSAID = non-steroidal anti-inflammatory drug; sulfi = sulfasalazine.*
variations. The problem of detecting differentially expressed genes can be overcome by performing supervised clustering. To this end we divided the patients into four main groups: CD, SpA with chronic gut inflammation, SpA without chronic gut inflammation, and controls. Discriminant analysis can reduce N-dimensional data into a more visual 2-D or 3-D plot, with prior knowledge of groups (fig 1). With this approach, the above-defined groups became clearly separated, indicating that our full dataset contains genes that can differentiate between these disease states.

Identification of genes whose differential regulation is common to both SpA with chronic gut inflammation and CD

By using an independent t-test, we identified 123 genes that are expressed differentially between CD and control (p<0.01). With this set we were unable to discriminate SpA patients from controls, although three out of four of the SpA patients with chronic gut inflammation clustered together, indicating the presence of alterations similar to those observed in CD. Thus it was logical to screen for genes modulated commonly in CD and control on one hand, and SpA, SpA with chronic gut inflammation and control on the other hand. In order to include a larger number of genes in this analysis, the statistical significance level was lowered from p<0.01 to p<0.05. This led to the identification of two sets of genes whose expression pattern discriminates CD from control (p<0.05, n=630) and SpA from control (p<0.05, n=464). The latter significance level was determined by ANOVA, in which SpA patients with chronic gut inflammation were defined as a distinct group. The set of 95 genes that are differentially expressed in both CD and SpA could distinguish the three disease groups (fig 2A, table 2). In addition, SpA patients with chronic gut inflammation cluster together and are more related to the CD cluster than to the control/SpA cluster, but they remain a separate entity (fig 2A). Principal component
analysis using this set of 95 genes, another way of representing the data, clearly discriminates our patient groups (fig 2B). Based on the identification of a set of genes involved in CD and also implicated in SpA, we attempted to identify genes that might render these individuals more susceptible to develop CD.

**Genes within the CD/ SpA chronic cluster**

Genes whose expression is aberrant in both CD and SpA with chronic gut inflammation are represented in table 2 (†: up-regulated; ↓: down-regulated, *p*<0.05). Among them, two genes had already been described in the context of CD. Acyl-coenzyme A oxidase 1 (ACOX1), which is the first enzyme of the fatty acid beta-oxidation pathway, donates electrons directly to molecular oxygen, thereby producing hydrogen peroxide. The enzymatic activity of ACOX1 was diminished in both inflamed and non-inflamed areas in CD. Our observation of ACOX1 transcript down-regulation corroborates this report, and indicates a fault at the level of transcription or mRNA stability.

Glutathione peroxidase 2 (gastrointestinal glutathione peroxidase, giGSH-Px) is one of the four types of selenium-dependent glutathione peroxidases. Its exclusive expression in the gastrointestinal tract indicates that it functions as a barrier against the absorption of dietary hydroperoxides, and as protection against damage from endogenously formed hydroxyl peroxides. Its activity is increased in ulcerative colitis patients in the active and in the remission stages. In CD, plasma levels of giGSH-Px are increased. We found that this gene is over-expressed in normal colon tissue in CD and SpA patients with a history of chronic gut inflammation, and so it can act as a marker expressed at non-pathological sites in the intestine in CD and CD susceptible SpA patients.
### Table 2 Ninety-five ESTs that cluster CD and SPA patients with chronic gut inflammation

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Genes with aberrant expression in both CD and SPA patients with chronic gut inflammation (↓ down-regulated and ↑ upregulated) as compared to the control and SpA population (p<0.05). Genetic markers are cited for genes that are located within or near (500 kb) one of the CD loci, together with the score supporting linkage of the loci (LOD: logarithm of the ratio of the odds that two loci are linked, NPL: non-parametric LOD score).
CHAPTER 7

DISCUSSION

Clinical study of intestinal abnormalities in SpA patients has previously relied on cytokine profiles and immunological alterations. In addition to analyzing individual proteins, genome-wide transcript profiles can be analyzed by microarrays. Global gene expression analysis in non-inflamed colon tissue was used to find genes that are differentially expressed in both CD patients and SpA patients with a history of chronic gut inflammation. Previous studies of gene expression in IBD have focused on biopsies of actively inflamed tissues.\textsuperscript{21-23} The use of samples from non-inflamed areas from CD patients offers the possibility of identifying early markers for CD, which would permit prediction of the evolution to CD in SpA patients. Moreover, changes in the expression of genes that are regulated during inflammation would be more prominent than the subtle alterations in non-inflammatory genes, although it cannot be ruled out that this procedure will also pick up genes whose differential expression is a consequence and not a cause of the disease. Additionally, looking at basal gene expression may allow us to take into account genetic influences, since gene expression is highly heritable.\textsuperscript{24} Therefore, future studies on markers for CD should concentrate primarily on genes that are located near one of the known loci for CD (table 2). Genes located within a region linked to CD or IBD in general (if multipoint linkage was performed), or within five centimorgan (cM) of the markers that are linked to CD or IBD (in case of two point linkage) should be considered first. Using a model for early CD when identifying CD susceptibility genes can circumvent the heterogeneity of the disease, because probably only a very small number of CD genes will be implicated in SpA.

Array analysis is a rapid procedure for studying the expression of many genes in no more than several samples. Because the number of samples is limited, and the number of genes explored is usually large, false-positive results will obviously arise.
Nevertheless, array analysis enables us to explore gene expression with different computational tools. To confirm the importance of a set of genes associated with a phenotype, complementary techniques such as quantitative RT-PCR (qPCR) are mandatory. Thus, arrays are not simply a way to find single differentially regulated genes; they can be used to compare global gene expression in distinct groups.

We show that SpA patients have an aberrant gene expression profile in comparison to healthy controls, indicating that alteration of gene expression in the colon of SpA patients is a biologically relevant concept. We identified a set of genes that are differentially expressed in both CD and SpA patients who are at higher risk of developing CD. Based on the expression of these 95 genes, SpA patients with subclinical chronic gut inflammation cluster with CD, confirming the clinical association between the two inflammatory disorders. We also suggest a number of candidate genes for mutation screening. We are currently verifying a selection of genes by qPCR, and exploring the involvement of genes that are differentially expressed in both CD and SpA patients with a history of chronic gut inflammation, in order to find early (genetic) markers for CD in SpA patients.

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REFERENCES


FIGURE LEGENDS

Fig 1: Discriminant analysis of all patients using unfiltered data, illustrated in two directions. Four groups are clearly separated. CD (●), SpA without chronic inflammation (●), SpA with chronic inflammation (●) and controls (●).

Fig 2A: Complete Linkage clustering based on a set of 95 genes whose expression is deviant in CD patients and in SpA patients with chronic gut inflammation as compared to healthy controls. Two main clusters mark a SpA/control cluster and a CD/SpA with chronic inflammation cluster.

Fig 2B: Principal Component Analysis-view using a set of 95 genes whose expression is deviant in CD patients and in SpA patients with chronic gut inflammation. CD (●), SpA without chronic inflammation (●), SpA with chronic inflammation (●) and controls (●).
fig 1:
fig 2A:
fig 2B:
CHAPTER 8

Identification of new candidate genes for CD by linking gene expression variation with known susceptibility genes

Reduced metallothionein expression in colonic Crohn’s disease: evidence for MTF1 as a new disease-modifying gene.


Submitted
REDUCED METALLOTHIONEIN EXPRESSION IN COLONIC CROHN’S DISEASE: EVIDENCE FOR MTF1 AS A NEW DISEASE-MODIFYING GENE

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ABSTRACT

Background & aims: Crohn’s disease (CD) is a multifactorial disorder involving both genetic and environmental factors. This study aimed at identifying and characterizing new candidate susceptibility genes for CD by integrating known disease loci with gene expression variation in non-inflamed colon biopsies of CD patients.

Methods: Sixteen CD patients and 11 controls were subjected to microarray analysis. Expression of metallothionein (MT) was analyzed in intestine and blood by quantitative PCR and immunohistochemistry. MT-knockdown HT29 cells were generated by small interfering RNA. MT1M, and its transcriptional regulator MRE-binding transcription factor 1 (MTF1), were screened for mutations by sequencing.

Results: Eighteen differentially expressed baseline genes were identified. We focused on the reduced expression of MT in intestine and blood of CD patients with colonic involvement. We showed that MT induction was not impaired in these patients. To model lowered MT expression in epithelial cells, we created MT-knockdown HT29 cells. These showed a reduced IL8 secretion in response to bacterial challenge, suggesting deficient inflammatory responses in the CD colon. No mutations were found in MT1M exons and its promoter region. A polymorphism in MTF1 (IVS1-128A>T) was associated with disease location. Gene-gene interaction of MTF1 and CARD15 predisposes individuals to a high risk for ileal disease.

Conclusions: Combining microarray screening with genetic linkage data is an effective tool for identifying novel candidate susceptibility genes. We showed that deficient basal MT expression in CD patients with colonic involvement is genetically determined. The IVS1-128A>T polymorphism in MTF1 is linked to disease location and serves as a new disease-modifying gene.
INTRODUCTION

The strong influence of genetic determinants in Crohn’s disease (CD, MIM 266600) has been shown by familial clustering, and by the high concordance rate in monogenic twins. First-degree relatives of affected individuals show a 20 to 50-fold higher risk for developing CD. Moreover, affected siblings frequently develop the disease at similar ages, and concordance rates reach 80% for disease site, behaviour and presence of extra-intestinal manifestations.\(^1\)\(^2\)

The mode of inheritance of CD is complex, and the number of genes predisposing to CD or modifying its course is currently unknown. However, it is not expected that a single risk gene is sufficient for disease development.

Two complementary approaches are used to identify determinants underlying genetically complex traits such as CD: candidate gene association studies and genetic linkage. In the candidate gene approach, genes that might be involved are screened for polymorphisms in a limited number of patients, and then frequencies in large populations of patients and controls are compared. In genetic linkage, candidate regions are identified by scanning the entire chromosome with microsatellite markers. These so-called disease loci can rarely be narrowed down to less than a few megabases. Nevertheless, if the candidate region contains a manageable number of genes, a candidate gene is chosen on the basis of a known property, e.g. function or tissue-specific expression. Unfortunately, mapping CD loci is hampered by phenotypic variability, genetic heterogeneity across populations, uncontrolled environmental influences, reduced penetrance, and limited statistical power in such studies. Nevertheless, several CD loci have been corroborated by more than one independent study. The most frequently identified locus for CD is 16q12 (IBD1), which led to the mapping of the first gene to be firmly associated with CD, called CARD15.\(^3\)\(^4\). Today, there is evidence for more than 20 loci, but the identity of the causative genes remains largely unknown.\(^5\) In addition, success in finding susceptibility genes has been limited by the modest effect of individual genes on the
complex phenotype. We applied a complementary approach to identify new potential susceptibility genes. Because much of the variation in gene expression is genetically transmitted, we evaluated variation of gene expression in unaffected biopsies from CD patients, and combined these results with known linkage data. For this purpose, we looked at gene expression in biopsies taken from non-inflamed regions of the colon from CD patients and healthy controls. We selected genes that show variation in baseline expression and that are also located near a locus for CD. We focused on characterizing one of the candidate genes, metallothionein (MT), and the consequences of its altered expression. This led to the identification of a new disease-modifying gene, \textit{MTF1}, located at 1p33 (\textit{IBD7}).

\section*{MATERIALS AND METHODS}
\textbf{Patients and biopsies.} CD was diagnosed according to clinical, endoscopic and histological criteria. Patients were classified according to the Vienna classification. Disease location, however, was defined as the maximal spread of inflammation during the entire follow-up. We thus defined three subgroups: pure colonic involvement (C), both ileal and colonic involvement (IC), and ileal involvement only (I). For RNA extraction, 3 colon biopsies from each of 54 CD patients and 30 controls were collected during colonoscopy. All biopsies were taken from endoscopically normal regions of the sigmoid, immediately placed in RNAlater (Ambion, Cambridgeshire, UK) and stored at -80°C. For immunohistochemical analysis, colon biopsies were collected from 22 CD patients and 8 controls. Additional ileal biopsies were collected from 12 CD patients and 8 controls. Biopsy specimens were immersed in 4% formalin (Labonord, France).

RNA extraction. Total RNA was extracted from biopsies using the RNeasy Mini Kit (Qiagen, Westburg BV, The Netherlands) with on-column DNase treatment (Qiagen). Needle
homogenization was performed. The quality of RNA used for microarray analysis was checked on the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Total RNA from 3 ml of whole blood was extracted using the RNeasy Midi Kit (Qiagen) following lysis of red blood cells with erythrocyte lysis buffer (Qiagen). Concentration and purity (ratio $\text{OD}_{260}/\text{OD}_{280}$ between 1.8 and 2.2) of the RNA used for quantitative real-time PCR was checked on a spectrophotometer (UV-1601, Shimadzu Benelux, Belgium).

**Microarray hybridization, scanning and analysis.** The construction of the focus microarray chip, containing 6,779 expressed sequence tags and specifically designed for the study of colonic gene expression, has been described. Total RNA (5 µg) was amplified using a modified protocol for *in vitro* transcription. Probe labelling, hybridization, washing and scanning were carried out at the MicroArray Facility (MAF, Leuven, Belgium) of the Flanders Interuniversity Institute for Biotechnology (VIB) as described on http://www.microarrays.be/service.htm. Images were analyzed with ArrayVision (Imaging Research Inc., Canada). Each hybridization was repeated in a dye swap experiment. Spot intensities were measured, corrected for local background, and those that exceeded the background by more than two standard deviations (SD) were included in the analysis. For each gene, ratios of red (Cy-5) to green (Cy-3) intensities ($I$) were calculated and normalized via a Lowess Fit of the $\log_2$ ratios $[\log_2(\text{I}_{\text{Cy-5}} / \text{I}_{\text{Cy-3}})]$ over the $\log_2$ total intensity $[\log_2(\text{I}_{\text{Cy-5}} \times \text{I}_{\text{Cy-3}})]$.

A mixture of RNA from 5 CD patients, 5 non-CD inflammatory controls and 5 healthy controls served as reference RNA for comparison of the microarray datasets. This reference sample provides a positive hybridization signal at each probe element on the microarray, which is essential when calculating and comparing fluorescence ratios. The data were imported into GeneMaths® XT (Applied Maths, Belgium). Weighted mean ratios and their
corresponding error (pixel SD) were calculated from the dye swap. Data were normalized over all arrays, and missing values were imputed using k-nearest neighbour algorithm (20 neighbours). GeneMaths® XT was used to perform all subsequent analyses.

**Quantitative real-time PCR (qPCR).** One μg of total RNA was converted to single stranded complementary DNA (cDNA) by reverse transcription (Superscript, Gibco, Invitrogen, Belgium) with oligo dT priming. One tenth of the cDNA was used in real-time quantification using the SYBR green kit (Eurogentec, Belgium) and 300 nM of each primer. A two-step program was run on the iCycler (BioRad Laboratories, Belgium). Cycling conditions were 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melting curve analysis and direct sequencing of amplicons on the ABI3700 analyzer (Perkin Elmer, Applied Biosystems, Belgium) confirmed primer specificities. All reactions were run in duplicate and normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) levels. GAPDH was chosen after checking the expression stability of a set of housekeeping genes in biopsies of CD patients and controls using the Genorm software. Primers were designed using the Beacon Designer software (PREMIER Biosoft International, USA). Sequences of all primer sets are listed in Table 1.

**Immunohistochemistry.** Colon and ileum biopsies fixed in formalin were embedded in paraffin according to standard procedures. Sections were rehydrated by serial immersion in xylene and ethanol. Immunostaining was performed on a NexES IHC automated staining system (Ventana Medical Systems, France) using a 1:10 dilution of a mouse monoclonal anti-metallothionein antibody (clone E9, Zymed Laboratories, Sanbio, The Netherlands). An isotype-specific irrelevant antibody (X0931, DakoCytomation, Belgium) was used to control for non-specific binding of the primary antibody. Slides were blindly scored by two
pathologists. Semi-quantitative scoring was based on staining intensity and the number of positive cells.

**Induction assays.** The mononuclear cell fraction (PBMC) was isolated from 30 ml of venous blood by density gradient centrifugation in Ficoll-Pacque (Amersham Biosciences, The Netherlands). PBMCs were seeded in 6-well plates at $10^6$ cells per well in RPMI medium (Gibco, Invitrogen) supplemented with 10% FCS. The next day, they were stimulated with 200 $\mu$M ZnSO$_4$ (Sigma, Belgium), 2 $\mu$M dexamethasone (Sigma), 10 ng/ml recombinant human IL6 (Peprotech, Campro Scientific, The Netherlands), 1000 U/ml human recombinant TNF (produced in-house) or 200 U/ml human recombinant IFN$\gamma$ (Biosource Europe, Belgium). The cells were lysed in 350 $\mu$l RLT buffer (Qiagen) after 3, 6 or 24 hours. To induce oxidative stress, PBMCs were incubated with 100 $\mu$M H$_2$O$_2$ (Sigma), which was washed off with PBS after 10 minutes. Cells were lysed after 2, 4 or 6 hours. Total RNA was isolated using the Rneasy Mini Kit (Qiagen), and converted to cDNA for subsequent qPCR. Normalized Ct values were corrected for background at each time point (e.g. dCt$_{\text{induced, t1}}$ - dCt$_{\text{un-induced, t1}}$), and plotted against time (0, 3, 6 and 24 hours, or 0, 2, 4 and 6 hours for H$_2$O$_2$). Induction was calculated as the area under the curve (AUC) for the three time points (AUCs are shaped as triangles and rectangles, and their summation or integration gives the same result):

$$AUC = \int_0^3 (m_1 x) dx + \int_3^6 (m_2 x + b) dx + \int_6^{24} (m_3 x + c) dx,$$

where $m_1 =$ the slope of the 0-3 line, $m_2 =$ the slope of the 3-6 line, $m_3 =$ the slope of the 6-24 line, $b =$ the y-intercept of the 3-6 line, and $c =$ the y-intercept of the 6-24 line.

HT29 human colon epithelial cells (American Type Culture Collection (ATTC) HTB38) and MT-knockdown cells (HT29MTkd) were seeded in 24-well plates at $10^6$ cells per well in RPMI supplemented with 10% FCS. The following day, cells were infected in triplicate at
multiplicities of infection (MOI) of 100 with adherent-invasive Escherichia coli strain LF82, isolated from a patient with CD, (a gift from A. Darfeuille-Michaud)\textsuperscript{11} for 1 hour, followed by gentamycin treatment (100 \( \mu \text{g/ml} \)) for 1 hour. Supernatants were collected after 8, 24 and 48 hours of infection/induction.

**Small interfering RNA (siRNA).** The short hairpin RNA (shRNA) fused to an H1 promoter was synthesized by PCR on the pSUPER vector (Tronolab, Switzerland), using primers: \(5'\)-

\[
\text{CAA TCT CTT GAA TTG CAC TTG CAG GAG CCG GGG GGA TCT GTG GTC TCA TAC AGA ACT TAT AA-3'}\]

and \(5'\)-

\[
\text{CCA TCG ATT TCC AAA AAC CGG CTC CTG CAA GTG CAA TCT CTT GAA TTG C-3'}\]. The sequence in bold is a 19-mer that specifically targets MT, and is present in MT1B, MT1E, MT1H, MT1J and MT1M. The PCR fragment was cloned in pLVTH-siGFP (Tronolab, Switzerland). To produce virus for delivery of the shRNA, HEK293T cells (ATCC CRL1573) were transfected by the calcium phosphate method with 3 \( \mu \text{g} \) pCMV-d8.91 (Tronolab, Switzerland), 1.5 \( \mu \text{g} \) PMDG2 (Tronolab, Switzerland) and 1.5 \( \mu \text{g} \) of the shRNA construct. After 48 hours, supernatant containing viral particles was harvested, and put through a 0.45 \( \mu \text{m} \) filter. One day before viral transduction, HT29 cells were seeded at 5x10\(^4\) cells per well in a 24-well plate. They were overlaid with viral supernatant, and centrifuged for 1 hour at 32°C. This procedure was repeated with 72-hour viral supernatant. Cells expressing high levels of GFP were subsequently sorted with the EPICS altra cell sorter (Beckman Coulter, The Netherlands).

**Cytometric Bead Array immunoassay.** Concentrations of IL1\(\beta\), IL6, IL8, IL10, IL12p70 and TNF in the culture supernatants were measured simultaneously using Cytometric Bead Array (CBA) (Human Inflammation Kit, Pharmingen, Becton Dickinson, Belgium) according to the manufacturer’s instructions. Concentrations of the cytokines were obtained by
comparing the mean fluorescence intensity of the samples with that of the corresponding standard curves. Flow cytometric analysis was performed using a BD FACS scan. Data acquisition and analysis was done using BD CBA software.

**IL8 ELISA.** IL8 concentrations in supernatants were assessed in dilution series by sandwich ELISA. Microtiter plates (Nunc, USA) were coated with 2 μg/ml anti-IL8 antibody (Pharamingen) in PBS for 2 hours at RT, and non-specific binding sites were blocked with 0.1% casein-PBS at 4°C overnight. Dilution series of the supernatant (1:2) were incubated for 2 hours at RT in 0.1% casein-PBS, and detected with 1:1000 biotinylated anti-IL8 antibody (Pharamingen) and 1:500 streptavidin-HRP (Pharmingen) in 0.1% casein-PBS for 1 hour. Substrate was added and the reaction was stopped after 30 minutes with 1 M H2SO4. Plates were read at 450 and 595 nm in a microplate reader (BioRad).

**Mutation screening of MT1M and MTF1.** Genomic DNA was extracted from whole blood using the Qiagen blood and cell culture DNA kit. The sequence encompassing the MT1M gene and ~600 bases upstream of the transcription start site was PCR-amplified in 25 randomly selected CD patients and 6 controls (forward primer: 5’-GAG GCC GAC CAG TGT TC-3’, reverse primer: 5’-AGT CTC TGG ATG AAA ATG TGA G-3’). The PCR product was sequenced with BigDye Terminator v3.1 Cycle Sequencing kit and analyzed on an ABI3700 analyzer (Applied Biosystems) with the former and the following primers: exon 1: 5’-CCC AGC CCA GCC CAG GAC CG-3’; exon 2: 5’-TCA CTG CCC ACT GCG TTT TTC TC-3’; exon 3: 5’-CAA GTC TAC TGC TAC CTC TC-3’.

Mutation analysis of MTF1 was performed in 95 randomly selected CD patients using flanking intronic primers for all exons and overlapping primers for 1 kb of the upstream regulatory region. PCR primers were designed using the SNPbox software\(^2\). A total of 28
primer sets were used (primer sequences are available on request). PCR fragments were sequenced with BigDye Terminator v3.1 Cycle Sequencing kit (Perkin-Elmer, Applied Biosystems), and analyzed on an ABI3730 DNA analyzer (Applied Biosystems). The sequencing trace files were analyzed for the presence of variants using novoSNP.

**CARD15 and MTF1 genotyping.** The 3 CD-associated *CARD15* polymorphisms R702W (SNP8), G908R (SNP12) and 1007fs (SNP13), as well as *MTF1* IVS1-128A>T, were genotyped in a cohort of 222 randomly selected CD patients and 63 controls using RFLP-PCR as previously described. *MTF1* IVS1-128A>T genotyping was also done by RFLP-PCR. A fragment containing the polymorphism was amplified with the following primers: forward 5'-TAA GAC TCA ATT GAT TCA GGG ACG AGA GC-3', reverse 5'-CAG TTG TGA GAA ATG AAA ACG TAA TGA C-3'. The PCR product was precipitated with SeeDNA (Amersham Biosciences), digested with *Dra*I, and restriction fragments were analyzed on a 2% agarose gel. The presence of the IVS1-128T allele abolishes a *Dra*I site, resulting in a band of 271 bp, instead of 251 bp in the presence of the IVS1-128A allele.

**Statistical analysis.** Statistical analyses were performed using SPSS software (SPSS inc., USA). Differences between groups in immunohistochemical scores and induction AUCs were calculated using the Mann-Whitney U test. Correlations between metric data were calculated using Pearson’s Rho, and those between rank data with Spearman’s Rho. Rank data for histological scores were 0-1 = 1, 1-2 = 2, 2-3 = 3 and for RNA levels <1,000 = 1, 1,000-10,000 = 2, >10,000 = 3. Odds ratios and their 95% confidence intervals (CI) were calculated with Pearson’s χ² test. Logistic regression was used to evaluate independence and interaction between parameters. Significance of differences in ELISA IL8 concentrations between time-series were calculated using a general linear model, error bars are calculated from 3 dilutions.
Two-tailed probabilities were calculated, and $P$-values of less than 0.05 were considered statistically significant.

RESULTS

Identification of potential candidate genes for CD. Gene expression in normal colon biopsies of 16 CD patients and 11 healthy controls (Table 2) was analyzed on a focus microarray chip containing 6,779 expressed sequence tags. We aimed at identifying potential candidate susceptibility genes for subsequent validation and mutation screening. This process was performed in two steps. First, we characterized genes that were differentially expressed between CD patients and controls using an independent t-test ($P<0.01$, $N=240$). We then selected the genes that were located near a chromosomal region that has been linked to CD in at least two independent studies (Table 3). We also included the loci that were found in a study performed on a Flemish cohort\textsuperscript{15}, because the patients in our study live in this area of Belgium. We employed an arbitrary distance of 5 megabases around markers that showed the highest linkage. The exact chromosomal locations of the markers and genes were explored using the genome browser at http://genome.ucsc.edu. In this way, 18 genes were selected (Table 4), and all clones of the respective genes were sequence verified and annotated correctly.

Metallothioneins are down-regulated in colon, ileum and whole blood of CD patients with colonic involvement.

Metallothionein RNA expression. In the microarray screen we found two closely related metallothionein (MT) transcripts, MT1F and MT1M, that were down-regulated in CD patients. Although other genes may show lower $P$-values, we chose to focus on these genes,
because they are located near the *IBD1* locus, which has been linked to CD in almost every linkage analysis, and which contains *CARD15*. Metallothioneins are involved in a variety of metal scavenging processes, and they possess both bactericidal and anti-inflammatory activities\(^\text{16,17}\). In humans, at least 18 MT gene isoforms exist (*MT1A-MT1X, MT2A, MT3* and *MT4*), but only *MT1* and *MT2* are inducible. We performed a detailed expression study on different MT isoforms using qPCR on an independent population of 38 CD patients and 19 controls. Because of the high sequence homology between MT isoforms, we first determined whether both MT1M and MT1F were specifically down-regulated. To this end, we designed primers for MT1M and MT1F, as well as for MT1E, MT1J and MT2A (Table 1). Primer specificities were confirmed by the presence of a single melting peak after denaturation of the amplicons, and by direct sequencing. The expression of MT1M in CD patients with colonic involvement (subgroups C and IC) was significantly less than in patients with pure ileal involvement (*P*=0.0244, Figure 1) and in controls (*P*=0.0076). Furthermore, the expression of MT1M in all biopsies tested was significantly correlated with expression of the other isoforms tested (Table 5). This indicates that at least these MT isoforms share the same regulatory machinery for basal expression in the colon. The highest expression in colon biopsies was observed for MT1E, followed by MT2A>MT1J>MT1F>MT1M. Basal transcript levels of MT1F were measured in whole blood of 15 CD patients and 13 controls, and were found to be down-regulated in CD patients with colonic involvement (*P*=0.026, Figure 2). MT1M levels were too low for reliable quantitative measurement. None of the patients and none of the controls had elevated C-reactive protein levels at the time of blood sampling. Therefore, changes in MT expression due to systemic inflammation is not likely\(^\text{18}\).
Metallothionein protein expression. MT protein expression was evaluated semi-quantitatively in colon and ileum biopsies of CD patients and controls using immunohistochemical staining with a mouse monoclonal anti-metallothionein antibody (cloneE9, Zymed Laboratories). This antibody however, cannot distinguish between MT isoforms, because all of them have the epitope it recognizes. Overall, the expression of MT in colon was significantly lower than in the ileum ($P=0.042$). No conclusions could be drawn about MT expression in the colon, because the scores rarely reached 2. Compared to controls, a significant decrease in MT protein expression was found in CD patients with colonic involvement (subgroups C and IC) in ileum biopsies, again indicating a possible fundamental defect in MT expression in these patients (data not shown). A correlation was found between RNA expression as assessed by qPCR and the immunohistochemical score of protein expression (Spearman’s Rho: 0.826, $P=0.001$, $N=12$). This finding is interesting because in each individual, the biopsies used for RNA extraction and those used for staining were not obtained at the same time, again indicating a stable inherent basal expression of MT. Epithelial cells were primarily positive for MT staining. The strongest expression was found at the base of the villi, within paneth cells, and in rapidly proliferating epithelial cells in the crypts (Figure 3).

Metallothionein induction in response to oxidative stress is higher in CD patients. Because MT expression is decreased in the colon, ileum and whole blood of CD patients with colonic involvement, we examined whether this was due to impaired induction. We stimulated PBMC isolated from 10 CD patients (subgroups C: 2, IC: 4, I: 4) and 9 controls with known MT inducers (10 ng/ml IL6, 1000 U/ml TNF, 200 μM ZnSO4, 200 U/ml IFNγ and 2 μM dexamethasone), and measured MT1M expression by qPCR. No difference in induction was found between CD patients and controls. However, induction following
exposure to 100 μM H₂O₂ for 10 minutes was significantly higher in CD patients, independently of the disease location ($P=0.0015$, Figure 4).

**Colonic epithelial cells defective in metallothionein expression secrete less IL8 in response to LPS or adherent-invasive E. coli.** Because we observed MT expression mainly in epithelial cells, we investigated whether a low basal expression of MT in epithelial cells has consequences upon challenge with bacteria. We used small interfering RNA (siRNA) to create HT29 colonic epithelial cells deficient in MT expression (HT29MTkd). Although the siRNA sequence was targeted against MT1B, MT1E, MT1H, MT1J and MT1M, the RNA expression of most MT isoforms was lowered approximately twofold as assessed by qPCR (Figure 5). Diminished protein expression was shown by immunofluorescence using the monoclonal anti-metallothionein antibody (data not shown). Cytokine secretion in response to stimulation with adherent-invasive E. Coli (AIEC LF82) was tested with the Human inflammation kit (Becton Dickinson). We could only measure IL8 secretion after bacterial challenge with this technique. The secretion of IL8 was significantly lower in HT29MTkd cells than in normal HT29 cells ($P<0.001$, Figure 6).

**Screening for mutations in the MT1M gene.** We screened the complete MT1M gene (~1.4 kb) and its promoter region (~600 bases upstream of the transcriptional start site), using NM_176870 as reference sequence, in 25 CD patients and 6 controls. Two intronic polymorphisms, IVS1-366C>T and IVS2-49C>T, and one 3’ untranslated region (3’UTR) polymorphism, c.286A>G, were found. The absence of promoter or coding mutations in MT1M that would cause its down-regulation was not too surprising, since the down-regulation was not confined to MT1M. Better candidates for mutation screening are probably upstream factors regulating all MT isoforms, and so we proceeded in that direction.
A polymorphism in the first intron of the \textit{MTF1} transcription factor gene is associated with ileal disease. One of the best-characterized transcription factors regulating MT expression is MRE-binding transcription factor 1 or \textit{MTF1}, an essential zinc finger protein that binds to specific DNA motifs termed metal-response elements (MRE). Furthermore, this protein is responsible for both basal and inducible expression of MT\textsuperscript{19}, and the gene is located at 1p33 (\textit{IBD7}), a locus that has been linked to CD in a Flemish population\textsuperscript{15}. We show that expression of the transcription factor MTF1 in blood of CD patients and controls correlates with the expression of MT1F (Pearson’s Rho: 0.572, \(P=0.001\), \(N=20\)). All 11 exons and the promoter region (~1000 bp upstream of the transcription start site) were screened for mutations in 95 CD patients, using NM\_005955 as a reference sequence. Two missense mutations were found: c.198C>G (Asp63Glu) in 4 patients, and c.1253G>A (Glu385Lys) in 2 patients. The functional relevance of these mutations still needs to be investigated. Moreover, a potential polymorphism at the splice site junction between exons 8 and 9, c.1270A>G, was frequently found. Yet no alternative splice variant could be detected in cDNA from blood of patients with different genotypes (data not shown). Here, we focused on a polymorphism in the first intron of \textit{MTF1}, IVS1-128A>T, because of its potential influence on gene expression\textsuperscript{20}. No difference in frequency between a cohort of 222 CD patients and 63 controls was detected (data not shown). However, genotype-phenotype analysis revealed that IVS1-128A>T had considerable influence on the location of disease: the IVS1-128T allele was associated with ileal disease (subgroups I and IC). In the presence of the IVS1-128T allele, 139/166 patients (84%) had ileal disease, compared to 38/56 (68%) of those with the AA genotype (OR: 2.4, CI: 1.216-4.891, \(P=0.011\)). Because we observed that \textit{CARD15} mutations were also highly correlated with ileal disease (subgroups I and IC, \(P<0.0001\)), which has also been observed by others\textsuperscript{21-23}, we investigated whether the
combined presence of \textit{CARD15} and \textit{MTF1} risk genotypes had a substantially higher impact on disease location than either of them alone. The odds ratio for developing ileal disease (subgroups I and IC) in \textit{CARD15} mutation carriers is 4.2 (CI: 1.980-9.098) and in IVS-128T allele carriers 2.4 (CI: 1.216-4.891). Moreover, logistic regression revealed significant interaction between the two genetic markers: carriage of an IVS-128T allele increased the risk of ileal disease by 12% in \textit{CARD15} wild type patients (from 60 to 72%, Figure 7), and in carriers of \textit{CARD15} mutations by 23% (from 74 to 97%). This means that both genes contribute to the location of inflammation, and mutually interact.

The IVS-128A>T polymorphism is located in the first intron of \textit{MTF1}, which is located within the 5’UTR. It is not contained within a CpG island. A search for transcription factor binding sites using the transcription factor database (TRANSFAC) revealed a potential binding site for GATA binding protein 4 (GATA4).

Based on these findings, we speculate that carriers of the IVS-128AA genotype express less tissue MT compared to carriers of the IVS-128T allele. Therefore, the genotype was weighed against the level of MT1M expression in biopsies of all patients and controls of whom the genotype was known. We could not show a significant association of MT1M expression and genotype, although the expected trend does exist (IVS-128AA (\(N=11\)) vs. IVS-128AT and TT (\(N=32\)), \(P=0.195\)).

\textbf{DISCUSSION}

Identification of susceptibility genes, their interaction, and their relationship to specific clinical manifestations is an important step in understanding CD and developing improved clinical management or therapy. We used an alternative, hypothesis-independent strategy to identify novel candidate genes. Transcriptome analysis of non-inflamed colon biopsies of CD patients and controls revealed 18 novel potential candidate genes localized to CD loci.
Many genome-wide linkage studies of CD have been performed\textsuperscript{24}. This led to the identification of the first gene strongly associated with CD, \textit{CARD15}\textsuperscript{3-4}. Nevertheless, despite its strong association with CD, \textit{CARD15} is difficult to relate causally to CD. Polymorphisms show very limited penetrance, occurring in approximately 40\% of patients (predominantly those with ileal disease), as well as in 15\% of healthy individuals. Furthermore, linkage to chromosome 16 was still observed in CD patients not carrying common \textit{CARD15} mutations\textsuperscript{25-27}. An alternative explanation is that polymorphisms in \textit{CARD15} are not in themselves causal, but modify the immune response in inflammatory lesions elicited by some other mechanism. An interesting theory based on evolutionary benefit was suggested by Hugot and co-workers\textsuperscript{28}. They stated that a mutated CARD15 protein would have been beneficial during the outbreak of the plague in Europe, but it somehow represents a disadvantage in the pathogenesis of CD. The identification of \textit{CARD15} and the insights into its role in innate immunity and CD pathology highlight the importance of mapping susceptibility genes\textsuperscript{29}.

Two of the 18 potential candidate genes we identified belong to the family of closely related metallothioneins (MT). They are located in tandem within the \textit{IBD1} locus and arose by non-processed gene duplications\textsuperscript{30}. Furthermore, they are involved in protecting cells against toxic levels of metal ions, radicals and bacterial infections. Four MT classes exist in humans, but only MT1 and MT2 isoforms are inducible by cytokines, hormones, metals and stress in general\textsuperscript{31}. We showed that MT was significantly down-regulated in CD patients with colonic involvement as compared to healthy controls. In addition, detailed expression analysis of different MT isoforms revealed that their expression is highly correlated at basal level. Low RNA expression levels were linked to reduced protein expression. Furthermore, we provide evidence that a low expression level of MT in CD patients with colonic involvement results from a genetic predisposition rather than from an early inflammation event. Indeed, the down-regulation was not only found in colon but also in ileum biopsies, both sampled from non-
inflamed areas, as well as in whole blood samples regardless of C-reactive protein levels. Remarkably, MT RNA levels correlated with protein expression in biopsies that were not time matched, contributing to the idea that MT expression is stable and potentially inherited. Two studies have previously shown an up-regulation of MT in CD\textsuperscript{32,33}, while we and others found a down-regulation\textsuperscript{34-37}. Moreover, MT quantification in CD based on radioimmunoassay\textsuperscript{38}, microarray\textsuperscript{39} and silver-saturation assay\textsuperscript{40} also reported a down-regulation.

We further demonstrated that although basal MT levels were reduced in CD patients with colonic involvement, they are still inducible by conventional stimuli in peripheral blood. Unexpectedly, MT induction with oxidative stress was higher in CD patients, independent of disease location. Relative overreaction in CD patients might reflect the fact that basal low levels of MT result in more free hydroxide radicals that can activate the MT transcription factor, MTF1, resulting in the overall increased transcription of MT\textsuperscript{41}.

In order to clarify the potential functional consequence of reduced basal levels, we generated MT-knockdown colonic epithelial HT29 cells using small interfering RNA. We report that, in response to bacterial challenge with a CD-associated \textit{E. coli} strain\textsuperscript{11}, reduced expression of MT in epithelial cells correlated with reduced IL8 secretion. This observation is in accordance with the recent finding that IL8 up-regulation is impaired in response to acute trauma to colon and skin of CD patients\textsuperscript{42}. IL8 is a potent chemoattractant and activator of neutrophils, which respond rapidly to different types of infections, and play an essential role in the inflammatory response. They release antimicrobial peptides as well as reactive oxygen intermediates that may cause tissue damage. We hypothesize that the intestinal epithelial cell lining of CD patients with colonic involvement expresses less MT and initially secretes less IL8 in response to bacteria. This might secure an exaggerated secondary, compensatory immune response.

It is unlikely that polymorphisms in the MT genes themselves would be responsible for their
low expression, since we showed that they are down-regulated as a group. Yet, in our knockdown model for MT, we see a similar decrease in expression of MT isoforms that were not targeted with the short hairpin RNA. Thus, the possibility of a positive feedback loop for MT expression via its own transcriptional regulator cannot be ruled out. We could not find coding or promoter polymorphisms in MT1M, but other isoforms need to be screened. Alternatively, epigenetic mechanisms such as DNA methylation might be responsible for the down-regulation of MT genes in CD patients.

In searching for mutations in MTF1 (IBD7), a transcription factor that regulates basal expression of MT, we focused on a polymorphism within the first intron, IVS1-128A>T, because regulating regions are frequently found there. This polymorphism predisposes to inflammation at specific sites in the intestine. Disease location is a phenotypic characteristic that remains stable over time. Similar to CARD15, the association of MTF1 with ileal disease offers a new candidate disease-modifying gene, rather than a disease-predisposing gene. In addition, the two genes interact to bring about ileal disease.

The links of CARD15 and MTF1 with disease location might reflect the functions of these proteins at their sites of expression: CARD15 is expressed in paneth cells of the ileum, and the expression of MT in ileum is much higher than in colon. Both proteins are probably crucially involved in maintaining a low bacterial count in the ileum. A fully functional CARD15 or MTF1 might be crucial in this process. However, they might be disadvantageous in the colon, where bacterial load is high. In the colon, a weakly functional CARD15 can be beneficial, because inappropriately intense immune responses will not be elicited. Greater MT expression in the colon can also be beneficial because it can lead to efficient clearance of mucosal infection.

The association of the IVS1-128T allele in MTF1 with higher MT expression needs investigation. We could not show an association in our samples, however, we did not make
associations in selected subgroups, because of the limited number of samples. *In vitro* assays using reporter gene constructs will provide direct information of the transcriptional functionality of these alleles. We must keep in mind, however, that to fully understand the genetic contribution of IVS-128A>T in CD, the haplotype structure surrounding this polymorphism needs to be determined. The mutation causing altered transcription of MTF1 could be located elsewhere within the same haplotype block. MTF1 regulates basal expression of M1. Therefore, a difference in MTF1 expression will be directly linked to altered MT expression. Indeed, we showed a correlation between MT1F and MTF1 levels in peripheral blood. The IVS-128T allele is located within a potential binding site for GATA4, a transcription factor that is expressed in the ileal enterocytes in the small intestine, but not in the colon. Therefore, a primary goal in this context is to study GATA4 binding to the *MTF1* gene.

Loss of MTF1 in mouse embryonic fibroblasts results in enhanced collagen deposition, which is an important complication of CD. In these fibroblasts, transforming growth factor-beta is activated. This protein has potent anti-inflammatory properties, but at the same time it drives the process of fibrosis in the deeper layers of the gut. Nonetheless, we could not find an association between the IVS-128A>T polymorphism and stricturing disease, indicating that additional risk factors influence this particular phenotype. Interestingly, though, the IVS-128T allele in *MTF1* is associated with CD of the ileum, which has a higher likelihood of stricturing than does colonic CD.

We used microarray screenings in unaffected tissues for human genetic studies. A similar study was performed by Lawrance and colleagues, with the exception that they used moderately inflamed resected colonic tissue. We used non-inflamed tissue to target basal differences in gene expression due to genetic variation, and not due to inflammation-related events. Significant evidence was found for the genetic transmission of variation in gene
expression. The variation in expression level is highest among unrelated individuals, and smallest between monozygotic twins, indicating that germ-line differences contribute to variation in gene expression.

In summary, screening unaffected colon biopsies by microarrays proved to be useful in the identification of new candidate genes for CD. A cluster of MT genes located at IBD1 is consistently down-regulated in CD patients with colonic involvement. Subsequently, we identified a new disease-modifying gene, MTF1, that is associated with ileal disease, and together with CARD15 serves as a good predictor of disease location in CD patients.

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## CHAPTER 8

### TABLES

#### Table 1. Sequences of qPCR primer sets

<table>
<thead>
<tr>
<th>Gene symbol</th>
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<th>Forward primer</th>
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### Table 2. Clinical characteristics of CD patients analyzed by microarrays

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<th>Sex</th>
<th>Familial CD</th>
<th>Age of onset</th>
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<th>Disease location</th>
<th>CARD15 status</th>
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A1: <40 years; A2: ≥ 40 years; B1: non-stricturing, non-penetrating; B2: stricturing; B3: penetrating; disease location is defined as maximal extension of inflammation during total follow-up of patients with I: ileal involvement only, C: colonic involvement only, IC: ileal and colonic involvement; mutant CARD15: carriage of at least one mutant allele for SNP8, SNP12 or SNP13 (according to Hugot et al. 2001); wt CARD15: not carrying a mutant allele for SNP8, SNP12 or SNP13; 5-ASA: 5-aminosalicylates.
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<td>IBD14 14p2.1-26.2</td>
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<td>Hampe et al. 2002</td>
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<td>Cho et al. 1998</td>
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<td>African CD</td>
<td>Hampe et al. 2002</td>
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<td>D16S409</td>
<td>Ashkenazi Jewish CD</td>
<td>Brought et al. 2001</td>
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<td>Brought et al. 2001</td>
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<td>Study Type</td>
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<td>IBD1</td>
<td>16q12.1</td>
<td>D16S411</td>
<td>American non-Jewish CD</td>
<td>Ohmen et al. 1996</td>
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<td>IBD1</td>
<td>16q12.1</td>
<td>D16S411-D16S419</td>
<td>multicenter (IBDIGC)</td>
<td>Cavanaugh et al. 2001</td>
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<td>IBD1</td>
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<td>16q12.1</td>
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<td>European CD</td>
<td>Hampe et al. 1999</td>
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<td>IBD1</td>
<td>16q12.1-12.2</td>
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<td>Hugot et al. 1996</td>
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<td>IBD1</td>
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<td>Curran et al. 1998</td>
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<td>IBD1</td>
<td>18p11.31</td>
<td>D18S62</td>
<td>Flemish IBD</td>
<td>Vermeire et al. 2004</td>
</tr>
<tr>
<td>IBD6</td>
<td>19p13</td>
<td>D19S591-GATA21G05</td>
<td>Canadian CD</td>
<td>Rieux et al. 2000</td>
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<td>IBD6</td>
<td>19p13.3-13.2</td>
<td>D19S1034-D19S586</td>
<td>American IBD</td>
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<td>IBD6</td>
<td>20p12.3</td>
<td>D20S192</td>
<td>Flemish IBD</td>
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<td>22q11.23-12.1</td>
<td>D22S315-D22S421</td>
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<td>IBD6</td>
<td>Xp21.1-21.2</td>
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<td>DXS1202-DXS1214</td>
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<td>Xq21.32</td>
<td>DXS1203</td>
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Table 4. Differentially expressed genes in colon biopsies of CD patients located near a locus for CD

<table>
<thead>
<tr>
<th>Unigene</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Chromosomal location</th>
<th>Expression in CD</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Hs.486246</td>
<td>PHTF1</td>
<td>putative homeodomain transcription factor 1</td>
<td>1p13</td>
<td>↑</td>
<td>0.000099</td>
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<tr>
<td>Hs.77955</td>
<td>MEF2D</td>
<td>MADS box transcription enhancer factor 2, polypeptide D</td>
<td>1q22</td>
<td>↓</td>
<td>0.0035</td>
</tr>
<tr>
<td>Hs.106674</td>
<td>BAP1</td>
<td>BRCA1 associated protein</td>
<td>3p21.31-p21.2</td>
<td>↓</td>
<td>0.0017</td>
</tr>
<tr>
<td>Hs.438691</td>
<td>GMPPB</td>
<td>GDP-mannose pyrophosphorylase B</td>
<td>3p21.31</td>
<td>↓</td>
<td>0.0013</td>
</tr>
<tr>
<td>Hs.302047</td>
<td>PLCB3</td>
<td>phospholipase C-like 3</td>
<td>3q25.31</td>
<td>↓</td>
<td>0.0048</td>
</tr>
<tr>
<td>Hs.318567</td>
<td>NDRG1</td>
<td>N-myc downstream regulated gene</td>
<td>8q24.3</td>
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<tr>
<td>Hs.84072</td>
<td>TM4SF3</td>
<td>transmembrane 4 superfamily member 3</td>
<td>12q12.1</td>
<td>↑</td>
<td>0.0046</td>
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<tr>
<td>Hs.85951</td>
<td>XPO1</td>
<td>exportin, tRNA (nuclear export receptor for tRNAs)</td>
<td>12q14.1</td>
<td>↑</td>
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<tr>
<td>Hs.419776</td>
<td>NAP1L1</td>
<td>nucleosome assembly protein 1-like 1</td>
<td>12q21.1</td>
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<tr>
<td>Hs.159481</td>
<td>GALGT</td>
<td>UDP-N-acetyl-alpha-D-galactosamine</td>
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<td>Hs.437377</td>
<td>MT1F</td>
<td>metallothionein 1F</td>
<td>16q22.2</td>
<td>↓</td>
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<td>Hs.188518</td>
<td>MT1M</td>
<td>metallothionein 1M</td>
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<td>Hs.100914</td>
<td>CEP192</td>
<td>Centrosomal protein 192kDa</td>
<td>19p11.21</td>
<td>↑</td>
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<tr>
<td>Hs.512640</td>
<td>PRKCSh</td>
<td>protein kinase C substrate 80K-H</td>
<td>19p13.2</td>
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<td>Hs.134074</td>
<td>SLC35D1</td>
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<td>Hs.437</td>
<td>TCF15</td>
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<td>20p13</td>
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<tr>
<td>Hs.102336</td>
<td>ARHGAP8</td>
<td>Rho GTPase activating protein 8</td>
<td>22q13.31</td>
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<tr>
<td>Hs.28491</td>
<td>SAT</td>
<td>spermidine/spermine N1-acetyltransferase</td>
<td>Xp22.1</td>
<td>↑</td>
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Table 5. Correlations of MT isoform expression in colon biopsies of CD patients and controls

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<tr>
<th></th>
<th>MT1E</th>
<th>MT1F</th>
<th>MT1J</th>
<th>MT1M</th>
<th>MT1A</th>
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<td>MT1E</td>
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<td>0.631</td>
<td>0.496</td>
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<td>&lt;0.01</td>
<td>0.058</td>
<td>0.058</td>
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<tr>
<td>N</td>
<td>65</td>
<td>55</td>
<td>66</td>
<td>15</td>
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<tr>
<td>MT1F</td>
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<td>0.589</td>
<td>0.378</td>
<td>0.784</td>
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<tr>
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<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
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<tr>
<td>N</td>
<td>55</td>
<td>55</td>
<td>62</td>
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<tr>
<td>MT1J</td>
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<td>0.609</td>
<td>0.805</td>
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<td></td>
<td></td>
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<tr>
<td>N</td>
<td>53</td>
<td>13</td>
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<tr>
<td>MT1M</td>
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</table>
FIGURES

**Figure 1.** MT1M expression is down-regulated in colon biopsies of CD patients with colonic involvement. CD colitis: subgroups IC + C (N=20), CD ileitis: subgroup I (N=18), control (N=19).

**Figure 2.** MT1F expression in blood is down-regulated in CD patients with colonic involvement. CD colitis: subgroups IC + C (N=11), CD ileitis: subgroup I (N=4), control (N=13).
Figure 3. Metallothionein staining of representative samples of non-inflamed ileal biopsies of (A) a control and (B) a CD patient. Control ileum shows strong immunoreactivity for MT in epithelial cells, with stronger reactivity in the proliferative cells of the crypts and at the base of the villi. In CD patients, MT expression is significantly decreased. Bar = 100µm.
Figure 4. MT1M RNA induction in response to oxidative stress is higher in CD patients \((N=10)\) than in controls \((N=9)\), irrespective of the disease location \((P=0.0015)\).

Figure 5. The RNA expression of different MT isoforms in HT29MTkd cells is downregulated in comparison to HT29. The solid line represents a twofold change in expression. The short hairpin RNA was targeted at MT1B, MT1E, MT1H, MT1J and MT1M (black bars), but the other isoforms tested (MT1A, MT1F, MT1G, MT1X and MT2A) also show reduced expression.
Figure 6. IL8 secretion in response to bacterial challenge is lower in MT-knockdown cells (HT29MTkd) than in HT29 cells. +AIEC: stimulated with adherent-invasive E. coli LF82 MOI 100; unst: unstimulated (P<0.001).

Figure 7. The presence of the MTF1 IVS-128T allele and CARD15 mutations contribute to ileal disease. Percentages represent number of patients positive or negative for CARD15 mutations that are positive or negative for the IVS-128T allele. Logistic regression revealed a gene-gene interaction between CARD15 and MTF1.
CHAPTER 9

General discussion and summary
Crohn’s disease (CD) and spondyloarthropathies (SpA) are considered to be mutually related, complex, multigenic disorders. A disturbed immunological response towards unknown environmental factors results within genetically predisposed individuals in the development of chronic, uncontrolled intestinal and/or joint inflammation (1-3).

Over the last decade, important progress was made regarding genetic research of both disease entities. Genome screening led to the identification of several susceptibility loci containing multiple possible candidate genes (4-8).

Linkage and association studies contributed to the discovery of CARD15 on chromosome 16q as the first CD-related gene (9-11). Three CARD15 gene polymorphisms have been associated with CD and can be found in 30 to 45% of CD patients. Functional studies showed a disturbed immunological response of the host cell towards bacterial cell wall components in the presence of these genetic variants (12-15). A dysregulated mucosal homeostasis with a decreased production of pro-inflammatory cytokines and anti-bacterial defensins can lead to an increased influx and the intramucosal persistence of intestinal bacteria or microbial antigens, leading to secondary, chronic gut inflammation (16, 17). These findings confirm the role of a reduced tolerance towards bacterial flora in the development of this chronic intestinal disease, as already demonstrated in numerous previous animal and human studies (18-20).

The relation between HLA-B27 carriership and the risk for SpA, in particular AS, is well known (21). However, the role of this genetic factor is much less important in CD-associated AS and asymptomatic SI (22, 23). Recently, several non-MHC related susceptibility loci, including a locus at 16q, have also been identified in SpA populations (7, 8).

Several clinical and immunological findings point to an important link between CD and SpA (2, 24). Articular inflammation (peripheral and/or axial arthropathy) is common in CD and is classified as spondyloarthropathy (25). On the other hand, (subclinical) gut inflammation, with similar histological features as in CD, has been described in two thirds of SpA patients. The presence of histological, chronic inflammatory lesions in ileum and/or colon of SpA patients was identified as a risk factor for the future development of clinically overt Crohn’s disease (26-33). Furthermore, several immunological alterations have been found in intestinal mucosa and synovium of CD and SpA patients, leading to the presumption of (re)circulation of inflammatory cells between gut and joint (34-40).
The aim of the present work was to investigate the role of genetic alterations in the gut-joint axis of CD and SpA.

1. *CARD15* polymorphisms predispose to the presence of subclinical, chronic gut inflammation in patients with SpA.

Following the identification of a linkage region in ankylosing spondylitis on chromosome 16, several studies have been performed to investigate the *CARD15* gene as a putative susceptibility gene for SpA or AS (41-49). No associations with these CD-associated polymorphisms could be found in these populations. However, the presence of subclinical chronic gut inflammation was not taken into account, whereas exactly this subgroup of SpA patients is at risk to develop clinical CD (50).

In Chapter 2 we showed that the presence of chronic gut inflammation in SpA patients is associated with the CD-related polymorphisms in the *CARD15* gene. This finding provided for the first time genetic evidence for the relation between CD and SpA. Furthermore, it suggests a possible role for a genetically disturbed handling of intestinal bacteria or bacterial products in the etiopathogenesis of this chronic rheumatic disorder. In the past, the hypothesis of a possible infectious (enterogenous) trigger for the development of SpA has been put forward on numerous occasions. Both bacterial antigens and DNA have been found in synovial fluid of mainly reactive arthritis patients, but also in patients with undifferentiated SpA, AS and PsA (51-55). Furthermore, carriership of *Klebsiella pneumoniae* in the gut was once identified as a possible risk factor for the future development of AS (56). A disturbed immunological response, as demonstrated in the presence of *CARD15* polymorphisms, may lead to an augmented influx of bacteria from the gut lumen and may contribute to an intestinal origin of articular inflammation, in particular since inflammatory cells and bacterial antigens presumably circulate between gut and joint.

The majority of SpA patients with gut inflammation remain asymptomatic and do not develop clinically overt CD (50). This finding raises the question whether *CARD15* polymorphisms are not merely linked with the development of (subclinical) gut inflammation than to clinical CD as such.
2. The role of \textit{CARD15} polymorphisms in the development of articular inflammation of CD.

The exact pathogenesis of extraintestinal manifestations in IBD is still not clarified (57). The circulation of enterogenous bacteria or antigens between the gut and organs at distance might also be important in IBD, in particular in view of the recent findings on the functional role of \textit{CARD15} polymorphisms. This hypothesis is corroborated by the finding that in ReA, where intestinal bacteria play an essential role in the development of articular inflammation, also other extraintestinal and extraarticular foci of inflammation can often be found (skin, conjunctiva, iris, urethra and heart) (58).

Previous genotype-phenotype studies could not demonstrate an association between extraintestinal manifestations and the presence of \textit{CARD15} polymorphisms (59-63). However, only few data are available on the association with specific articular manifestations and in particular no information was available on axial involvement (59, 60). Sacroiliitis, often asymptomatic and not HLA-B27 related, is considered to be the most frequent extraintestinal manifestation of CD (22). Moreover, a study of relatives of AS patients suggested a non-HLA-B27 related genetic factor in the link between intestinal and axial inflammation (64).

In \textbf{Chapter 3}, we investigated the association of \textit{CARD15} polymorphisms with different rheumatologic manifestations of CD. This study showed a higher prevalence of radiological sacroiliitis in CD patients with \textit{CARD15} polymorphisms. Similar to previous association studies, we found no association with peripheral arthritis (59, 60). The possible link between these genetic variants and axial inflammation is also reflected by previous findings in SpA patients. Chronic gut inflammation in SpA, associated with \textit{CARD15} polymorphisms as demonstrated by the previous study, is mostly seen in patients with axial involvement (65). Moreover, the presence of chronic ileitis and/or colitis in non-AS SpA patients is even considered to be a risk factor for the future evolution towards AS (66).

Data on the association of radiological sacroiliitis with specific phenotypes of the disease are scarce. Therefore we performed a multicenter study of this frequent extraintestinal manifestation in cooperation with Leuven and Liège in order to investigate potentially associated clinical features and the relationship with \textit{CARD15} polymorphisms (\textbf{chapter 4}).

We found an association between the presence of radiological SI and the development of peripheral arthritis. Furthermore, CD patients with clinical AS had a higher chance of
developing uveitis. These findings suggest common characteristics in the origin of these extraintestinal manifestations.

In this second study, we could however not confirm the link between \textit{CARD15} polymorphisms and sacroiliitis. Reproducibility of genetic association studies is a considerable problem in genetic research of complex, multigenic diseases. Potential confounding factors leading to inconsistent results between studies, include lack of statistical power, population stratification, genetic heterogeneity, environmental interactions, genotype and phenotype quality control and the choice of statistical models \cite{67-70}. Looking at the different association studies concerning \textit{CARD15} polymorphisms, conflicting results often arose, even within ethnically similar populations \cite{5, 13}.

Both studies in this thesis included phenotypically and ethnically similar populations of CD patients, who were enrolled independent of the presence or history of articular manifestations. \textit{CARD15} genotyping and scoring of the sacroiliac radiographs have been blindly performed and the known associations between \textit{CARD15} polymorphisms and ileal and fibrostenosing disease were confirmed in both cohorts. Furthermore, the results of the univariate analysis showing an association between sacroiliitis and the presence of \textit{CARD15} variants in the first study, were confirmed by multivariate analysis. The lack of explanation for the inconsistent findings in these studies reflects once more the problem of genetic association studies of complex diseases. When performing such studies, one should strive for large, ethnically and phenotypically homogeneous and well defined patient populations or, preferably, for 2 independent and well balanced cohorts within one and the same study.

Despite the inconsistent results on sacroiliitis and \textit{CARD15} polymorphisms, the genetically predestined circulation of inflammatory cells and insufficiently cleared micro-organisms or bacterial components, as a cause of inflammation at distance of the gut, remains an interesting hypothesis in both IBD and SpA \cite{20, 38}. In view of the multigenic character of both disease entities, presumably various genetic defects play a role in a disturbed handling of intestinal flora and the transport of bacterial antigens to other organs. It is still unclear how exactly components of the bacterial flora might be transported from the intestinal mucosa via the blood circulation to organs at distance.
3. **CARD15** polymorphisms induce a disturbed cellular and humoral immunological response towards microorganisms.

*CARD15* plays an important role in the innate immune response of the host towards intraluminal microorganisms (71, 72). The intracytoplasmic protein *CARD15* acts as a receptor for muramyl dipeptide, a component of peptidoglycan, which can be found in the cell wall of most bacteria (73). A disturbed immunological response of the host cell towards this bacterial antigen has been associated with the CD-related *CARD15* polymorphisms in several functional studies (*in vitro* as well as *ex vivo*) (12-15). However, the results from these studies were predominantly focussed upon the (rare) homozygous carriers of these genetic variants, more specifically of the frameshift mutation. Single heterozygotes are much more common and until now no clear functional defect could be demonstrated in such patients.

In chapter 5 we investigated the role *CARD15* polymorphisms in the response of peripheral blood monocytes, isolated from CD patients, towards adherent-invasive *E. coli* (AIEC). This bacterial strain, specifically associated with CD, was isolated from the ileum of CD patients and is able to invade the host cell and to survive and even replicate intracellularly (74-79). After infection of the monocytes with these AIEC, a disturbed early cytokine secretion (IL-1β, IL-6 and IL-10) was observed in patients carrying *CARD15* polymorphisms. Although the effect was most pronounced in homozygotes, we were also able to demonstrate for the first time a functional defect in the (much more frequent) single heterozygous carriers of these genetic variants. Further research will have to show whether this early disturbed immunological response can also be found in other cell types (intestinal macrophages, dendritic and epithelial cells) and after infection with other invasive or non-invasive bacteria.

This study also reflects the importance of synergy between *CARD15* and TLRs (80-83). As opposed to infection with AIEC, which can stimulate monocytes via both *CARD15* and TLRs, no clearly altered response could be found after stimulation with MDP alone. Van Heel et al recently showed that TLR-agonists trigger a stronger response in the presence of *CARD15* stimulation and that this synergistic effect is lost in homozygous carriers of *CARD15* polymorphisms (84, 85).

After binding of (AIEC-derived) MDP and LPS to respectively *CARD15* and TLR4 receptors of *CARD15* mutant monocytes/macrophages, this abrogated *CARD15*-TLR4 synergy can significantly contribute to the observed downregulation of the inflammatory monocyte
response to AIEC. In combination with the reduced expression of antibacterial defensins (as previously described by Wehkamp et al) in CARD15 polymorphism carriers, a reduced secretion of IL-1β and IL-6, as observed in the present study, may in turn via defective neutrophil recruitment and infiltration facilitate the mucosal influx of intestinal bacteria, leading to a secondary inflammatory response (figure 1) (16, 86-89). The lower (and hence more pro-inflammatory) IL-10/TNFα ratio after AIEC infection of these monocytes also directly contributes to this ultimate mucosal inflammation. Whether an increased mucosal influx of intestinal bacteria also leads to the circulation of bacterial antigens towards organs at distance is yet unknown but remains an interesting working hypothesis for future studies. Further research will be focussed upon the correlation of a disturbed immunological response towards AIEC and the mucosal influx and circulation of bacteria or bacterial components with the development of extraintestinal manifestations.

Figure 1. Hypothetical diagram of the functional role of CARD15 polymorphisms in the pathogenesis of Crohn-related mucosal inflammation in the presence of AIEC.

CARD15 polymorphisms also seem to influence humoral response. We found a clear association between the presence of these genetic variants and antibodies directed towards Saccharomyces cerevisiae in CD patients (chapter 6). ASCA IgA and IgG are considered to be specific serological markers for CD, but these antibodies can also be detected in patients with SpA (90-94). In this study, we found the highest ASCA titers in homozygotes and
compound heterozygotes, but also heterozygous carriers of \textit{CARD15} polymorphisms were clearly more ASCA positive compared to wild-type patients. A genetic influence on the origin of these antibodies against baker’s or brewer’s yeast was already suggested by several family and twin studies demonstrating higher ASCA titers in unaffected relatives and twins of CD patients (95-100). A similar association between \textit{CARD15} polymorphisms and ASCA has also been described in another study with similar ethnic background (101).

It is still unclear whether ASCA represent cross-reactive auto-autobodies or whether they are merely the consequence of an aberrant response toward baker’s or brewer’s yeast (102). This uncertainty around the exact role of ASCA in CD (or SpA) makes it difficult to speculate on the mechanism whereby \textit{CARD15} polymorphisms can lead to the formation of these antibodies. Nevertheless, in view of the known association between these genetic variants and an altered immune response towards micro-organisms and bacterial components, a disturbed (secondary serological) response towards (components of) \textit{Saccharomyces cerevisiae} might well be possible.

4. The search for new molecular targets in the association between CD and SpA.

Clinical findings and common immunological alterations in CD and SpA clearly show an association between these two chronic inflammatory disorders (24, 103). Moreover, SpA with subclinical chronic gut inflammation could actually serve as a unique model for studying early CD (2). In order to confirm the association between CD and SpA at the gut transcriptome level, we used macro- and micro-arrays. Gene expression was analyzed in macroscopically non-inflamed colonic biopsies from CD patients, SpA patients (with or without chronic gut inflammation) and healthy controls, in order to detect differentially expressed genes (\textbf{Chapter 7}). First of all, we found that the gut transcriptome in the global SpA population was different from that in healthy controls. Furthermore, SpA patients with subclinical chronic gut inflammation cluster with CD patients, suggesting similarities in genetic expression profiles and corroborating the evidence for the relation between CD and SpA. We presented a set of 95 genes, which were differentially expressed in the gut of CD patients and SpA patients with subclinical chronic gut inflammation.
Since the variation in gene expression can be genetically determined, the search for differentially expressed genes could lead to the discovery of new putative candidate susceptibility or disease-modifying genes (104). Results of transcriptome analysis in CD patients and healthy controls were linked with replicated susceptibility loci for CD, identifying 18 potential candidate genes located within these loci (Chapter 8). We focused upon 2 metallothionein (MT) isoforms MT1F and MT1M, encoded by genes within the IBD1 locus (chromosome 16q) and involved in metal scavenging processes and bactericidal and anti-inflammatory activities (105, 106). Basal MT expression was significantly downregulated in the blood and ileal and colonic biopsies of CD patients with colonic involvement, suggesting that a genetic predisposition is more likely than a secondary inflammatory event. Reduced expression of MT in MT-knockdown colonic epithelial HT29 cells correlated with reduced IL-8 secretion, a potent chemo-attractant and activator of neutrophils, playing an essential role in the inflammatory response. The reduced expression of MT in intestinal epithelial cells and a disturbed IL-8 secretion in response to bacteria might lead to an exaggerated secondary, compensatory immune response. Mutation screening revealed a polymorphism (IVS1-128A>T) within the \textit{MRE-binding transcription factor 1} or \textit{MTF1} gene (IBD7 locus), encoding the main transcription factor regulating MT expression (107). In blood of CD patients and controls, the expression of the transcription factor MTF1 was significantly correlated with the expression of MT1F. The IVS1-128A>T polymorphism had considerable influence on the location of the disease. In particular, the presence of the IVS1-128T allele was associated with ileal disease in a cohort of 222 CD patients and this association was even corroborated in the simultaneous presence of \textit{CARD15} polymorphisms. The correlation between the presence of this \textit{MTF1} polymorphism and the expression of MTF1 and metallothioneins still needs to be investigated further.

\textbf{Summary}

Next to the known clinical and immunological links between CD and SpA, we demonstrate in this work a genetic connection between these two disorders. Carriership of CD-related \textit{CARD15} polymorphisms in SpA patients is associated with the presence of chronic gut inflammation, similar to CD. Whether these genetic variants in CD and SpA also play a direct role in the origin of inflammation at distance of the gut, is not certain. The frequent association between axial involvement, peripheral arthritis and uveitis in CD, suggests however common etiological factors underlying these different extraintestinal manifestations.
In view of the multigenic character of CD and SpA, presumably several genes play a role in the disturbed handling of bacterial flora and the possible circulation of antigens between the intestine and the joint. Further research in both disease entities is mandatory to investigate how bacterial components are transported to extraintestinal organs. Functional study of isolated monocytes of CD patients demonstrated for the first time a disturbed immunological response in single heterozygous carriers of \textit{CARD15} polymorphisms, representing the largest group of mutation carriers. Monocytes of these patients showed an aberrant early cytokine secretion after infection with AIEC, a bacterial strain specifically associated with CD. Moreover, these polymorphisms also seem to induce a disturbed humoral response, reflected by the association with anti-\textit{Saccharomyces cerevisiae} antibodies.

We also confirmed the link between CD and SpA at the transcriptome level. Remarkable similarities were noted in the genetic expression profile of non-inflammatory colonic biopsies of CD patients and SpA patients with chronic intestinal inflammation using array analyses. Finally, we demonstrated that the search for differential gene expression may lead to the identification of new disease-modifying genes with a role in the pathogenesis of the disease. Metallothioneins are substantially down-regulated in CD patients with colonic involvement. Furthermore, we identified a polymorphism in \textit{MTF1}, a gene for the main transcriptional regulator for MT, which also was related with disease location in CD.

In conclusion, this work provides new proof for the close (etiopatho)genetic relationship between CD and SpA, presents new insights in the functional role of CD-associated \textit{CARD15} polymorphisms and describes an alternative approach (via differential gene expression) to identify new potential susceptibility or disease-modifying genes.

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SAMENVATTING
De ziekte van Crohn en spondyloarthopathieën worden beschouwd als onderling verwante, complexe, multigene aandoeningen. Een gestoord immunologisch antwoord op ongekende omgevingsfactoren geeft in genetisch voorbeschikte personen aanleiding tot de ontwikkeling van chronische, ongecontroleerde darm- en/of gewrichtsinflammatie.

Gedurende de laatste decade is er aanzienlijke vooruitgang geboekt in het genetisch onderzoek van beide ziekte-entiteiten. Uitgebreide genoomscreening heeft geleid tot de identificatie van verschillende susceptibiliteitsloci, waarin tal van mogelijke kandidaatgenen verborgen zitten.

Genetische linkage- en associatiestudies droegen vervolgens bij tot de ontdekking van CARD15 op chromosoom 16q als eerste Crohn-gerelateerd gen. Drie CARD15 polymorfismen werden met CD in verband gebracht en komen voor in 30 tot 45% van de CD patiënten. Functionele studies toonden aan dat deze genetische varianten aanleiding geven tot een gestoorde immunologische respons van de gastheercel op bacteriële celwandcomponenten. Een ontregelde mucosale homeostase met verminderde productie van pro-inflammatoire cytokines en anti-bacteriële defensines kan leiden tot een grotere influx en intramucosaal persisteren van intestinale bacteriën of microbiële antigenen met secundaire, chronische darminflammatie tot gevolg. Deze bevindingen bevestigden de rol van een verminderde tolerantie tegen bacteriële flora in de ontwikkeling van deze chronische darmziekte, zoals reeds voordien aangetoond in talrijke dierexperimentele en humane studies.

Het verband tussen HLA-B27 dragerschap en het risico op SpA, meer bepaald AS, is goed gekend. De rol van deze genetische factor is echter veel minder sterk in CD-geassocieerde AS en asymptomatische SI. Recent werden nu ook verschillende non-MHC gerelateerde susceptibiliteitsloci geïdentificeerd in SpA populaties, onder andere op chromosoom 16q.

Meerdere klinische en immunologische gegevens bevestigen de link tussen CD en SpA. Zo kunnen CD patiënten een axiale en/of perifere arthropathie ontwikkelen welke onderdeel uitmaken van het SpA concept. Anderzijds treft men echter bij twee derden van de SpA patiënten ook (subklinische) darminflammatie aan, met gelijkaardige histologische kenmerken als bij CD. De aanwezigheid van chronisch inflammatoire kenmerken in ileum en/of colon van SpA patiënten vormt dan ook een verhoogd risico voor het later ontwikkelen van een klinisch manifeste ziekte van Crohn. Daarnaast werden ook verschillende immunologische
wijzigingen aangetroffen in de intestinale mucosa en synovium van CD en SpA patiënten, welke een (re)circulatie van inflammatoire cellen tussen darm en gewricht doen vermoeden. Het was de bedoeling van dit werk om de rol van genetische alteraties in het verband tussen gewricht en darm in CD en SpA te onderzoeken.

1. **CARD15** polymorfismen predisponeren tot subklinische chronische darminflammatie in patiënten met SpA.

Na de identificatie van een susceptibiliteitslocus op chromosoom 16q voor AS, hebben tal van associatiestudies de rol van CARD15 polymorfismen in SpA en AS nagekeken. Een verhoogde prevalentie van deze genetische varianten kon in deze populaties niet worden aangetoond. Er werd hierbij echter geen rekening gehouden met de aan- of afwezigheid van chronische darminflammatie, nochtans de risicofactor bij uitstek voor het ontwikkelen van CD in deze reumatologische populaties.

In **hoofdstuk 2** tonen we aan dat de aanwezigheid van subklinische chronische darminflammatie in SpA geassocieerd is met CD-gerelateerde polymorfismen in het CARD15 gen. Deze bevinding levert voor het eerst ook genetische argumenten voor het verband tussen CD en SpA en suggereert een potentiële rol voor een gestoorde omgang met intestinale bacteriën of bacteriële bestanddelen in de etiopathogenese van deze chronische reumatische aandoeningen. Reeds lang wordt de hypothese van een mogelijk infectieuze (enterogene) trigger voor het ontstaan van SpA naar voor geschoven. Zowel bacteriële antigenen als DNA werden teruggevonden in het synoviaal vocht van voornamelijk ReA patiënten, doch ook bij patiënten met ongedifferentieerde SpA, AS en PsA. Daarenboven werd dragerschap van *Klebsiella pneumoniae* in de darm ooit als mogelijke risicofactor voor de ontwikkeling van AS naar voor geschoven. Een gestoorde immunologische afweer in de darmmucosa, zoals aangetoond bij CARD15 polymorfismen, zou kunnen leiden tot een verhoogde locale influx van bacteriën en een intestinale oorsprong van artculaire inflammatie, temeer daar inflammatoire cellen en bacteriële antigenen vermoedelijk kunnen circuleren tussen darm en gewricht.

Het merendeel van de SpA patiënten met darminflammatie ontwikkelen geen klinisch manifeste CD. Meteen rijst dan ook de vraag of CARD15 polymorfismen niet eerder met (subklinische) intestinale inflammatie geassocieerd zijn dan met echte CD op zich.
2. De rol van CARD15 polymorfismen in de ontwikkeling van articulaire inflammatie in CD.

De exacte pathogenese van extra-intestinale manifestaties in IBD is nog steeds niet opgehelderd. De circulatie van enterogene bacteriën of antigenen tussen de darm en organen op afstand lijkt echter ook in IBD potentieel belangrijk, mede gezien de recente bevindingen rond de functionele rol van CARD15 polymorfismen. Deze hypothese wordt versterkt door de bevinding dat in ReA, waar enterogene bacteriën een primordiale rol spelen in de ontwikkeling van gewrichtsinflammatie, vaak ook nog andere extra-articulaire en -intestinale haarden van inflammatie worden aangetroffen (huid, conjunctiva, iris, urethra en hart).


In hoofdstuk 3 onderzochten we de associatie van CARD15 polymorfismen met verschillende reumatologische manifestaties van CD. Deze studie toonde een hogere prevalentie van radiologische sacroilitis in CD patiënten met CARD15 polymorfismen. Conform vroegere associatiestudies, vonden we geen verband met perifere artritis. De mogelijke link tussen deze genetische varianten en axiale inflammatie komt ook tot uiting bij SpA patiënten. Chronische darminflammatie is bij deze patiënten, zoals in de vorige studie aangetoond, geassocieerd met CARD15 polymorfismen en komt duidelijk het meest voor bij patiënten met axiale aantasting. Bij niet-AS SpA patiënten vormt de aanwezigheid van chronische ileitis en/of colitis zelfs een belangrijke risicofactor voor de latere evolutie naar AS.

Gegevens over de associatie van radiologische sacroilitis met specifieke fenotypes van CD zijn schaars. Bijgevolg werd in samenwerking met Leuven en Luik een multicentrische studie rond deze frequentie extra-intestinale manifestatie opgezet, waarbij gekeken werd naar eventueel geassocieerde klinische kenmerken en het verband met CARD15 varianten (hoofdstuk 4). We vonden een verband tussen de aanwezigheid van radiologische SI en het
ontwikkelen van perifere artritis. Daarenboven hadden CD patiënten met klinische AS eveneens een verhoogde kans op het doormaken van uveitis. Deze gegevens suggereren gemeenschappelijke kenmerken in het ontstaan van deze extra-intestinale manifestaties.

In deze 2de studie kon het verband tussen CARD15 polymorfismen en sacroiliitis weliswaar niet worden bevestigd. Reproduceerbaarheid van genetische associatiestudies blijkt een aanzienlijk probleem in het huidig genetisch onderzoek van complexe, multigene aandoeningen. Talrijke factoren, zoals genetische heterogeniteit, statistische power, omgevingsfactoren, inhomogene populaties, gebrek aan kwaliteitscontrole op geno- en fenotypering evenals de keuze van statistische modellen kunnen leiden tot inconsistentie in de resultaten tussen studies. Ook in CARD15 associatiestudies werden frequent tegenstrijdige bevindingen beschreven zelfs in etnisch gelijkardige populaties.


Onafgezien van de inconsistente resultaten over het verband tussen sacro-iliitis en CARD15 polymorfismen, blijft de genetisch voorbeschikte circulatie van inflammatoire cellen en onvoldoende geklaarde micro-organismen of bacteriële componenten, als oorzaak van inflammatie op afstand van de darm een interessante werkhypothese in zowel IBD als SpA. Gezien het multigene karakter van beide ziektebeelden spelen vermoedelijk meerdere genetische defecten een rol in de gestoorde omgang met de intestinale flora en het transport van bacteriële antigenen naar andere organen. Het is evenwel nog onduidelijk hoe componenten van de bacteriële flora eventueel vanuit de intestinale mucosa via de bloedbaan naar organen op afstand worden getransporteerd.


In hoofdstuk 5 onderzochten we in CD patiënten de rol van *CARD15* polymorfismen op de respons van perifere bloedmonocyten tegen adherent-invasieve *E. coli* (AIEC). Deze bacteriële streng, specifiek met CD geassocieerd, werd geïsoleerd uit ileum van CD patiënten en is in staat in de gastheercel binnen te dringen, te overleven en zelfs te vermenigvuldigen. Na infectie van de monocyten met deze AIEC, werd een gestoorde vroegtijdige cytokinesecretie (IL-1β, IL-6 en IL-10) waargenomen in patiënten met *CARD15* polymorfismen. Hoewel het effect meest uitgesproken was in homozygoten, kon voor het eerst ook een functioneel defect worden aangetoond in de (veel frequenter) heterozygote dragers van deze genetische varianten. Verder onderzoek zal moeten uitwijzen of dit vroeg gestoord immunologisch antwoord ook kan worden aangetroffen in andere celtypes (intestinale macrofagen, dendritische cellen en epitheelcellen) en na infectie met andere, al dan niet invasieve bacteriën.

Deze studie weerspiegelt ook het belang van synergie tussen *CARD15* en TLRs. In tegenstelling tot AIEC, die monocyten kunnen stimuleren via zowel *CARD15* als TLRs, kon geen duidelijk gestoorde respons worden teruggevonden na stimulatie met MDP alleen. Van Heel et al toonden recent aan dat TLR-agonisten een sterkere respons uitlokken in aanwezigheid van *CARD15* stimulatie en dat dit synergistisch effect verdwijnt bij homozygote dragers van *CARD15* polymorfismen.

Na binding van het MDP en LPS van de AIEC bacterie aan respectievelijk *CARD15*- en TLR4 receptoren in monocyten/macrofagen met een mutant *CARD15*-gen, kan het ontbreken
van CARD15-TLR4 synergie in aanzienlijke mate bijdragen tot de geobserveerde downregulatie van de inflammatoire respons van deze monocyten op dit micro-organisme. Samen met de gedaalde expressie van antibacteriële defensines in dragers van *CARD15* polymorfismen (beschreven door Wehkamp et al), kan een verminderde secretie van IL-1β en IL6, zoals waargenomen in de huidige studie, via een deficiënte recrutering en infiltratie van neutrofielen leiden tot een verhoogde mucosale influx van intestinale batteriën met een secundaire, inflammatoire respons tot gevolg. De lagere (en dus meer pro-inflammatoire) IL-10/TNFα ratio na infectie van deze monocyten met AIEC draagt dan eveneens rechtstreeks bij tot de uiteindelijke mucosale ontstekingsreactie. Of een toegenomen mucosale influx van intestinale batteriën ook leidt tot de circulatie van batteriële antigenen naar organen op afstand is nog onduidelijk maar vormt een interessante werkhypothese voor toekomstige studies. Verder onderzoek zal zich toespitsen op de correlatie van een gestoorde immunologische respons tegen AIEC en de mucosale influx en circulatie van batteriën of batteriële bestanddelen met het ontwikkelen van extra-intestinale manifestaties.

*CARD15* polymorfismen lijken echter ook een invloed te hebben op de humorale respons. We vonden een duidelijk verband tussen de aanwezigheid van deze genetische varianten en antilichamen tegen *Saccharomyces cerevisiae* in patiënten met CD (Hoofdstuk 6). ASCA IgA en/of IgG worden beschouwd als specifieke serologische merkers voor CD doch deze antilichamen worden ook aangetroffen bij patiënten met SpA. In deze studie vonden we de hoogste ASCA titers bij homozygoten en compound heterozygoten, maar ook heterozygote dragers van *CARD15* polymorfismen waren duidelijk meer ASCA positief vergeleken met wild-type patiënten. Een genetische invloed op het ontstaan van deze antilichamen tegen bakkers- of brouwersgist werd vroeger reeds gesuggereerd door verschillende familie- en tweelingstudies, die in onaangegetaste verwanten en tweelingen van CD patiënten hogere ASCA titers beschreven. Een gelijkaardig verband tussen *CARD15* polymorfismen en ASCA werd ook in een andere studie met gelijkaardige ethnische achtergrond beschreven.

Het is tot op heden onduidelijk of ASCA kruisreactieve auto-antilichamen vertegenwoordigen dan wel het gevolg zijn van een aberrant antwoord tegen bakkers- of brouwersgist. Deze onzekerheid over de exacte rol van ASCA in CD (of SpA) maakt speculatie omtrent het mechanisme waarbij *CARD15* polymorfismen leiden tot de vorming van deze antilichamen moeilijk. Niettemin lijkt, gezien het verband tussen deze genetische varianten en een gestoorde immuunrespons tegen micro-organismen en batteriële componenten, een gestoord
(secundair serologisch) antwoord tegen (componenten van) *Saccharomyces cerevisiae* zeker mogelijk.

4. Onderzoek naar nieuwe moleculaire targets in het verband tussen CD en SpA.


Gezien variatie in genetische expressie ook genetisch bepaald kan zijn, kan de zoektocht naar differentieel tot expressie gebrachte genen leiden tot de ontdekking van mogelijk nieuwe kandidaattgenen met een rol in de pathogenese of het ziekteverloop van deze aandoeningen. Resultaten van transcriptoanalyses in CD patiënten en gezonde controles werden gelinkt met gereguleerde susceptibiliteitsloci voor CD. Aldus werden 18 potentiële kandidaattgenen binnen deze loci geïdentificeerd (Hoofdstuk 8). De aandacht werd toegespitst op 2 metallothioneine (MT) isovormen MT1F en MT1M, gecodeerd door genen in de IBD1-locus (chromosoom 16q) en betrokken bij “metal scavenging” processen en bactericide en anti-inflammatoire activiteiten. De basale MT expressie was significant gedaald in het bloed en ileum- en colonbiopten van CD patiënten met colonaantasting, suggestief voor een genetische predispositie eerder dan inflammatie als oorzaak. Een verminderde expressie van MT in MT-knockdown colonepitheel HT29 cellen correleerde met een verlaagde secreet van IL-8, een sterk chemo-attractant voor en activator van neutrofielen, welke een essentiële rol vervullen in de inflammatoire respons. De gedaalde expressie van MT in intestinale epitheelcellen en de
gestoorde IL-8 secretie in het antwoord op bacteriën kan leiden tot een overdreven secundaire, compensatoire immunologische respons. Mutatiescreening bracht een polymorfisme (IVS1-128A>T) in het MRE-binding transcription factor 1 of MTF1 gen (IBD7 locus) aan het licht. Dit gen codeert voor de belangrijkste transcriptiefactor in de regeling van MT expressie. In bloed van CD patiënten en controles was de expressie van de transcriptiefactor MTF1 significant gecorreleerd met de expressie va MT1F. Het IVS1-128A>T polymorfisme had een belangrijke invloed op de localisatie van de ziekte. De aanwezigheid van het IVS1-128T allel was specifiek geassocieerd met ileale aantasting in een cohorte van 222 CD patiënten en dit verband werd zelfs nog versterkt in de gelijktijdige aanwezigheid van CARD15 polymorfismen. De correlatie tussen de aanwezigheid van dit MTF1 polymorfisme en de expressie van MTF1 en metallothioneines dient echter nog verder te worden onderzocht.

Globale samenvatting
In dit werk tonen we aan dat er, naast klinische en immunologische links, ook een genetisch verband bestaat tussen CD en SpA. Dragerschap van Crohn-gerelateerde CARD15 polymorfismen is ook in SpA patiënten geassocieerd met de aanwezigheid van chronische darminflammatie. Of deze genetische varianten in CD en SpA ook rechtstreeks een rol spelen in het ontstaan van inflammatie op afstand van de darm, staat niet vast. Het frequent samengaan van axiale aantasting, perifere artritis en uveitis in CD patiënten geeft wel aan dat gemeenschappelijke etiologische factoren aan de basis liggen van verschillende extraintestinale manifestaties. Gezien het multigene karakter van CD en SpA spelen vermoedelijk meerdere genen een rol in een gestoorde omgang met bacteriële flora en het mogelijk circuleren van antigenen tussen darm en gewricht. Verder onderzoek in beide ziektebeelden is onontbeerlijk om na te gaan hoe bacteriële componenten naar extra-intestinale organen worden getransporteerd.

Functioneel onderzoek in geïsoleerde monocyten van CD patiënten toont voor het eerst aan dat ook in heterozygote dragers van CARD15 varianten, de grootste groep van mutatiedragers, een gestoorde immunologische respons kan worden genoteerd. Monocyten van dergelijke patiënten vertonen een aberrante vroegtijdige cytokinesecretie na infectie met AIEC, een bacteriële streng die specifiek met CD werd geassocieerd. Bovendien blijken deze polymorfismen ook een gestoord humoraal antwoord te induceren, zoals blijkt uit de associatie met anti-Saccharomyces cerevisiae antilichamen.

Het verband tussen CD en SpA werd verder eveneens op transcriptoomniveau bevestigd. Gebruik makend van array analyses, werden opvallende gelijkenissen opgemerkt in het
genetische expressieprofiel van niet-inflammatoire colonbiopten in CD patiënten en SpA patiënten met chronische darminflammatie. We toonden ook aan dat de zoektocht naar differentieel tot expressie gebrachte genen kan leiden tot de identificatie van nieuwe kandidaagenen met een rol in de pathogenese en het ziekteverloop. De expressie van metallothioneines is aanzienlijk verlaagd in CD-patiënten met colonaanstasting. Verder werd in CD een polymorfisme geïdentificeerd in MTF1, het gen voor de belangrijkste transcriptionele regulator van MT, dat eveneens met de ziekte-localisatie in verband stond. Samengevat kunnen we stellen dat dit werk nieuw bewijs levert voor de (etiopatho)genetische link tussen CD en SpA, nieuwe inzichten brengt in de functionele rol van CD-geassocieerde CARD15 polymorfismen en een alternatieve benadering beschrijft om (via differentiële genexpressie) nieuwe, potentiële kandidaat susceptibiliteits- of “disease-modifying” genen te identificeren.
Curriculum Vitae

Harald Peeters was born on March 8th 1972 in Veurne, Belgium. He obtained his degree of medical doctor magna cum laude in 1998 at the Ghent University. He started training in internal medicine in October 1998 at AZ Sint-Lucas, Gent, followed by one year at AZ Maria Middelares, Gent, and a third year at the Ghent University Hospital. In 2001, he started his spezialisation for gastroenterologist with a four year period of scientific research under the promotership of Prof. Dr. M. De Vos and co-promotership of Prof. Dr. F. De Keyser, resulting in the thesis presented here. For this work he was supported by a concerted action grant GOA2001/12051501 of the Ghent University. Since October 2005 he continues his clinical training in gastroenterology which will end in September 2006.

The author is recipient of research grants of the Flemish Society of Crohn’s Disease and Ulcerative Colitis (CCV) (2002) and the Flemish Society of Gastroenterology (VVGE) (2002) and received the “Inflammatory Bowel Disease Prize” at the Belgian Week of Gastroenterology in 2004. Since 2003 he is an active member of the Belgian IBD Research Group.

List of publications

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Abstracts


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