GENETIC AND MOLECULAR DETERMINANTS IN INFLAMMATORY BOWEL DISEASE

Francesca Bresso

Stockholm 2006
To Aste

Μηδέν ἄγαν
Temple of Apollo, Delphi.
ABSTRACT

Inflammatory Bowel Disease, IBD, are polygenic disorders that present itself to the clinician as a sum of interacting events arising from multiple factors of genetic, immunological and environmental origin, resulting in a chronic relapsing inflammation that manifests itself in two major forms, ulcerative colitis (UC) and Crohn’s disease (CD). Shortage of reliable biomarkers for correct diagnosis and thus appropriate treatment regime is still a major problem for the clinically active physician. The disclosure of CARD15/NOD2 as a susceptibility gene for CD, represents, therefore, an important observation towards a better understanding of the pathogenesis of IBD and its integrated diagnosis. The objective of my thesis was to further our understanding of the patho-physiology of IBD. In paper I, the contribution of CARD15/NOD2 polymorphisms in explaining concordance of Crohn’s disease in monozygotic twins was evaluated. Although total allele frequency of these mutations was higher in concordant twin pairs compared to discordant pairs, statistical significance was not observed. Thus, other CARD15/NOD2 polymorphisms or additional genes are likely to contribute to the disease. In paper II, we assessed polymorphisms in the CARD15/NOD2 gene and in the TNFα promoter, in order to explain variation in individual disease phenotypes. The results indicate that certain CARD15/TNFα allelic combinations can affect TNFα gene expression, which potentially can contribute to interindividual variation in susceptibility to, and manifestation of, IBD. One trademark of IBD is disruption of the intestinal barrier homeostasis. In paper III, we performed a population genetic study and evaluated whether the ABC transporter cystic fibrosis transmembrane conductance regulator (CFTR), known to be a recognition receptor for bacteria, could be a putative candidate gene in IBD. While the ΔF508 CFTR heterozygosity is markedly underrepresented in Crohn's disease patients from Italy and Sweden, stratification for disease location revealed an absence of ΔF508 carriers among Scottish CD patients with right-sided colitis. Our data are in line with the possibility that a mutated CFTR may exhibit a protective role in CD. In some instances, IBD may progress to colorectal cancer. It was therefore of great interest to learn that the bile acid ursodeoxycholic acid (UDCA) was reported to reduce preneoplastic lesions in IBD patients. In paper IV, microarray analysis was performed on a colon epithelial cell line stimulated with UDCA to identify target genes. A cluster of UDCA regulated genes was identified and one gene, the NSAID-activated gene-1 (NAG-1), a divergent member of the TGFβ superfamily is of particular interest. Complementary experimental data support that NAG-1 may take part in the UDCA mediated antiproliferative effect. In conclusion, my work adds new information of our understanding of IBD and may help along to develop better and accurate diagnosis and in improving treatment regimens of IBD related preneoplastic lesions.
LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:


III. Francesca Bresso, Johan Askling, Marco Astegiano, Brunello Demarchi, Nicoletta Sapone, Mario Rizzetto, Paolo Gionchetti, Karen M. Lammers, Annalisa de Leone, Gabriele Riegler, Elaine R. Nimmo, Hazel Drummond, Colin Noble, Leif Torkvist, Anders Ekborn, Marco Zucchelli, Robert Löfberg, Jack Satsangi, Sven Pettersson, and Mauro D’Amato. A potential role for the common cystic fibrosis ΔF508 mutation in Crohn’s disease. Accepted for publication in Inflammatory Bowel Diseases, 2006

IV. Francesca Bresso, Esther Edlundh-Rose, Mauro D’Amato, Alexandra Are, Gedimia Grecius, Agne Linden, Urban Sjöqvist, Joackim Lundeberg, Velmurugesan Arulampalam, Robert Löfberg, and Sven Pettersson. Ursodeoxycholic acid (UDCA) mediated cell cycle arrest of adenocarcinoma cells likely operates through induction of NAG-1. Manuscript
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<th>Full Form</th>
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<tbody>
<tr>
<td>5-ASA</td>
<td>5-aminosalicylate</td>
</tr>
<tr>
<td>APC</td>
<td>Adenomatous polyposis of the colon</td>
</tr>
<tr>
<td>ACF</td>
<td>Atypical crypt foci</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CAC</td>
<td>Colitis associated colorectal cancer</td>
</tr>
<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>DCA</td>
<td>Deoxycholic acid</td>
</tr>
<tr>
<td>DLG</td>
<td>Discs large, drosophila, homolog of</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine rich repeat</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-Inflammatory drug</td>
</tr>
<tr>
<td>NAG-1</td>
<td>Non-steroidal anti-Inflammatory drug activated gene-1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ B</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>PSC</td>
<td>Primary sclerosing cholangitis</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute carrier</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>UDCA</td>
<td>Ursodeoxycholic acid</td>
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</table>
1 INTRODUCTION
1.1 CLINICAL CLASSIFICATION AND DISEASE FEATURES IN IBD: WHERE ARE WE?

Clinical descriptions of acute and chronic diarrhoea with or without blood go back thousands of years (Hippokrates of Kos c.460-c.370 BC). However, we had to wait until the 15th century for the first description of chronic inflammatory diarrhoea, when the hospital of S. Maria Nuova in Florence allowed dissections not only by Leonardo da Vinci but also by Antonio Beniviene (1443–1502). In his 15 autopsies, published posthumously, he compared anatomopathological findings with the clinical course of his deceased patients. “XCV” had “gripes in the intestines, called by the Greeks dysenteria . . . apt to ulcerate the lining of the intestines and thus the excrement comes down bloodstained and mucous.” Both “XCV” and “XCVI,” with similar symptoms, and in addition wasting and fatality with “entails . . . internally eroded,” may have had chronic inflammatory bowel disease. In 1761, Morgagni reported the history of a young man prone to attacks of diarrhoea for decades, with fever and a rectal abscess that discharged spontaneously who had “mesenteric lymphadenopathy . . . erosions, ulceration and perforation of the extremity of the ileum and the nearest point of the colon to the extent of two hands breadth”, very much close to a detailed description of Crohn’s disease, although typhoid can not be excluded.

Throughout the 19th century, there was increasing recognition that there were non-infective causes of colonic ulceration. Allchin, in 1885, reported a typical clinical and autopsy case of “follicular ulceration of the colon”. He pleaded that “the term dysentery . . . should not at once be applied in an adjective form to any diarrhoea depending upon ulceration of the colon, when the factors for the production of the specific disease are, so far as we can recognize, wanting”. In the early 1900s, Lockhart-Mummery, surgeon at St. Mark’s hospital in London stated quite simply, “The most important advance in our knowledge of these cases has been due to the invention of the electric sigmoidoscope.” The first complete description of ulcerative colitis, indeed, was given by Sir Arthur Hurst, with description of the associated sigmoidoscopic appearances. Nevertheless, he considered ulcerative colitis to be primarily infective dysentery in which other factors had occurred secondarily, thus establishing a chronic disease process. In the same years, the landmark publication of Crohn, Ginzburg, and Oppenheimer called attention to “terminal ileitis” as a distinct and chronic entity. “Regional enteritis” and “granulomatous enterocolitis” defined the segmentarity of the disease, the involvement of disparate site of the gastrointestinal tract, including small and large bowel, and the presence of granuloma. In the end, Crohn’s disease was adopted to encompass the many clinical presentations of this pathologic entity. (Citations referred in Roman numerals at the end of the references)
Despite several years of advances in treatment, pathogenesis of inflammatory bowel disease (IBD) is not completely understood, as reflected by a lack of adequate clinical classification of diagnosis and phenotypes. IBD is, indeed, still defined as idiopathic, chronic, relapsing, inflammatory disorders of the gastrointestinal tract, traditionally classified as UC (UC) and Crohn’s disease (CD), with approximately 10% of patients having indeterminate colitis (Figure 1).\(^1\) Appreciation of disease course and clinical phenotypes, as well as developments in genetics have led to an understanding that IBD may not simply be UC or CD, but rather a heterogeneous group of diseases precipitated by a complex interaction of environmental, genetic, and immunoregulatory factors.\(^2\) During the last few years, several classifications have been suggested for the identification of phenotypic subgroups.\(^3-5\) The Montreal classification was recently introduced as a revision of the previous ones. Although considering age of onset, disease location, and disease behaviour as the predominant phenotypic elements, this classification slightly increased flexibility in grouping disease locations and in the timing of phenotype definition (Table 1).\(^5\) For the first time, Montreal Working Party proposed a subclassification system for UC as well, incorporating assessment of disease extent and severity of an individual relapse of disease, of fundamental importance in the decision of therapeutic strategy. However, the needs for a classification of longitudinal disease progression, or disease behaviour over time, that is, the frequency of disease relapse and course of disease during the natural history, and the risk for malignant transformation, are clearly still unmet. Indeed, genetic and molecular determinants of the diseases, eventually based on pathogenetic mechanisms, would be of critical importance in integrating present clinical classifications, defining IBD heterogeneity, contributing in the identification of IBD different phenotypes and in the delineation of individualized treatments.
Table 1: Montreal Classification for Crohn’s disease, extent and severity of ulcerative colitis

<table>
<thead>
<tr>
<th>Crohn’s disease</th>
<th>Ulcerative colitis</th>
<th>Ulcerative colitis</th>
<th>Ulcerative colitis</th>
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<tbody>
<tr>
<td></td>
<td>Extent</td>
<td>Anatomy</td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis</td>
<td>E1 Ulcerative proctitis</td>
<td>Involvement limited to the rectum</td>
<td></td>
</tr>
<tr>
<td>A1 below 16 y</td>
<td>E2 Left sided UC (distal UC)</td>
<td>Involvement limited to a proportion of the colorectum distal to the splenic flexure</td>
<td></td>
</tr>
<tr>
<td>A2 between 17 and 40 y</td>
<td>E3 Extensive UC (pancolitis)</td>
<td>Involvement extends proximal to the splenic flexure</td>
<td></td>
</tr>
<tr>
<td>A3 above 40 y</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1 ileal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2 colonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L3 ileocolonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4 isolated upper disease*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Behaviour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 non-stricturing, non-penetrating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2 stricturing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B3 penetrating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p perianal disease modifier</td>
<td></td>
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</tbody>
</table>

*L4 can be added to L1–L3
*p” is added to B1–B3 when concomitant perianal disease is present.

**1.2 GENETICS: NEW INSIGHT IN IBD PATHOGENESIS**

We believe that genetic epidemiology and functional genomics represent a turning point in the understanding of the pathogenesis of IBD and may provide novel biomarkers able to integrate the existing clinical classifications. Evidence for the role of genetic factors in the development of IBD has come from epidemiological data, which include ethnic and racial differences in disease prevalence, familial aggregation, twin studies, and association with other syndromes resembling IBD or other diseases with a recognizable genetic susceptibility. Population-based studies demonstrated that approximately 5–10% of all individuals affected with IBD report a family history, which indicates that having other family members with the disease strongly influences the development of IBD. First-degree relatives of affected individuals show approximately 20–50-fold and 10–20-fold increased risk of developing CD and UC compared with the general population, respectively. Moreover, the affected siblings frequently present at similar ages and concordance rates, reaching up to 80% for disease site, behaviour and presence of extraintestinal manifestations. A total of 75% of multiply affected families with IBD appear concordant for disease type (i.e., either all affected patients within a family have CD or all have UC), with the
remaining 25% being mixed (i.e., having one member with CD and another with UC), which is consistent with a model of disease pathogenesis involving multiple susceptibility genes, some of which are common to both CD and UC, while others are separately linked to one disease or the other. An earlier age of onset for normal cases of IBD compared with those cases in which no known family history exists was observed.6-8

Twins studies are a powerful tool to disentangle the relative contribution of genetic and environmental factors in complex diseases. A significantly higher concordance rate among monozygotic than among dizygotic twin pairs has been observed. Monozygotic twin concordance in CD was reported between 42 and 58%, whereas dizygotic twin concordance was not significantly different from that for all siblings. The monozygotic and dizygotic concordance for UC was lower, ranging from 6–17% and 0–5%, respectively, suggesting that the genetic contribution is weaker, although present, in UC compared with CD. 9-11

The development of a linkage map of the human genome with informative microsatellite markers has enabled hypothesis-free scanning of the human genome for loci associated with susceptibility to simple monogenic and polygenic disease. Many susceptibility loci have been implicated in inflammatory bowel disease, with varying degrees of replication and statistical support. Nine loci, termed IBD 1–9, have been replicated. Whereas some of them seem specific to CD (e.g., IBD1 on 16q-OMIM 266600) or UC (e.g., IBD2 on 12q-OMIM 601458), others seem to confer susceptibility to inflammatory bowel disease overall (e.g., IBD3 6p-OMIM 604519).12

1.2.1 IBD susceptibility genes

By positional cloning and candidate gene approach, a number of IBD susceptibility genes have been recently identified. Some of these genes, coding for intracellular bacterial receptors such as the NODs (CARD1513, 14 and, more recently, CARD415), scaffolding proteins involved in epithelial integrity (DLG516), epithelial transporters (SLC22A4/517 and MDR118, 19) and pattern recognition molecules such as TLRs, are involved in host-microbe homeostasis and maintenance of intestinal barrier (Table 2).20 These findings, together with animal model of IBD where bowel inflammation does not develop in germ-free conditions, support the hypothesis that IBD is the result of an abnormal immune response toward commensal bacteria in genetically susceptible individuals.21

Table 2: Intestinal barrier and IBD susceptibility genes

<table>
<thead>
<tr>
<th>IBD susceptibility genes</th>
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<tbody>
<tr>
<td>CARD15/NOD2</td>
</tr>
<tr>
<td>CARD4/NOD1</td>
</tr>
<tr>
<td>TLRs</td>
</tr>
<tr>
<td>DLG5</td>
</tr>
<tr>
<td>OCTN1/OCTN2</td>
</tr>
<tr>
<td>MDR1</td>
</tr>
<tr>
<td>CFTR</td>
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</tbody>
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4
1.2.1.1 CARD15/NOD2: the IBD gene

In 2001, by means of positional cloning and candidate gene approach, NOD2/CARD15 (nucleotide-binding oligomerization domain 2/caspase recruitment domain family member 15), corresponding to the IBD1 locus on chromosome 16, was discovered as the first gene to be associated with CD. The 3 most common variants, and the most studied, are two missense mutations, Arg702Trp and Gly908Arg, and a frameshift mutation Leu1007fsinsC (Figure 3). Of the patients with CD, 10–30% are heterozygous and 3–15% homozygous or compound heterozygous for the common CARD15 mutations; the corresponding proportions from the control population are 8–15% and 0–1% respectively. The three mutations seem to have differing effects on the risk of developing the disease, in particular, for carriers of Leu1007fsinsC, Gly908Arg, and Arg702Trp, risk was increased by a factor of four, three, or two, respectively, whereas the risk for simple homozygote varied between thirty-eight and forty-four. Furthermore, the contribution of CARD15 (as defined by the three most common sequence variants) to disease susceptibility shows clear ethnic variation, being much lower in northern Europe than elsewhere in Europe, and being not at all a feature of CD in Japanese and Asian populations. Most studies also show association between CARD15 mutations and CD phenotype. Within the range of clinical presentations of CD, phenotypic associations that involve the ileum are the best replicated; furthermore, double-dose carriers are unlikely to have purely colonic CD. The difficulty in interpreting results of individual studies stems from differing phenotypic definitions and the instability of CD phenotype over time.

NOD2 belongs to the phylogenetically conserved Nod-like receptors (NLR) protein family (Figure 2). While mutations within the nucleotide-binding domain have been associated with two other granulomatous diseases: Blau's syndrome and early-onset sarcoidosis, most disease-associated CARD15 mutations in CD affect the leucine-rich region (LRR) of the gene, and therefore the recognition of its ligand. NOD2 functions as an intracellular receptor for muramyl dipeptide (MDP), a component of peptidoglycan that is present in cell walls of gram positive and negative bacteria. NOD2 is expressed in antigen presenting cells such as macrophages and dendritic cells and in intestinal epithelial cells, where expression at protein level is low or undetectable. The expression of NOD2 is regulated by bacterial flagellin interacting with TLR5 and by pro-inflammatory cytokines, such as TNF. NF-κB-binding sites in the CARD15 promoter are involved in the response to TNF stimulation. That means that, when NOD2, previous activation by MDP, activates NF-κB and induces TNF, NOD2 can upregulate itself, in a positive feedback. Within the cells of the intestinal epithelium, the greatest concentration of CARD15 mRNA has been recorded in the Paneth cells of the small intestine. This finding could have important implications for disease pathogenesis, since the greatest numbers of Paneth cells are found in highest concentrations within the ileum, and CARD15 mutations are associated with ileal CD. In addition, Paneth cells synthesise and secrete several antibacterial proteins, notably the defensins. Patients with ileal CD have an α-defensin deficiency, which is most pronounced in those carrying CARD15 variants. Moreover, cryptidins, mouse correspondent to α-defensins in human, are very low in Nod2−/− mice, as well. These finding provides a coherent theory whereby Paneth cells (which express both CARD15...
and α-defensins) could be the common link for the occurrence of CD of the ileum. Studies in human epithelial cells, disease-related CARD15 mutations are associated with reduced NFκB activation in transfection studies, and decreased capacity to restrict proliferation of Salmonella enterica serovar typhimurium in monolayer culture. However, CARD15 mechanism of action is not completely clear and current data from mouse and human studies remain discrepant. Several other components of the innate immune pathway interact with and modify CARD15 function, such as GRIM-19, NEMO, and RIPK2, and many other not yet identified.

**Figure 2: CARD15/NOD2**

Like other members of this family, NOD2 presents a tripartite domain structure that consists of the following: a carboxy-terminal LRR domain, which is involved in ligand recognition; a central NOD (nucleotide-binding oligomerisation domain), which facilitates self-oligomerization and has ATPase activity; and two amino-terminal domains (CARDs- caspase activation and recruitment domain) that are composed of protein–protein interaction cassettes, and that are responsible for activation of NF-κB and/or caspases (modified from Hugot et al.)

1.2.1.2 Other susceptibility genes involved in intestinal barrier homeostasis

**NOD1/CARD4** maps within a region of previously defined IBD linkage on chromosome 7p14.3, confirmed by both subsequent genome wide scans and a genome scan meta-analysis. The effect is reported to influence susceptibility to IBD overall rather than to UC or CD specifically. Interestingly, allelic variation in the same single nucleotide polymorphism confers susceptibility to asthma, another chronic barrier disease. NOD1 has structural similarity to NOD2, but NOD1 ligand is the peptidoglycan derived peptides γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP). Like NOD2, NOD1 is expressed in large and small bowel, plays a role in up-regulation of the pro-inflammatory transcription factor NF-κB and regulates mucosal barrier function and bacterial killing.

The IBD5 region on chromosome 5q31 is one of only two loci widely confirmed to be associated with CD in multiple independent cohorts. Although many populations have demonstrated association with IBD5, there remains uncertainty as to the causal variant within the region. A recent report identified polymorphisms in SLC22A4 (OCTN1) and SLC22A5 (OCTN2), carnitine and organic cations transporters, as being responsible for the IBD5 association.

Stoll et al. refined the previously defined linkage region on 10q23 and used positional cloning to identify genetic variants in DLG5 associated with IBD. Stratifying the study sample according to the presence of risk-associated variants of CARD15, a
significant difference in association of the 113A variant of DLG5 with CD in affected individuals carrying the risk-associated CARD15 alleles versus those carrying non-risk-associated CARD15 alleles was found. This suggested a complex pattern of gene-gene interaction between DLG5 and CARD15, reflecting the complex nature of polygenic diseases. DLG5 is a scaffolding protein with multiple protein interaction domains that has been reported to be involved in maintaining cell shape and polarity as well as located at sites of cell–cell contact.\(^{61}\) It can link the vinexin–vinculin complex and beta-catenin at sites of cell-cell contact and therefore may play an important role in stabilizing epithelial barrier.\(^{62, 63}\)

The ATP-binding cassette, subfamily B, member 1 (\(ABCB1\)) gene (also known as the multidrug resistance 1 (\(MDR1\)) gene), which encodes P-glycoprotein 170, is located on chromosome 7q21.1, in a region for which there is evidence for linkage from the index UK genome-wide scan.\(^{45}\) P-glycoprotein 170 functions as an ATP-dependent efflux transporter pump and is widely expressed on epithelial surfaces, in particular in the gut, where high constitutive expression is noted.\(^{64}\) Although the physiological function of the protein remains controversial, it seems most likely to have a role in protection of the epithelium against xenobiotics, which is consistent with the theory that gene-bacterial interaction is at the centre of disease pathogenesis.\(^{65}\) The \(ABCB1\) knockout mouse model spontaneously develops enterocolitis when specific pathogens are excluded, although not in germ-free conditions.\(^{66}\) In both CD and UC, microarray data has shown that detoxification genes, ATP-binding cassette transporters (including \(ABCB1\)), are strongly downregulated in unaffected colonic tissue.\(^{67}\) The \(MDR1\) exonic single nucleotide polymorphisms C3435T and G2677T have been shown to correlate with activity/expression of P-glycoprotein 170.\(^{18, 19, 65, 68-72}\) There is now a wealth of genetic studies investigating these candidate polymorphisms in IBD, with the overall trend toward increased susceptibility to UC. Furthermore, both protective and susceptible haplotypes have been shown, suggesting that \(ABCB1\) controls disease susceptibility in a bidirectional way.\(^{71, 72}\) Moreover, phenotypic associations with disease extent are reported.\(^{18, 19, 65, 68-72}\)

The TLR class of receptors has a key role in maintenance of epithelial homoeostasis in the gut. Mice deficient in TLR signalling are more susceptible than wild type mice to colitis induced by DSS. Lipopolysaccharide—the major component of the outer membrane of gram negative bacteria—binds to TLR4, which is expressed on intestinal epithelial cells, and its expression is increased in people with IBD, although whether as cause or effect is uncertain. Mutations in TLR4, TLR2, TLR1 and TLR6 have been observed to be associated with IBD, although not in all the studies.\(^{73-76}\) TLR5 specifically recognises the pathogen-associated molecular pattern flagellin, a common antigen present on most motile bacteria in the gut. A strong serological response to flagellin is reported in several animal models of colitis and colitis can be induced by transferring flagellin-specific T cells to immunodeficient animals. Preliminary data suggest that carriage of a dominant negative TLR5 polymorphism (TLR5-stop) seems to protect against CD and results in a substantial decrease in flagellin-specific IgA and IgG.\(^{75-77}\)
1.2.1.3  Cystic fibrosis transmembrane conductance regulator: novel IBD gene?

The common CARD15 variants and the other mentioned genes contribute only a small part to the overall genetic susceptibility to CD. Other genetic or environmental factors, or both, are needed for full disease penetrance. The cystic fibrosis transmembrane conductance regulator (CFTR) might be a novel candidate gene for IBD.

CFTR is the product of the gene (chr.7q31.2) mutated in patients with cystic fibrosis (CF), and this lethal genetic disease affects 1 in every 2500 Caucasians in the United States. CFTR is an integral membrane glycoprotein composed of 1480 amino acids, primarily localized to the lumen-facing, or apical, membranes of epithelial cells in the airway, intestine, reproductive tissues, and exocrine glands. It functions as a cAMP-regulated Cl− channel that is responsible for transepithelial salt and water transport (Figure 3). As its name implies, CFTR also acts as a conductance regulator, exerting modulatory influences over a plethora of other ion channels, transport proteins, and processes critical in the maintenance of epithelial barrier homeostasis, such as mucins secretion. The biological significance of the CFTR chloride channel is demonstrated by the fact that several human diseases are attributed to altered function of CFTR, among which cystic fibrosis and secretory diarrhoea are the two major disorders. The most common CF mutation is the deletion of 3 nucleotides, resulting in the deletion of a single phenylalanine residue at position 508 (ΔF508) on the protein molecule, and is responsible for ~70% of CF alleles. It is estimated that approximately half of the CF patients are homozygous for the mutation ΔF508. This allele encodes an unstable and inefficiently folded CFTR protein, the major consequence being the failure of the mutant protein to be correctly processed and delivered to its proper cellular location in the plasma membrane. As a result, the mutant protein is retained in the endoplasmic reticulum and rapidly targeted for degradation. Another major disorder involving CFTR is secretory diarrhoea, which is caused by excessive activation of this chloride channel by bacterial enterotoxins, such as Vibrio cholerae toxin, in the gut. The central role of CFTR in certain forms of secretory diarrhoea has been presented as possible explanation for the CFTR heterozygote advantage. Indeed, it is puzzling that mutations in the CFTR gene are maintained in certain human populations at high frequencies (e.g. up to 4–5% for the ΔF508 mutation in individuals of European descent), although, before modern medical management, CF was a lethal disease usually by the age of 2 years. The CFTR role in host-microbe interaction is even more complex and may further contribute in the explanation of the heterozygote advantage above mentioned. Indeed, CFTR function as a pattern recognition receptor for different bacteria, such as S. typhi and P. aeruginosa. S. typhi, but not the related murine pathogen S. typhimurium, uses CFTR for entry into epithelial cells. Cell lines lacking functional CFTR, expressing the most common ΔF508 CFTR mutation internalize fewer serovar typhi bacteria than do cell lines expressing normal CFTR. Additionally, transgenic mice heterozygous for the murine ΔF508 Cftr allele translocate 86% fewer serovar typhi to the submucosa after inoculation into the gastrointestinal lumen compared with wild-type mice, and mice homozygous for the ΔF508 Cftr allele essentially translocate no serovar typhi cells to the gastrointestinal submucosa. CFTR is stored principally in the cytoplasm in subapical membrane vacuoles, and both intestinal commensal bacteria and S.typhi itself, has been shown to increase CFTR expression on cell membrane. The receptor site on CFTR for serovar typhi prePilS protein (the soluble precursor form of the structural pilin) has been determined to be the first extracellular loop,
encompassing amino acids 103–117. After mediating bacterial self-association, the pili act to attach the bacterial clumps to CFTR in the membrane of gut epithelial cells. These sequential type IVB pilus-mediated events cannot be performed by (for example) S. enterica serovar typhimurium, which may explain why only serovar typhi causes enteric fever in humans. CFTR is an epithelial cell receptor for P. aeruginosa, as well, and binds to the conserved outer-core oligosaccharide of the bacterium. Binding of CFTR to P. aeruginosa promotes internalization of the organism by epithelial cells, resulting in shedding of the cells with the internalized microbes and clearance by mucociliary transport. In addition, Esen et al. showed that CFTR-dependent epithelial cell ingestion of P. aeruginosa was associated with the activation of the Src-like tyrosine kinases and the consequent tyrosine phosphorylation of several eukaryotic proteins. Internalization elicited cellular responses independent of binding, and these responses are associated with activation pathways of inflammation such as NF-κB nuclear translocation.

**Figure 3**: Cystic fibrosis transmembrane conductance regulator: multiple functions in epithelial barrier (modified from www.cfgenetherapy.org.uk)

1.3 ROLE OF INTESTINAL BARRIER IN IBD PATHOGENESIS

An impairment of intestinal barrier in genetically predisposed patients is thought to result in the exposure of bacterial luminal components to innate and adaptive immune cells, which ultimately may trigger and propagate inflammatory responses thus leading to IBD. If we look back to what Sir Arthur Hurst suggested as pathogenetic model for UC, we can find surprising similarities. He considered UC to be primarily infective dysentery (we instead talk of “dysbiosis”) in which other factors had occurred secondarily, thus establishing a chronic disease process (aberrant immune response, genetic susceptibility, dysfunction in the intestinal barrier). However, a definitive conclusion if intestinal barrier dysfunction has a pathogenetic role, or is only responsible for maintaining the inflammatory reaction, or itself is a consequence of inflammation has not been achieved yet.
1.3.1 Dysbiosis

In the last decades, indeed, the focus of interest in microbial aetiology of IBD has shifted from infectious (e.g. Mycobacterium) to commensal agents. There is abundant evidence that commensal bacteria are involved in the perpetuation and maintenance of human IBD and in experimental colitis. In CD, faecal stream diversion reduces inflammation and induces mucosal healing in the excluded intestinal segment, whereas infusion of intestinal contents quickly reactivates the disease. IBD patients can respond favorably to antibiotic and probiotic treatment, although a few observations suggest that commensal bacteria probably have a more important role in CD than in UC, and the dominant bacterial stimuli may be different in ileal and colonic CD. Several groups have documented alterations in luminal or adherent microbial commensal flora in patients with IBD (dysbiosis). The faecal flora has been reported to differ in patients with IBD and in healthy subjects, as well as from patients with CD, UC and infectious colitis, suggesting that the modifications of the flora are not only because of the ecological changes induced by colitis. Increase in faecal concentration of B. vulgatus and enterobacteria and a decrease in faecal lactobacilli and bifidobacteria have repeatedly been observed in CD. Furthermore, in ileal specimens, adherent-invasive E. coli strains were found in 21.7% of CD chronic lesions vs. 6.2% of controls. The biofilm, a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface, has recently been identified as sources of many recalcitrant bacterial infections. Swidsinski et al found adherent bacterial biofilms in practically all IBD patients who had no recent history of antibiotic or 5-ASA treatment. The biofilm, composed prevalently by bacteroides, in untreated IBD patients was thick, dense, and adherent. However, if changes of bacterial flora are primary or secondary to inflammation is yet to be defined. In at least eleven different animal models, colitis and immune activation fail to develop in the absence of commensal bacteria, and multiple animal models of colitis respond to antibiotics and probiotics. Monoassociation of IL-10−/− mice with the commensal bacteria E. faecalis and E. coli induced phenotypically distinct forms of colitis. E. faecalis-induced colitis was slow in onset and involved the distal colon, with severe transmural colitis accompanied by dysplasia and duodenal obstruction after 24 weeks of monoassociation. By contrast, E. coli monoassociation led to relatively early (3 weeks) onset of a mild-to-moderate inflammation that was at its most severe in the cecum. Dual association with both commensal bacterial species rapidly (by 1 week) induces severe pancolitis with dysplasia after 5 weeks. Gnotobiotic HLA B27 transgenic rats selectively develop colitis when monoassociated with Bacteroides vulgatus or B. thetaiotaomicron, but not B. distasonis or B. fragilis. Preliminary studies show that Klebsiella monoassociation induces moderate pancolitis and Bifidobacterium animalis monoassociation causes distal colonic and duodenal inflammation. These results demonstrate that even a traditionally probiotic bacterial species can induce inflammation in a susceptible host. An exception to the requirement of bacteria to induce intestinal inflammation is provided by the dextran-sulphate (DSS)-induced colitis model, which (uniquely) worsens in the absence of commensal bacteria. These studies suggest how both bacterial species and host specificity may contribute to colitis and location of the inflammation.
1.3.2 Abnormal intestinal barrier

There is a layer of mucus lining the gastrointestinal tract, which acts as both a lubricant and as a physical barrier between luminal contents and the mucosal surface. The production of mucus is altered in IBD, showing a thinner than normal colonic mucus layer in UC and a thicker than normal layer in CD. Mucins, the building blocks of the mucus gel, determine the thickness and properties of mucus. It has been demonstrated that the degree of sulphation and sialylation and the length of the oligosaccharide chains all vary in IBD. As mucins are strategically positioned between the vulnerable mucosa and the bacterial contents of the bowel, changes in mucin structure and/or quantity probably influence their protective functions and therefore constitute possible aetiological factors in the pathogenesis of IBD. Genetic variants of the membrane-bound MUC3A have been suggested to be involved in predisposition for UC and CD. Moreover, the Muc2−/− mice have an increased susceptibility to cytotoxic luminal agents like DSS. Treatment with DSS led to fulminant colitis within days, which was much more severe in each aspect than in wild-type mice treated with DSS. Furthermore, Muc2−/− mice displayed aberrant intestinal crypt morphology and altered cell maturation and migration. Most notably, the mice frequently developed adenomas in the small intestine that progressed to invasive adenocarcinoma, as well as rectal tumors, indicating that Muc2 plays an essential role in epithelial protection. MUC2 is the major colonic mucin in IBD, present as immature form in areas of goblet cell depletion. However, if changes of mucins are primary or secondary to inflammation is yet to be defined.

Permeability can refer to the degree to which the epithelium is permissive to the passage of luminal substances or can refer more broadly to all of the protective functions of the intestinal mucosa. Increased permeability to inert macromolecular proteins has long been described in patients with CD, can predict relapse and may precede histological inflammation. Buhner and colleagues report that the increased permeability in CD is associated with the presence of variants in CARD15. This finding support a model where CARD15 variants could be the underlying genetic defect that causes subclinical inflammation and hence permeability disturbances. Other clinical investigations of large pedigrees has suggested that the pattern of increased permeability in healthy relatives of patients with CD follows an autosomal recessive mode of inheritance and that subclinical inflammation could also be an independent underlying trait in these families. However, the trend towards increased permeability in spouses of CD patients suggests that environmental factors contribute to this phenomenon, as well. While smoking is known to have strong effects on lung mucosa, its effects on intestinal mucosa are not as straight forward. Interestingly, a study of 242 IBD pedigrees showed that in siblings with similar genetic susceptibility to IBD, smokers tended to develop CD and non-smokers tended to develop UC. Other environmental factor modulating intestinal barrier are NSAIDs drugs that increase permeability and can induce disease reactivation. Increased permeability in CD is related to disease activity, and recently Suenaert and colleague reported that treatment with anti-TNFα antibody largely restores the gut barrier in CD. This could imply that increased permeability in CD is secondary to the inflammatory process. It remains scientifically unproven whether permeability is primary to an increase in inflammation or vice versa. Data from animal model of colitis are still inconclusive. The entry of bacteria into mucosa lying may perturb the
homeostasis and prone for an inflammatory reaction. This was observed in studies of transgenic mice that were genetically engineered to have faulty N-cadherin function, and thus created leaky "tight" junctions between epithelial cells in spatially defined areas of the intestine. This allowed bacteria in the mucosal microflora to breach the epithelial barrier and cause inflammation in affected areas, but not in areas where tight junctions were intact. These mice developed intestinal inflammation and dysplasia. In another model, mice deficient in the multi-drug resistant protein (mdr1a) spontaneously developed colitis. These IBD-like symptoms resolved under antibiotic treatment, indicating that bacteria critically affected the epithelium. Mice carrying a homozygous knockout mutation in the trefoil factor-3 gene proved very sensitive to developing colitis, whereas supplementation of these knockout mice with trefoil factor-3 reverted the disease.

1.3.3 Pattern recognition receptors and inflammation

Activation of innate host defense mechanisms is based on the rapid recognition of conserved molecular patterns in microbes by pre-formed receptors recently recognized (toll-like and NOD-family receptors). Several pieces of evidence suggest a possible role of pattern recognition receptors dysfunction in IBD pathogenesis. Interestingly, expression of select TLRs and NODs is increased in the colonic mucosa of patients with IBD and allelic variants have been associated with IBD. Commensal-derived flagellin appears to initiate only limited immune responses via TLR-5 in normal intestinal epithelial cells, but turns into a dominant antigen in CD. Furthermore, flagellin-specific T effector cells induce severe colitis after adoptive transfer into SCID mice, highlighting the potential pathogenic role of broken immune tolerance in IBD. In addition, CpG motifs derived from bacterial DNA, a ligand for TLR-9, exacerbate inflammation in DSS colitis model and promote Th1 responses. However, CpG DNA motifs from probiotic bacteria exerted beneficial effects in the same model. These findings and a recent study from Jijon et al., showing that probiotic DNA is able to inhibit deleterious effects of Salmonella-derived DNA in intestinal epithelial cells, suggest that environmental and ligand-specific factors modify the outcome of TLR-mediated signaling. Watanabe et al. reported evidence that NOD2 normally serves as a negative regulator of peptidoglycan (PGN)-derived TLR-2 signaling. NOD2 mutants appeared to insufficiently inhibit PGN-driven TLR-2 stimulation ultimately leading to Th1 responses. The mechanisms by which NOD2 may inhibit TLR-2 signalling have not been defined yet, but reportedly do not involve direct interaction between the two receptors.

Two recent studies in NOD2 knockout mice (NOD2−/−) and mice with a truncated NOD2 protein (NOD2m/m) have yielded different perspectives in the interaction between NOD2 and the TLR-2 response. These animal models substantiate the concept that variance in the NOD2 protein is not sufficient to induce intestinal disease; both NOD2−/− and NOD2m/m mice did not show ‘spontaneously’ intestinal pathology, but rather required a disrupted barrier function (induced by DSS) and luminal microflora for the onset of colitis. Of note, NOD2−/− mice did not show increased susceptibility to DSS-induced colitis while the NOD2m/m mice exhibited increased morbidity after DSS treatment. The latter may due to increased IL1β secretion and NF-κB signalling in NOD2m/m mice. However, NOD2−/− mice exhibited severe
deficiency in adaptive immune response and subsequent specific antibody production. NOD2⁻/⁻ mice also showed diminished expression of defensin-related cryptidins and decreased protection against infection from enteric bacterial pathogens.

1.4 FROM INFLAMMATION TO CANCER

Although Virchow suggested already in the nineteenth century that chronic inflammation might give rise to malignancy, the link between inflammation and cancer was never quite understood. The 're-discovery' of an inflammation-cancer connection can be attributed, in part, to epidemiological studies that identified chronic infections and inflammation as major risk factors for various types of cancer, such as infection of hepatitis B virus and hepatitis C virus and hepatocellular carcinoma, Helicobacter pylori and gastric cancer. Chronic inflammatory bowel diseases are, indeed, thought to increase the risk of colorectal cancer by approximately 1% per year, another excellent example of tumor development in inflammation.¹⁴⁰,¹⁴¹ Although several clinical factors have been identified, their ability to define IBD subgroups of patients with higher risk for colon cancer is far from being adequate.¹⁴² A direct consequence is that all the IBD patients with more than 8 years of disease duration are strongly suggested to be included in colonoscopic surveillance programs, indeed not the ideal preventive approach to cancer control either.¹⁴³ Thus, biomarkers able to better recognize IBD patients with high risk for colon cancer and eventually molecular determinants for identification of a more personalized chemopreventive treatment and its effect are highly warranted.

1.4.1 Colitis associated colon cancer: feared IBD subphenotype

Patients with IBD, both UC and CD, have an increased risk of colon cancer (CRC). In UC, the probability of colon cancer is 2% after 10 years of disease, 8% after 20 years, and 18% after 30 years.¹⁴⁴ In CD the risk estimates have been controversial, but a recent meta-analysis of six studies reported an overall increased risk of both colon cancer and small bowel cancer.¹⁴⁵ Compared with sporadic colorectal carcinoma, colon cancer arising in patients with IBD has several distinguishing clinical features. Colitis-associated colorectal cancer (CAC) affects individuals at a younger age than the general population. They often have a mucinous or signet ring cell histology and there is a higher rate of two or more synchronous primary CRCS.¹⁴⁶ Regardless of the underlying condition, essentially all CRCs develop from a dysplastic precursor lesion. In sporadic CRC, the dysplastic precursor is on the adenomatous polyp (adenoma). In contrast, dysplasia in patients with IBD can be polypoid or flat, localized, diffuse, or multifocal and, once found, marks the entire colon as being at heightened risk of neoplasia, thereby warranting surgical removal of the entire colon and rectum. It is widely believed that genetic instability of cancer cells drives tumorigenesis by producing new pools of mutations in which selection and subsequent clonal expansion occurs. Emerging evidence suggests that in CAC, the frequency of chromosomal instability (CIN) (85%) and microsatellite instability (MSI) (15%) is roughly the same as in sporadic colon cancer.¹⁴⁷ Aneuploidy and telomere shortening have been described in non-dysplastic tissue of UC patients.¹⁴⁸-¹⁵⁰ Results of studies of MSI have been highly variable and even discordant. Evidence shows that MSI in IBD might be different from that in sporadic CRC. For example,
chromosomal location of microsatellite instability in sporadic CRC tends to differ from those sites altered in UC.\textsuperscript{151} In sporadic CRC, hMLH1 promoter hypermethylation accounts for 80% of the cases of MSI, whereas in UC cancer the estimated frequency is around 46%.\textsuperscript{152} Indeed, methylation is increasingly being recognized as being important as a mechanism contributing to the genetic alterations in CAC. Methylation of CpG islands in several genes seems to precede dysplasia and is more widespread throughout the mucosa of UC patients.\textsuperscript{153} Distinguishing features of CAC, furthermore, are differences in the timing and frequency of molecular alterations (Figure 4). For example, APC loss of function, considered to be a very common early event in sporadic colon cancer, is much less frequent and usually occurs late in the colitis-associated dysplasia-carcinoma sequence.\textsuperscript{154} In the setting of colitis, p53 mutations occur early, while present in later stages of adenoma-carcinoma sequence, and are often detected in mucosa that is nondysplastic or only indefinite for dysplasia.\textsuperscript{155} In carefully mapped colectomy specimens, p53 mutation occurred before aneuploidy, which, in turn, preceded p53 loss of heterozygosity, indicating that chronic inflammation predisposes to these early mutations.\textsuperscript{156} In UC patients, several inflammation-associated genes such as cyclooxygenase-2, nitric oxide synthase-2, and NF-kB are also increased in inflamed mucosa and remain elevated in colonic neoplasms.\textsuperscript{140, 157}

So far, dysplasia, family history of CRC and, probably, backwash ileitis, together with duration, extent and early onset of disease and severity of inflammation are the only factors available in clinical practice to define the subgroup of IBD patients with increased risk for malignant transformation.\textsuperscript{158-160} Of particular interest is the subgroup of IBD patients affected by primary sclerosing cholangitis (PSC). PSC is a chronic inflammatory disorder of the biliary tree.\textsuperscript{161} It is slowly progressive, developing the complications of portal hypertension and chronic liver failure.\textsuperscript{162} The association of PSC with UC and CD has been described since 1960s and define a well characterized IBD subphenotype.\textsuperscript{163} Indeed, PSC is preferably associated with UC and PSC patients with CD almost always have extensive colitis or ileocolitis but not isolated ileitis. Moreover, the prevalence of PSC is higher in patients with substantial colitis (5.5%) than in patients with distal colitis (0.5%).\textsuperscript{164} It is well known that patients with UC have an increased risk of developing colorectal carcinoma.\textsuperscript{165} In a study from Sweden the absolute cumulative risk of developing colorectal dysplasia/cancer in the PSC/UC group was 9%, 31%, and 50%, respectively, after 10, 20, and 25 years of disease duration.\textsuperscript{165, 166} PSC patients with UC remain at an increased risk for developing colorectal cancer/dysplasia also after they have undergone orthotopic liver transplantation;\textsuperscript{167} in addition, UC patients with PSC seem to have a higher risk of neoplastic transformation in the pouch mucosa than UC patients without PSC.\textsuperscript{168} The reason for the increased risk of development of colorectal neoplasia in PSC patients is obscure. It has been speculated that secondary bile acids, such as deoxycholic acid (DCA), play a role in the carcinogenesis of the colorectal mucosa in PSC.\textsuperscript{169} This is supported by the fact that right-sided cancers seem to be more common in patients with PSC compared with patients with UC alone.\textsuperscript{170} Patients with PSC have increased colonic concentrations of DCA. Similarly, UC patients with colorectal neoplasia have higher faecal bile acid concentrations than UC patients without dysplasia.\textsuperscript{171} Interestingly, ursodeoxycholic acid (UDCA), a more hydrophilic bile acid, commonly used in treatment of cholestatic diseases, has been shown to have chemopreventive effect, decreasing the risk for malignant transformation in IBD patients with PSC, as discussed below.\textsuperscript{172,173-176}
1.4.2 Inflammation and cancer

Activated NF-κB is found in inflamed mucosal biopsies of patients with IBD, both in macrophages and in epithelial cells, and TNFα is found in increased concentrations in the mucosa, serum and stool of patients with IBD. A fundamental role of these two players has been suggested in CAC. In an animal model for CAC (azoxymethane and dextran sulphate (AOM-DSS) treated mice), selective inactivation of the IKKβ gene within enterocytes resulted in an 80% decrease in tumor multiplicity. As tumor size was not affected, it can be concluded that IKKβ-dependent NF-κB in enterocytes contributes to tumor initiation or early tumor promotion, rather than tumor growth and progression. Indeed, analysis of enterocyte IKKβ-deleted mice shortly after exposure to AOM plus DSS revealed increased apoptosis of enterocytes, including pre-neoplastic cells in which AOM led to mutational activation of the β-catenin pathway. Enhanced apoptosis is probably caused by defective induction of Bcl-XL. However, when IKKβ was deleted in myeloid cells (for example, mature macrophages, dendritic cells and neutrophils), tumor multiplicity was reduced by only 50%, although tumor size was also reduced. Indeed, deletion of IKKβ in myeloid cells, but not in enterocytes, diminished the proliferation of AOM-exposed enterocytes. The myeloid-specific mutation, however, had no effect on apoptosis of AOM-exposed enterocytes. These results led to the conclusion that IKKβ-driven NF-κB contributes to the development of CAC through two distinct cell-type-specific mechanisms: in enterocytes it activates anti-apoptotic genes and thereby suppresses the apoptotic elimination of pre-neoplastic cells, whereas in myeloid cells it promotes the production of cytokines that act as growth factors for pre-malignant enterocytes. One of these growth factors was subsequently identified as IL-6, which is encoded by an NF-κB target gene. The inhibition of IL-6 signalling with antagonistic anti-IL-6 receptor antibodies inhibited tumor growth with little effect on tumor multiplicity, thereby resembling IKKβ ablation in myeloid cells. Curiously, in the early stages of the carcinogenic protocol, IL-6 is produced by lamina propria myeloid cells, whereas at the end of the CAC protocol it is mainly expressed by tumor-infiltrating T cells. Another proinflammatory cytokine involved in cancer is TNFα. Despite being named for its ability to induce tumor necrosis, which is an activity that is mostly mediated through increased vascular permeability and subsequent vascular collapse, there is ample evidence that TNFα acts as a tumor promoter in several models of experimental cancer.
evidence that TNFα is also produced by cancers. General or cell selective deletion/inhibition of TNFα reduces the incidence of experimental cancers. An interesting genetic link between TNFα and malignancy was recently identified in renal cancer, where the tumor suppressor gene is a translational repressor of TNFα.

1.4.3 Chemoprevention

The most effective strategy to prevent colorectal cancer is prophylactic colectomy but it is seldom accepted as a routine preventive method. After 8 years of disease duration IBD patients are at high risk of cancer and require surveillance colonoscopy, with multiple biopsies taken and graded by a pathologist. Considering the real effect, risk, expense, and sampling error of endoscopic surveillance, it is not an ideal preventive approach to cancer control either. As lesions are not always endoscopically visible, it takes 33 or more biopsies to have 90% confidence of finding dysplasia, if it is present. And once dysplasia is found, its grading is subject to intraobserver and interobserver variability. Therefore, the development of safer and more effective methods for reducing the risk of colorectal cancer would be of substantial benefit to IBD patients. The most attractive method is perhaps to prevent CRC using specific, cheap and non toxic drugs. Recent findings have indicated that several compounds, such as dietary calcium, vitamin D and folate may modulate and inhibit colon carcinogenesis. In addition, non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the incidence of cancer. The main, but not unique, target of NSAIDs is COX2, and specific COX2 inhibitors were found to be efficacious in reducing tumor load in patients with familial adenomatous polyposis. Increasing evidences suggest that NSAIDs regulate gene expression, which may be responsible, at least in part, for their activity. Among NSAIDs target gene, non-steroidal anti-inflammatory drugs activated gene (NAG-1), a newly identified member of the TGF-β superfamily, has antiproliferative and proapoptotic activities in colon and gastric cancer cells, and seems to be another effector for NSAIDs antitumorogenic effect. However, the long-term use of NSAIDs is associated with significant toxicity and aggravates symptoms of colitis in IBD patients. In this group of patients, nevertheless, evidence is accumulating that 5-aminosalicylic acid (5-ASA) may prevent colorectal neoplasia. 5-ASA reduced the number of atypical crypt foci by over one-third, effectively reduced tumor number and load, increased the rate of tumor apoptosis, and reduced the rate of tumor cell proliferation in the AOM CRC animal model. In FACS analysis, 5-ASA, like other NSAIDs, produces an increase in the proportion of cells in G0/G1 and a concomitant decrease in the proportion of cells in S phase. Although promising results have been obtained from a wide variety of preclinical experimental studies, epidemiological findings and a few human clinical trials, further investigations are required to define cost/benefit of their clinical application. Prospective, randomized clinical studies, however, requires large number of individuals, clinical and endoscopic resources and can be very expensive. Thus, understanding of mechanism of action of putative chemopreventive compound may help in deciding about drug pharmacological relevance.

1.4.4 Role of UDCA in chemoprevention of CAC

Chronic inflammation by disruption of the mucosal barrier function and the concomitant immune hyperactivation by the microflora is presumed to represent a
central event that is permissive for the progressive transformation of colon epithelial cells in inflammatory bowel disease. IBD patients with concomitant PSC is a well characterized subgroup which has an increased risk for CRC. Ursodeoxycholic acid, UDCA, in affordable formulations, is widely used for cholesterol gallstones dissolution and in the treatment of chronic cholestatic liver diseases, such as primary biliary cirrhosis and PSC, with good compliance and little or no side effects. UDCA is the 7β-hydroxy epimer of chenodeoxycholic acid, is a naturally occurring hydrophilic bile acid present in trace amounts in human bile, and is a very promising chemopreventive candidate (Figure 5). Evidence for the chemopreventive effect of UDCA was provided in animal models for CAC and supported in clinical trials. The study by Tung et al. investigated the relationship between UDCA use and colonic dysplasia or adenocarcinoma in UC patients with PSC. Overall, 44% of the patients developed dysplasia, 32% in the UDCA group vs. 72% of those not taking UDCA. Comparing the effects of supplemental dietary ursodeoxycholic acid to cholic acid, used as tumor promoter, and to piroxicam, a chemopreventive agent, in the AOM model of experimental colonic carcinogenesis, UDCA prevented DCA dependent tumor promotion and its tumor suppressive effects exceeded that of dietary piroxicam. UDCA can inhibit the development and growth of aberrant crypt foci (ACF) that are the earliest identifiable putative premalignant precursors of AOM and human CRC, during either tumor initiation or in the promotion/progression phase. The effects of UDCA on colitis-related colorectal carcinogenesis in DSS mice model have been evaluated with similar results and the prevalence of dysplasia showed an inverse relationship to the UDCA concentration in the faecal water. Moreover, UDCA was tested in combination with sulindac, a NSAID with chemopreventive properties, for prevention of adenomas in the Min mouse model of adenomatous polyposis. Ursodeoxycholic acid caused a dose-dependent decrease in the number of intestinal tumors. Unlike sulindac and other nonsteroidal anti-inflammatory drugs, which are quite beneficial in the distal intestine but are somewhat less effective in the proximal small intestine, UDCA had equal efficacy throughout the entire intestine. Combined treatment with low-dose sulindac was less toxic, and was more effective than either agent alone for the prevention of tumors throughout the entire intestine. Recently, a phase III, double-blind placebo-controlled trial of UDCA was performed to evaluate its ability to prevent colorectal adenoma recurrence. One thousand two hundred and eighty five individuals who had undergone removal of a colorectal adenoma within the last 6 months were randomly assigned to daily treatment with UDCA (8-10 mg/kg of body weight) or with placebo for 3 years or until follow-up colonoscopy. UDCA treatment was associated with a statistically significant reduction in the recurrence of adenomas with high-grade dysplasia. Despite accumulating evidence of chemoprevention by UDCA, the mechanisms of action are still unclear. UDCA is a molecule with pleiotropic effects. Its capacity of decreasing the total amount of toxic hydrophobic acid in the bile and in the faecal water is considered one of the possible mechanisms by which UDCA can decrease malignant transformation. Other and more direct cytoprotective effects such as stabilizing membranes and acting as an antioxidant and an immunomodulator, have been described. In general, UDCA displays activities that are distinct, even in opposition, to those exhibited by DCA. DCA contributes to colon carcinogenesis by altering intracellular signalling of a variety of transcription factors, like AP-1 and NF-κB, UDCA inhibits them. Indeed, it is possible that these two bile acids can act on
some of the same signalling pathways, but with different modulating effects, or may act through different receptors.

**Figure 5:** *Ursodeoxycholic acid (UDCA)*
2 AIMS

An appropriate IBD classification would have potential benefits with respect to patient diagnosis and counselling, and assessment of prognosis. IBD phenotypes and severe subphenotype, such as development of CAC, might be easily and unequivocally identified, and, therefore, more personalized therapeutic strategies might be defined. Indeed, we believe genetic and molecular determinants might integrate and complete clinical classification, contributing to the answer of the above mentioned needs. As a gastroenterologist, it was exciting to participate in this research:

- Assessing the potential role of NOD2/CARD15 polymorphisms in explaining concordance of CD in monozygotic twins and evaluating a potential functional interaction between polymorphisms in NOD2/CARD15 and TNFα genes that might further our understanding on genotype-phenotype correlation

- Exploring the role of cystic fibrosis transmembrane conductance regulator in contributing to susceptibility and phenotype of IBD

- Determining biomarkers to monitor the effects of UDCA chemoprevention and the mechanisms underlying its antitumorogenic activity in IBD associated colon cancer
3 RESULTS

3.1 ROLE OF NOD2/CARD15 POLYMORPHISMS IN EXPLAINING CONCORDANCE OF CROHN’S DISEASE IN MONOZYGOTIC TWINS (PAPER I)

A high concordance rate has been observed in monozygotic twin pairs with CD. In addition, the phenotype of CD is similar in concordant monozygotic twin pairs and shows features that have been associated with CARD15/NOD2 mutations. It can be hypothesised that concordant pairs carry an increased load of CD susceptibility genes. The present study reports for the first time CARD15/NOD2 mutations in a population-based cohort of monozygotic twins, where at least one twin in each pair suffers from CD, and aims to assess whether these variants explain disease concordance.

CARD15/NOD2 polymorphisms were identified in three of 29 twin pairs. Within each of these pairs, both twins carried the same variant. One concordant and one discordant pair carried a single copy of the Arg702Trp variant (simple heterozygotes) and another concordant pair a single copy of the frameshift mutation (Leu1007fsinsC). Thus, five of 38 (13%) twins with CD carried any of the CARD15/NOD2 mutations, corresponding to a total allele frequency for CARD15/NOD2 polymorphisms of 6.6% (Arg702Trp 3.9%, Gly908Arg 0% and Leu1007fsinsC 2.6%). One healthy twin sibling carried a Arg702Trp variant.

The mean age at diagnosis was 25.5 years (median 23.0 years; range 12–52 years) in the concordant twins and 33.1 years (median 33.5 years; range 16–60 years) in the discordant ones ($p = 0.07$). Ileal involvement at the time of diagnosis was more common in the concordant than in the discordant twins, 17/18 versus 11/20 ($p = 0.009$). Only two of the nine concordant and one of the twenty discordant pairs carried any of the CARD15/NOD2 variants. Thus, the total allele frequency of these mutations was 4.4 times higher in twins in concordant pairs than in twins in discordant pairs, 11.1% versus 2.5% ($p = 0.06$).

Six of 192 healthy blood donors carried a single copy of the Arg702Trp variant and four a single copy of the frameshift mutation. The total allele frequency was 2.6% in the healthy controls (1.6%, 0% and 1.0% for the three alleles, respectively).

3.1.1.1 Comments and reflections, including technical limitations of the study

Considering that the Swedish twin cohort for IBD is one of the very few in the world, it was a privilege to have the chance to study it. This cohort is population based and was identified by running the Swedish twin registry against the Swedish hospital discharge registry. This implies that twins not hospitalized for their disease are excluded and that a possible selection bias towards more severe IBD exists. On the other hand, no national register for outpatient clinics exists in Sweden, and therefore improvement of the recruitment system would be difficult.

To assess whether CARD15/NOD2 variants explain disease concordance, monozygotic twins were considered and allelic frequencies in concordant or discordant twins for CD
evaluated. In addition to this type of study design, another interesting approach to evaluate our hypothesis would be to study the occurrence of CARD15/NOD2 in dizygotic twins concordant for CD. Occurrence of CARD15/NOD2 variants would then be higher than expected in concordant dizygotic twins. However, in the Swedish cohort only one concordant dizygotic twin pair with CD exists. Therefore, it is not possible to study allelic variants in this subgroup due to the rare occurrence of dizygotic concordant twin pairs with CD.

The low total allele frequency in the Swedish monozygotic twins with CD as well as in the general population, 6.6% and 2.6%, respectively, suggests that the three CARD15/NOD2 mutations are of less significance in Sweden compared to other European Countries. These findings are supported by the observed allelic frequencies of all three variants in the CD group (varying from 4.3% - 8.4%) and in the healthy controls (2.6%) in recently published papers. The reproducibility of mutations frequencies, furthermore, can be seen as guarantee of low rate of genotyping error, that might represent a major concern, especially in an underpowered study. Well aware of this risk, we applied several precautions: samples, randomly located in plate, were genotyped blindly; two researchers independently evaluated the results of the electrophoresis run on agarose gel and uncertain samples were re-genotyped and re-run. Furthermore, we observed that one discordant twin pair carried CARD15/NOD2 variant, but only one of them developed the disease. No differences could be observed regarding exposure to childhood and environmental factors, such as early and frequent gastrointestinal infection or smoking habit, in the discordant monozygotic twin pair carrying the Arg702Trp polymorphism, as it might be expected. This emphasizes the importance of other environmental factors in the aetiology of CD.

Despite the limited number of recruited and genotyped monozygotic twins, we observed that the CARD15/NOD2 mutations was found more often in concordant than in discordant twins. These findings suggest that these polymorphisms contribute to concordance of CD in monozygotic twins and support the hypothesis that discordant monozygotic twins are under an increased load of CD susceptibility genes as compared to discordant monozygotic twins. However, these three CARD15/NOD2 polymorphisms alone do not explain concordance for CD in monozygotic twins and other polymorphisms in CARD15/NOD2 or other genes are needed.

3.2 FUNCTIONAL INTERACTION BETWEEN POLYMORPHISMS IN NOD2/CARD15 AND TNFA GENES IN GENOTYPE-PHENOTYPE CORRELATION (PAPER II)

Polymorphisms in the CARD15/NOD2 gene and in the promoter region of the TNFα gene are associated with susceptibility to and modulate the phenotype of CD. The molecular mechanisms for this genotype–phenotype correlation are yet to be elucidated. CARD15 is an intracellular receptor for bacterial muramyl dipeptide (MDP), and can elicit an inflammatory response via activation of the NF-κB pathway. MDP is also known to induce the expression of pro-inflammatory cytokines including TNFα, through a still poorly characterized signalling pathway. We sought to determine whether CARD15-mediated NF-κB activation can contribute to MDP-induced TNFα
production and, consequently, if polymorphisms in both genes affect the control of such induction.

HEK293 cells were co-transfected with CARD15 and a TNFα promoter-luciferase construct (TNFCC-Luc), and stimulated with MDP. While CARD15 alone induced only a very small increase in TNFα promoter reporter activity, MDP stimulation produced up to a threefold induction, which correlated with the amount of transfected CARD15. No effect was detected when MDP was added to the cells in the absence of CARD15, confirming that the latter is entirely responsible for the induction.

We focused on a region of the promoter, spanning nucleotides −879 to −845, where both the transcription factors NF-κB and OCT1 have been shown to bind. Since this is also the region where the SNPs −863 and −857 map, a −863C–857C-containing sequence was used, which is referred to as the wt sequence and is expected to allow the binding of NF-κB, but not that of OCT1. Transfection with CARD15 induced the formation of a complex that was much more abundant after MDP stimulation. This complex disappeared when a NF-κB consensus sequence was used for competition, and was shifted when an anti-p65 antibody was added to the binding reaction. This result suggests therefore that CARD15/MDP activation of NF-κB can result in the binding of this transcription factor to the TNFα promoter sequence analyzed here.

We next sought to determine the effect of both CARD15 1007fs variant and TNFα promoter polymorphisms in three haplotypic combinations (with the three corresponding luciferase reporter constructs TNFCC-Luc, TNFAC-Luc, and TNFCT-Luc). Either CARD15 wt or CARD15 1007fs mutant were then co-transfected with each TNFα construct in MDP-stimulated and not stimulated HEK293 cells. CARD15 1007fs mutant was not able to induce TNFα promoter activation in any of the combinations tested. The three TNFα promoter constructs responded differently to CARD15 wt and the −863A–857C haplotype showed a significantly higher induction.

3.2.1.1 Comments and reflections, including technical limitations of the study

Although it would have been better to choose a colonic epithelial cell line, HEK293, from embryonic kidney cells, were used being a good tool in transfection experiments with MDP. In fact, they are easy to transfect, and, since they do not express CARD15/NOD2 or TLR2 or TLR4 normally, we could be sure that CARD15, when transfected, is entirely responsible for the MDP mediated induction of TNFα that we observe in our experiment.

When the CD-associated CARD15 1007fs variant was analyzed, induction of TNFα promoter activity was found to be defective. Indeed, the CARD15 1007fs mutant was not able to induce TNFα promoter activation in any of the combinations tested. Our finding is in line with the hypothesis that CARD15 mutation results in a loss of function, based on in vitro experiments performed with cell lines transfected with wild type and mutant CARD15 variants, and supported by the Nod2−/− mouse model by Kobayashi et al.41

The three TNFα promoter constructs responded differently to CARD15 wild type, where the −863A–857C haplotype showed a significantly higher induction. Similar results were obtained by Higuchi et al. who showed the same pattern of activation of
the three TNFα promoters, with the haplotype -863A–857C showing the strongest transcriptional activity.218
In our experiments, both CARD15 and TNFα promoter polymorphisms have been shown to affect the induction of TNFα gene transcription after MDP stimulation, so that diverse CARD15/ TNFα allelic combinations could give rise to different levels of TNFα production. This finding might therefore contribute in explaining the association between CARD15 and TNFα variants with disease susceptibility and clinical phenotypes, such as disease activity, location and behavior, extraintestinal manifestations, and response to steroid treatment.219-224

3.3 CFTR, A PATTERN RECOGNITION MOLECULE CONTRIBUTING TO SUSCEPTIBILITY AND PHENOTYPE OF IBD (PAPER III)

Chronic inflammation of the gastrointestinal tract is thought to result from an impairment of gut epithelial barrier and a dysregulated immune response to the intestinal bacterial flora in genetically susceptible individuals. 21 In the intestine, CFTR, product of the gene mutated in patients with cystic fibrosis, has been shown to regulate both secretion/absorption functions and mucus production,225 and contribute to the innate immune responses to the intestinal flora, by directly interacting with bacteria and their components.84 We therefore aimed to evaluate its possible role in IBD susceptibility.

To test the CF locus as a candidate IBD susceptibility gene, a total of 2568 individuals (935 CD, 781 UC and 852 controls) from three independent cohorts of Italian, Swedish and Scottish consecutive IBD patients and controls were enrolled in this study.

We initially genotyped the CFTR ΔF508 mutation in 504 IBD patients (306 CD and 198 UC) and 358 controls from Italy. This mutation was under-represented in the CD group, where it occurred at a 8-fold lower frequency compared to controls (p = 0.021), whilst the frequency of ΔF508 did not significantly differ between UC patients and controls, and was comparable to what expected for a general population from Italy (2.1%).226

To consolidate this finding, we genotyped two additional independent cohorts of IBD patients and controls from Sweden and UK (Scotland). Despite its expected lower frequency in this population,227 an inverse association with CD was observed for ΔF508 also in the cohort from Sweden, where not a single heterozygote was found among CD patients (p = 0.027 vs. controls). However, this result could not be replicated in the Scottish cohort, where the ΔF508 mutation was equally distributed in the three groups of CD, UC and controls individuals.

In the Scottish population, phenotype-genotype association was found between CFTR mutation and disease location, indeed, no ΔF508 carriers suffered of right-sided colitis (p = 0.023 vs. all other locations). Of note, the right colon was spared from inflammation also in the only other CFTR ΔF508 positive CD subject identified in the Italian population.
One of the common problems in case-control association project is the power of the study. Well aware of this possible limitation, we started extended international collaborations based on several centers in three different countries, Italy, Sweden and the UK. Thanks to the commitment of the clinicians and enthusiasm of the patients, 2568 individuals (935 CD, 781 UC and 852 controls) were enrolled. This was indeed the prerequisite for a successful study. However, as such, multicentric collaborations may imply several problems, such as misclassification of diagnosis and disease phenotypes. To reduce these uncertainties, only specialist centers with gastroenterologist trained in IBD and experienced in clinical studies were involved in the project. Inclusion criteria for the case group required that only definite diagnosis of CD or UC were accepted and no indeterminate colitis was included.\textsuperscript{1,5}

Considering the key role of intestinal barrier homeostasis in IBD pathogenesis, CFTR represents a good candidate gene for IBD. CFTR locus resides in a region on chromosome 7 previously associated to IBD in genome wide scans.\textsuperscript{45} Interestingly other IBD genes mapping to this region have already been identified. However, their physical distance from CFTR strongly argues against the existence of linkage disequilibrium with this locus and the observed effect should therefore be entirely ascribed to the studied mutation. Currently, more than 1000 mutations of CFTR are registered with the Cystic Fibrosis Gene Analysis Consortium (CFGAC; www.genet.sickkids.on.ca/). In our study, the genotyped mutation is the most common and results in a lack of protein presentation on the cell membrane, as all class 2 mutations.\textsuperscript{228} If the biological effect of CFTR mutations resides in the lack of presentation of the protein functioning as bacterial receptors, it might be speculated that all the mutations of the same class might influence CD susceptibility, adding clinical relevance to our finding. As already mentioned in paper I, several measures were respected to limit the risk of genotyping errors: genotyping was performed blindly; cases and controls samples were randomly located in plate from the Italian population; two researchers evaluated the results of the PAGE analysis. Uncertain samples were re-genotyped and re-run. Indeed, observed \( \Delta F508 \) mutation frequencies are consistent with Hardy-Weinberg equilibrium, and carriers’ frequency in controls matched the expected frequency in general populations.

The \( \Delta F508 \) mutation occurs at a relatively low frequency in the general population and, from our results, even at a lower frequency in CD patients, thus hampering the identification of associations with highly significant P values and limiting the application of Bonferroni analysis for correction of multiple comparisons. Replication studies in independent populations are best suited to circumvent this problem, and three independent populations were tested in our studies.\textsuperscript{229} Population heterogeneity is a serious issue in any population-based study of complex disease, and the Scottish population has been presented as an example of genetic heterogeneity in several studies, as mentioned in the paper. However, even in this population, association of \( \Delta F508 \) heterozigosity with protection towards right sided colitis has been shown.

In conclusion, in our study, we observed that CFTR heterozygous carriers have an estimated 88\% reduced risk of developing CD, and in particular of right sided colitis. Although our results need to be confirmed in other independent populations and may have a hardly predictable relevance from a clinical perspective, we believe that they might significantly contribute to further our understanding of CD pathogenesis.
3.4 MOLECULAR DETERMINANTS OF THE CHEMOPREVENTIVE EFFECT OF UDCA IN IBD ASSOCIATED COLON CANCER (PAPER IV)

Colorectal cancer is a serious complication of chronic inflammation of the colon in IBD. In patients with severe colitis and associated primary sclerosing cholangitis, the cumulative risk of cancer reaches up to 50% after 25 years of disease duration. Ursodeoxycholic acid, UDCA, commonly used in PSC treatment, has been observed as having chemopreventive properties both in colitis associated and adenoma associated colon cancer. 174, 202 In this study, we set out to assess molecular determinants of UDCA that might be useful in monitoring its chemopreventive effect, as well as to increase our understanding of its antitumorogenic mechanisms.

Colonic epithelial SW480 adenocarcinoma cells, were treated with either 500 µM UDCA or ethanol (as a control) for 2, 10, 24 and 48 hours and gene expression analysis was performed using the KTH 29.8k human cDNA microarrays. A total of 31 genes showed a greater-than-two-fold magnitude of change, of which 22 were induced and 9 were repressed. Validation experiments by RT-PCR confirmed eighteen of the inducible (82%) and 9 of the repressed genes (67%) to be bona fide target genes of UDCA. All 18 genes, except GADD45β which was induced only at 2 hour, were up-regulated after 10 hours of UDCA treatment, and remained induced even after 24 hours. Generally, the down-regulated genes showed a more transient pattern of UDCA-dependent repression, with significant inhibition at 10 hours of treatment.

Cell cycle analysis on SW480 cells treated with UDCA, ethanol or DCA for varying times showed that only UDCA inhibits proliferation through induction of G1 arrest. As expected,230, 231 no significant increase in apoptosis was observed in either UDCA or DCA treated cells under these experimental conditions.

Among the UDCA target genes, NAG-1, non-steroidal anti-inflammatory drug-activated gene, also known as macrophage-inhibiting cytokine-1 (MIC1) and growth differentiation factor 15 (GDF15), was the only gene with an early up-regulation. Interestingly, NAG-1 modulates cell cycle inducing G1 arrest.232 As shown in Figure 2a, UDCA induces NAG-1 expression as early as 2 hours after treatment and increases up to 6-fold at 48 hours. NAG-1 protein levels appear to show a modest increase 24 and 48 hours after treatment, but increase noticeably up to 72 hour of incubation with UDCA, but not DCA or ethanol (Figure 6).

**Figure 6:** UDCA dependent increase of NAG-1 protein levels
Rhode et al described the upregulation of NAG-1 as a downstream effect of HSPA2 depletion in HeLa cells and breast cancer cells (MCF7).\textsuperscript{233} To further clarify mechanisms governing the UDCA-induced induction of NAG-1, we analysed the effects of UDCA on HSPA2 expression. While no changes in RNA level were seen (data not shown), a progressive reduction of HSPA2 protein was clearly observed 24 hours after stimulation with UDCA, but not DCA or ethanol. Considering the discrepancy in kinetics of NAG-1 mRNA expression and HSPA2 decrease, it may be likely that UDCA dependent transcriptional activation may be responsible for the early NAG-1 up-regulation and the latter event of HSPA2 depletion might function to further increase NAG-1 protein levels.

The breast adenocarcinoma cell line MCF7 was chosen to assess putative applications for UDCA as a general chemopreventive agent. Following 24 hours treatment, UDCA induced a G1 arrest, which was still sustained at 72 hours. In addition, the UDCA target gene NAG-1 was up-regulated at the RNA level (up to 5-fold increase after 24 hours of UDCA stimulation). UDCA treatment also elicits a decline in the protein levels of HSPA2. However, the drop in protein level is much more noticeable at 48 and 72 hours after UDCA treatment, while exposure to DCA does not change HSPA2 levels. Thus all the criteria used to determine the effectiveness of UDCA in SW80 cell is recapitulated in another adenocarcinoma cell line of non-colonic origins. Hence, the indication that UDCA has an antiproliferative effect in breast cancer cell line opens novel pharmacological possibilities in chemoprevention.

NAG-1 was identified by PCR-based subtractive hybridization, from an NSAID induced library in cyclooxygenase negative cells, as a divergent member of the TGF-β superfamily. NAG-1 is up-regulated in human colorectal cancer cells by several NSAIDs, and it is considered one of the effector of their chemopreventive activity.\textsuperscript{232} We therefore evaluated the possible synergistic effect on NAG-1 expression of the concomitant stimulation of colonic cancer cells with both UDCA and sulindac (50 µM). After 48 hours stimulation, sulindac alone did not influence NAG-1 mRNA levels, neither did DMSO in which sulindac was dissolved, while UDCA induced as expected an increase in NAG-1 expression of about 9 times in magnitude. Furthermore, a synergistic effect was observed when UDCA and sulindac were added together, with an increase of NAG-1 expression of about 15 times in magnitude (Figure 7). Morphological changes occurred as well. Already at 24 hours decrease in cell proliferation and increased number of dead cells was observed when UDCA and sulindac was added compared to controls. And after 48 hours stimulation, the morphological differences were even more pronounced resulting in very sparse cells when treated with both UDCA and sulindac.
3.4.1.1 Comments and reflections, including technical limitations of the study

To retain a degree of homogeneity, we decided to start our investigation on colonic cancer cell lines, SW480 cells, instead of using directly bioptic materials. We plan also to analyze other colon cancer cell lines, such as HT116. Ultimately, our aim is to validate our findings on bioptic samples from IBD patients with high risk of developing CRC and treated with UDCA and from controls. Indeed, thanks to the active interest of gastroenterologists (RL and US) who contributed in the identification of UDCA as possible chemopreventive drug, bioptic material is already available.

Although the pharmacokinetics and dynamics of UDCA is well known, the concentration of this bile acid in the faecal aqueous phase is not fully defined. The concentrations chosen to stimulate our cells is the highest shown having no proapoptotic effect and it is compatible with the one used in others studies.

We can conclude that the microarray analysis of UDCA chemopreventive effect on colonic cancer cell lines indeed succeeded in identifying UDCA target genes and have suggested possible UDCA regulated pathways which can inhibit cell proliferation. These results have somewhat enhanced our understanding of the antiproliferative effects of UDCA and may eventually lead to novel combinatorial chemopreventive strategies. The expression of UDCA target genes may be used to monitor treatment efficacy. Ultimately, the results from these studies may support initiatives to widen the use of UDCA in the prevention of other types of tumors.
4 GENERAL DISCUSSION

In my work presented in this thesis, I have been trying to explore a common practical problem in gastroenterology, that of the diagnosis of inflammatory bowel disease, with particular attention to one of its feared complications, colitis associated colon cancer. Clinical, anatomic and histologic features have traditionally guided the distinction between Crohn’s disease and ulcerative colitis, although no gold standard exists. A differential diagnosis between Crohn’s disease and ulcerative colitis is often not easy. Furthermore, appreciation of disease course and clinical phenotypes, as well as developments in subclinical serological markers, have led to an understanding that IBD is much more than simply Crohn’s disease or ulcerative colitis, but indeed it encompasses a broad spectrum of diseases of polygenic origin that probably reflect differences in diseases pathogenesis. Due to this disease heterogeneity, an appropriate clinical classification of IBD is not straightforward, and may result in critical errors in management, in inappropriate or even contraindicated treatment. For this reason, diagnosing IBD and definition of its phenotypes continue to be a more than occasional challenge to the practicing, especially young, gastroenterologist (personal experience).

In this context, genetic epidemiology and functional genomics, thanks to tremendous advances in biotechnology and bioinformatics, can be similarly commented as Lockhart-Mummery did “The most important advance in our knowledge of these cases has been due to the invention of the electric sigmoidoscope” in the early 1900s. Recognizing the importance of genetics in pathogenesis and clinical phenotype of inflammatory bowel disease is a landmark in understanding these diseases. Positional cloning and candidate gene approach may not only provide new insights in IBD pathogenesis but identify genetic determinants of IBD susceptibility and phenotype, and therefore integrate clinical classification in the delineation of IBD heterogeneity. The discovery of a set of genetic markers, for instance CARD15/NOD2, may present for the first time possibilities to classify disease phenotypes according to molecular and signalling pathways rather than based only on clinical knowledge and experience of disease manifestations which are maybe more subjective. It is enough to think, for example, of the difficulties in defining Crohn’s disease behaviour when both fistula and stenosis are present, or in deciding about the right timing for colectomy when dysplasia is found in ulcerative colitis. In the course of my project, in my attempt to answer to the need of an integrated classification, I found myself exploring patho-physiological aspects of IBD, which really became the core of my research.

In the first paper of our thesis, we show that the three common CARD15/NOD2 polymorphisms are more frequent among concordant than discordant monozygotic twins with Crohn’s disease, supporting our hypothesis that concordant monozygotic twins are under an increased load of susceptibility genes. However, in this study, these three CARD15/NOD2 polymorphisms alone, not surprisingly, are not sufficient to explain Crohn’s disease concordance. It is possible in fact that the low number of genotyped monozygotic twins hampers statistical significance or indeed the contribution to IBD susceptibility and phenotype of NOD2 is low, as suggested by the low frequency of NOD2 allelic variant in the Swedish population. Thus, other polymorphisms in CARD15/NOD2 or other genes are needed in explaining disease concordance in Swedish monozygotic twins. IBD is a complex genetic disease with different susceptibility genes and probably modifier genes, involved in determining its
onset and phenotype. In the second paper, gene-gene interaction between polymorphisms in the CARD15/NOD2 gene and in the promoter region of the TNFα gene, a strong positional and functional candidate gene for IBD, was evaluated as possible explanation for interindividual variation in susceptibility to, and manifestation of, Crohn’s disease. The three TNFα promoter haplotypes we tested responded differently to CARD15, possibly via different modulation of NF-κB binding ability. In particular, TNFα promoter haplotype (-863A–857C) showed the strongest transcriptional activity. Our result parallel the data obtained by Higuchi et al.218 Here by transfection of Raji cells and stimulation with concanavalin A, an identical pattern of activation for the three TNFα promoters was found. Notably, an association of the 857C allele with IBD was recently reported, which correlated with a higher TNFα production in lipopolysaccharide-stimulated whole blood from -857C genotyped individuals, although this association was independent of the haplotypic context in the population studied, and the genetic effect was attributed to the single-site polymorphism.220 Thus, gene-gene interaction, specific for each patient, may modulate expression of a proinflammatory cytokine (TNFα) and its production levels and therefore affect IBD susceptibility or clinical manifestations, such as location and behaviour, or response to steroid treatment.235 Furthermore, when the CD-associated CARD15 1007fs variant was analyzed, induction of TNFα promoter activity was found to be defective. Remarkably, it has been recently demonstrated that TNFα and NF-κB can induce the expression of CARD15 in different cell types, and the existence of a positive feedback loop can therefore be envisaged, where TNFα production is amplified via the CARD15-mediated NF-κB signalling described here.38, 236 Our result is in line with the hypothesis that CARD15 mutation results in loss of function, based on in vitro experiments performed with cell lines transfected with wild type and mutant CARD15 variants, and supported by the Nod2−/− mouse model by Kobayashi et al.41 These animals are susceptible to bacterial infection via the oral route and show a decrease expression of a subgroup of intestinal anti-microbial peptides, known as cryptidins, which correspond to human defensins. Paneth cells, where expression of NOD2 is high, synthesise and secrete these defensins. Patients with ileal Crohn’s disease have an α-defensin deficiency, which is most pronounced in those carrying CARD15 variants.40, 237 These findings suggest a coherent theory whereby Paneth cells could be the common link for the occurrence of Crohn’s disease of the ileum. Conflicting with the above-mentioned results, however, is the fact that high levels of both NF-κB and TNFα are found in Crohn’s disease patients. So far data from mouse and human studies remain discrepant and more work is needed to shed light on CARD15 role in IBD pathogenesis.
CARD15/NOD2 appears to be an intracellular receptor for bacterial compound and as such its association to IBD stimulated and renovated interest in exploring intestinal barrier dysfunction and the perturbation of gut interactions with bacteria in disease pathogenesis. A role for the intestinal flora and its interactions with the host was already proposed from the analysis of animal models of IBD, where the disease is critically dependent on the presence of luminal bacteria, and does not develop in animals raised under germ-free conditions. However, no specific connection with pattern recognition receptors was recognized before the finding of NOD2.
The cystic fibrosis transmembrane conductance regulator (CFTR), as the name suggest, has regulatory functions in intestinal barrier homeostasis.225 This protein not only regulates secretion/absorption functions and mucus production, but functions as pattern
recognition receptor for bacteria, such as Salmonella typhi and Pseudomonas aeruginosa. In the third paper, therefore, we analyzed the potential role of CFTR as a novel candidate gene for IBD. Indeed, we could show association of ΔF508 heterozygosity and Crohn’s disease in two of the three tested populations, while no differences in carrier frequencies were observed in ulcerative colitis patients compared to controls. Moreover, in the third population we genotyped a protective effect of ΔF508 mutation towards right-sided colitis was observed. Interestingly, CFTR mutation confers a specific protection for Crohn’s disease, not for ulcerative colitis, and in particular toward inflammation of the proximal colon. As mentioned in the introduction, commensal bacteria may contribute more to features of Crohn’s disease than in ulcerative colitis, as proposed by antibiotic treatment efficacy in inducing disease remission, bacterial load and composition appear to be different in ileal and colonic Crohn’s disease, and it is known that bacterial flora distributes differently through the whole colon. It is also known that the reduction of CFTR molecules on apical cellular membrane, due to gene mutations, may limit bacterial adhesion and translocation to the submucosa, as well as activation of inflammatory response. Therefore, it is possible to speculate that CFTR mutation may reduce the load of specific bacteria, maybe selectively located in right colon, interacting with colonic epithelium and thus confers protection toward inflammation in this tract of the intestine. This finding might as well pinpoint how alternative subphenotypes of disease (such as left-right colon disease location in Crohn’s disease) might be appreciated when more biological hypothesis are explored, although not commonly listed in classical clinical classification, and might help in formulating novel pathogenetic speculations. However, our results have to be validated and the protective role of ΔF508 heterozygosity for Crohn’s disease, and in particular for inflammation of the right colon, further assessed. In this regards, the study of the effect of CFTR mutation in IBD animal model might be considered. As mentioned in the introduction, IL-10−/− mice is a commonly used mice model for inflammatory bowel disease, where onset of colitis is dependent on the presence of luminal bacterial flora. In particular, monoassociation of IL-10−/− mice with different commensal bacteria induced phenotypically distinct forms of colitis. E. faecalis-induced colitis was slow in onset and involved the left colon, by contrast, E. coli monoassociation led to relatively early onset of a mild-to-moderate inflammation that was at its most severe in the right colon. Well known is the ΔF508 mouse model for cystic fibrosis, where heterozygous offspring ΔF508+/− appears phenotypically normal and healthy. Interestingly, both IL-10−/− mice and ΔF508 mice have been obtained on the same C57BL/6J background. Therefore, to further evaluate the protective role of ΔF508 heterozygosity for right-sided colitis, it might be possible to crossbreed IL-10−/− mice with ΔF508+/− to obtain IL-10−/− mice carrier for ΔF508. Indeed, this model might be expected to be less susceptible to colitis compared to IL-10−/− mice. Furthermore, these animal models might be useful in the identification of bacterial species that specifically induce right-sided colitis and selectively interact with CFTR. It is known that both Salmonella and Pseudomonas interact with CFTR, but it is possible that other bacteria, pathogens or commensal, might recognize CFTR as pattern recognition molecule. Interestingly, a similar protective role of CFTR heterozygosity has been proposed for asthma, although with contrasting results. As mentioned in the introduction, other genes previously associated to asthma, such as NOD1/CARD4, turned out to play a role in inflammatory bowel disease as well. A recent clinical study showed that both
ulcerative colitis and Crohn’s disease patients have a greater likelihood of having asthma, than population controls. All together, these findings support the general concept of chronic inflammatory barrier diseases of polygenic origin, grouping IBD and asthma, as well as other diseases such as psoriasis, that may share at least some of the susceptibility genes. Some of our preliminary results contribute to support this hypothesis. In collaboration with Prof. J. Kere, in fact, we evaluated the neuropeptide S receptor (NPSR1) gene, as a candidate gene for IBD susceptibility. NPSR1 maps in a region of chromosome 7 previously linked to IBD and has been recently associated to asthma. NPSR1, as well as its ligand neuropeptide S, is found on the epithelia of several organs including the small and large intestine, and its expression seems to increase with inflammation, as previously shown in asthmatic patients. We tested NPSR1 polymorphisms for association with IBD, and verified whether the expression of its two major isoforms (NPSR1-A and NPSR1-B) is altered in the intestine of IBD patients. NPSR1 haplotypes were genotyped in 1509 subjects from two of the three cohorts of IBD patients and controls already tested for CFTR (Italy and Sweden). Significant associations were detected in both cohorts. A global analysis of the whole dataset identified strong association of the NPSR1 haplotype block with Crohn’s disease (CD) (P = .0065), ulcerative colitis (UC) (P = .0014) and IBD (P = .0005). Individual predisposing and protective effects were identified mainly for the risk haplotype H2 in CD (P = .0004) and the non risk haplotype H8 in UC (P = .0001). NPSR1 mRNA and protein levels were increased in IBD patients compared to controls, and the risk haplotype H2 correlated with higher expression of both NPSR1-A (P = .004) and NPSR1-B (P = .01) mRNAs. These results provide evidence for an involvement of NPSR1 in the genetic susceptibility to both Crohn’s disease and ulcerative colitis, and extend previous findings on this gene in asthma by implicating NPSR1 polymorphism in the predisposition to other chronic inflammatory disorders of the barrier organs (M. D’Amato et al submitted to Gastroenterology 2006).

Although responsible for the severe disease cystic fibrosis, CFTR mutations have a high frequency in the general population, and heterozygote advantage has been postulated. Indeed, we do not believe that ΔF508 heterozygosity protective effect for Crohn’s disease might explain CFTR heterozygote advantage. However, we might speculate that CFTR mutations, modulating a pattern recognition molecule and epithelial permeability/mucus production, might have been under a positive selective pressure in host-microbe interaction. In mouse models of cystic fibrosis, in fact, CF heterozygous animals are less susceptible to S. typhi infection and respond less to cholera toxin than wild type mice.

Our findings resemble NOD2 data in several respects, although with clear differences. In fact, both CFTR and NOD2 mutations have been associated with Crohn’s disease, but while NOD2 variants increase disease susceptibility, CFTR’s protects. NOD2 mutations are associated with ileal location of the disease, and double-dose carriers are unlikely to have purely colonic disease, CFTR mutation protects towards proximal colitis. Both CFTR and NOD2 function as pattern recognition receptors and have been described to modulate NF-κB activity. Both CFTR and NOD2 have relatively low allelic frequency in the general population. This definitely hampers their clinical relevance, although a better scenario is suggested for NOD2, since its polymorphisms frequency in the disease group is higher than in controls. Thus, in conclusion, although of limited clinical relevance, our results might underline the complex role of CFTR in intestinal barrier homeostasis, reinforce the interest in understanding its functions, and
eventually contribute in understanding of Crohn’s disease susceptibility and phenotypic manifestations.

Chronic inflammation by disruption of the mucosal barrier function and the concomitant immune hyperactivation by the microflora is presumed to represent a central event that is permissive for the progressive transformation of colon epithelial cells in inflammatory bowel disease. Ulcerative colitis and Crohn’s colitis have an increased risk of developing colon cancer. High risk patients are difficult to identify and prevention strategies are lacking. Interestingly, ursodeoxycholic acid, UDCA, 7β-hydroxy epimer of chenodeoxycholic acid, normally present in the bile, has been suggested to have chemopreventive effects in colitis associated colon cancer. So far, however, only limited clinical experiences and animal models supported the potential chemopreventive effect of UDCA, and we believe that better understanding of its mechanism of action might provide novel and more effective pharmacological applications. Thus, in the last part of my project, gene expression pattern of UDCA in colon cancer cell line have been evaluated and molecular determinants of the chemopreventive effect of UDCA have been studied. A similar microarray experiment, designed to evaluate expression profile of primary rat hepatocytes incubated with UDCA, was performed by Castro et al., using Affymetrix GeneChip Rat 230A arrays. There, a total of 96 genes, of which 28 up-regulated (> 1.5-fold) in UDCA-treated cells versus controls, were identified as statistically significant. None of the UDCA regulated gene reported in our experiment was present among the genes identified in primary hepatocytes. There might be several explanations. Indeed, different microarray platforms, Affimetrix GeneChip (in situ-synthesized oligonucleotide microarrays) versus spotted microarrays, and different cells origins, primary rat hepatocytes and human colon adenocarcinoma cell line, seem to be the most important. Furthermore, there is evidence suggesting that UDCA effects differ in different cell lines and tissues. For instance, UDCA has an antiapoptotic activity in hepatocytes, while an antiproliferative effect in colon cell lines. However, in both experiments, morphological changes and gene expression kinetics of the treated cells compared to controls occurred at later time points, suggesting that UDCA may have broad and varying spectrum of secondary transcriptional leading.

In our system, the identification of UDCA target gene lead us to delineate putative biomarkers of UDCA antiproliferative effect and perhaps of its chemopreventive activity in vivo. These genes, indeed, might be useful in monitoring UDCA treatment response in IBD patients with high risk for colon cancer. Furthermore, UDCA target genes suggested possible molecular pathway responsible for UDCA mechanism of action. Among UDCA target genes, for example, NAG-1 is consistently upregulated and our preliminary results suggest it to be one of the effector of its antiproliferative role. Indeed, NAG-1, a member of the TGFβ superfamily, has known antinflammatory and antitumorogenic activities. In particular, it induces cell-type specific apoptosis and has antiproliferative effect, inducing cell cycle arrest in G1. In our SW480 based system and at the tested concentration, UDCA does not mediate apoptosis but induces G1 arrest in the cell cycle. To further evaluate the role of NAG-1 in mediating UDCA antiproliferative effect, several experiments still need to be performed, including the validation of the UDCA activity on the cell cycle previous depletion of NAG-1 via RNA interference, study of NAG-1 promoter and transcription factors that can be recognized as UDCA regulated, and, eventually the evaluation of NAG-1 levels in biopic material from IBD patients treated with UDCA and controls.
Interestingly, NAG-1 is also upregulated by NSAIDs and appears to be responsible, at least in part, of their chemopreventive activity.232 The burden of NSAIDs side effects is well known, and limits their application as chemopreventive drugs in clinical practice. The identification of NAG-1 as target of UDCA thus may open the possibility of combinatorial therapy that may reduce the risk for NSAIDs adverse effect although maintaining their efficacy. Interestingly, our preliminary results showed a synergistic upregulation of NAG-1 by combinatorial stimulation with UDCA and sulindac. This result might as well explain, at least in part, the synergistic antitumorogenic effect observed in APC Min animal model treated with UDCA and sulindac.201 Indeed, in this model combined treatment with low-dose sulindac was less toxic, and was more effective than either agent alone for the prevention of tumors throughout the entire intestine. Extending the meaning of this finding, it is possible to speculate that we might obtain an increase chemopreventive efficacy with combinatorial use of UDCA and 5-ASA, very well known antinflammatory drug with antitumorogenic activity in IBD patients. Indeed, clinical trials might be warranted.

When comparing the UDCA dependent RNA expression kinetics of NAG-1 with the corresponding upregulation of its protein level, a discrepancy in kinetics becomes evident. Indeed, while after 72 hours UDCA stimulation NAG-1 mRNA starts decreasing, the protein level reaches its maximum. Although it might be possible that this discrepancy is due to different half time of RNA and protein, it might as well be that a second UDCA dependent pathway contributes to maintain elevated NAG-1 protein levels. As elegantly demonstrated by Rhode et al, NAG-1 is responsible for the antiproliferative effect of HSPA2 depletion in MCF7.233 Thus, we evaluated the possible effect of UDCA on HSPA2 protein level. Interestingly, our results suggest that UDCA might mediate HSPA2 depletion, in a time and dose dependent way. Furthermore, a noticeable HSPA2 depletion is evident after 72 hours of UDCA stimulation, leading to the speculation that it might at least in part explain the sustained high protein level of NAG-1 despite mRNA decrease at late time points.

Notably, all the reported results were specifically obtained after UDCA stimulation, whilst DCA did not show similar effects (nor did ethanol). Ethanol was used as control since UDCA is dissolved in it, while DCA was chosen to verify that the possible results obtained after UDCA stimulation were not caused by a general property of bile acids, but were specifically induced by UDCA. In general, UDCA displays activities that are distinct to those exhibited by DCA.204-208 Depending on their physicochemical properties, such as hydrophobicity, bile acids can perturb differently membrane structure by alteration of membrane microdomains, cellular uptake and their apoptotic activity. Only deoxycholic acid and chenodeoxycholic acid, the most hydrophobic bile acids, induce apoptosis in the human colon cancer cell line HCT116, while UDCA, one of the most hydrophilic bile acid, does not.254, 255 Moreover, while DCA induces hyperproliferation and apoptosis, UDCA inhibit DCA induced apoptosis and causes arrest the cell cycle in colon-derived tumor cells.230, 254 While the more hydrophobic bile acids (DCA, CDCA, LCA) recognize farnesoid X receptor (FXR), a member of the nuclear receptor superfamily that regulates bile acid homeostasis, and TGR5, a G-protein-coupled receptor, as respectively intracellular and membrane receptors, UDCA does not fully induce their activity.256, 257 This might indeed means that other receptors yet to be identified might account for UDCA functional activities.

Another interesting finding is that UDCA antiproliferative effect is not confined to colon cancer cells, but affects breast cancer cell line with the same efficacy. We found
this intriguing. While the promoting effect of bile acids to colon cancer is well established, their role in breast carcinogenesis is less known. It has been proposed that deoxycholic acid, which is derived from the bacterial degradation of the primary bile acid, cholic acid, in the colon, may be involved in the aetiology of breast cancer.\(^\text{258, 259}\) Several studies have reported differences in faecal bile acids excretion or in the composition of the major faecal bile acids in breast cancer patients, but the results have been equivocal.\(^\text{260-262}\) Deoxycholic acid is found in human breast cyst fluid at concentrations about 50 times greater than those in plasma and in the plasma of postmenopausal women with breast cancer, while no differences in UDCA concentration was observed.\(^\text{263-266}\) Deoxycholic acid promotes the growth, oestrogen receptor and oestrogen-regulated proteins of MCF-7 human breast cancer cells.\(^\text{267}\) The FXR was detected in normal and tumor breast tissue, and activation of FXR by high concentrations of deoxycholic acid induced MCF-7 apoptosis.\(^\text{268}\) Furthermore, UDCA derivates inhibit MCF7 cell proliferation.\(^\text{269}\) Thus it is possible to speculate that UDCA might be further considered for chemoprevention in breast cancer as well, opening a complete new pharmacological application for this, affordable, safe and well tolerated bile acid.

In conclusion, as a young gastroenterologist, the opportunity to explore inflammatory bowel disease with powerful scientific tools and to approach research was exciting. Indeed, I do not think I understood what lab research meant at the very beginning of my PhD. I have just felt that yes, it was interesting, though humble work was required, but it was a wonderful chance to learn. It resulted in much more. It revealed to be not only a scientific journey into IBD pathogenesis, my main interest in gastroenterology, but a life experience. I came in contact with a completely new universe, with different dynamics and a different language, something that I normally perceive as one of the best mirrors of a culture. And I learned to respect it, to enjoy it, to want to be part of it, and to identify with it, although still being gastroenterologist. And here some of the problems came. Some identity problems, some confusion in what translational research really means. I do not think I found the answer yet, I think this is just a promising beginning.

Although the results of my research might have a limited impact on clinical practice, the whole research process has surely modified my being gastroenterologist, and developed my curiosity as a person. Results, I do not know where I am, probably somewhere in the middle (meden agan), but I am committed to keep on learning (gnozi sauton).
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Γνώθι σαυτόν
Temple of Apollo, Delphi.