Genetic Risk Factors of Inflammatory Bowel Disease

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PhD Thesis by

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Summary in Danish

Kronisk inflammatorisk tarmsygdom dækker over sygdommene colitis ulcerosa og morbus Crohn. Studier har vist, at en række faktorer er involveret i inflammationsprocessen ved kronisk inflammatorisk tarmsygdom. Medfødt genetisk prædisposition, immunsystemet og miljømæssige elementer er alle medvirkende faktorer i udvikling og forløb af sygdommene. Sygdommene har historisk set været en byrde overvejende i den vestlige verden, men de seneste årtier har incidensen været stigende i Asien. Den hurtige stigning tydeliggør betydningen af miljømæssige påvirkninger i ætiologien af kronisk inflammatorisk tarmsygdom.

Variationer i CARD15 genet, der koder for et protein der genkender bakteriedele, var det første gen der viste sig at prædisponere til morbus Crohn, men ikke til colitis ulcerosa. De seneste års intensive genom associations studier har bidraget betydeligt med nye genetiske risikofaktorer. Nogle af disse gener er fælles for sygdommene, men flere er specifikke for enten colitis ulcerosa eller morbus Crohn. Desuden viser flere studier, at der er store etniske forskelle i udbredningen af de fundne genetiske risikomarkører. Tre varianter i CARD15 genet der prædisponerer for morbus Crohn er eksempelvis ikke nær så hyppige i Skandinavien sammenlignet med andre europæiske lande, og er ikke til stede i Asien. Der er også store etniske forskelle i miljøpåvirkninger, hvilket gør det kompliceret at finde sande genetiske og miljømæssige risikofaktorer. Tobaksrygning er den bedst undersøgte miljømæssige faktor. Det har vist sig at rygning forøger risikoen for at udvikle morbus Crohn, men at det har en beskyttende effekt mod udvikling af colitis ulcerosa.

I et case-control studie undersøgte vi hyppigheden af tre CARD15 variationer i den danske befolkning. To af de tre hyppige variationer var associeret med morbus Crohn, men var ret sjældne i Danmark sammenlignet med andre europæiske lande.

Patienter med kronisk inflammatorisk tarmsygdom har en øget permeabilitet over tarmvæggen. Det ledte til vores hypotese om at en lav aktivitet af enzymer, der omsætter fremmedstoffer lokalt i tarmen, kunne forårsage skade på tarmepitelet. Dette var en mulig risikofaktor for udvikling af kronisk inflammatorisk tarmsygdom. Flere af disse enzymer (samlet under betegnelsen xenobiotika metaboliserende enzymer), har hyppige gen polymorfier der påvirker enzymaktiviteten. Vi undersøgte om nogle af disse polymorfier var associeret med kronisk inflammatorisk tarmsygdom i Danmark, men vi fandt ingen evidens for at det var tilfældet. Vi undersøgte også for en mulig interaktion mellem gen polymorfierne og rygning. Vi fandt enkelte genvariationer, hvor der var en risikoforskel for rygere og ikke rygere. Eksempelvis fandt vi en genvariation kodende for manglende aktivitet af et enzym (GSTM1), der forstærkede den beskyttende effekt af rygning på udvikling af colitis ulcerosa.

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CARD15 variationer er altså ikke særligt udbredte i Danmark, men de prædisponerer til udvikling af morbus Crohn. Generelt set kunne vi konkludere, at genetiske polymorfier i xenobitotika metaboliserende enzymer ikke ser ud til at spille en afgørende rolle for udvikling af kronisk inflammatorisk tarmsygdom. Enzymerne er vigtige i omsætningen af medikamenter brugt til behandling af kronisk inflammatorisk tarmsygdomme. Det kan betyde at bestemmelse af genetiske polymorfier i xenobitika metaboliserende enzymer er relevant i forbindelse med medicinsk behandling af sygdommene og fremtidige studier er planlagt for at afdække dette.

The present thesis is partly based on three studies, which are referred to in the text by Roman numerals I-III. The studies have been carried out in the period from 2005-2010 at the Department of Clinical Biochemistry, Section of Molecular Diagnostics, Aalborg Hospital, and Mech-Sense, Department of Gastroenterology, Aalborg Hospital in collaboration with Centre for Sensory-Motor Interactions (SMI), Aalborg University.

I: Ernst A, Jacobsen B, Østergaard M, Okkels H, Andersen V, Dagiliene E, Pedersen IS, Thorsgaard N, Drewes AM, Krarup HB. Mutations in CARD15 and smoking confer susceptibility to Crohn's disease in the Danish population. Scand J Gastroenterol. 2007 Dec;42(12):1445-51.

II: Ernst A, Andersen V, Østergaard M, Jacobsen BA, Dagiliene E, Pedersen IS,
Drewes AM, Okkels H, Krarup HB. Genetic variants of glutathione S-transferases mu, theta, and pi display no susceptibility to inflammatory bowel disease in the Danish population. Scand J Gastroenterol. 2010 Sep;45(9):1068-75.

III: Ernst A, Andersen V, Østergaard M, Jacobsen BA, Pedersen IS, Drewes AM, Okkels H, Krarup HB. Common polymorphisms in the microsomal epoxide hydrolase and N-acetyltransferase 2 genes in association with inflammatory bowel disease in the Danish population. Eur J Gastroenterol Hepatol. 2011;23:269-74.

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Anja Ernst, March 2011

Abbreviations

BCRP: Breast cancer resistance protein
CARD: CAspase Recruitment Domain
CARD15: CAspase Recruitment Domain 15
CD: Crohn's Disease
GST: Glutathione S-transferase
GSTM1: Glutathione S-transferase μ
GSTM1*0: Glutathione S-transferase μ null genotype
GSTP1: Glutathione S-transferase π
GSTP1 105 low: Glutathione S-transferase π 105 low activity genotype
GSTP1 114 low: Glutathione S-transferase π 114 low activity genotype
GSTT1: Glutathione S-transferase θ
GSTT1*0: Glutathione S-transferase θ null genotype
GWA: Genome Wide Association
IBD: Inflammatory Bowel Disease
MDR1/ABCG1: MultiDrug Resistance p-glycoprotein
mEH: microsomal Epoxide Hydrolase
MLPA: Multiplex Ligation dependent Probe Amplification
NAT2: N-acetyltransferase 2
NFkB: Nuclear Factor kappa Beta
NLRs: Nucleotide oligorimisation Like Receptors
OR: Odds Ratio
PCR: Polymerase Chain Reaction
SNP: Single Nucleotide Polymorphism
TLRs: Toll-Like Receptors
UC: Ulcerative Colitis

Chapter   1.

Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are two related diseases that belong to a larger group of illnesses called chronic inflammatory bowel diseases (IBD). Studies indicate that the inflammation in IBD involves a complex interaction of several factors, among these inherited genetic susceptibility, the immune system, and environmental factors. However, relatively little is known about genetic risk factors and the interaction between genetic and environmental factors.

1.1 INFLAMMATORY BOWEL DISEASES

IBD is mainly used to describe CD and UC but other forms such as indeterminate colitis also exist. CD and UC are characterised by an abnormal immune response. CD and UC differ by both the localisation of and the nature of the disease[1]. CD usually involves the colon and ileum, but may involve any part of the gastrointestinal tract. All layers of the intestine may be involved, and there can be normal healthy bowel in between patches of diseased gut. In UC the gastrointestinal involvement is limited to the colon and the rectum. The rectum is nearly always involved and the lesion extends proximally in a continuous pattern[1].

1.1.1   Incidence and prevalence

The prevalence and incidence of IBD have historically been higher in developed countries[2, 3].The incidence of CD and UC has recently been investigated in different geographical regions of Denmark. For women the incidence of CD was approximately 10 pr 100.000 and for men 8 pr 100.000 in the two regions[4, 5]. The incidence of UC was in the range of 13-17 pr 100.000 for both men and women in the two distinct regions of Denmark[4, 5]. The prevalence of CD and

UC was 151 and 294 pr 100.000 inhabitants in Northern Jutland on the 31st of December 2002, which gave an estimate of 25000 Danish IBD patients[5].

1.1.2   Symptoms and diagnosis

A diagnosis of IBD is usually made in young adults. Two recent Danish studies revealed that the majority of IBD diagnoses is made in the period between the late teenage years and into the thirties[4, 5]. The diagnosis of CD or UC is established by finding characteristic intestinal ulcerations and excluding alternative diagnoses, such as enteric infections. CD patients typically present with diarrhoea, abdominal pain, fever and weight loss. UC patients typically present with rectal bleeding, diarrhoea, and abdominal pain. Active disease in UC is characterized by the endoscopic appearance of superficial ulcerations and bloody stools are common. The diagnosis of IBD can be made only when other reasonable alternatives in the differential diagnosis have been excluded[6, 7].

1.1.3   Treatment and prognosis

IBD is a chronic disease, but the activity will fluctuate between disease flare-up and times of remission. The quality of the social life of a patient may be tremendously affected. Symptoms may range from mild to severe, and therapy is dependent on both severity of disease, location of disease, and disease associated complications. The medical therapy includes anti-inflammatory and immunosuppressive drugs and biological drugs[6, 7]. The response to medical treatment and tolerance of the medicaments vary greatly between individuals. The main goal of disease treatment is to induce remission of disease, and for the patient to remain in remission. Colorectal cancer risk is an important concern for patients with UC or CD[8]. In general, the longer a person has had IBD, the greater is the risk of developing colorectal cancer. Surveillance using colonoscopy is used to detect early dysplasia in IBD patients.

1.2 GENES IN COMPLEX DISEASES

Where monogenic diseases have recognisable inheritance patterns for recessive, autosomal dominant and gender-linked diseases which allows for exact calculation of risk of disease, the inheritance pattern of complex diseases are less evident. Complex diseases are associated with the effects of multiple genes and also in combination with environmental factors such as lifestyle factors.

Genetic risk factors of complex diseases are usually found by association studies. Hypothesis generated studies have been used for decades to investigate whether candidate genes are associated with disease. The completion of the Human Genome Project revealed the human genetic profile to consist of more than 3 billion base pairs. An estimate of between 20.000-25.000 genes was found which

means that only a small percentage (1.0-1.4%) of the genome sequence encodes proteins[9]. The genomes of human individuals are more than 99% identical, leaving approximately 1% of the human genome responsible for both normal genetic variation and genetic predisposition of diseases. The International HapMap Project, a project investigating the genetic variation between populations of different ethnic origin, was conducted in parallel with the Human Genome Project[9]. The information from these studies is used in search of genetic predisposition of diseases. The HapMap Project has made an estimate that more than 10 million Single Nucleotide Polymorphisms (SNPs) are present in the genome[10]. Many of the polymorphisms are common functional polymorphisms influencing the phenotype of the individual. Hence, the polymorphisms are responsible for variations in the population.

1.2.1   Common variants vs. rare variants in common diseases

According to the common disease - common variant hypothesis, the risk of contracting common diseases is influenced by genetic polymorphisms that are relatively common in the population[11, 12]. Genome Wide Association (GWA) studies has made the search for susceptibility genes without any prior assumptions of the genes possible. GWA studies use a large number of well-spaced SNPs to provide almost complete coverage of the human genome. The function of the disease associated variant is often unknown. The causative gene variant may then be found by scanning nearby genes that could possibly be related to the disease of interest. There is also the possibility that the common variants may act as modifiers of the effect of other rare variants[11, 12]. However, some precautions may be taken. When using the GWA approach very large study populations are needed because the variants in general contribute with only a modest disease risk. The significance levels must take into account the large number of multiple testing to avoid large number of false positives. Replication studies are needed to eliminate false positives found in preliminary studies.

The common disease – rare variant hypothesis argues that rare relatively high penetrant genetic variants contribute to common diseases[13]. For rare variants the functional effect lies within the rare variant. The rare variants are often population specific due to the founder effect, and replication studies of rare variant associations are difficult because of the rarity of the variant. Rare variants are found by sequencing regions in functionally relevant genes. Hence, selection of genes to investigate is extremely essential when searching for rare disease causing variants.

GWA studies uncovering common variants vs. trying to identify rare disease associated variants are two different ways to identify genetic susceptibility of disease. Pros and cons have been made concerning both approaches and the discussion will probably continue in upcoming years[12, 13].

1.3 GENES IN INFLAMMATORY BOWEL DISEASES

A positive family history of IBD is the greatest independent risk factor of developing disease. Twin studies have shown a higher concordance rate among monozygotic than among dizygotic twin pairs proving a genetic influence on occurrence of IBD. The monozygotic disease concordance was approximately 50% for CD and 18% for UC[14-18]. Thus, the genetic contribution to disease seems to be more pronounced with regard to CD than to UC.

1.3.1   Disease associated genes

As IBD is characterized by altered epithelial barrier function and defects in the immune response, genes involved in the immune response, especially the innate immune response, are likely candidates as risk factors of IBD. Genes that directly or indirectly affect the epithelial barrier are also possibly candidates when searching for genetic risk factors of IBD.

Huge progress has been made in unraveling the genetic background of IBD. The recent year's GWA studies have made an immense contribution to the number of known genetic risk factors of IBD, but the contribution to disease risk is in general low. More than 100 loci are now known for both UC and CD, where some of them are shared[19-21]. Many of the identified susceptibility genes cluster into known cellular processes of immunity and autophagy[22-24]. It is intriguingly exciting when more than one gene in a pathway is associated with disease susceptibility. Several genes involved in the differentiation of the T helper cells Th17 have shown to be associated with IBD. Genes such as IL12B, JAK2, STAT3, CCR6 and IL23R[20, 22, 25, 26]. Most of these are shared for CD and UC, but CCR6 are specific to CD[23].

Specific genetic variants in ATG16L1 and IRGM involved in autophagy have shown disease susceptibility to CD [22, 27, 28]. A possible interaction between ATG16L1 and the first identified CD susceptibility gene CARD15 strengthen the importance of autophagy related genes in CD[29-31].
Genes involved in epithelial barrier function such as ECM1, LAMB1 have shown susceptibility specific to UC and not to CD[22, 23, 26].

A tremendous job is still ahead unraveling the causal genes and genetic variants within susceptibility loci identified by GWA studies.

1.3.2   CARD15

The innate immune system serves to immediately recognize pathogen associated molecular patterns and control infection by inducing pro-inflammatory cytokines and chemokines that recruit inflammatory cells[32]. Pattern recognition receptors are proteins expressed by epithelial cells and cells of the innate immune system. Pattern recognition receptors have a key role in maintaining the integrity of the epithelial barrier[33, 34]. Two distinct systems of pattern recognition receptors

have been investigated in susceptibility to IBD; the membrane bound Toll-Like Receptors (TLRs) and the cytoplasmic Nucleotide-binding oligomerisation domain Like Receptors (NLRs)[35]. The receptors initiate immunity by recognizing different molecular patterns such as bacteria cell wall components[34].

The TLRs are important initiators of immunity by recognizing different pathogen associated molecular patterns shared by bacteria[36]. The action of TLRs upon recognition of microbial patterns is an initiation of a signaling cascade that triggers immunity by a pro-inflammatory pathway[37]. Several TLRs, TLR2, TLR4, TLR5 and TLR9, have been associated with IBD[32, 36, 38]. TLR4 is the most intensively investigated TLR with respect to IBD and a large meta-analysis have reported two polymorphisms in the gene to cause susceptibility to both UC and IBD in Caucasians[36]. The data suggest that TLRs are crucial for initiation and progression of IBD, but mutations in a single TLR gene are insufficient to explain the complex pathogenesis of IBD.

The NLRs are like the TLRs important in regulation of pro-inflammatory pathways in response to bacteria by inducing signaling pathways initiating an immune response[39-41]. The NLRs consist of three domains; a C-terminal leucine rich repeat, a central domain and a variable N-terminal domain which is responsible for the diversity of the NLRs[42].

A caspase recruitment domain (CARD) is the N-terminal domain in the NLRs nod1 and nod2. These receptors are alternatively named CARD4 and CARD15 due to changes in the nomenclature. CARD15 was the first IBD susceptibility gene identified, and it has been found only to be a risk factor of CD[43, 44]. Few variations have been reported in African and Asian populations, but at least 30 variations may be seen in Caucasians[45, 46]. The majority of variations are specific for each individual but three variations occur more frequently and may in some populations be considered rather as polymorphisms and not mutations. The three common variants are two SNPs and one frame-shift mutation which account for up to 82% of all CARD15 mutations found[46]. The three variations are located within the C-terminal region of the protein responsible for ligand recognition[42]. Recognition of bacterial components by CARD15 proteins activate the Nuclear Factor kappa Beta (NFkB) pathway which initiates transcription of proinflammatory cytokines and antimicrobial peptides such as defensins[35, 40, 47] This is illustrated in figure 1 on the following page.
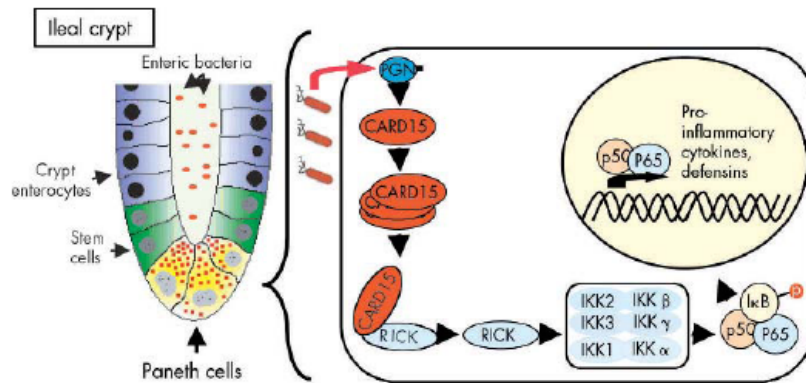
Figure 1. Action of CARD15

CARD15 is activated by binding of degradation products of peptidoglycans (PGNs) derived from bacterial cells walls. Their presence triggers CARD15 oligomerisation and recruitment of RIP-like interacting CLARP kinase (RICK) via CARD-CARD interaction. RICK then activates
the nuclear factor kB inhibitor (IkB) kinase complex (IKK) via phosphorylation of IKKc. The IKK complex next phosphorylates IkB resulting in nuclear factor kB (NFkB) translocation to the nucleus and transcriptional activation of NFkB responsive genes such as proinflammatory cytokines or
defensins. Modified from Gasche et al[47].

The exact mechanism of how CARD15 variations contribute to CD is not fully understood. It has been debated whether the contribution to CD pathogenesis happens either through a loss of function or a gain of function mechanism. The loss of function explanation relies on a diminished response upon recognition of microbial patterns leading to a less effective recruitment and function of innate cells. Based on this hypothesis otherwise harmless commensal bacteria will increasingly translocate into the intestinal mucosa leading to an activation of the adaptive immune system and ultimately a chronic inflammation[34]. The gain of function explanation states initiation of a hyper-response upon recognition of microbial patterns leading to an excessive innate immune response, despite of normal levels of microbial patterns present[34, 48].

Prevalence of the three CARD15 variants have shown great ethnic differences, indicating that genetic susceptibility differ between populations of different ethnic origin[49, 50]. Heterogeneity exist even between the European countries, i.e. CARD15 variants are less frequent in Northern Europe[49, 51]. Regional diversity of CARD15 variants in Europe indicates the existence of

selection pressure within a region. Natural selection within a certain geographical region along with genetic drift can ultimately lead to elimination, or in this particular case fixation, of a specific variant[52].

1.4 GENE ENVIRONMENT INTERACTIONS

More than genetic susceptibility is responsible for developing IBD. The environmental contribution to IBD is evident by the fact that incidence and prevalence of IBD are increasing in areas which have historically been low incident areas[20]. Environmental factors play a crucial role in disease pathogenesis and the interaction between susceptibility genes and different environmental factors has been shown to influence the risk of developing IBD[53, 54].

All sorts of environmental factors such as breastfeeding, childhood infections, use of oral contraceptives, appendectomy, smoking and hygiene have been investigated in search for association with IBD[53, 55-57]. Most research has resulted in contradictory results. Appendectomy has shown some protective effect against UC[53], but the most intensively investigated factor is tobacco smoking. Smoking has consistently proven to be a risk factor for CD, while on the contrary current smoking has shown to be a protective factor against UC[53, 55, 58, 59]. Differences in associated genes between smoking and non-smoking CD patients point towards complex gene environment interactions [53, 54, 60].

1.4.1 The xenobiotica metabolising enzyme system

The human body is exposed to a wide array of xenobiotics; from environmental components and pharmaceuticals to endogenously produced reactive substances. The body comprises a complicated enzymatic biotransformation system which detoxifies these substances[61, 62]. The majority of the detoxification reactions take place in the liver but a great amount of detoxification occurs in the gastrointestinal tract as well[63]. The enzymes are highly polymorphic displaying wide phenotypic variation. Impaired ability to remove reactive substances from the body may play a role in the aetiology of chronic conditions i.e. autoimmune diseases by gene environment interactions[61].

The intestinal epithelial barrier constitutes the largest and most important barrier against the external environment. The crucial function of the epithelial barrier is to allow absorption of nutrients and water, while maintaining an effective defence against luminal toxins and antigens. The permeability of the epithelial barrier is regulated by multiple factors such as cytokines, immune cells, apoptosis and exogenous factors such as xenobiotics[61, 64, 65]. Increased intestinal permeability have been shown not only in IBD patients, but also in healthy first degree relatives[66-68]. Xenobiotica metabolising enzymes and cellular efflux transporters are critical components in maintaining intestinal barrier integrity by

removing or detoxifying reactive metabolites of xenobiotics which make these enzymes candidates as risk factors[69].

The consequence of biotransformation is in most cases detoxification; however, metabolism of some xenobiotics generates metabolites that are more reactive than their substrate compound. The biotransformation system involves several enzyme systems that are commonly divided into two phases; phase I and phase II. The phase I enzymes are responsible for oxidation, reduction or hydrolysis and can be either detoxifying or activating[63]. The phase II enzymes exert primarily detoxifying potential by conjugation[61]. The export of xenobiotics and conjugates out of the cell may be considered phase III biotransformation[61]. The efflux transporters (phase III) mediates the transport of xenobiotics and conjugated compounds back into the gut lumen or into the lymph for transport back to the liver[70, 71]. The transporters play a pivotal role in drug resistance but are also involved in protecting tissue from xenobiotic accumulation and toxicity. Two members of this group of transporters were included in the present study; p-glycoprotein encoded by MDR1/ABCB1 (MDR1), and the breast cancer resistance protein encoded by BCRP/ABCG2 (BCRP)[72]. Figure 2 depicts the route towards excretion for xenobiotics with different characteristics.

Phase I	Phase II	Phase III
Lipophilic xenobiotica → Product of oxidation, reduction and hydrolysis	→ Hydrophilic metabolites	→ Excretion
CYP mEH*	GSTs* NATs* SULT	MDR1* BCRP*
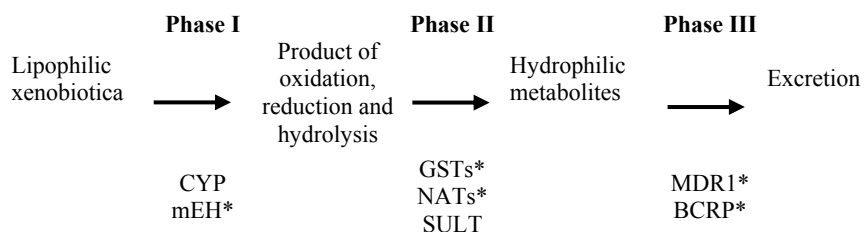
Figure 2. The road towards excretion of xenobiotics. * denotes enzymes covered in this thesis.

1.4.2   Glutathione S-transferases

Substrates of the Glutathione S-transferase (GST) enzyme family could be by-products of either free radical damage generated during oxidative stress such as fatty acid hydro peroxides or diol-epoxide by-products derived from polyaromatic hydrocarbons originating from incomplete combustion of tobacco smoking[73-77]. The GST enzymes share several substrates derived from tobacco smoke. Hence, GST genotype may have a modifying effect on smoking. A previous study found genetic GST variants encoding low (Glutathione S-transferase π (GSTP1 105)) and missing activity (Glutathione S-transferase μ (GSTM1*0)) to have a modifying effect on smoking increasing the level of inflammation[78].

1.4.3 microsomal Epoxide Hydrolase

Microsomal Epoxide Hydrolase (mEH) plays an important role in both the activation and detoxification of by-products of polycyclic aromatic hydrocarbons from exogenous chemicals such as tobacco smoke. Two common functional polymorphisms exist in the mEH gene. One, the 113 polymorphic site, resulting in a 40% decrease in enzymatic activity, another, the 139 polymorphic site, resulting in a 25% increase in enzymatic activity[79, 80]. This phase I metabolising enzyme exhibit a dual role and is capable of both detoxification of epoxide intermediates and of participating in metabolic activation of intermediates into more reactive epoxides[81, 82]. Figure 3 illustrates the dual role of mEH by the metabolisation of benzo(a)pyrene.
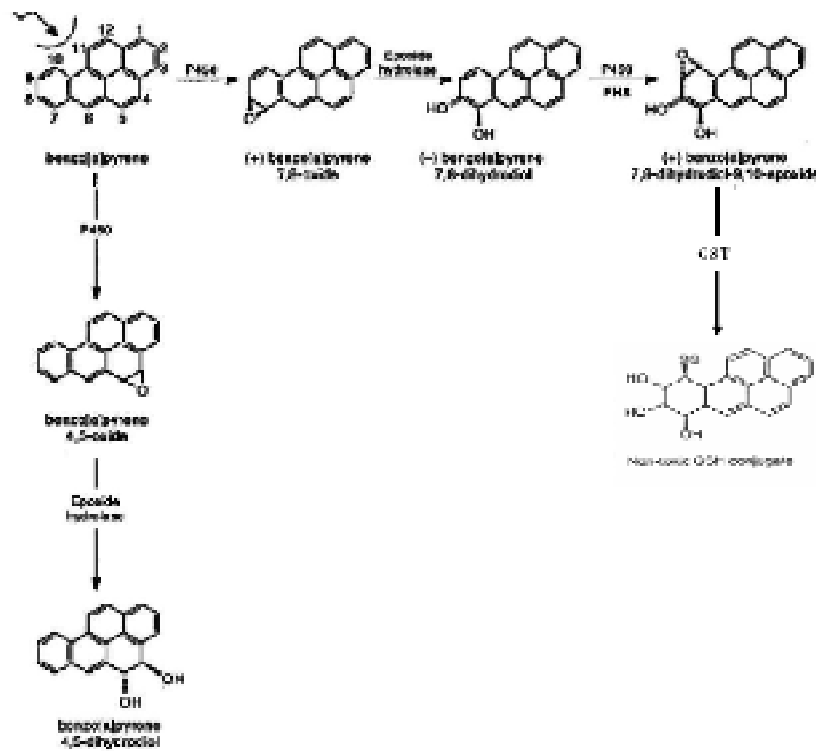
Figure 3. Metabolisation of Benzo(a)pyrene

mEH is involved in the metabolisation of two benzo(a)pyrene derived intermediates. The vertical reaction to the left leads towards benzo(a)pyrene4,5dihydrodiol, a less reactive molecule. In the horizontal reaction

mEH gives rise to benzo(a)pyrene7,8dihydrodiol which act as substrate for generation of the highly reactive benzo(a)pyrene7,8dihydrodiol-9,10epoxide. The vertical reaction to the right is the detoxifying conjugation reaction with glutathione by an enzyme of the GST family.

1.4.4   N-acetyl transferase 2

The N-acetyltransferase 2 (NAT2) enzyme is important in the biotransformation of a number of aryl amines and heterocyclic amines derived from foods, tobacco smoking or other environmental substances[83]. It is possible that individuals with slow NAT2 acetylator status have a decreased ability to metabolise xenobiotics leading to accumulation which leads to increased permeability in the gastrointestinal tract. The accumulation of xenobiotics are believed to induce an autoimmune mechanism and NAT2 slow acetylator status has been shown to be associated with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and diabetes mellitus[84-86]. A Japanese study has shown an association between NAT2 slow acetylator status and CD[87].

1.4.5   P-glycoprotein and Breast Cancer Resistance Protein

The enzymes P-glycoprotein and Breast cancer Resistance Protein (BCRP) function as intestinal transporter of xenobiotics and are highly expressed in the gut[88, 89]. P-glycoprotein encoded by the Multi Drug Resistance protein (MDR1) gene has shown decreased levels of expression in inflamed tissue in active UC[89-91]. Genetic variants of the MDR1 gene exist and the main focus has been on two polymorphic sites in the gene. The two functional genetic variants have been shown to correlate with the activity and expression of the enzyme, and the two polymorphisms have been associated with IBD in different populations but with conflicting results[90, 92-94].

BCRP has shown decreased levels of expression in the inflamed mucosa of patients with active UC as was the case for p-glycoprotein[89, 90]. BCRP as a possible susceptibility gene of IBD has not been as intensively investigated as MDR1. A Hungarian study showed no association between two variants of the BCRP gene and IBD[95].

1.5 HYPOTHESIS

CARD15 was the first gene found to confer susceptibility to CD[43, 44]. The variations in CARD15 have shown to display wide ethnic variation, and even display heterogeneity within Western populations and between European countries[49, 51]. The CARD15 variants have consistently shown to display susceptibility only to CD and not UC[28, 96]. Hence, the hypothesis was that

CARD15 variations were associated with CD and not with UC in our population, and that phenotypic associations would be likely.

High levels of oxidative stress along with increased intestinal permeability have been observed in IBD[97-99]. Reactive molecules play a central role in altering the intestinal permeability. Hence, impairment in the biotransformation system responsible for detoxification of reactive substances might be important in the aetiology of IBD. This led to the hypothesis that genetic polymorphisms responsible for low or missing activity of detoxification enzymes are risk factors of IBD. This hypothesis is supported by the common disease – common variant hypothesis[11, 12]. Polymorphisms in detoxification enzymes have been associated with autoimmune disease i.e. systemic lupus erythematosus and rheumatoid arthritis[84, 86]. However, only a few contradictory studies have focused on polymorphisms in detoxification enzymes as possible risk factors of IBD.

1.6 AIMS

The overall objective of this study was to clarify the extent of three relatively common variations in the CARD15 gene in susceptibility to IBD in our cohort. It was also intended to make a comparison with other Western populations and to determine whether the CARD15 mutations were important regarding disease phenotype (study I). The other main objective of this study was to estimate the significance of the highly polymorphic xenobiotica metabolising enzymes of the GST family (study II), the mEH (study III) and the NAT2 (study III) in susceptibility to IBD in our cohort. Whether the polymorphic enzymes were important for disease phenotype and whether an interaction with smoking was present was also assessed (I, II, III).

The aims were:

1. To assess the significance of three common variations in the CARD15 gene in susceptibility to IBD, and to determine whether any genotype-phenotype correlations were present.
2. To assess the significance of having a genotype encoding low or no enzymatic activity of Glutathione S-transferase μ (GSTM1), Glutathione S-transferase θ (GSTT1) and Glutathione S-transferase π (GSTP1) in susceptibility to IBD, and to determine whether any genotype-phenotype correlations were present.
3. To estimate whether interactions were present between the GST enzymes in susceptibility to IBD.
4. To assess the significance of having the slow acetylator genotype of NAT2 in susceptibility to IBD, and to determine whether any genotype-phenotype correlations were present.

5. To assess the significance of low activity genotype of mEH in susceptibility to IBD, and to determine whether any genotype-phenotype correlations were present.

6. To determine the role of smoking behaviour in susceptibility to CD and UC, and to investigate a possible modifying effect of smoking on GST genotypes, NAT2 acetylator status and mEH genotypes in susceptibility to CD and UC.

Chapter 2.

Methods

2.1 STUDY DESIGN

The present epidemiologic study was conducted as an observational study of the case-control type. This was concerned with the frequency of exposure (genotype or smoking) in IBD patients (cases) and people without the disease (controls). This type of study is the predominant design when searching for aetiology and causal associations in diseases. Association between exposure and disease was expressed as odds ratios (OR) which represent the odds of exposure in cases divided by the odds of exposure in controls.

2.1.1   Strength and limitations of the study

A great advantage when choosing the case-control design was the possibility to examine many exposures in the same study. A great strength of this study design was that of being able to recruit cases fast. Three hundred and eighty-eight patients with CD and 565 patients with UC were included in the study. The patients were recruited from the outpatient clinics between January 2004 and March 2005 from three Danish hospitals in the cities of Aalborg, Viborg and Herning. A senior registrar reviewed the case record and only patients fulfilling the diagnostic criteria of CD and UC as proposed by Binder were included[100]. Patients with indeterminate IBD were excluded, and patients under the age of 18 were also excluded from the study.

   The collection of patients was biased by the fact that colectomised patients were not included in the study group because these patients no longer attended routine follow-up consultations. These patients are estimated to represent 10-15% of the patients. A control group of 796 healthy blood donors representative of the general Danish population were recruited from Viborg County during the same period. All participating patients and controls gave written consent and the local Ethical Committee at Aalborg and Viborg County approved the protocol (VN2003/5).

The purpose of the control group was to provide an estimate of the frequency of genetic polymorphisms and smoking status in subjects in the population without the disease. A major challenge in the case-control study design was the selection of a representative control group. The controls in this study were blood donors recruited at Viborg Hospital. Blood donors tend to be healthier than the general population which may be reflected in their genetic profile (discussed in paper III). Recall bias is common regarding exposure in case-control studies. In the present study genetic polymorphisms represent the exposure, thus recall bias was not an issue. Uncertainty of whether the former smokers were smokers at the time of diagnosis, or whether they quit smoking prior to being diagnosed with IBD, may have biased the results when separating smoking into three groups of current smokers, former smokers, and never smokers. To avoid bias the current smokers and former smokers were grouped together. For a few sub-analyses the ever smoker group were separated into current and former smokers.

2.2 GENOTYPING ASSAYS

Two different Polymerase Chain Reaction (PCR) based methods were used to determine the genotype of the different genes in the individual papers I, II and III.

2.2.1 PCR gel based assays

Two steps are included in the PCR gel based assays: PCR amplification by use of specifically designed primers, followed by visualisation using gel electrophoresis. A housekeeping gene is usually used as an internal control of amplification. Primers with similar annealing temperatures are designed for the gene of interest as well as the control gene and are run for 35-45 cycles depending on the assay.

2.2.2 Real-time PCR based assays

The real-time PCR assay visualises the exponential PCR amplification as it progresses, whereas in traditional PCR, results are collected after the reaction is complete. In real-time PCR the quantity of the PCR product is directly proportional to the amount of template.

2.2.2.1 Allelic discrimination assay

The real-time allelic discrimination assay was used to genotype SNPs (I, II, III). As for the PCR gel based assays a specific set of primers was designed. Besides the primers, two specific probes which recognised the two possibilities at the polymorphic site of the gene of interest were designed. TaqMan technology from Applied Biosystems was used for genotyping in the present studies (I, II, III). The two probes specific for each of the two possible alleles of the polymorphic site was

labelled with two different fluorescent dyes at the 5´ end (V and F fig. 4). The 3´end was labelled with a quencher (Q, fig. 4), which absorbs the fluorescent emission. The probe anneals to the DNA sequence complementary to its sequence and it is incorporated into the DNA strand. This separates the fluorophore from the quencher and fluorescent emission occurs.
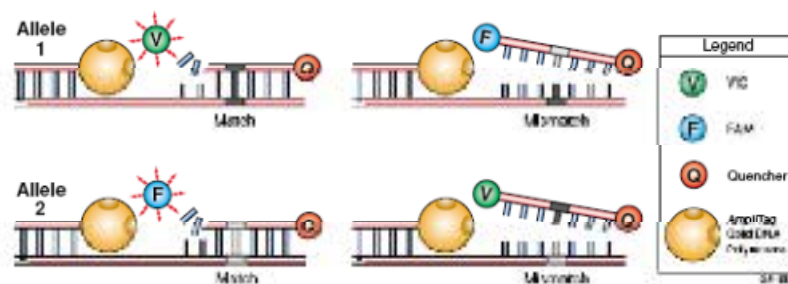
Figure 4. Taq-Man based allelic discrimination

Top: Vic-labelled probe recognises its target allele 1 and is incorporated during the amplification of DNA. The FAM-labelled probe does not recognise allele 1. Bottom: The FAM-labelled probe recognises its target allele 2 and is incorporated during the amplification of DNA. The Vic-labelled probe does not recognise allele 2. Figure from manual supplied with the HT7900 Real-time apparatus from Applied Biosystems.

This emission represents the amplification of products in real-time. Figure 5 shows a signal for both fluorescent dyes. Hence, this sample is heterozygous for the polymorphic site. If the sample had been homozygous for either the wild type or the variant, only one curve would show. For allelic discrimination assays a scatter plot is produced for each run, which allows for a fast check of the results. Figure 6 (on the following page) represents the genotype callings made by the system for an allelic discrimination run from the genotyping of NAT2 (III).
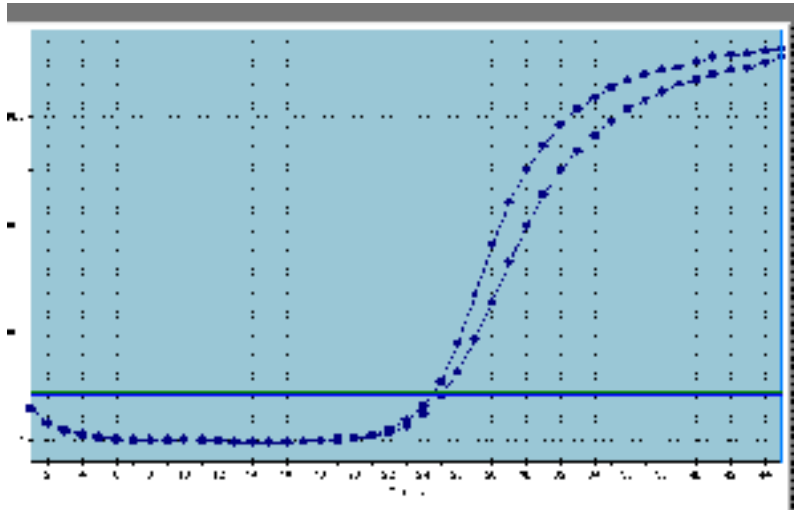
Figure 5. Amplification curves of real-time assay

Amplification curves for a sample being heterozygous for the polymorphic site investigated. Each curve represents a signal for one specific fluorescent dye.
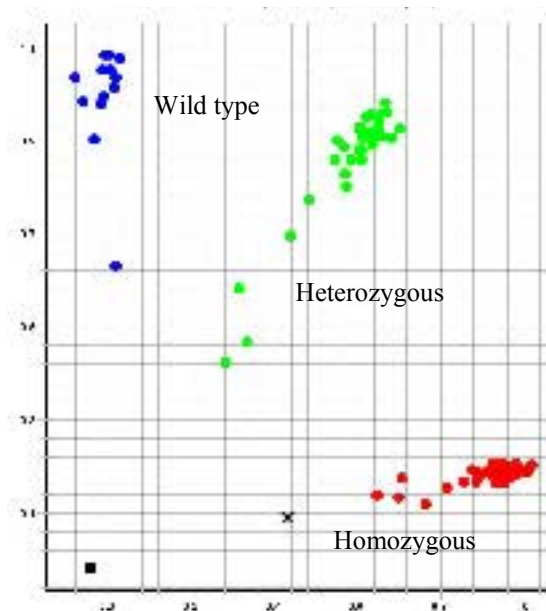
Figure 6. Allelic discrimination scatter plot

Scatter plot of allelic discrimination run. Circles in upper left corner represent the wild type genotype, circles in the lower right corner represent the homozygous variant genotype, and circles along the diagonal represent the heterozygous genotype. The black square is a negative control. The x is a genotype not called by the system.

2.2.2.2   Relative quantification assay

In the relative quantification real-time assay a target gene and a reference gene are amplified in the same tube. For the method to be valid, the efficiency of the target gene amplification and the efficiency of the reference gene amplification must be approximately equal. This method compares the threshold cycle of one target gene to a reference housekeeping gene in a single sample.

2.3 GENOTYPING ASSAYS USED IN THE THESIS

2.3.1   Genotyping of CARD15

The allelic discrimination design was chosen for genotyping of the three common CARD15 variants (I). The primers and probes used as well as the concentrations and the run parameters are described in detail in paper I. Direct sequencing of the three common variants was used for validation of the assay using primers from King et al Human Mutation Supll. Online Nov. 2005.
Another approach for genotyping CARD15 on the entire cohort could be direct sequencing. This would have been the method of genotyping if searching for rare private for the genotyping of the entire cohort, which would have been the preferable technique if we were searching for rare private mutations in our cohort.

2.3.2   Genotyping of GSTT1

The PCR gel based assay was used only to genotype GSTT1 (II). A primer pair specific for the commonly deleted region in GSTT1 was designed. This resulted in an amplification product of 480 bp when the GSTT1 was present on either one or both alleles. Hence, having the GSTT1*0 genotype lead to no amplification. A primer pair specific for amplification of a 299 bp product of the β-globin gene was used as an internal control of amplification. The primers, reaction mix and incubation information are listed in paper II. The PCR products were separated by size using agarose gel electrophoresis and visualised by an intercalating dye. Figure 7 shows an image from the genotyping of GSTT1 (II).
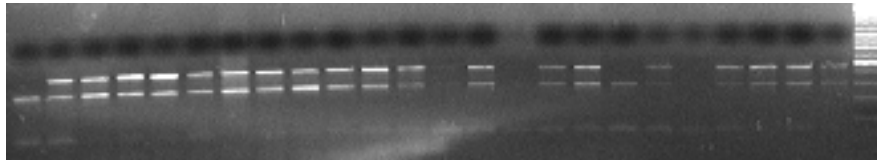
Figure 7. Image of the GSTT1 genotyping

Lanes 1 and 18 showing only the 299 bp β-globin product represents the null variant GSTT1 genotype (GSTT1*0). Lanes 2-17, and 19 show both the 299 bp β-globin product and the 480 bp GSTT1 product. These individuals are positive for GSTT1, thus they have one or two GSTT1 alleles. Lanes 21-24 are positive controls for GSTT1. Lane 25 is a molecular size marker. The signals from lane 13, 15 and 20 were weak and the samples were repeated.

2.3.3   Genotyping of GSTM1

An in-house PCR gel based technique was available for genotyping of GSTM1 but the assay was not as robust as for GSTT1. The annealing temperature had to be changed constantly and it was difficult to interpret the result from the gel image. Hence, it was decided to use a real-time PCR based assay for genotyping GSTM1 to make sure the results were valid.

This design was used for genotyping of GSTM1 which exhibit a variant where the entire gene is deleted[101] (II). The method is somewhat similar to the allelic discrimination method but in this study one of the labelled probes anneal to the GSTM1 gene, if present, and the other probe to a reference gene. With this type of assay determining copy number variation is possible, but we did not use this opportunity. The first published improved real-time PCR methods to determine copy number variation in GSTM1 and GSTT1 relied on the use of fixed concentrations of sample DNA and triple determinations of each sample[102, 103]. For the present study the main interest was to identify the persons homozygous for the GSTM1 deletion variant (GSTM1*0) and no discrimination were made between copy number variation of GSTM1 (being homozygous or heterozygous for the GSTM1 allele) (II). Thus, only single determination was necessary. Recently a high-throughput single determination genotyping method of copy number variation was published[104]. The determination of copy number variation in GSTM1 could become relevant for future studies examining the role of GSTM1 genotype in relation to adverse effects of azathioprine treatment in IBD patients[105, 106]. Another approach to determine the copy number variation of GSTM1 is to use the Multiplex Ligation dependent Probe Amplification (MLPA) by MRC-Holland which offers a panel containing several xenobiotica metabolising enzymes[107].

2.3.4   Genotyping of GSTP1

The allelic discrimination design was chosen for genotyping of the two specific GSTP1 polymorphic sites (II). The primers and probe sequences are listed in paper II. The reaction set-up and run parameters were identical to those for the CARD15 genotyping and are also described in paper II.

2.3.5   Genotyping of mEH

For genotyping of the two mEH polymorphic sites the allelic discrimination design was chosen (III). Commercially available assays from Applied Biosystems specific for the two polymorphic sites were used and the run parameters are listed in paper III. The allelic discrimination assay was chosen in preference to a conventional PCR gel based assay due to a report of genotyping errors from a PCR based assay in a previous publication[108]. This publication showed an association between low activity of mEH and CD. Due to a questioning of their results the group came up with a reanalysis of their cohort using a real-time PCR based assay and was not able to reproduce the positive association between low activity of mEH and CD[109, 110].

2.3.6   Genotyping of NAT2

The NAT2 genotypes can be divided into three phenotypic categories of rapid, intermediate and slow acetylators. The NAT2 acetylator status is determined by a number of SNPs. A combination of six relatively common SNPs in NAT2 was used to decide the acetylator status in study III. Haplotype determination for the large study group used in this thesis was laborious. The haplotype determination was performed by hand, but in many cases several haplotypes were possible. The NAT2PRED web server is an extremely useful tool in assigning individual acetylator status from the SNPs without having to determine the haplotypes[111]. The NAT2PRED is based on a dataset of 1377 individuals (94% Caucasians). The performance was high with sensitivity and specificities ranging between 99.6 and 100% for determining the three acetylator phenotypes[111]. The ability of the NAT2PRED web server has been assessed by a study including 8489 individuals from 56 populations with different geographic origin[112]. The conclusion from this study was that the server correctly identified the slow acetylator phenotype with more than 99% sensitivity in all populations outside Sub-Saharan Africa where another variant, the 191 G>A SNP, plays an important role[113]. The classification error rate found in the evaluation study implied that the NAT2PRED is poor at distinguishing between fast and intermediate acetylators. The classification error rate was though not high in European populations[112]. The data were submitted for each of the six SNPs for all the participants in this study and the results were returned by email. The results listed the probability of all three acetylation phenotypes for each individual. The intermediate and fast acetylators

were grouped for data analysis in our study as suggested by the NAT2PRED evaluation study[112].

Table 1 depicts the acetylation calls (prediction) for 10 samples from our cohort. The prediction for index 10 was not very secure. In total 7 of 1716 calls were in the range of 0.5-0.6 all resulting in a rapid genotype call. (See Table 1 footnotes for further information).

Table 1. Acetylation callings from the NAT2PRED webserver

index	genotype	p(R)	p(I)	p(S)	prediction
1	1,1,1,1,1,1	**0.97995**	0.01221	0.00784	R
2	3,1,1,3,1,1	0.00116	0.00126	**0.99758**	S
3	3,1,1,3,1,1	0.00116	0.00126	**0.99758**	S
4	1,3,3,1,3,1	0.00164	0.00118	**0.99718**	S
5	1,3,2,1,3,1	0.00164	0.00118	**0.99718**	S
6	1,3,3,1,3,1	0.00164	0.00118	**0.99718**	S
7	1,2,2,1,2,1	0.00100	0.99696	0.00203	I
8	3,2,2,1,2,2	0.00001	0.00126	**0.99874**	S
9	1,3,3,1,3,1	0.00164	0.00118	**0.99718**	S
10	1,1,2,1,1,1	**0.61518**	0.14525	0.23957	R

Index is the number of the individual. Genotype cover the genotypes for each of the six polymorphic sites separated by a comma: 1 is wildtype homozygous, 2 is heterozygous, 3 is homozygous for the variant. p(R), p(I) and p(S) are the probabilities for the three callings Rapid, Intermediate and Slow acetylator status. Prediction is the actual call made by the server: R=Rapid, I=Intermediate and S=Slow. The results were returned by email.

2.3.7   Genotyping of MDR1 and BCRP

Genotyping of MDR1 and BCRP on our study population was performed by one of our collaborators, MSc. PhD, Mette Østergaard at Viborg Hospital. An allelic discrimination assay was used to genotype the polymorphic sites G2677T/A and C3435T and intron variant G-rs3789243-A SNPs of MDR1 and C421A in BCRP. The primers and probes used are listed in table 2 which is a modified version from the original paper[72]. The concentrations and annealing temperatures are also described in table 2. The allelic discrimination was performed in a 25 ml reaction volume containing 1XTaqMan Universal Master Mix (Applied Biosystems,Foster City, Calif., USA), primer and probe concentrations were as described in table 2, and 1μl DNA was added. The reaction conditions were: 2 min at 50ºC, 10 min 95ºC, followed by 50 cycles of 20 s denaturation at 95ºC, 60 s annealing/elongation at an assay-specific temperature, according to table 2.

Genotype controls were selected among control samples and verified by sequencing analyses, using the BigDye Terminator v3.1 Cycle Sequencing kit

from Applied Biosystems according to the manufacturer's recommendations. For each SNP, 20 samples randomly selected within each of the three genotype groups were repeated to confirm reproducibility. Deviant amplification plots and dual-colour scatter plots in MDR1 2677 runs were repeated and then subjected to sequencing analysis in order to analyse for the presence of the rarer 2677A variant.

Table 2. Genotyping of MDR1 and BCRP

Primers and probes		Concentration Primer/Probe (nM)	Annealing temperature (ºC)
MDR1-2677forward	5´GTTGTCTGGACAAGCACTGA	300/150/100*	60
MDR1-2677reverse	5´ATCAATCATATTTAGTTTGACTCACC		
MDR1-2677G	HEX-CCAG**C**ACCTTCTAGT-BHQ1		
MDR1-2677T	FAM-CCCAG**A**ACCTTCTAGTT-BHQ1		
MDR1-3435forward	5´GCCTATGGAGACAACAGCCG	300/200	62
MDR1-3435reverse	5´CGATGAAGGCATGTATGTTGGC		
MDR1-3435C	HEX-AAGAGAT**C**GTGAGGGC_BHQ1		
MDR1-3435T	FAM-AAGAGAT**T**GTGAGGGC_BHQ1		
Grs3789243A forward	5´ACCTTACAAATAAGTCTCAACATTCTCTG	900/200	62/72**
Grs3789243A reverse	5´CCCAGACAATAAGCCCAAGATCC		
rs3789243G	HEX-AACGACGC**C**CCATAA-BHQ1		
rs3789243A	FAM-CAACGACGC**T**CCATAAATT-BHQ1		
BCRP-421forward	5´ATGTTGTGATGGGCACTCTG	300/150	62
BCRP-421reverse	5´CATGATTCGTCATAGTTGTTGCA		
BCRP-421C	HEX-AAACTTA**C**AGTTCTCAGCA_BHQ1		
BCRP-421A	FAM-AAACTTA**A**AGTTCTCAGCA_BHQ1		

BHQ=black hole quencher. Reaction concentration of primers and probes, as well as the annealing/elongation temperature are given. *Wild-type-specific probe 150 nM, variant (mutant)-specific probe 100 nM; **elongation was split in the MDR1 rs3789243 assay; annealing at 62ºC for 30 s, followed by elongation at 72ºC for 30 s. Primers were purchased from DNA Technology (Aarhus, Denmark) and probes from Proligo. The reactions were run on a Stratagene Mx3000 machine (Stratagene, La Jolla, Calif., USA), using the allelic discrimination feature of the MxPro software (Stratagene).

Chapter 3.

Results - Risk factors of IBD

3.1 SMOKING

Smoking has been found to be an independent risk factor of CD and current smoking to have a protective effect against UC, which was also true for this study population[58, 59](I). Ever smoking was associated with CD with an OR of 1.8 (1.4-2.3), (*P*<0.001), whereas current smoking had a protective effect against UC with an OR of 0.7 (0.5-0.9), (*P*=0.015) (Table 3). The score of the ORs were similar to what has been found in a large number of studies[114]. Extensive research has been made concerning the dual effect of smoking on IBD. A recent publication shared some thoughts as to why current smoking has a protective effect against UC[55]. Intestinal permeability is known to be high in UC patients[67]. The mucus thickness has shown to be shallower in UC compared with CD[55]. Nicotine has shown to enhance mucosal production, thus this could possibly strengthen the epithelial barrier in UC hereby decreasing the intestinal permeability[55]. Smoking reduces rectal blood flow, which may cause less recruitment of pro-inflammatory mediators to the rectum, thus protecting against sustained inflammation.

Table 3. Association between smoking behaviour and IBD in the cohort.

Smoking status	*OR (95%CI) P-value*	
	CD	*UC*
Ever smoker	1.8 (1.4-2.3) *P*<0.001	1.1 (0.9-1.4) *P*=0.28
Current smoker	2.6 (2.0-3.5) *P*<0.001	0.7 (0.5-0.9) *P*=0.015

OR=Odds ratio, 95%CI=95% confidence interval, *P* =P value.
CD=Crohn´s disease, UC=Ulcerative colitis.

3.2 CARD15

We found that harboring at least one CARD15 variant to be associated with CD but not UC in the Danish population (I). The OR for a CD patient carrying one CARD15 variant was 1.9 (1.3-2.8), *P*<0.001 (I).   A gene-dosage effect was observed in the population raising the OR to 21.1 (4.9-91.2), *P*<0.001 for carrying two CARD15 variants (I). In comparison the ORs were 2.4 (2.8-8.0) and 6.7 (4.1-10.9) respectively in a large meta-analysis of 79 studies (in which our study was also included)[115]. The confidence interval for carrying two variants was wide in our study compared with that of the meta-analysis. These numbers suggest that the OR of 6.7 for CD patients carrying two variants is more likely than our OR of 21.1 (Table 4).

The susceptibility to CD is inherent in the R702W SNP and in the 1007insC frame-shift mutation, whereas the rare G908R SNP was not associated with CD in our population (I).

The frequencies of the three variants in the control group of our population was similar to those of a recent publication of more than 38 000 healthy Danes (Table 4)[116]. When comparing the frequencies with a meta-analysis of 3500 healthy Caucasians our findings are similar to the findings in other Northern European countries, which in general has a lower CARD15 variant frequency than other European countries[49].

Table 4. Distribution of CARD15 variants in the Danish population

CARD15 variants	n, (%)		
	CD	UC	HC
Ernst et al (I)			
R702W alleles	*40 (5.5)	36 (3.3)	44 (2.8)
G908R alleles	11 (1.5)	9 (0.8)	16 (1.0)
1007insC alleles	**46 (6.4)	12 (1.1)	19 (1.2)
0 variant	365 (79.4)	504 (89.8)	718 (90.3)
1 variant	^61 (15.9)	56 (10.0)	75 (9.4)
2 variants	^^18 (4.6)	1 (0.2)	2 (0.3)
Least 1 variant	^^^79 (20.6)	57 (10.2)	77 (9.7)
Yazdanyar et al 2009[116]			
0 variants			38592 (87.0)
1 variant			4838 (12.5)
2 variants			164 (0.4)
Least 1 variant			5002 (13.0)

*R702W allele frequency in CD patients versus controls: OR=1.9 (1.3-3.0) *P*=0.02.
**1007insC allele frequency in CD patients versus controls: OR=5.3 (3.1-9.1) *P*<0.001.
^CARD15 1 variant genotype frequency in CD patients versus controls OR=1.9 (1.3-2.8) *P*<0.001.
^^CARD15 2 variants genotype frequency in CD patients versus controls OR=21.1 (4.9- 91.2) *P*<0.001.
^^^CARD15 at least 1 variant genotype frequency in CD patients versus controls OR=2.4 (95% confidence interval (1.7-3.4) *P*<0.001). OR=Odds ratio, 95%CI=95% confidence interval, *P* =P value.
CD=Crohn´s disease, UC=Ulcerative colitis.

Compatible with the high expression of CARD15 in the Paneth cells of the ileum, the CARD15 variants were associated with ileal involvement in CD. The OR was 2.6 (1.5-4.5), *P*= 0.001, for carrying at least one CARD15 variant (I). When considering the three CARD15 variants separately, the association with ileal involvement of CD reached only statistical significance for the 1007insC frame-shift mutation (I). This association between CARD15 variants and disease location in CD patients have been demonstrated in the majority of association studies[117-

120]. A weak association was found between CARD15 variants and less than 40 years of age at disease onset with an OR of 2.0 (1.0-4.0), P=0.038. Smoking was found to confer risk of CD and to display a protective effect of UC, but no modifying effect of smoking on CARD15 genotype was found for either CD or UC (I). Summarised study I found an association between two relatively common CARD15 variants (R702W and 1007insC) and CD. The association was strongest for the 1007insC mutation and a gene-dosage effect was observed. CARD15 seemed to influence disease phenotype by affecting disease onset and the 1007insC variant was associated with ileal involvement in CD. No direct association or phenotypic associations was found between CARD15 variants and UC (I).

3.3 XENOBIOTICA METABOLISING ENZYMES

3.3.1 Glutathione S-transferase family

Genotyping of GSTM1 resulted in very similar frequencies within the three groups of CD patients, UC patients and healthy controls in our population (II) (Table 5). The findings were similar to the findings in three previous European studies[108, 121, 122] (Table 5). An Indian study found a significant association between GSTM1*0 and UC[123]. The frequency of the GSTM1*0 genotype in the Indian UC patients (61%) was similar our study group (53%), but the difference appear to reside in the control populations where the GSTM1*0 genotype has shown to be less frequent in the general Indian population (30%)[124, 125]. (Table 5).

The frequencies of the Glutathione S-transeferase θ null variant genotype (GSTT1*0) were also similar between the three groups of CD, UC and healthy controls in our population (II) (Table 5). Higher frequencies of GSTT1*0 were found in two previous European study populations (II). The GSTT1*0 frequency is generally lower in Scandinavian populations, thus, the outcome of the studies were the same, with no difference in GSTT1*0 frequency between IBD patients and healthy controls[108, 121, 126]. In the previously mentioned Indian study a strong association was found between GSTT1*0 and both UC and IBD[123]. Ethnic differences are expected in susceptibility genes, but the fact that very few IBD patients were recruited in the Indian study could very likely have biased the results. Further research into whether GSTM1*0 and GSTT1*0 genotypes are truly associated with IBD in the Indian population are needed.

The distribution of GSTP1 low activity genotypes (GSTP1 105 low and GSTP1 114 low) found in our cohort, were in agreement with previous findings in Caucasian populations[108, 126] (Table 5). Research regarding other diseases has shown GST genotypes to be risk factors of disease only when present in combination and not as a single gene[73]. Hence, it could be expected that combinations of several of the GST genotypes might be necessary to induce susceptibility to IBD. However, no association was found between any combination of having GSTM1*0, GSTT1*0 and GSTP1 low activity genotypes and IBD in our study (II).

Early onset disease has been associated with high familial prevalence of CD, hence, suggesting a stronger genetic contribution in this group of patients[15, 51, 127]. We were not able to replicate a Swedish finding of an association of GSTM1*0 and early onset of UC[122]. With regard to phenotypic association in general we found no association between GST genotypes and the phenotypic behaviours of early onset of disease, localisation of disease and severity of disease in our study population (II). Neither did we find any indication of GST single gene or a combination of several of the GST genotypes to cause susceptibility to IBD in our study population (II). Hence, GST genotypes do not seem to play an important role in susceptibility to IBD.

Table 5. GST genotypes among IBD patients and healthy controls in different populations

Genotype	Distribution of GST genotypes n, (%)		
	CD	UC	HC
GSTM1*0			
Ernst et al (II)	215 (56)	296 (53)	417 (53)
Hertervig[122]	65 (60)	101 (56)	219 (49)
Duncan et al[121]	68 (62)	112 (49)	203 (54)
De Jong et al[108]	82 (54)		74 (50)
Mittal et al[123]	9 (45)	52 (61)	49 (30)
GSTT1*0			
Ernst et al (II)	66 (17)	82 (15)	104 (13)
Duncan et al[121]	17 (16)	52 (24)	47 (18)
De Jong et al[108]	23 (15)		30 (20)
Mittal et al[123]	18 (90)	77 (91)	26 (16)
GSTM1*0 &GSTT1*0			
Ernst et al (II)	36 (9)	44 (8)	50 (6)
Duncan et al[121]		10 (13)	
De Jong et al[108]	*(7)		*(13)
Mittal et al[123]	4 (20)	28 (33)	8 (5)

CD=Crohn´s disease. UC=ulcerative colitis. HC=healthy controls.
GSTM1*0:GSTM1 null genotype.
GSTT1*0: GSTT1 null genotype.
GSTM1*0 & GSTT1*0: GSTM1 null and GSTT1 null genotype.
*only percentages available in paper.

By-products from tobacco smoke are likely substrates of the GST enzymes and current smoking seems to have a protective effect against UC. This was supported in the current study where an interaction was found between GSTM1*0 and smoking for UC patients, with GSTM1*0 genotype strengthening the protective effect of smoking (II). One might speculate the GSTM1 active genotype could

eliminate or reduce the protective effect of smoking in UC, whereas the GSTM*0 genotype does not interrupt the protective effect of tobacco smoking.

3.3.2   N-acetyltransferase 2

We were not able to replicate a Japanese finding of a positive association between NAT2 slow acetylator status and CD(III)[87]. In our study the frequency of NAT2 slow, intermediate and rapid acetylators was in agreement with the findings from another Caucasian study population (II)[128]. Highly different frequencies of NAT2 acetylator status are found between different ethnic groups. The NAT2 rapid acetylator genotype is present in less than 10% in Caucasian populations and in populations of African descent[129, 130]. The NAT2 slow acetylator genotype varies between 50-65% in these populations[131](III). In Asian populations the NAT2 slow acetylator genotype is less frequent ranging between 10-25% but with great differences between countries[131] (Table 6). An overrepresentation of NAT2 slow metabolisers have been shown among aryl-amine exposed bladder cancer patients compared with healthy controls in Caucasian populations[131]. In contrast, the NAT2 slow metabolisers have been shown to be underrepresented among aryl-amine exposed bladder cancer patients in a Chinese population. This indicates that pathways other than NAT2 could be involved in the metabolism of aromatic amines[132]. This example demonstrates that genetic risk factors may only be risk factors in some ethnic populations not only because of interaction with environmental exposure differences, but possibly also depending on the general prevalence of the genetic variant in the given population.

Table 6. NAT2 acetylator status in healthy controls of different populations

NAT2 acetylator status in healthy controls of different populations n, (%)			
(Origin of population)	*Rapid*	*Intermediate*	*Slow*
Ernst et al (II) (Caucasian)	44 (6)	300 (38)	443 (56)
Kiyohara et al[84] (Japanese)	^(30)	^(49)	^(21)
Inatomi et al[131] (Japanese)*			10 (7)
Su et al[131] (Taiwan)*			13 (13)
Kim et al[131] (Korean)*			24 (11)

* Taken from Golka et al[131] ^only percentages avaliable in paper

We found no evidence of NAT2 playing a key role in phenotypic characteristics of disease regarding either UC or CD (III). Nor was any interaction between NAT2 and smoking found in susceptibility to IBD (III). In conclusion, NAT2 does not seem to be important in susceptibility to IBD in the Danish population (III).

3.3.3   microsomal Epoxide Hydrolase

We found no association between either of the two mEH polymorphic sites 113 or 139 or a combination of the polymorphisms with IBD (III) (Table 7). The findings are in agreement with two previous European studies using the same genotyping procedure[109, 110].

Table 7. Distribution of mEH genotypes

	mEH polymorphic sites (n, (%))						
	mEH 113 genotypes				**mEH 139 genotypes**		
	High	*Intermediate*	*Low*		*High*	*Intermediate*	*Low*
CD	177 (47)	167 (44)	36 (9)	CD	21 (6)	118 (32)	235 (63)
UC	286 (52)	225 (41)	42 (8)	UC	25 (5)	190 (35)	335 (61)
HC	373 (47)	359 (45)	61 (8)	HC	42 (5)	284 (36)	466 (59)
	mEH combined genotypes						
	High	Intermediate	Low				
CD	67 (18)	166 (44)	142 (38)				
UC	101 (19)	278 (51)	167 (31)				
HC	156 (20)	363 (46)	274 (35)				

CD=Crohn´s disease. UC=Ulcerative colitis. HC=Healthy controls.

Percentages may not add up to exactly 100% due to rounding.

The dual role of mEH meant that we had to consider the possibility of both low and high activity mEH genotypes as possible risk factors. Analysing the mEH high activity genotype against low and intermediate activity genotypes an association with diagnosis of CD before age 40 was found with an OR of 2.2 (1.1-4.2), *P*=0.02. The association was expected to be stronger among smokers, because of the dual role of mEH towards benzo(a)pyrene of tobacco smoke, but when analysing ever smokers isolated the association did not reach statistical significance (II). This finding could possibly be biased by the small numbers in this sub-grouping of patients. Thus, the results indicate that mEH may influence the age at disease onset among CD patients but further research on a larger population is needed to clarify this. No other phenotypic associations were found.

Dealing with mEH a trend towards a modifying effect of smoking on low mEH activity genotype was found for both CD and UC patients. Thus, smokers with a low activity mEH genotype may have a higher risk of developing IBD compared with never smokers (III). Hence, mEH may be important in susceptibility of IBD in combination with environmental factors.

3.3.4   Xenobiotic transporters MDR1 and BCRP

In the xenobiotica transporter gene MDR1 two functional polymorphic sites were genotyped, the G2667T/A and C3435T variants, and an intron-variant G-rs3789243-A[72]. No association was found between the two functional variants, G2667T/A and C3435T, and either UC or CD[72]. The intron-variant G-rs3789243-A was weakly associated with CD which was in contrast to the associative finding with UC found in a Scottish study[94] (Table 8). Combining the three variants into different haplotypes did not reveal an association with IBD either. Encouraged by the conflicting findings regarding MDR1 as a susceptibility gene of IBD, two meta-analyses have been made both showing the C3435T polymorphic site to influence susceptibility to UC but the effect is small[133, 134] (Table 8). The two meta-analyses failed to show an association between the tri-allelic G2677T/A polymorphic site and IBD. No disease phenotypic associations were found for the genetic variants in the meta-analyses. The findings indicate that the contribution to IBD from the MDR1 gene may vary among different populations and may also be dependent on population specific environmental factors[135].

Our study was confirmative of these findings with very similar allele frequencies of the MDR polymorphic variants investigated. The BCRP variant investigated was not associated with IBD supporting the Hungarian study, hence BCRP does not seem to play an important role in aetiology of IBD[72, 95].

Table 8. Associations between MDR1 variants and IBD.

MDR1 variant	OR (95%CI) P-value		
	CD		UC
C3435T			
Østergaard, Ernst et al[72]	0.8 P=0.12	(0.6-1.1)	0.8 (0.6-1.1) P=0.15
Onnie[133]	1.0 P>0.05	(0.9-1.1)	1.1 (1.0-1.2) P=0.013
Annese[134]	-		1.1 (1.1-1.3) P=0.003
G-rs3789243-A			
Østergaard, Ernst et al[72]	1.4 P=0.05	(1.0-1.9)	1.3 (1.0-1.5) P=0.09
Ho et al[94]	1.2 P=0.67	(0.7-2.0)	1.8 (1.1-2.9) P=0.04

OR=Odds ratio, 95%CI= 95% confidence interval, P =P value.
CD =Crohn´s disease, UC=Ulcerative colitis.

Chapter 4.

Conclusion and perspectives

The frequencies of the CARD15 variants were similar to the findings from other Northern European countries. Harbouring at least one of three common CARD15 variants was associated with CD but not UC in the Danish population, and a stronger association was found for being homozygous for the CARD15 variants compared with being heterozygous (I). The susceptibility was inherent in two of the three common CARD15 variants, the R702W and the 1007insC, the latter displaying the strongest susceptibility to CD. CD patients carrying at least one CARD15 variant were more likely to have ileal disease than CD patients with no CARD15 variants (I). A weak phenotypic association was found between carrying at least one CARD15 variant and early onset CD (I). No disease susceptibility and no phenotypic associations were found between CARD15 and UC (I). Smoking was found to confer risk of CD and current smoking to be a protective factor of UC, but no modifying effect of smoking on CARD15 genotype was found for either CD or UC (I).

GSTM1, GSTT1 and GSTP1 did not confer susceptibility to either CD or UC, and no disease phenotypic associations were found (II). Combining members of the GST family did not reveal any association with either CD or UC (II). A modifying effect of smoking was found on GSTM1 genotype. Harbouring the GSTM1*0 genotype seemed to strengthen the protective effect of current smoking with regard to UC (II).

NAT2 did not confer susceptibility to either CD or UC and no disease phenotypic associations were found (III). No modifying effect of smoking was found on NAT2 acetylator status, thus NAT2 does not seem to play an independent role in IBD (III).

No association was found between either of the two polymorphisms in mEH or the combined mEH genotype and IBD (III). One possible phenotypic association was found, that of mEH high activity genotype and early onset CD (III). An indication of a possible modifying effect of smoking on mEH genotype was found for both CD and for UC (III).

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4.1 PERSPECTIVES

It is a general opinion that IBD is based on a dysfunctional immune response, but the exact mechanism is unknown. Increased intestinal permeability also plays an important role in IBD but one might speculate what comes first; intestinal permeability leading to enhanced bacterial sensing which induces an immune response, or an exaggerated immune response which results in a great production of reactive by-products which may alter the intestinal barrier? The extremely complicated nature of the immune system makes it very difficult to find isolated genetic variants which contribute independently to disease pathogenesis. This particular thesis focused on genetic susceptibility from variants in enzymes of the xenobiotica metabolising system, which is also a very complicated system comparable with the immune system. A combination of various genetic variants is possibly responsible for the genetic susceptibility to IBD and individual genetic variants confer only a modest disease risk which may be very difficult or impossible to demonstrate. Environmental factors also have a substantial role in disease susceptibility to complex diseases. Exposure to environmental factors might be very difficult to measure which further complicates the finding of true risk factors of IBD. IBD is currently emerging in Asia which leaves us with a great opportunity to investigate the shift in environmental exposures in that part of the world. Hopefully this will add knowledge to how environmental factors influence disease aetiology. The CARD15 variants have shown susceptibility to CD only in the Western world and not in the Asian world. This indicates that the genetic susceptibility to IBD differ in different populations. Identification of specific genetic susceptibility genes is important and may help decode the pathways essential to disease pathogenesis. The many susceptibility genes that have been uncovered from the GWA studies have shown to participate in specific pathways, which may lead to a better understanding of the disease and may also improve the treatment. The xenobiotica metabolising enzymes investigated in this thesis did not prove to cause susceptibility to IBD. This may however, be important in predicting which patients might develop adverse effects in response to treatment with certain pharmaceuticals. GSTM1 has demonstrated its importance in relation to adverse effects of treatment with pharmaceuticals containing azathioprine[105, 106]. Looking for copy number variation in GSTM1 in relation to adverse effects of azathioprine treatment is something we plan to investigate in the near future. NAT2 slow acetylator status has shown to be associated with adverse effects of sulfasalazine treatment of IBD in Asian populations[136, 137]. We already have the NAT2 acetylator status of the large group of IBD patients included in this thesis and we will probably look into this also.

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