A cohort of anti-TNF treated Danish patients with inflammatory bowel disease, used for identifying genetic markers associated with treatment response

Steffen Bank

This review has been accepted as a thesis together with three previously published papers by University of Aarhus 17th of June 2014 and defended on 27th of August 2014.

Tutors: Vibeke Andersen, Ulla B. Vogel, Paal Skytt Andersen and Bjørn Nexø Andersen.

Official opponents: Morten Vatn, Henrik Krarup and Marianne Hokland.

Correspondence: Department of Medicine, Viborg Hospital, Heibergs Allé 4, 8800 Viborg, Denmark.

E-mail: stb@mb.au.dk

Dan Med J 2015;62(5):B5087

THE 3 ORIGINAL PAPERS ARE

I. Steffen Bank, Paal Skytt Andersen, Johan Burisch, Natalia Pedersen, Stine Roug, Julie Galsgaard, Stine Ydegaard Turino, Jacob Broder Brodersen, Shaista Rashid, Britt Kaiser Rasmussen, Sara Avlund, Thomas Bastholm Olesen, Hans Jürgen Hoffmann, Marianne Kragh Thomsen, Vibeke Østergaard Thomsen, Bjørn Andersen Nexø, Ulla Vogel and Vibeke Andersen
Effectiveness of anti-tumour necrosis factor-α therapy in Danish patients with inflammatory bowel diseases. Dan Med J. 2015;61:A4994.

II. Bank S, Nexø BA, Andersen V, Vogel U, Andersen PS. High-quality and -quantity DNA extraction from frozen archival blood clots for genotyping of single-nucleotide polymorphisms. Genet Test Mol Biomarkers 2013;6:501-503.

III. Steffen Bank, Paal Skytt Andersen, Johan Burisch, Natalia Pedersen, Stine Roug, Julie Galsgaard, Stine Ydegaard Turino, Jacob Broder Brodersen, Shaista Rashid, Britt Kaiser Rasmussen, Sara Avlund, Thomas Bastholm Olesen, Hans Jürgen Hoffmann, Marianne Kragh Thomsen, Vibeke Østergaard Thomsen, Morten Frydenberg, Bjørn Andersen Nexø, Jacob Sode, Ulla Vogel and Vibeke Andersen
Associations between functional polymorphisms in the NFκB signaling pathway and response to anti-TNF treatment in Danish patients with inflammatory bowel disease. Pharmacogenomics J. 2014;14:526-34.

INTRODUCTION

Inflammatory bowel diseases

Chronic inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), are complex diseases that result from the interaction of numerous genetic and environmental factors [1]. CD and UC are chronic diseases, but the disease activity will fluctuate between flare-ups and times of remission and symptoms may range from mild to severe. The incidence of CD in Denmark is approximately 8 and 10 pr 100.000 for men and women, respectively. The incidence of UC is approximately 13-17 pr 100.000 for both men and women [2, 3]. The prevalence of CD and UC has been estimated to be 25.000 or 0.5% of the Danish population [3].

CD and UC have important negative consequences for the individual, by reducing the quality of life, and for the society as a consequence of expenses for medical treatment, medicine and absence from work.

Symptoms: For patients with CD symptoms at diagnosis commonly include diarrhoea for several weeks, abdominal pain and may include blood in stools, fever and/or weight loss. For patients with UC symptoms at diagnosis commonly include bloody diarrhoeas for several weeks and may be accompanied by abdominal pain, fever and/or weight loss. Patients with CD or UC are most frequently diagnosed in late adolescence or early adulthood [4, 5].

Diagnosis: There is no test that alone can be used to diagnose the patient with CD or UC. Clinical evaluation and a combination of biochemical, endoscopic, histological, magnetic resonance (MR) or computed tomography (CT) investigations are used to establish the diagnosis of CD and UC. Biochemical tests include measurements of C-reactive protein (CRP) and fecal-calprotectin (F-calprotectin) which are used as markers for inflammation. Microbial testing for infectious diarrhoea including Clostridium difficile toxin is recommended to exclude bacterial causes of the symptoms [4, 5].

In CD the inflammation often involves the colon and/or ileum but may occur anywhere along the gastrointestinal tract from the mouth to the anus. The inflammation in CD is typically discontinuous and may include fistulas (connection of two body cavities). The inflammation in patients with UC usually starts in the rectum and extends in a continuous manner to affect a variable extent of the colon. In UC the inflammation does not extend into the deeper layers of the bowel wall, whereas in CD inflammation can be transmural [4, 5].

The location and distribution of the inflammation is usually determined by endoscopy and is divided into three phenotypes according to the Montreal classification [6, 7]. For CD the locations are classified as terminal ileum (L1), colon (L2) and ilecolon (L3) and for UC the distributions are classified as proctitis (involvement limited to the rectum), left-sided (up to the splenic flexure) and extensive (proximal to the splenic flexure (including pancolitis)).

Risk factors: A family history of CD or UC is the strongest risk factors for the onset of both CD and UC underscoring the genetic component of the diseases. In familial cases of UC there is a slight female preponderance and young age at onset compared to sporadic cases [4, 5]. Twin studies indicate that the genetic

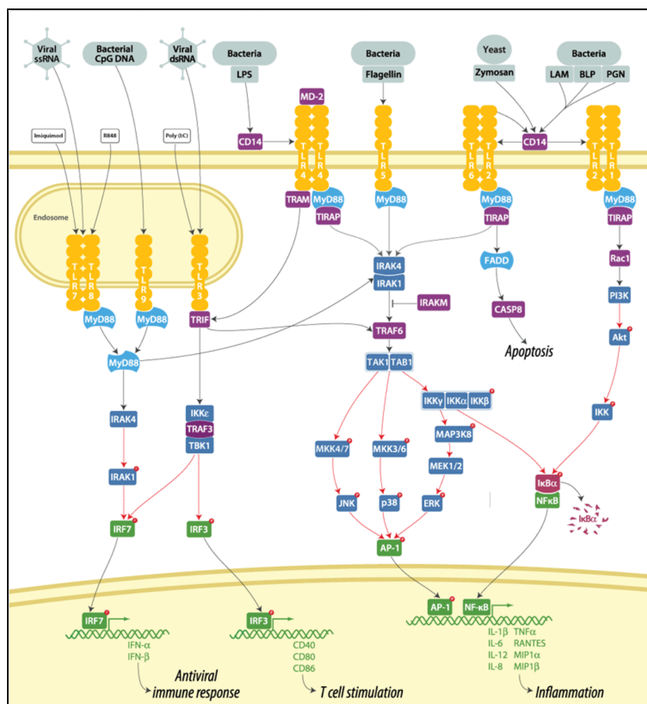

Figure 1
Simplified overview of the the NFκB pathway.

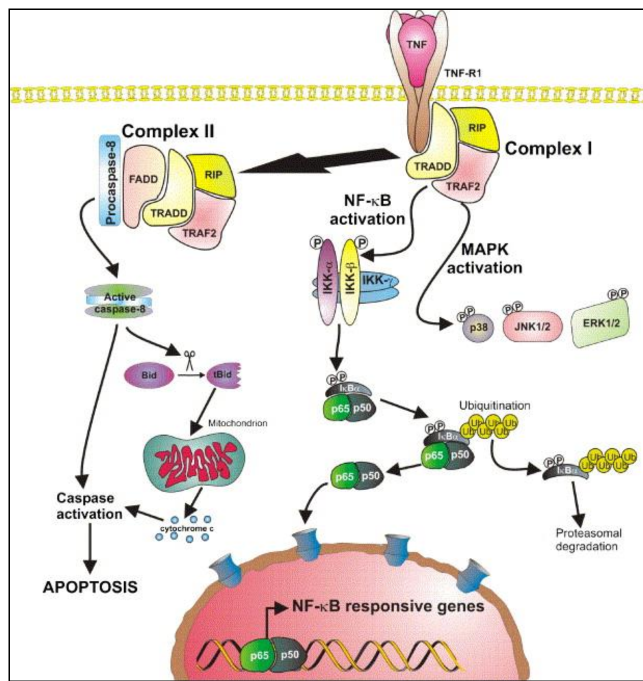

Figure 2
Simplified overview of the the TNF-α pathway.

contribution to disease is more pronounced for CD than UC [8-12].

Smoking has different effect on the risk of CD and UC. Smoking increases the risk of CD and may increase the risk of relapses. However, smoking has a protective effect on the development and severity of UC. In contrast, former smokers have a higher risk of developing UC [13].

Patients diagnosed with UC at a young age (<16 years) tend to have a more aggressive disease and higher risk of colectomy than patients diagnosed at an older age (>40 years) [4, 5].

Inflammation, the NFκB pathway

The inflammation in CD and UC result from the interactions of numerous genetic and environmental factors. One theory is that bacteria in the bowel can initiate an inflammation, which becomes chronic in genetic susceptible individuals [1].

NFκB is a transcription factor, which regulates many physiological processes including the innate- and adaptive-immune response, apoptosis and inflammation [14]. In many inflammatory diseases, such as inflammatory bowel disease, NFκB is found to be chronically active [15-17]. NFκB can be activated by Toll like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMP) that are broadly shared by pathogens but distinguishable from host molecules such as bacterial or viral DNA (TLR9), flagellin (TLR5) or lipopolysaccharide (LPS) (TLR2 and TLR4) (Figure 1) [18].

For example, LPS from Gram-negative bacteria cell membranes can be bound by CD14 in the presence of lipopolysaccharide-binding protein (LBP), which together with MD-2 interacts with TLR4 (Figure 1). TLR4 is a membrane bound protein, which upon LPS stimuli forms homodimers and activates an intracellular kinase cascade. This kinase cascade ultimately activates the IKK-complex, which phosphorylates and degrades the NFκB inhibitor IκBα [18]. NFκB (p50-RelA or p50-p65) is shuttled from the cytosol to the nucleus after IκBα degradation. Here it initiates the expression of pro- and anti-inflammatory cytokines including the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [19].

The TNF-α pathway

Tumor necrosis factor alpha (TNF-α) is a cytokine synthesized as a 212-amino acid transmembrane pro-polypeptide arranged in stable homotrimers [20, 21]. At the cell surface, pro-TNF-α is biologically active and is able to induce immune responses via intercellular signaling. In addition, pro-TNF-α can undergo proteolytic cleavage by ADAM17 at its Ala76-Val77 amide bond, which releases a soluble 17 kDa homotrimer to the extracellular domain [22].

TNF-α can bind to two receptors, TNF-R1 and TNF-R2. TNF-R1 is expressed in most tissues, and can be activated by both the membrane-bound and soluble TNF-α, whereas TNF-R2 is found only in cells of the immune system and respond to the membrane-bound form of the TNF-α. The binding of TNF-α to TNF-R1 can activate three pathways, the NFκB, MAPK and apoptosis pathway (Figure 2). The MAPK pathway is involved in cell differentiation, proliferation and is generally pro-apoptotic. The apoptosis pathway induces programmed cell death.

TNF-α can feedback activate the NFκB pathway through a kinase cascade resembling, though distinct from, the TLR pathway where the IKK complex is activated and IκBα is phosphorylated and degraded (Figure 2) [18].

Background for the use of predictive markers

Ineffective medical therapy can have negative consequences for the individual and the society. For the individual, ineffective medical therapy does not resolve the symptoms which can have economical and social consequences as well as reduce the patient's quality of life. In addition, the medical therapy may result in serious side effects. For some disease, such as cancer, the disease may evolve while the patient is treated with ineffective medicine which can be fatal. For the society ineffective medical therapy can

prolong the patient's absence from work, increase expenses for medical therapy and medical care.

A test predicting response (personalized medicine) will benefit both the individual patient as well as the society. For the patients who are predicted not to benefit from the medical treatment, other medical or surgical alternatives are usually available. Predictive markers can be clinical, biochemical or genetic or a combination of clinical, biochemical and genetic markers. There has been some success in implementing predictive biomarkers in medical treatment of patients mainly within treatment of patients with cancer [23, 24].

A test that can predict response to anti-TNF (infliximab and adalimumab) among patients with CD and UC has not yet been implemented in the clinic. Only the most severely ill patients with CD and UC are treated with anti-TNF, however, approximately one-third of the patients have minimal or no response. Genetic variations in individual patient's may explain why some benefit from the therapy and others do not. Genetic variations may be used to predict how individual patients respond to anti-TNF therapy and may give a better understanding of the underlying biological mechanisms involved in response.

Anti-TNF therapy

Infliximab:

The TNF-α level is increased in the blood, stool and colonic tissue among patients with CD and UC [25-28]. Furthermore, TNF-α is a key pro-inflammatory cytokine in inflammation and tissue destruction in CD [29]. Therefore, TNF-α seemed an attractive biological target for developing medicine for treating patients with CD (and other autoimmune diseases). Development of the mouse-human chimeric monoclonal antibody infliximab (Remicade) (or cA2 as it was known) started in the early 90ties [30]. Mice models and cell line studies showed, that infliximab effectively neutralized TNF-α by binding and inactivating soluble TNF-α and by binding to transmembrane TNF-α, which leads to apoptosis [31-33]. Further studies showed, that infliximab significantly reduced lesions and inflammation in patients with CD [34, 35]. Infliximab was the first drug which on short-term induced such improvements and was therefore a breakthrough in the management of CD [29].

In the late 90ties the first large clinical control study showed, that a single infusion induced significant improvements in 65% of patients with CD with severe luminal disease compared to 17% in the placebo group four weeks after treatment initiation [36]. Shortly after, in 1998, infliximab was approved by the US Food and Drug Administration (FDA) for the treatment of CD [37]. Later, infliximab was also proven effective for short- [38] and long-term [39] treatment of fistualizing CD. These initial clinical trials were followed by the larger ACCENT1 and ACCENT2 clinical trials, which proved that infliximab was effective for short and long term treatment of CD compared to placebo [40, 41]. Numerous studies have since confirmed the efficacy of infliximab [42-45].

Active Ulcerative Colitis Trial 1 and 2 (ACT 1 and ACT 2) were the first large trials of infliximab among moderate to severe diseased patients with UC [46]. The response rate 8 weeks after treatment initiation was 64-69% among the infliximab treated patients with UC compared to 29-37% in the placebo group [46]. For long-term maintenance therapy every eight week, 45% of the infliximab treated patients with UC still had clinical response 54 weeks after initiation compared to 20% in the placebo group [46]. Infliximab was approved by the US Food and Drug Administration (FDA) for the treatment of UC in 2005.

Other anti-TNF drugs:

Other anti-TNF drugs, like adalimumab (Humira) and certolizumab pegol (Cimzia), have been developed following the success of infliximab. Adalimumab, a fully humanized monoclonal antibody, was shown to have higher efficacy for short- and long-term treatment of both fistulizing and luminal CD than placebo in the large clinical trial CHARM [47]. Adalimumab was approved by the U.S. Food and Drug Administration (FDA) in 2008 for treatment of CD and for treatment of UC in 2012. The administration of adalimumab differs from infliximab as the former is injected by the patient every 14 day, whereas the latter is given intravenous at the hospital as maintenance therapy every 8 week.

Evaluation of response:

The majority of the studies evaluating treatment response to anti-TNF among patients with IBD have been conducted with infliximab among patients with CD. Most of the prospective studies used the Crohn's Disease Activity Index (CDAI) or Harvey-Bradshaw Index (HBI) for evaluating luminal disease activity usually after 4 to 10 weeks [43, 48-50]. The CDAI uses the number of stools, abdominal pain, general well being, complications and antidiarrhoeal drugs to score the patients. The HBI is a simpler version of the CDAI. In most trials only patients with a CDAI >220 at baseline were included. The patients were then categorized as responders, if they had a decrease in CDAI of > 100 (or > 70 in some studies) and in remission if the CDAI was < 150 [4].

For fistulizing CD the Perianal Disease Activity Index (PDAI) has been used [51]. A few studies have examined adalimumab among patients with CD using the CDAI, HBI or PDAI indexes as effect measures [52, 53].

Only a few prospective studies have evaluated the efficacy of anti-TNF in patients with UC. They used the Mayo Scoring System, which is based on stool frequency, rectal bleeding, endoscopy findings and the physician's global assessment for evaluating response [46].

For retrospective evaluation of treatment response among both patients with CD and UC a few studies have used "The simple 3-step scale" [54-56].

Side effects:

Usually, 5-10% of the patients have side effects to infliximab infusions [36, 39, 40, 47]. However, patients receiving placebo are just as likely to have side effects [36, 39, 40, 46]. The majority of side effects include skin rash, hypertension, shortness of breath, headache, fatigue and low grade fever. However, more severe side effects such as serum sickness, anaphylactic reaction, cancer and even death have been linked to infliximab [29, 39, 54]. In patients with latent Mycobacterium tuberculosis (TB) infection, active tuberculosis may develop after treatment initiation with infliximab. Therefore, it is recommended that the patients are screened for latent TB infection before treatment initiation [57].

PREDICTORS OF RESPONSE

Clinical markers

Age and duration of disease:

In a large clinical study among adalimumab treated patients with CD, a disease duration of less than two years was associated with a higher response rate than patients with a longer disease duration [58]. Likewise, in pediatric patients with CD the best response to infliximab was seen in children with a disease duration less than one year [59]. The significantly higher response rates in the REACH trial of infliximab among children with CD [60] (as compared to adult response rates) may also be a result of earlier intervention or a younger age of treatment [61]. Among patients

with UC response to infliximab has also been associated with younger age at treatment initiation [62]. It has been hypothesised, that the reason for worse treatment outcomes in long lasting disease may be due to formation of fibrosis [63]. However, other studies did not find an association between duration of disease or age at treatment and treatment response [64-66].

Disease location and distribution:

Patients with isolated colonic CD (L2) have been reported to have higher response rates [61, 65] and those with intestinal strictures and prior surgery have been reported to have a lower response rate [61, 67]. In addition, a study found that fistulizing CD patients had a higher response rate and a significantly longer duration of response compared with patients with luminal disease [66]. Others have not found an association between disease location and response [64, 68].

Smoking:

There is no consensus on how smoking impacts treatment efficacy [69-71]. The discrepancies between the existing studies might relate to differences in the definition of smoking status. Two studies defining smoking as more than 5 cigarettes per day found that smoking was associated with non-response [69, 70], whereas the definition was less clear in a larger study, where no association was found [71]. Smoking has also been associated with shorter duration of response [64].

Concomitant medication:

A large double-blinded clinical trial among patients with CD evaluated the efficacy of infliximab monotherapy, azathioprine (immunosuppressant) monotherapy and the two drugs combined. After 26 weeks 57%, 44% and 30% treated with both drugs, infliximab monotherapy and azathioprine monotherapy were in remission, respectively [72]. The effect of combining infliximab with immunosuppressants has also been associated with fewer infusion reactions [54].

Concurrent use of immunosuppressive medications compared to infliximab monotherapy has also been found to be associated with response after short term treatment in another study, however, there was no difference on long term duration of response [64].

Biomarkers

C-reactive protein (CRP):

The level of CRP is a widely used marker of inflammation. Several studies have examined if there are association between CRP level and response to anti-TNF. In one study considering all levels of CRP, there was no association between CRP level and response [65]. In other studies, which only included patients with at least twice the upper limit of the normal range pre-treatment (CRP > 20 mg/L), a decrease of 25% or more in CRP level was associated with beneficial response [48, 49, 73].

The synthesis of CRP is inducible by TNF-α and CRP levels may reflect levels of non-neutralized TNF-α [74]. However, whether an elevated CRP is truly predictive of response to anti-TNF or simply a marker that symptoms are truly due to active inflammatory disease remain to be proven [63].

Serology:

ANCA (Anti-Neutrophil Cytoplasmic Antibodies) is a family of antibodies related to inflammatory diseases [75]. Perinuclear ANCA (pANCA), speckled ANCA (sANCA) and anti-Saccharomyces cervisiae antibody (ASCA) have been the subject of several investigations of anti-TNF response among patients with CD and UC. Positive sANCA pre-treatment has been associated with beneficial response to infliximab among patients with CD in one study [76] but the association was not replicated in a larger study [65].

Positive pANCA and negative ASCA pre-treatment has been associated or marginally associated with non-response to infliximab in three studies involving 279 patients with CD ($p = 0.07$) [77], 100 patients with UC ($P = 0.05$) [62] and 89 pediatric patients with CD or UC ($p = 0.0003$) [42].

Immunochemistry:

A study has reported an association between the C-allele of rs1143634 in IL1B and higher IL-1β blood level pre-treatment and a lower response rate to infliximab therapy among patients with CD [78].

Furthermore, TNF-α level has been investigated as a possible marker to anti-TNF therapy. In a small study among patients with fistulizing CD, TNF-α, IL-1ß and IL-6 blood levels were measured before and after treatment with infliximab. In this 10 week study, patients who did not respond to infliximab had higher baseline TNF-α levels [79]. Larger studies among patients with CD did not find a relationship between treatment response and TNF-α blood level [78, 80].

Measuring TNF-α blood level by enzyme-linked immunosorbent assay (ELISA) is difficult, with a very low level of TNF-α close to or under the detection limit [74]. However, a functional assay based on direct estimation of circulating TNF-α bioactivity has been developed and tested in two cohorts of 20 and 50 patients with rheumatoid arthritis. A high level of circulating bioactive TNF-α was associated with beneficial response to infliximab with a positive predictive value of 90% [81, 82]. However, the level of TNF-α protein measured by ELISA was similar in responders and non-responders, indicating that it is important to measure bioactive TNF-α [81, 82].

Gene-expression profiling; Transcriptional biomarkers:

Microarray technology can be used to measure gene expression (mRNA) e.g. in blood or mucosal tissue. Mucosal gene expression pre-treatment was measured among pediatric patients with UC and identified TNFSRF11B, STC1, PTGS2, IL-13Ralpha2 and IL-11 as markers of non-response. All proteins encoded by these genes are involved in the adaptive immune response. These markers can separate responders from non-responders with 95% sensitivity and 85% specificity [83]. However, the study was conducted in a small cohort and has not been replicated.

Genetic markers

Genetic variation:

In the human genome there are many genetic variations. Genetic variations start as mutations, which are inherited in the population. The variations consist of two or more genetic variations in a single locus in the genome. Genetic variation in the genome include deletions, insertions, inversions, repeats, copy number variations (CNV) or single nucleotide polymorphisms (SNP). Deletions and insertions are single- or multiple nucleotides that are present or absent in a genomic locus in individuals. Inversions are sections of DNA inserted as an inverted sequence. Repeats are regions of the genome which have been duplicated. CNV are two or more nucleotides directly adjacent to each other repeated multiple times. Single-nucleotide polymorphisms (SNPs) are single nucleotide (A, T, C or G) variations in a genomic locus and usually consist of two possible nucleotide variations at a single locus. It is estimated that there are 10 to 30 million SNPs in humans [84].

Most genetic variations have no known biological effect but some cause a change in phenotype. The phenotypic change caused by a SNP can be a result of a change in a codon, which changes an amino acid in the protein. The amino acid can be part of a catalytic site, a binding site, a post-translational modification site or be important for the protein folding and thus activity. A

SNP can also introduce a frameshift change or premature stop codon, which often results in an inactive protein. A SNP in the gene coding region can furthermore change how the mRNA is processed if the SNP alters a splicing site, how the mRNA fold or the half-life of the mRNA. The SNP can also be in the promoter region, the region which regulates how the gene is expressed, and thereby change how much the gene is expression by e.g. changing the binding affinity to transcription factors. In addition, a SNP in a regulatory region such as an enhancer or silencer far away from a gene can also alter expression.

Polymorphisms associated with response:

A large number of genetic studies among patients with CD have examined the associations between SNPs, insertions or deletions and response to infliximab. However, most of them studied only a few SNPs and in underpowered cohorts of less than 100 patients. Response to adalimumab has been studied in two small cohorts among patients with CD but no associations were found [52, 53]. Patients with UC have been examined in three small cohorts of mixed CD and UC patients [42, 78, 85].

In cohorts of more than 100 patients polymorphisms in TNFRSF1A (36 A>G (P12P) (rs767455) [50, 86], TNFRSF1B (587 T>G (rs60195947) [87], rs1061624 A>G [86], 6528 T>C (rs976881) [88] and rs652625 T>A [88]), ADAM17 (rs12469362 T>C, rs1056204 A>C, rs10495565 A>G and rs446248 A>G) [43], FasL (-843 C>T (rs763110)) [48, 88], FAS (-670 C>T (rs1800682)) [48], Caspase-9 (93 C>T (rs4645983)) [48] and FCGR3A (rs396991 T>G (F158V)) [49, 70, 89] have been found to be associated with response. These genes are involved in TNF-α signaling (TNFRSF1A, TNFRSF1B, ADAM17 (TNF receptor 1 and 2 and the protease involved in cleavage and release of membrane bound TNF-α, respectively)) or apoptosis signaling (FasL, FAS and Caspase-9). FCGR3A may also be involved in TNF-α signaling as the receptor binds the Fc portion of immunoglobulin G and has been found to affect the infliximab binding affinity and apoptosis activity in vitro [89]. A study among 75 patients with CD found that a haplotype in the LTA gene was associated with non-response [76] which in part may be explained by the TNFA -857 C>T polymorphism as it is in linkage with the LTA haplotype [90].

Some polymorphisms have been studied in numerous cohorts which makes a meta-analysis possible. Almost all of the studies used CDAI for evaluating efficacy at 4 to 10 week after treatment initiation in European or Japanese cohorts of patients with CD. Two studies indicates that the rare allele of TNFRSF1A (36 A>G, rs767455) was associated with non-response (n = 166 and 80) [50, 86] but this result was not replicated in a larger cohort (n = 543) [87]. Three studies (n = 145, 344 and 102) [49, 70, 89] found an association between homozygote rare allele carriers of FCGR3A (rs396991 T>G (F158V)) and non-response, whereas three studies (n = 189, 106 and 41) [45, 73, 91] did not find an association. Meta-analyses of these two polymorphism may help determine if they are associated with response to infliximab.

The polymorphisms TNFRSF1B (587 T>G (rs60195947)) [50, 87], TLR4 (rs4986790 A>G (D299G)) [69, 92], NOD2 (rs2066844 C>T (R702W); rs2066845 G>C and rs2066847 Del > C) [52, 53, 69, 71, 93-95] and TNFA (-238 G>A (rs361525); -308 G>A (rs1800629) and -857 C>T (rs1799724)) [45, 80, 87, 91] have been examined in two or more cohorts, but none of them found an association. Some of these negative results may be caused by lack of power to find an association in the small cohorts.

The only polymorphism where two cohort studies show consistency was for the rare allele of FasL (-843 C>T (rs763110)), which was associated with non-response in both studies [48, 88].

Hypothesis free genome wide association studies (GWAS) have been performed on two small cohorts of 10 and 89 patients with CD or UC [42, 96]. In a study with 89 pediatric patients with CD or UC, polymorphisms in BRWD1 (rs2836878), PHACTR3 (rs6100556), ATG16L1 (rs2241880), ICOSLG (rs762421), FAM19A4 (rs4855535), TACR1 (rs975664), 5q31 (rs2188962), CDKAL1 (rs6908425) and HLA-DAQ1 (rs2395185) were associated with response [42]. These genes are involved in apoptosis (BRWD1 and PHACTR3), autophagy (ATG16L1), cell adhesion (ICOSLG), regulation of immune cells (FAM19A4), risk factor for gastrointestinal disorders (TACR1) or have unknown function (5q31, CDKAL1 and HLA-DAQ1).

Summary

The discrepancies between the observations can be a result of how age, smoking and CRP level are defined and differences between short and long term treatment for concomitant medication. For all observations sample size matters and can explain some of the discrepancies between the studies.

Clinical markers such as young age at treatment initiation and heavy smoking may be useful markers for predicting response to anti-TNF. There is less consensus regarding the usefulness of disease location and concomitant medication, although concomitant medication with immunosuppressant has been found to have a beneficial effect on short term response. Biochemical markers such as positive pANCA and negative ASCA pre-treatment may also be useful markers. There is not enough data to determine if protein or mRNA pre-treatment level can be used as markers for anti-TNF response. Many of the genetic polymorphisms found to be associated with response to anti-TNF among patients with CD are in genes involved in the TNF-α (TNFRSF1A, TNFRSF1B and ADAM17) or the apoptosis (FasL, FAS and Caspase-9) signaling pathway.

HYPOTHESIS, AIMS AND DESIGN

Hypothesis

Genetically determined variation in the activity of genes in the TNF-α and NFκB pathways and in other cytokines than TNF-α is associated with response to anti-TNF therapy among patients with CD and UC. Moreover, there are shared biological mechanisms involved in response among patients with CD and UC.

Aims

To identify polymorphisms associated with response to anti-TNF therapy among patients with CD or UC, and to use the associated polymorphisms in applied science to create a predictive model of response and in basic science to get a better biological understanding of the mechanisms involved in response.

Design

First, a cohort of previously naïve anti-TNF treated patients with CD or UC was established by retrospectively collecting blood and clinical data from Danish hospitals in a biobank. Blood clots were collect from three Danish laboratories after routine screening for Mycobacterium tuberculosis and the blood samples collected were pooled and used to represent a single cohort of patients. Patients with CD or UC were identified by linking CPR-numbers from the blood samples to The National Patient Registry. Response rate, clinical- and biochemical-markers associated with response in the established cohort were compared with previous published cohort studies as a quality control of the retrospectively collected clinical data and to look for novel markers (paper I). Next, commercial DNA extraction kits were evaluated to achieve the highest DNA yield from the blood clots (paper II). Finally, a

case-only study was used to compare genotypes between responders and non-responders to anti-TNF therapy (paper III).

A candidate gene approach was used with focus on polymorphisms in the TNF-α and NFκB pathways. In addition, polymorphisms in genes which have been shown to be associated with CD and/or UC, polymorphisms in other inflammatory cytokins and non-functional polymorphisms in key genes in the NFκB pathway where there were no known functional polymorphisms were included. Furthermore, polymorphisms with a known biological effect were identified through a litterature search, as they allow biological interpretation of the results based on increased or decreased gene or protein activity.

MATERIALS AND METHODS

Manuscript I

Ethical Considerations:

The study was conducted in accordance with the Declaration of Helsinki and was approved by the local Regional Ethics Committees (M20100153 and S-20120113) and the Danish Data Protection Agency (J. 2010-41-4719 and 2008-58-035). The Ethics Committees gave suspension for obtaining written informed consent.

Collecting biological samples:

Prior to anti-TNF therapy routine screening for Mycobacterium tuberculosis (TB) is performed as the treatment may lead to activation of latent tuberculosis. In Denmark the TB screening is usually carried out at major laboratory centres. Statens Serum Institut was the only Danish laboratory offering the QuantiFERON-TB test for diagnosing latent Mycobacterium tuberculosis infection until January 2011 where Aarhus University Hospital introduced the test. The remaining blood clots after TB analyses were collected from 01.09.2009 to 30.03.2011 at Statens Serum Institut (SSI, Copenhagen, Denmark) and from 01.01.2011 to 30.03.2011 at Department of Respiratory Diseases B and Department Clinical Microbiology, Aarhus University Hospital (Aarhus, Denmark). The blood clots were frozen at -20°C for later analyses. The blood collected from the three laboratories were pooled and used to represent a single cohort of patients.

Identification of patients with CD and UC:

Each blood sample had a unique serial number which was used to identify the unique personal identification number of Danish citizens (CPR-number). The CPR-numbers were linked to The National Patient Registry and all International Classification of Diseases, Tenth Revision, (ICD-10), codes were extracted. Patient records from patients with ICD-10 codes of K50-K63, which included intestinal diseases, from 18 medical departments were examined.

Database design and content:

In order to facilitate data acquisition, to ensure data quality, as well as to enable centralization of data storage, a secure web-based database (https://trialpartner.dk) was constructed by Datamanagement (Datamanagement, Aarhus, Denmark). The data registered in the database were stored on two independent servers.

Data were collected retrospectively from patient records. Data registered in the database included disease (CD or UC), gender, year of birth, age at diagnosis, disease location and behaviour according to the Montreal classification, smoking status, the date of the first infliximab (Remicade; Centocor, Malvern, PA, USA) or adalimumab (Humira; Abbott Laboratories, North Chicago, IL, USA) treatment, treatment indication, C-reactive protein (CRP), F-calprotectin and treatment efficacy (as described later). Furthermore, concomitant medication with azathioprine or methotrexate up to four weeks and 5-aminosalicylates, intravenous or peroral glucocorticoids or antibiotics up to two weeks before the first infliximab or adalimumab treatment were registered. Finally, the date and indication for treatment termination (no effect, remission or adverse events) were registered.

Treatment protocol:

Indication for treatment was: Patients with moderate or severe disease who did not respond to prednisolone or immunosuppressive treatment. As a standard, patients were treated with 5 mg/kg infliximab intravenously starting at week 0, 2, 6 and every 8 weeks thereafter. As a standard, adalimumab was administered with 160 mg subcutaneously in week 0, 80 mg in week 2, and 40 mg every second week thereafter [97, 98].

Treatment efficacy:

Efficacy was assessed using physician's global assessment focusing on normalisation of bowel frequency and absence of blood with defecation. The simple 3-step scale used in previous studies was used to estimate efficacy 8 and 22 weeks after anti-TNF treatment initiation in naïve patients and reflected the maximum response during the period [54-56]. Patients with CD or UC with luminal disease were categorized as having: (A) no response, meaning no improvement or worsening of symptoms; (B) partial response, meaning some improvement of symptoms or reduction of steroid dose without worsening of symptoms; (C) response, meaning absence or almost absence of all clinical symptoms without increasing the steroid dose. Patients with fistulising CD were categorized as having: (A) no response, meaning no improvement or worsening of symptoms; (B) partial response, meaning reduced secretion or discomfort from fistulas or closure of one or some of the fistulas; (C) response, meaning closure of all fistulas evaluated by thumb pressure or no secretion [38].

Biological response:

A biological response was defined as a decrease in baseline CRP level of at least 25% within 22 weeks after treatment initiation [49, 73].

Manuscript II

DNA extraction:

Four different commercial purification kits were used to extract DNA from the blood clots: (1) Maxwell 16 Blood purification kit (Promega, Southampton, United Kingdom), (2) PureGene (Qiagen, Hilden, Germany) with and without glycogen, (3) QIAamp DNA Micro kit (Qiagen, Hilden, Germany) and (4) Nucleospin 96 Blood kit (Macherey-Nagel, Düren, Germany). The frozen blood clot was thawed at room temperature for 15 minutes and 100 µl to 300 µl total blood was extracted without being homogenized or otherwise pretreated. The DNA was extracted according to the manufacturers` instructions and dissolved in ddH2O.

DNA yield and quality:

Two µl purified DNA was used to measure the DNA concentration and the purity (A260/A280) of the extractions by NanoDrop-1000 (Thermo Scientific, Waltham, MA, USA).

Manuscript III

Identifying functional polymorphisms:

Only few studies have examined the biological effect of polymorphisms. To find functional polymorphisms in the NFκB and TNF-α pathway, relevant genes in those pathways first had to be found. By using the pathway databases http://www.genome.jp/kegg/pathway.html and http://www.wikipathways.org/index.php/WikiPathways relevant genes were found. Functional polymorphisms in relevant genes were found by searching pubmed

(http://www.ncbi.nlm.nih.gov/pubmed/) with “polymorphism AND gene-name AND (reporter gene OR luciferase OR ELISA OR enzyme-linked immunosorbent assay OR RT-PCR OR reverse transcriptase PCR OR EMSA OR electrophoretic mobility shift assay OR flow cytometry)”.

After an extensive literature search approximately 100 functional polymorphisms were found and 39 polymorphisms mainly in the NFκB and TNF-α pathway were chosen.

Single nucleotide polymorphisms studied:

The DNA was genotyped by KBioscience (KBioscience, Hoddesdon, United Kingdom) (http://www.lgcgenomics.com/). DNA was shipped in a volume of 150 µl and a concentration > 10 ng/µl. Each polymorphism examined by KBioscience required a volume of 1.5 µl.

The SNPs studied were TLR2 (rs4696480, rs1816702, rs11938228, rs3804099), TLR4 (rs12377632, rs5030728, rs1554973), TLR5 (rs5744168), TLR9 (rs187084, rs352139), LY96 (MD-2) (rs11465996), CD14 (rs2569190), MAP3K14 (NIK) (rs7222094), SUMO4 (rs237025), NFKBIA (IκBα) (rs696, rs17103265), NFKB1 (NFκB1) (rs28362491), TNFA (TNF-α) (rs1800629, rs1800630, rs1799724, rs361525), TNFRSF1A (TNFR1) (rs4149570), TNFAIP3 (A20) (rs6927172), IL1B (IL-1β) (rs1143623, rs4848306, rs1143627), IL1RN (IL-1RA) (rs4251961), IL4R (rs1805010), IL6 (rs10499563), IL6R (rs4537545), IL10 (rs1800872, rs3024505), IL17A (rs2275913), IL23R (rs11209026), IFNG (IFN-γ) (rs2430561), TGFB1 (TGF-β1) (rs1800469), PTPN22 (rs2476601), PPARG (PPAR-γ) (rs1801282) and NLRP3 (rs4612666).

Genotyping of TNFA (TNF-α) -857 C>T (rs1799724) and -863 C>A (rs1800630) failed due to their close proximity to each other. The 39 genotypes were replicated in 94 randomly selected samples and yielded >99% identical genotypes. The studied SNPs had a minor allele frequency of 5% to 48%.

Genotyping:

Competitive Allele-Specific Polymerase chain reaction (KASP™) is an end-point PCR technology used by KBioscience for genotyping. Two allele-specific forward primers (where the last nucleotide at the 3´-end is complementary to one of the two alleles) are mixed with a single reverse primer, KASP PCR master mix and DNA. Each of the two forward primers have a unique tail sequences which corresponds to the sequence in a FAM-dye or HEX-dye labeled oligo in the KASP PCR master mix. The FAM-dye and HEX-dye labeled oligos are prevented from emitting light by two complementary quencher labeled oligos in the KASP PCR master mix.
In the first round of PCR the 3´-end nucleotide in the allele specific forward primers determine which primer anneal to the single nucleotide polymorphism in the DNA template. A unique 5´ sequence (from the allele-specific forward primer) complementary to the FAM-dye or HEX-dye labeled oligo is thereby incorporated in the amplicon. In the second round of PCR the reverse primer creates the complementary strand to the amplicon. In the following PCR amplifications the FAM-dye or HEX-dye labeled oligo anneale to the complementary strand created by the reverse primer and the dye is thereby incorporated into the PCR product. This separates the FAM-dye or HEX-dye from the quencher labeled oligo. The PCR reactions will in the following rounds exponentially increase the amount of PCR product as well as the fluorescence emitted from the FAM-dye or HEX-dye.

The SNP in the DNA template is determined by the fluorescence read at the end of the PCR run. If both forward primers are incorporated into the PCR products (one primer anneal to each chromosome) light from both the FAM- and HEX- dye is detected and the patient is heterozygote. If only one primer is incorporated into the PCR product the patient is wildtype or variant homozygote (depending on the colour of the emitted light).

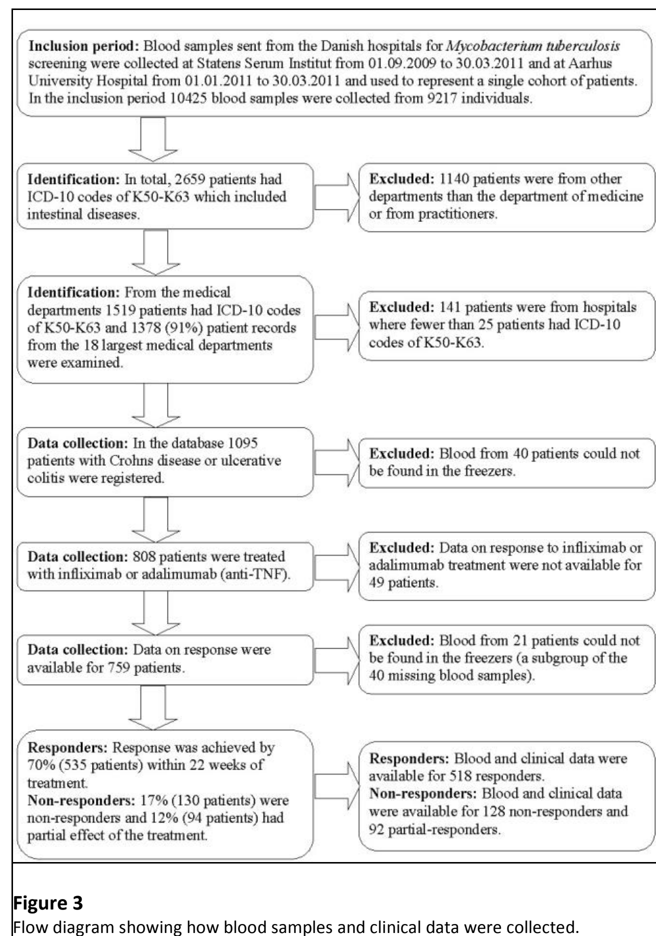

Figure 3
Flow diagram showing how blood samples and clinical data were collected.

Statistical analysis:

Logistic regression, crude and adjusted for age, gender and smoking status, was used to compare genotypes among responders versus non-responders and responders versus non- and partial-responders to anti-TNF therapy. Associations were expressed as odds ratios (OR) which represent the odds of exposure in responders divided by the odds of exposure in the non-responders.

A chi-square test or unpaired t-test was used to test if there was a statistically significant difference in response between patients with CD and UC, to examine if there were difference in secondary parameters between responders and non-responders and for haplotype analysis.

Statistical analyses were performed using STATA version 11 (STATA Corp., Texas, USA).

Power calculations:

The Genetic Power Calculator for case-control was utilized for power analysis of discrete traits [99]. The "high risk allele frequency" was set to 0.05, 0.10 or 0.48, the "prevalence" was set to 0.33 (responders vs. non-responders), D-prime was set to 1, type I error rate was set to 0.05.

RESULTS

Manuscript I

Blood and clinical data collection:

During the inclusion period, 10425 blood samples from 9217 individuals were collected as shown in Figure 3. In a pilot project, all patient records from Randers, Viborg and Herning hospitals (167 cases) from patients with ICD-10 codes of K50-K63 were

Characteristics	Crohn's disease (CD) Responder	Crohn's disease (CD) Partial responder	Crohn's disease (CD) Non-responder	p	Ulcerative colitis (UC) Responder	Ulcerative colitis (UC) Partial responder	Ulcerative colitis (UC) Non-responder	p	Inflammatory bowel disease (IBD) Responder	Inflammatory bowel disease (IBD) Partial responder	Inflammatory bowel disease (IBD) Non-responder	p
Efficacy - no (%)	362 (74)	63 (13)	67 (14)	-	173 (65)	31 (12)	63 (24)	-	535 (70)	94 (12)	130 (17)	-
Gender - no (%)												
Male	163 (78)	28 (13)	19 (9)		88 (66)	17 (13)	28 (21)		251 (73)	45 (13)	47 (14)	
Female	199 (71)	35 (12)	48 (17)	0.02	85 (63)	14 (10)	35 (26)	0.46	284 (68)	49 (12)	83 (20)	0.03
Anti-TNF treatment – no (%)												
Infliximab	302 (74)	54 (13)	50 (12)		173 (65)	31 (12)	63 (24)	-	475 (71)	85 (13)	113 (17)	
Adalimumab	60 (70)	9 (10)	17 (20)	0.12	-	-	-	-	60 (70)	9 (10)	17 (20)	0.54
Indication for anti-TNF therapy – no (%)												
Flare up	265 (73)	47 (75)	51 (76)	0.76	146 (84)	26 (84)	55 (87)	0.68	411 (77)	73 (78)	106 (82)	0.29
Fistulizing disease	48 (13)	12 (19)	9 (13)	1.00	-	-	-	-	48 (9)	12 (13)	9 (7)	0.60
Side effects to other medicine	11 (3)	1 (2)	2 (3)	1.00	14 (8)	0 (0)	2 (3)	0.25	25 (5)	1 (1)	4 (3)	0.63
Age, years												
Age at diagnosis, median (range)	24 (7-77)	23 (10-53)	27 (14-70)	0.17	31 (12-81)	32 (4-77)	31 (11-77)	0.64	26 (7-81)	25 (4-77)	29 (11-77)	0.07
Age at treatment, median (range)	33 (13-80)	30 (17-72)	40 (16-75)	0.03	38 (15-84)	42 (7-80)	37 (17-81)	0.75	35 (13-84)	34 (7-80)	38 (16-81)	0.04
Years from diagnosis to treatment initiation, median (range)	5 (0-44)	7 (0-41)	6 (0-35)	0.42	4 (0-37)	4 (0-37)	3 (0-33)	0.39	4 (0-44)	5 (0-41)	4 (0-35)	0.89
Location - no (%)												
Ileal (L1)	91 (25)	12 (19)	24 (36)	0.07	-	-	-	-	-	-	-	-
Colonic (L2)	124 (34)	24 (38)	23 (34)	1.00	-	-	-	-	-	-	-	-
Ileocolonic (L3)	130 (36)	24 (38)	16 (24)	0.07	-	-	-	-	-	-	-	-
Proctitis (E1)	-	-	-	-	22 (13)	3 (10)	14 (22)	0.10	-	-	-	-
Left side (E2)	-	-	-	-	77 (45)	14 (45)	28 (44)	1.00	-	-	-	-
Extensive (E3)	-	-	-	-	58 (34)	7 (23)	16 (25)	0.27	-	-	-	-
Data not available	17 (5)	3 (5)	4 (6)	-	16 (9)	7 (23)	5 (8)	-	-	-	-	-
Smoking history – no (%)												
Current smoker	102 (28)	21 (33)	23 (34)	0.38	20 (12)	1 (3)	1 (2)	0.02	122 (23)	22 (23)	24 (18)	0.34
Former smoker	40 (11)	8 (13)	5 (7)	0.40	37 (22)	7 (23)	13 (21)	1.00	77 (14)	15 (16)	18 (14)	1.00
Never smoker	94 (26)	22 (35)	11 (16)	0.12	42 (24)	6 (19)	13 (21)	0.61	136 (25)	28 (30)	24 (18)	0.11
Data not available	126 (35)	12 (19)	28 (42)	-	74 (43)	17 (55)	36 (57)	-	200 (37)	29 (31)	64 (49)	-
≥ 5 cigarettes/day	43 (12)	3 (5)	13 (19)	0.11	5 (3)	0	0	-	48 (9)	3 (3)	13 (10)	0.74
≥ 10 cigarettes/day	31 (9)	3 (5)	12 (18)	0.03	3 (2)	0	0	-	34 (6)	3 (3)	12 (9)	0.25
Concomitant medication – no (%)												
Azathioprine	104 (29)	27 (43)	15 (22)	0.30	31 (18)	8 (26)	13 (21)	0.70	135 (25)	35 (37)	28 (22)	0.43
5-aminosalicylates	30 (8)	2 (3)	5 (7)	1.00	68 (39)	9 (29)	25 (40)	1.00	98 (18)	11 (12)	30 (23)	0.22
Glucocorticoids	107 (30)	22 (35)	24 (36)	0.31	88 (51)	12 (39)	31 (49)	0.88	195 (36)	34 (36)	55 (42)	0.23
Methotrexate	6 (2)	3 (5)	0 (0)	-	1 (1)	1 (3)	0 (0)	-	7 (1)	4 (4)	0 (0)	-
Antibiotics	23 (6)	8 (13)	5 (7)	0.79	15 (9)	4 (13)	6 (10)	0.80	38 (7)	12 (13)	11 (8)	0.58
C-reactive protein (CRP) – no (%) of patients with available data												
Pre-treatment CRP ≥ 20 mg/L	74/216 (34)	16/49 (33)	14/46 (30)	0.73	33/97 (34)	6/24 (25)	14/47 (30)	0.71	107/313 (34)	22/73 (30)	28/93 (30)	0.53
≥ 25% decrease within 22 weeks	126/139(91)	29/34 (85)	21/28 (75)	0.03	57/68 (84)	9/15 (60)	12/22 (55)	0.01	183/207 (88)	38/49 (78)	33/50 (66)	0.01
F-calprotectin (F-cal) – no (%) of patients with available data												
Pre-treatment F-cal > 200 mg/kg	39/45 (87)	3/5 (60)	7/9 (78)	0.61	18/21 (86)	3/4 (75)	5/6 (83)	1.00	57/66 (86)	6/9 (67)	12/15 (80)	0.69
≥ 25% decrease within 22 weeks	25/29 (86)	1/2 (50)	0/4 (0)	0.01	10/13 (77)	3/4 (75)	1/2 (50)	1.00	35/42 (83)	4/6 (67)	1/6 (17)	0.01

Table 1

Clinical and demographic characteristics for anti-tumor necrosis factor-α (anti-TNF) naïve inflammatory bowel disease patients treated with anti-TNF.

	Crohn's disease (CD) Responder	Crohn's disease (CD) Partial responder	Crohn's disease (CD) Non-responder	p	Ulcerative colitis (UC) Responder	Ulcerative colitis (UC) Partial responder	Ulcerative colitis (UC) Non-responder	p	Inflammatory bowel disease (IBD) Responder	Inflammatory bowel disease (IBD) Partial responder	Inflammatory bowel disease (IBD) Non-responder	p
Adverse events leading to discontinuation – no (%)	33 (9)	8 (13)	9 (13)	0.27	17 (10)	4 (13)	5 (8)	0.80	50 (9)	12 (13)	14 (11)	0.62
Skin rash	10 (3)	1 (2)	3 (4)	-	6 (3)	-	1 (2)	-	16 (3)	1 (1)	4 (3)	-
Allergy, unspecified	8 (2)	2 (3)	3 (4)	-	2 (1)	3 (10)	1 (2)	-	10 (2)	5 (5)	4 (3)	-
Dyspnea	5 (1)	1 (2)	-	-	1 (1)	-	1 (2)	-	6 (1)	1 (1)	1 (1)	-
Joint and muscle pain	2 (1)	-	-	-	3 (2)	-	2 (3)	-	5 (1)	-	2 (2)	-
Infection, unspecified	1 (0)	1 (2)	1 (1)	-	1 (1)	-	-	-	2 (0)	1 (1)	1 (1)	-
Neuropathy	1 (0)	1 (2)	1 (1)	-	-	-	-	-	1 (0)	1 (1)	1 (1)	-
Serum sickness	-	-	1 (1)	-	-	-	-	-	-	-	1 (1)	-
Anaphylactic reaction	-	-	-	-	-	1 (3)	-	-	-	1 (1)	-	-
Cardiac failure	-	-	-	-	1 (1)	-	-	-	1 (0)	-	-	-
Other, unspecified	6 (2)	2 (3)	0 (0)	-	3 (2)	-	-	-	9 (2)	2 (2)	0 (0)	-

Table 2

Adverse events to anti-tumor necrosis factor-α (anti-TNF) therapy leading to treatment termination.

examined. Only patients from the medical departments had a diagnosis of CD or UC and were treated with infliximab or adalimumab. Therefore, only patient records from medical departments were examined. From the medical departments, 1519 patients had ICD-10 codes of K50-K63 and 1378 (91%) patient records from 18 hospitals were examined. Based on the patient records 1095 patients were registered as having CD or UC. Of these 808 had been treated with anti-TNF. However, data on response were not available for 49 patients and for the remaining 759 patients 21 blood samples could not be found for further genetic analysis.

Treatment efficacy:

Data on efficacy were available for 759 patients and included 535 (70%) responders, 94 (12%) partial responders and 130 (17%) non-responders (Table 1). Response rate was 65% (173 patients) and 74% (362 patients) among patients with UC and CD, respectively (OR: 0.66, 95% CI: 0.48-0.91, p = 0.01). Non-response rate was 24% (63 patients) and 14% (67 patients) among patients with UC and CD, respectively (OR: 1.96, 95% CI: 1.34-2.87, p = 0.001). Among patients with CD the response rate to infliximab and adalimumab treatment was 74% (302 patients) and 70% (60 patients), respectively (p = 0.12). The rate of non-response was 17% (48 patients) and 9% (19 patients) among females and males with CD, respectively (OR: 2.07, 95% CI: 1.13-3.81, p = 0.02).

Clinical markers associated with response:

Heavy smoking was associated with low response rate among patients with CD with 18% (12 patients) of the non-responders versus 9% (31 patients) of the responders smoking 10 or more cigarettes per day (OR: 2.33, 95% CI: 1.13-4.81, p = 0.03) (Table 1). A decrease in CRP levels by > 25% within 22 weeks after treatment initiation was not associated with response (p = 0.06). However, when only considering pre-treatment CRP > 20 mg/L (twice the upper limit of the normal range) a decrease of > 25% in CRP level within 22 weeks after treatment initiation, was associated with beneficial response (OR: 1.99, 95% CI: 1.08-3.68, p = 0.03). Young age at treatment initiation was associated with response among patients with CD, with a median age of 33 (13-80) and 40 (16-75) years among responders and non-responders,

respectively (p = 0.03). CD location, UC extension, treatment indication (flare-up, fistula or side effects to other medications), concomitant medication or overall smoking status was not associated with treatment response.

Adverse events:

As shown in Table 2, 76 (10%) of the 759 patients treated with infliximab or adalimumab terminated the treatment because of adverse events. The most severe adverse events were one case of serum sickness, one case of anaphylactic reaction and one case of cardiac failure. No deaths were reported.

Manuscript II

DNA extraction:

The DNA yields by the different purification kits tested are shown in Table 3. The highest yields were obtained using Maxwell 16 Blood purification kit with a median of 4.9 µg (range 0.8-25 µg) pr 300 µl total blood followed by PureGene with glycogen with a median of 0.65 µg (range 0.5-2.6 µg) pr 300 µl total blood (p < 0.001). The number of correct calls was > 99% for all of the extraction methods as described in Table 3.

The length of time the blood clots were stored did not have a significant effect on the DNA yield (p = 0.47).

Method	Blood (µl)	µg (median)	Range (µg)	µg / ml blood (median)	A_{260}/A_{280} (median)	Range (A_{260}/A_{280})	Real-time PCR Successful / Total (%)
Maxwell 16 Blood purification kit (Promega)[1]	300[2]	4.90	0.8-25	16.3	1.86	1.66-2.00	40633 / 41145 (99)
PureGene with glycogen (Qiagen)	300[2]	0.65	0.5-2.6	2.2	1.57	1.42-1.68	10 / 10 (100)
PureGene without glycogen (Qiagen)	300[2]	0.60	0.2-1.6	2.0	1.55	1.24-1.68	10 / 10 (100)
QIAamp DNA Micro kit (Qiagen)	100	0.55	0.2-1.6	5.5	1.72	1.50-2.02	10 / 10 (100)
Nucleospin 96 Blood kit (Macherey-Nagel)	200	0.38	0.1-0.5	1.9	2.01	1.73-2.60	6 / 6 (100)

[1]DNA was extracted from 1055 blood clot samples and 39 single nucleotide polymorphisms (SNPs) were genotyped.
[2]Can be scaled to larger volumes.

Table 3
Yield and A260/A280 ratio of DNA extracted from cryopreserved clotted blood using commercial kits.

Manuscript III

Study population:

Clinical data on response to anti-TNF therapy and blood samples were available from 256, 482 and 738 prior anti-TNF naïve patients with UC, CD and IBD, respectively. The biological effect of the studied SNPs and their association with anti-TNF therapy among patients with CD, UC or IBD are summarized in Table 4 and Figure 4.

Polymorphisms associated with response in CD:

Among patients with CD, 6 functional SNPs (TLR2 (rs1816702, rs3804099), TLR4 (rs5030728), TLR9 (rs352139), LY96 (rs11465996) and TNFRSF1A (rs4149570)) were associated with response when comparing responders with non-responders and this number increased to 7 SNPs when including partial-responders (IFNG (rs2430561)).

Polymorphisms associated with response in UC:

Among patients with UC, 8 SNPs (TLR2 (rs11938228, rs3804099), TLR4 (rs5030728), LY96 (rs11465996), CD14 (rs2569190), TNFAIP3 (rs6927172), IL1B (rs4848306) and IL6 (rs10499563)) were associated with response when comparing responders with non-responders and 11 SNPs (TLR2 (rs4696480), IL1RN (rs4251961) and IL17A (rs2275913)) were associated with re-sponse when including partial-responders.

Polymorphisms associated with response in IBD:

The polymorphisms showed the same direction of effect in both diseases except for the polymorphisms in LY96 (rs11465996).

When including all patients (IBD), 13 SNPs (TLR2 (rs11938228, rs3804099), TLR4 (rs5030728), TLR9 (rs187084, rs352139), LY96 (rs11465996), MAP3K14 (rs7222094), TNFA (rs361525), TNFRSF1A (rs4149570), TNFAIP3 (rs6927172), IL1B (rs4848306), IL6 (rs10499563)) and IL17A 197 (rs2275913) were associated with response among patients with IBD when comparing responders with non-responders. This number increased to 15 SNPs (TLR4 (rs1554973) and IFNG (rs2430561)) when including partial-responders.

Haplotype analysis:

Four haplotypes in TLR2, three in TLR4 and IL1B and two in TLR9 described 85%, 95%, 99% and 97% of the genotypes observed, respectively. The TLR2 haplotype 22 (rs4696480TT, rs1816702CC, rs11938228AA and rs3804099TT) (OR: 0.41, 95% CI: 0.19-0.86, p = 0.02) and the haplotype 12 (rs4696480TA, rs1816702CC, rs11938228CA and rs3804099CT) (OR: 0.48, 95% CI: 0.24-0.95, p = 0.04) were associated with non-response. Haplotype combination 33 was also associated with non-response, although not statistically significantly. Both haplotype 2 and 3 encompass the wildtype allele of rs3804099, and thus the haplotype analysis supports the found association between the variant allele of rs3804099 and beneficial response.

No associations were found for TLR4, TLR9 or IL1B.

DISCUSSION

Establishing the anti-TNF treated IBD cohort (Manuscript I)

In order to identify polymorphisms associated with response to anti-TNF therapy we first had to establish a cohort of previously naïve anti-TNF treated patients with CD or UC. To establish the cohort we needed to identify patients with CD or UC treated with anti-TNF, obtain clinical data on response and obtain biological samples.

Biological samples

Before patients are treated with anti-TNF they are usually screened for TB. By establishing a cooperation with the major laboratory centres in Denmark responsible for TB screenings we were able to collect biological samples from patients with CD or UC treated with anti-TNF. The advantage was that we were able to rapidly collect many biological samples without much effort thanks to our partners. Furthermore, biological samples from patients with rheumatoid arthritis or psoriasis treated with anti-TNF were also collected, as we collected blood from all patients screened for TB. This allowed spin off projects by establishing a cohort of anti-TNF treated patients with rheumatoid arthritis (a cohort of patients with rheumatoid arthritis has been established) and anti-TNF treated patients with psoriasis (in progress).

Patient identification

Patients with CD or UC were identified by linking CPR-numbers from the blood samples to The National Patient Registry and extracting data from patients with ICD-10 codes of K50-K63, which include all intestinal diseases.

Clinical data collection

By retrospective examining the patient's records clinical data could be collected. First, in a pilot study all patient records from all departments from three hospitals were examined. Only patients from the medical departments were diagnosed with CD or UC and treated with anti-TNF. Furthermore, in the pilot project we tried to calculate a score by using the CDAI, Mayo Scoring and by using "The simple 3-step scale" used in other retrospective studies, to evaluate response. However, the retrospective examination of the patient records did not include enough data to

Gene (SNP)	rs-number	Effect of the SNP	OR (95% CI)	Association
TLR2, *TLR4*, *TLR5* and *TLR9* (activates inflammation through the canonical NFκB pathway)				
A>T	rs4696480	*TLR2*, Unknown [100]	0.47 (0.23-0.95)[C]	Non-response (TT)[C]
C>A	rs11938228	*TLR2*, Unknown [100]	0.63 (0.41-0.98)[A]	Non-response (CA or AA)[A,C]
C>T	rs1816702	*TLR2*, rs1816702T increase receptor level [101]	2.02 (1.04-3.95)[B]	Response (CT or TT)[B]
597 T>C	rs3804099	*TLR2*, 597C decrease TNF-α, IL-1β & IL-6 level [102]	1.80 (1.15-2.81)[A]	Response (TC or CC)[A,B,C]
G>A	rs5030728	*TLR4*, Unknown [100]	1.45 (1.06-2.00)[A]	Response (GA or AA)[A,B,C]
T>C	rs1554973	*TLR4*, Unknown [100]	0.72 (0.52-0.99)[A]	Non-response (TC or CC)[A]
T>C	rs12377632	*TLR4*, Unknown [100]	-	No association
1174 C>T	rs5744168	*TLR5*, 1174T decrease TNF-α, IL-1β & IL-6 level [102, 103]	-	No association
-1486 T>C	rs187084	*TLR9*, -1486C & 1174G decrease expression [104]	1.99 (1.04-3.82)[A]	Response (TC)[A]
1174 G>A	rs352139	*TLR9*, -1486C & 1174G decrease expression [104]	0.48 (0.24-0.96)[A]	Non-response (AA)[A,B]
LY96 (MD-2 binds to TLR2 or TLR4 and is required for their activation to LPS stimuli)				
-1625 C>G	rs11465996	-1625G increase MD-2 & TNF-α level [105]	1.48 (1.00-2.19)[A]	Response (CG or GG)[A,B,C]
CD14 (binds LPS and transport it to TLR4)				
-159 G>A	rs2569190	-159AA increase CD14 level [106, 107]	0.54 (0.30-0.98)[C]	Non-response (GA or AA)[C]
MAP3K14 (NIK is a central kinase in the non-canonical NFκB pathway)				
T>C	rs7222094	rs7222094CC decrease NIK activity [108]	1.92 (1.00-3.68)[A]	Response (TC)[A]
SUMO4 (SUMO4 conjugates to IκBα and negatively regulates NFκB transcriptional activity)				
163 T>C	rs237025	163C increase NFκB1 expression [109]	-	No association
NFKBIA (IkBα is an inhibitor of NFκB1)				
2758 A>G	rs696	2758A increase expression [110]	-	No association
T>del	rs17103265	rs17103265del decrease expression [111]	-	No association
NFKB1 (NFκB1 (p50-RelA) is a transcription factor)				
-94 ins/del	rs28362491	-94del decrease expression [112]	-	No association
TNFA (TNF-α is a pro-inflammatory cytokine activated by NFκB1)				
-863 C>A	rs1800630	-863A increase expression [113]	-	Failed to genotype
-857 C>T	rs1799724	-857T increase TNF-α level [114]	-	Failed to genotype
-308 G>A	rs1800629	-308A increase expression [115]	-	No association
-238 G>A	rs361525	-238A decrease expression [116]	0.43 (0.19-0.97)[A]	Non-response (GA)[A]
TNFRSF1A (TNF receptor 1 (TNFR1) binds TNF-α and initiates a kinase cascade)				
-609 G>T	rs4149570	-609T increase expression [117]	2.07 (1.03-4.15)[A]	Response (TT)[A,B]
TNFAIP3 (TNF-α rapidly induced expression of *TNFAIP3* (A20) which inhibit NFκB activation and TNF-α mediated apoptosis)				
C>G	rs6927172	rs6927172G increase expression [118]	0.62 (0.42-0.92)[A]	Non-response (CG or GG)[A,C]
IL1B (pro-inflammatory cytokine activated by NFκB1)				
-3737 G>A	rs4848306	-3737A decrease transcription [119, 120]	1.85 (1.05-3.27)[A]	Response (GA or AA)[A,C]
-1464 G>C	rs1143623	rs1143623C decrease IL-1β level [120, 121]	-	No association
-31 T>C	rs1143627	-31C decrease expression [120-122]	-	No association
IL1RN (IL-1RA binds to the IL-1 receptor and inhibit IL-1β signaling)				
T>C	rs4251961	rs4251961C decrease IL-1RA level [123, 124]	0.42 (0.18-0.98)[C]	Non-response (TC or CC)[C]
IL4R (IL-4 receptor, IL-4 significantly inhibit IL-17 production)				
A>G (I50V)	rs1805010	rs1805010G increase IL-17 level [125]	-	No association
IL6 (pro- and anti-inflammatory cytokine activated by NFκB1)				
-6331 T>C	rs10499563	-6331C decrease expression [126]	2.26 (1.18-4.32)[A]	Response (TC or CC)[A,C]
IL6R (binds IL-6 and initiates a kinase cascade)				
C>T	rs4537545	rs4537545TT increase IL-6r and IL-6 level [127]	-	No association
IL10 (activated by NFκB1, capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ and TNF-α)				
-592 C>A	rs1800872	-592A increase expression [128]	-	No association
C>T	rs3024505	Unknown. Associated with IBD [129]	-	No association
IL17A (activated by NFκB1, pro-inflammatory cytokine, potent mediator in delayed-type reactions, induces the production of IL-1β, IL-6 & TNF-α)				
197 G>A	rs2275913	197A increase expression [130]	0.42 (0.18-1.00)[C]	Non-response (GA or AA)[A,C]
IL23R (IL-23 receptor, IL-23 induce the production of IL-17 and IFN-γ)				
G>A	rs11209026	rs11209026GG increase IL-17 serum level [131]	-	No association
IFNG (IFN-γ is a pro- and anti-inflammatory cytokine activated by NFκB1)				
874 T>A	rs2430561	874A decrease IFN-γ level [132]	1.66 (1.05-2.62)[A]	Response (TA or AA)[A,B]
TGFB1 (TGF-β1 is a cytokine which can inhibit the secretion and activity of many other cytokines including IFN-γ and TNF-α)				
-509 C>T	rs1800469	-509T increase expression [133]	-	No association
PTPN22 (involved in several signaling pathways associated with the immune response)				
1858 G>A	rs2476601	1858A decrease TNF-α in serum [134]	-	No association
PPARG (PPARγ is a transcription factor)				
C>G	rs1801282	rs1801282G decrease PPARγ mRNA level [135]	-	No association
NLRP3 (NALP3 is involved in the inflammasome)				
C>T	rs4612666	rs4612666T decrease expression [136]	-	No association

[A] Association among patients with IBD; [B] Association among patients with CD; [C] Association among patients with UC

Table 4
The biologic effect of the studied single nucleotide polymorphism (SNP), odds ratios (OR) and association with anti-TNF therapy among patients with Crohn's disease (CD), ulcerative colitis (UC) and combined inflammatory bowel disease (IBD).

calculate a score for most patients and response to anti-TNF therapy was therefore evaluated by using "The simple 3-step scale". Thus, a disadvantage of this retrospective approach was that it was not possible to collect all clinical data from all the patients.

Primary or long-term response

Anti-TNF response was assessed in every-day clinical practice in a cohort of 759 previously anti-TNF naïve Danish IBD patients sampled from 18 medical departments as summarized in Figure 3. The cohort should therefore be representative for Danish IBD patients in every-day clinical practice.

The cohort study was designed for evaluation of primary response, where response was evaluated as the maximum response within 22 weeks of treatment initiation. It could have been interesting to look for markers associated with long-term response or side effects. However, of the responders only 20 patient terminated treatment due to loss of effect and 50 patients terminated treatment due to side-effect. Furthermore, treatment was still on-going for 172 patients when the patient records were examined. In addition, the group of patients with side effects were inhomogeneous (Table 2) and genetic mechanisms involved in primary response, long-term response and side effects may differ. Therefore, if this cohort had been used to look for marker associated with long-term response or side effects it would have had limited power to detect such associations.

Validity of the cohort

The validation of our cohort was crucial for the validity of future studies on genetic variations associated with treatment response. To validate how the patients were divided into responders, partial- and non-responders in our study, we compared response rate and differences in clinical and biochemical parameters between responders and non-responders in our cohort with the results from other prospective [36, 40, 41, 137] and retrospective studies [54, 55, 138, 139]. In addition, clinical and biochemical markers may be used, alone or together with genetic markers, to predict response to anti-TNF therapy.

We found that anti-TNF treatment of CD and UC was effective with response rates of 74% and 65%, respectively. In our cohort study heavy smoking was associated with non-response whereas young age at treatment initiation was associated with beneficial treatment response among patients with CD. In addition, a decrease of 25% or more of pre-treatment CRP (> 20 mg/L) or F-calprotectin were associated with beneficial treatment response among patients with CD and UC. Furthermore, patients with UC were more likely to be non-responders than patients with CD, and females with CD were more likely to be non-responders than males with CD (Table 1). However, the clinical and biochemical markers associated with response to anti-TNF therapy in our study have to be interpreted with care as data were collected retrospectively and were not available for all patients.

The response rate to anti-TNF therapy in this retrospective study using the "The simple 3-step scale" was comparable to other retrospective studies for CD and UC [54, 55, 138, 139]. Compared to prospective clinical trials which used a score to evaluate response, the response rate in our study was to the high end [36, 40, 41, 137]. The time to evaluation of response differ in the studies which could influence the response rate. In addition, we excluded 49 patients treated with anti-TNF and many of those patients might have been non-responders which artificial could have increased the response rate in our cohort. The patients records from theses patients were either inadequate to evaluated response or the patients had other complication during anti-TNF treatment which shifted the focus away from the anti-TNF therapy.

Heavy smoking was associated with non-response among patients with CD in consensus with other studies [64, 65]. Among patients with UC smoking was associated with beneficial response to anti-TNF therapy although this is based on a very low number of patients in our study (Table 1). The associations found between smoking and response to anti-TNF therefore reflect how smoking effects patients with CD and UC, where smoking has a negative effect on patients with CD and a positive effect on patients with UC [13]. Smoking may therefore have a limited effect on response to anti-TNF therapy among patients with CD and UC but instead reflect a negative or positive effect on the disease itself.

In agreement with previous studies, we found that young age was associated with beneficial response among patients with CD [58, 61].

Patients with UC were more likely to be non-responders than patients with CD, which is in accordance with other studies [54]. The clinical data was collected similarly in this retrospective cohort for both patients with CD and UC. As the results have been confirmed in other studies it is likely that the difference in response between patients with CD and UC is due to biological differences.

In our study female patients with CD were more frequently non-responders than male patients with CD. Smoking status did not account for this gender difference. Interestingly, female patients with rheumatoid arthritis have also been found to be more likely to be non-responders to anti-TNF therapy than males [140]. The observations could reflect a biological difference between the genders. However, since the study was not based on a calculated score to evaluate response it could be speculated that psychology played a factor.

CRP and F-calprotectin were the only biochemical markers examined in our study and pre-treatment levels were not found to be associated with response to anti-TNF. However, a decrease of 25% or more of pre-treatment CRP (> 20 mg/L) or F-calprotectin levels was associated with beneficial response in accordance with other studies [48, 49, 65, 73]. Thus, the association between a decreased level of these inflammatory markers post-treatment and beneficial response further support the validity of how the patients have been divided into responders, partial- and non-responders in this retrospective study. However, as the associations are based on the level of CRP or F-calprotectin before and after treatment initiation, they can not be used to predict response.

In contrast to genotyping which are qualitative DNA analyses, biochemical analyses are qualitative analyses. Thus, in DNA analyses, the quantity of DNA is less important, as long as there is enough DNA for the analyses. In contrast, biochemical analyses rely on the quantity of protein and therefore uniform treatment and proper storage of the samples is important. Age, sex and ethnicity may effect the result of a biochemical analysis and a matched control group is therefore important. Biopsies can be used for biochemical analyses, however, for markers used to predict response to a drug, blood or urine samples are preferred as they are much less invasive than a biopsy [141, 142]. Finally, for predicting risk of disease, genetic markers may prove more convenient than protein or mRNA markers, as whole genome sequences may be available for all persons in the western world in the coming decades.

The blood clots we have collected after TB analysis are probably not well suited for downstream biochemical analyses, as the

samples have not been uniformly handled. Especially the difference in time our blood samples have been stored at room temperature pose a problem.

Other clinical markers including luminal location [64, 68] have been associated with response to anti-TNF. However, we were unable to confirm this finding. In addition, the use of azathioprine and methotrexate has been associated with beneficial response after short-term treatment and fewer infusion reactions among patients with CD and UC treated with anti-TNF [54, 64, 72, 143]. We were unable to confirm this result probably because of lack of power. However, our study was the first to evaluate the efficacy of both infliximab and adalimumab on treatment response among patients with CD and found that both were effective for treatment of patients with CD.

In conclusion, the response rate, difference in response rate among patients with CD and UC and clinical and biochemical markers associated with response to anti-TNF (smoking, age, CRP and F-calprotectin levels) in our retrospective cohort study reflected the findings in other studies. This indicates that the division of the patients into responders, partial- and non-responders in our study was reasonable and that the cohort therefore could be used as a solid foundation for further studies of genetic markers associated with response to anti-TNF therapy.

DNA extraction (Manuscript II)

The blood collected was left over clotted blood from TB screening. Clotted blood often result in low DNA yields and we therefore compared the efficiency of four commercially kits for extracting DNA. The Maxwell 16 Blood purification kit had almost ten-fold higher yield than the other commercial kits tested. However, the DNA yield obtained with any of the commercial kits tested varied considerably among individuals. Variations in leukocyte counts or in blood clot formation have been suggested to be associated with DNA yield [144, 145].

The DNA yield did not depend on how long the blood was cryopreserved which is supported by other studies where blood clots have been stored for up to 2.5 years without an effect on yield [146, 147].

These results show that DNA can be efficiently extracted from cryopreserved blood clot samples, even after prolonged storage. However, the retrospective approach of collecting blood after TB made it more laborious and expensive to extract the DNA and furthermore gave a lower yield compared to full blood which could have been collected in a prospective study.

Candidate genes (Manuscript III)

To examine if polymorphisms could be used to predict response to anti-TNF therapy we choose a candidate gene approach and furthermore wanted to include polymorphisms with a known biological effect. We chose to study polymorphisms in the NFκB and TNF-α pathway as TNF-α and NFκB feed-back activates each other, are elevated among patients with IBD and because anti-TNF inactivate soluble TNF-α. Furthermore NFκB and TNF-α are key players in the inflammatory pathway. We aimed at including 2 to 3 functional polymorphisms in every gene examined. However, the knowledge on the biological effect of SNP is limited and we therefore had to settle with a single functional SNP in some genes. A few SNPs outside of the NFκB and TNF-α pathway were chosen in an effort to identify other pathways which might be involved in response to anti-TNF and because some of these SNPs had been shown to be associated with risk of CD and/or UC.

Functional polymorphisms

One of the advantages of studying SNPs with a biological effect that modify the activity of the gene or gene product is that it allows us to make biological interpretation of our results, based on increased or decreased gene or protein activity. This can help us to understand some of the biological mechanisms involved in treatment response to a drug or susceptibility to a disease. Another advantage of studying functional SNP is that it would be expected that SNPs associated with susceptibility of a disease or response to a drug either had a biological effect or was linked to another SNP with a biological effect. It could therefore be expected that studying functional SNPs using a candidate gene approach would increase the likelihood of finding an association compared to examining polymorphisms with unknown (and possibly no) biological effect.

The disadvantage of focusing on functional SNPs is the limited knowledge on the biological effect of SNPs. It therefore requires an extensive literature search to identify the few SNPs with a biological effect in a pathway.

GWAS

An alternative approach to the candidate gene study is hypothesis free GWAS. The advantage of GWAS is that hundreds of thousands of SNPs spread throughout most of the human genome are examined. However, because of the high number of polymorphisms studied it is necessary to use strict and complex statistic methods which increase the risk of type II error (false negative). A practical challenge is that a fairly large amount of DNA is required (usually more than 1µg DNA compared to about 10ng for SNP studies). Furthermore GWAS are more expensive than candidate gene studies.

Polymorphisms associated with response and biological interpretation

In the inflammatory pathways, 37 SNPs in 26 genes were successfully genotyped and 19 of the functional polymorphisms in 14 genes were associated with response to anti-TNF therapy among patients with CD, UC or CD and UC combined (IBD) as shown in Figure 4.

As illustrated in Figure 5, genetically determined increased level of TLR2 (rs1816702) and MD-2 (LY96) (rs11465996) (required for TLR2 and TLR4 to respond to LPS) [101, 105] were associated with beneficial response among patients with CD, indicating that a higher activity of TLR2 was associated with a beneficial response among patients with CD. Among patients with UC genetically determined increased level of MD-2 (LY96) (rs11465996) and CD14 (rs2569190) [105-107] were associated with non-response, indicating that a high activity of TLR4 was associated with non-response among patients with UC. In addition, two SNPs in TLR2 (rs4696480 and rs11938228) and two SNPs in TLR4 (rs5030728 and rs1554973) with unknown biological effect were associated with response among patients with CD, UC or IBD as shown in Table 4. The TLR9 heterozygous genotype of -1486 T>C (rs187084) and the homozygous variant genotype of TLR9 1174 G>A (rs352139) were associated with beneficial response and non-response among patients with IBD, respectively. The 1486 T>C and 1174 G>A polymorphisms in TLR9 have only been shown to have a biological effect in haplotype context [104, 148], however, the haplotype analysis of TLR9 did not reveal any associations. Thus, the results indicate that TLR activity is important in determining response to anti-TNF therapy among

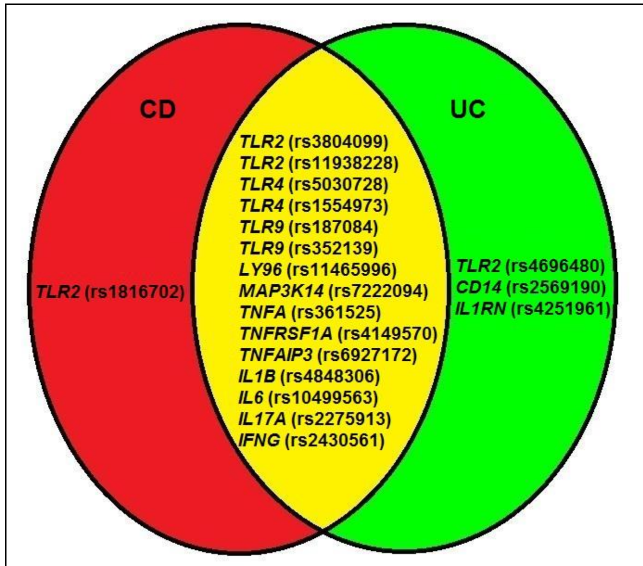

Figure 4
Polymorphisms associated with response to anti-TNF.
Thirty seven functional single nucleotide polymorphisms (SNPs) in 26 genes were successfully genotyped and 19 SNPs in 14 genes were associated with response to anti-tumor necrosis factor-α (TNF-α) therapy among patients with Crohn's disease (CD), ulcerative colitis (UC) or CD and UC combined. The 19 SNPs associated with response were in genes involved in regulation of NFκB through the TLR pathways (TLR2, TLR4, TLR9, LY96 (MD-2), CD14 and MAP3K14 (NIK)), TNF-α signaling (TNFA (TNF-α), TNFRSF1A (TNFR1) and TNFAIP3 (A20)) or cytokines regulated by NFκB (IL1B, IL1RN, IL6, IL17A and IFNG).

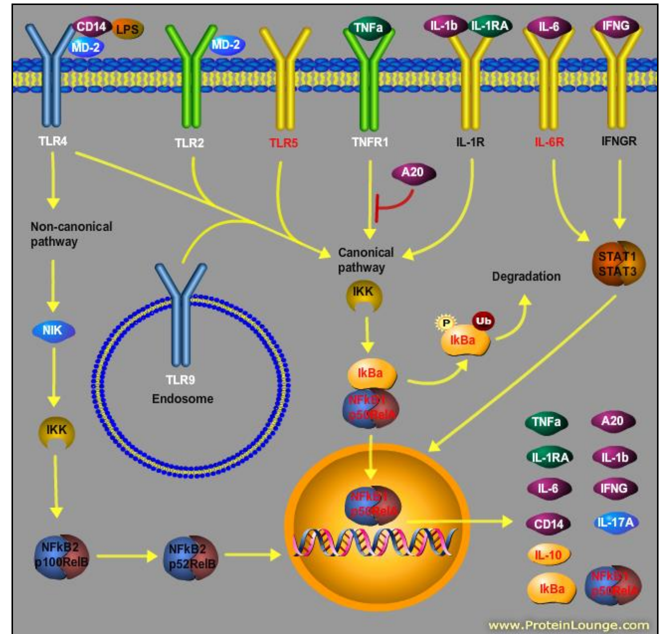

Figure 5
Simplified overview of the NFκB pathway highlighting the genes which were studied. Polymorphisms in genes associated or not associated with response to anti-TNF therapy among patients with IBD are written in white and red, respectively. Genes not studied are written in black.
Increased gene/protein activity was associated with beneficial response (green) or non-response (purple). The biological effect was unclear (TLR4, TLR9, NIK) or showed opposite direction of effect among patients with CD and UC (MD-2) (blue).
A significant association was only seen in CD14 and IL-1RA (inhibitor of IL-1β signaling) among patients with UC.
IBD: inflammatory bowel disease; CD: Crohn's disease; UC: ulcerative colitis.

patients with IBD.

Regarding the canonical and non-canonical NFκB pathway, functional polymorphisms in SUMO4, NFKBIA (IκBα) and NFKB1 (p50-RelA) were not found to be associated with response. However, the heterozygous genotype of rs7222094 T>C in MAP3K14 (NIK) was associated with beneficial response among patients with IBD. The biological effect of the heterozygous genotype is unknown [108], which make it difficult to interpret the association in MAP3K14 from a biological perspective. Further studies of the non-canonical NFκB pathway, e.g. by studying functional polymorphisms in LTA or TNFSF11 (RANKL) [149-151], could shed more light on any possible involvement of this pathway in anti-TNF therapy response.

The TNF-α signaling pathway showed that a genetically determined decreased expression of TNFA (TNF-α) (rs361525) [116] was associated with non-response among patients with IBD. Furthermore, a genetically determined increase expression of the TNF receptor 1 (TNFRSF1A) (rs4149570) [120] was associated with beneficial response among patients with CD and IBD. In addition, a genetically determined increased expression of TNFAIP3 (A20) (rs6927172) [118] was associated with non-response among patients with IBD. A20, encoded by TNFAIP3, is known to inhibit NFκB activation as well as TNF-α mediated apoptosis. Thus, the results indicate that polymorphisms in TNFA (TNF-α), TNFRSF1A (TNFR1) and TNFAIP3 (A20), which upregulate TNF-α signaling, were associated with beneficial response to anti-TNF therapy among patients with IBD.

Among cytokines regulated by NFκB, a genetically determined decreased expression of IL1B (rs4848306), IL6 (rs10499563) and IFNG (rs2430561) [119, 126, 132] were associated with beneficial response among patients with CD, UC and IBD. Furthermore, a polymorphism in IL1B (rs1143627) which has been shown to increase IL-1β level [121, 122], was borderline significantly associated with non-response among patients with IBD. In addition, a genetically determined decreased IL-1 receptor antagonist (IL-1RA) level (rs4251961) [123] was associated with non-response among patients with UC. IL-1RA binds to the IL-1 receptor and inhibit IL-1β signaling [18]. The IL-6 receptor is not regulated by NFκB and no association was found with the SNP studied in IL6R [127].

This could indicate that among patients with IBD, non-responders to anti-TNF therapy are more likely to have an inflammatory response mediated by other early pro-inflammatory cytokines than TNF-α such as IL-1β, IL-6 and IFN-γ. This suggests that drugs targeting IFN-γ, IL-1β or IL-6 could potentially be useful for treating patients, who do not respond to anti-TNF therapy. This interpretation is supported by the polymorphism in IL1RN, where a genetically determined high inhibition of IL-1β signaling (high IL1RA level) was associated with beneficial response. Furthermore, this interpretation is also supported by another study which has reported an association between the C-allele of rs1143634 in IL1B and higher serum IL-1β level and a lower response rate to infliximab therapy among patients with CD [78].

The variant allele of the 597 T>C polymorphism in TLR2 (rs3804099) was associated with beneficial response among patients with CD and IBD. The variant allele has been shown to decrease TNF-α level to approximately 67%, IL-1β level by 50% and IL-6 level by 40% of the wild type allele [102]. In the light of the other results, we expected a decreased TNF-α level to be associated with non-response and a decreased IL-1β and IL-6 level to be associated with beneficial. This indicates that the relative levels of the cytokines TNF-α, IL-1β and IL-6 are important in determining response to anti-TNF.

The variant allele of the 197 G>A polymorphism in IL17A was associated with non-response among patients with UC and IBD. The polymorphism has been shown to increase expression of IL-17 [130] indicating that high level of this cytokine may also be associated with non-response.

The functional SNPs studied in IL4R, IL10, IL23R, TGFB1, PTPN22, PPARG and NLRP3 were not associated with response to anti-TNF therapy.

Overall the results indicate that patients with genetically determined high TNF-driven inflammatory response benefit the most from anti-TNF therapy. Conversely, patients with genetically determined IL1B, IL6 and IFNG-driven inflammatory response seem to benefit the least form anti-TNF therapy. These patients might benefit from biological drugs targeting other cytokines such as IL-1β, IL-6 or IFN-γ or from a cocktail of several antibodies.

The results in this exploratory study should be interpreted with care. The data were collected retrospectively and secondary parameters were not available for all patients. In the study 37 SNPs were successfully genotyped. Associations between genotype and response were statistically analysed twice for each genotype, using logistic regression unadjusted and adjusted for age, sex and smoking status. The lowest p-value found was for rs3804099 in TLR2 with a p-value of 0.006. Therefore, if the results had been Bonferroni corrected for multiple tests no associations between the studied SNPs and response to anti-TNF would have been found. However, Bonferroni correction for multiple tests is very conservative and increases the risk of type II error (false negative) and reduces the statistical power. Another solution to test for false positive errors (type I errors) is to replicate the results in independent cohorts.

On the other hand, this study was rather large including 738 patients with IBD treated with anti-TNF. This gives the study more than 80% power to detect an odds ratio of 1.5 for polymorphisms associated with response to anti-TNF. In addition, blood and clinical data from these patients were collected at 18 large gastroenterological centres at basic and specialized hospitals in Denmark. Thus, the patients are representative of Danish patients with severe IBD.

Our study may over estimate the number of associations as no correction for multiple tests was made. However, our candidate gene study was explorative and used for generating new thesis that can be used in follow-up studies to select pathways and genes likely to be involved in anti-TNF response. Additional confirmation of these findings in independent cohorts should be performed before our results are applied in the clinic.

Consequences of predictability

The aim of using a predictive model for patient management is to improve the treatment of the individual by increasing the likelihood, that the patients benefit from the treatment and to avoid ineffective treatment with drugs that may have potentially serious side-effect.

Predictive models are based on statistical associations and it is therefore unlikely, that we will be able to completely separate responders from non-responders. A complete separation of the patients probably requires an understanding on a biological level. Thus, a consequence of using a predictive model is that there will be patients in the group who are predicted as non-responders, who would have had effect if treated. Similarly, although the response rate in the group of patients predicted to be responders will be higher than in an unselected group of patients, non-responders will still be treated.

There should be an alternative medical or surgical treatment available for the patients predicted to be non-responders. If there is no attractive alternative treatment then the physician may still choose to treat the patient predicted to be non-responder, as the patient might still benefit from the treatment. For some patients with CD and UC the only alternative to anti-TNF therapy is surgery and for these patients treatment with anti-TNF may be worthwhile even if the patients are predicted to be non-responders.

Before the patient can be predicted to be responder or non-responder, genetic or biochemical laboratory analyses are required which can delay the treatment. However, in the case of anti-TNF therapy a blood sample from the patient is routinely screened for TB prior to the treatment and genetic analyses could be conducted in parallel. A delay in the treatment with anti-TNF is therefore not expected to be a major issue.

Threshold for response

It is difficult to set a threshold for how high or low the response rate should be before the patient is treated, as several factors influence the decision of treatment. Cost of the medication, alternative therapy, side-effect of the medication and to the alternative therapy, and how or if the disease may evolve during ineffective treatment all influence the decision of treatment. All these variables are difficult or impossible to include in a model. Therefore, the best option in predicting treatment response may be to report the patients predicted response rate as a percent-value and let the treating physician make the final decision.

CONCLUSION

A cohort of 759 previously naïve anti-TNF treated patients with IBD was established with the purpose of identifying genetic markers predictive of anti-TNF treatment response in clinical practice. The cohort was validated by comparing response rate and differences in clinical and biochemical parameters between responders and non-responders in our cohort with the results from other prospective and retrospective studies. The response rate, difference between response rate among patients with CD and UC and clinical and biochemical markers associated with response to anti-TNF (smoking, age, CRP and F-calprotectin levels) in our retrospective cohort study reflected the findings in other studies. This indicates that the division of the patients into responders, partial- and non-responders in our study was reasonable and that the cohort therefore could be used as a solid foundation for further studies of genetic markers associated with response to anti-TNF therapy.

The clotted blood obtained after Mycobacterium tuberculosis screening often result in low DNA yields. We therefore compared the efficiency of four commercial kits for extracting DNA and found that high quality and quantity DNA can be extracted using the Maxwell 16 Blood purification kit (Promega).

We studied 39 mainly functional polymorphisms and identified 19 polymorphisms associated with response to anti-TNF treatment. Our results suggest that genes involved in the regulation of NFκB through the TLR pathways, genes regulating TNF-α signaling and cytokines regulated by NFκB are important predictors for the response to anti-TNF therapy among patients with IBD. Genetically strong TNF-mediated inflammatory response was associated with beneficial response to anti-TNF-therapy. In addition, patients with genetically determined high IL-1β, IL-6 or IFN-γ levels were less likely to respond, perhaps because the colonic inflammation was primarily driven by these pro-inflammatory cytokines. This could indicate that the cytokines IL-1β, IL-6 and

IFN-γ may be potential targets for treating patients with IBD who do not respond to anti-TNF therapy.

Thus, the aim of using the result in basic science to get a better biological understanding of the mechanisms involved in response have been met, although the results await replication. The aim of using the associated polymorphisms in applied science to create a predictive model of response among patients with CD or UC has not yet been achieved. A predictive model would be much stronger if it is validated in an independent cohort.

PERSPECTIVE

We plan to continue our search for genetic markers which can be used to predict the response of anti-TNF therapy, in the cohort we have established. Twenty new functional SNPs in the inflammasome pathway have been genotyped in the IBD cohort and are beeing analyzed. The inflammasome is a multiprotein complex which is responsible for activation of inflammatory processes among other by activating IL-1β and by inducing IFN-γ secretion.

In addition, we have received funding to study 16 functional SNPs in the apoptosis pathway. Finally, a hypothesis free GWAS study of the anti-TNF treated IBD cohort is in progress in collaboration with professor Andre Franke, Institut für Klinische Molekularbiologie, Kiel, Germany.

In a follow-up study we are establishing a replication cohort to confirm our results.

ABSTRACT

Introduction: Anti-tumor necrosis factor-α (TNF-α) is used for treatment of severe cases of inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC). However, one-third of the patients do not respond to the treatment. Genetic markers may predict individual response to anti-TNF therapy.

Methods: Blood sent for Mycobacterium tuberculosis screening was collected from 01.09.2009 to 30.03.2011. Patients with CD or UC (K50-K51) were identified by linking CPR-numbers from the blood samples to the National Patient Registry. Treatment efficacy reflected the maximum response within 22 weeks. DNA was purified by Maxwell 16 Blood purification kit (Promega) and 39 mainly functional single nucleotide polymorphisms (SNPs) were genotyping by LGC Genomics.

Results: A cohort of 492 patients with CD and 267 patients with UC treated with anti-TNF was established. Nineteen SNPs were associated with response.

Conclusion: The results suggest that polymorphisms in genes involved in activating NFκB through the TLR pathways, genes regulating TNF-α signaling and cytokines regulated by NFκB are important predictors for the response to anti-TNF therapy among patients with IBD. Genetically strong TNF-mediated inflammatory response was associated with beneficial response. In addition, the cytokines IL-1β, IL-6 and IFN-γ may be potential targets for treating patients with IBD who do not respond to anti-TNF therapy. These finding should be examined in independent cohorts before these results are applied in a clinical setting.

REFERENCES

1. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417-29.
2. Vind I, Riis L, Jess T et al. Increasing incidences of inflammatory bowel disease and decreasing surgery rates in Copenhagen City and County, 2003- 2005: a population-based study from the Danish Crohn colitis database. Am J Gastroenterol 2006;101:1274-82.
3. Jacobsen BA, Fallingborg J, Rasmussen HH et al. Increase in incidence and prevalence of inflammatory bowel disease in northern Denmark: a population based study, 1978-2002. Eur J Gastroenterol Hepatol 2006;18:601-6.
4. Van AG, Dignass A, Panes J et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Definitions and diagnosis. J Crohns Colitis 2010;4:7-27.
5. Dignass A, Eliakim R, Magro F et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. J Crohns Colitis 2012;6:965-90.
6. Satsangi J, Silverberg MS, Vermeire S et al. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006;55:749-53.
7. Silverberg MS, Satsangi J, Ahmad T et al. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. Can J Gastroenterol 2005;19:5-36.
8. Halfvarson J, Bodin L, Tysk C et al. Inflammatory bowel disease in a Swedish twin cohort: a long-term follow-up of concordance and clinical characteristics. Gastroenterology 2003;124:1767-73.
9. Jess T, Riis L, Jespersgaard C et al. Disease concordance, zygosity, and NOD2/CARD15 status: follow-up of a population-based cohort of Danish twins with inflammatory bowel disease. Am J Gastroenterol 2005;100:2486-92.
10. Orholm M, Binder V, Sorensen TI et al. Concordance of inflammatory bowel disease among Danish twins. Results of a nationwide study. Scand J Gastroenterol 2000;35:1075-81.
11. Spehlmann ME, Begun AZ, Burghardt J et al. Epidemiology of inflammatory bowel disease in a German twin cohort: results of a nationwide study. Inflamm Bowel Dis 2008;14:968-76.
12. Halme L, Paavola-Sakki P, Turunen U et al. Family and twin studies in inflammatory bowel disease. World J Gastroenterol 2006;12:3668-72.
13. Mahid SS, Minor KS, Soto RE et al. Smoking and inflammatory bowel disease: a meta-analysis. Mayo Clin Proc 2006;81:1462-71.
14. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol 2007;8:49-62.
15. Pallone F, Monteleone G. Regulatory cytokines in inflammatory bowel disease. Aliment Pharmacol Ther 1996;10:75-9.
16. Romagnani S. Th1/Th2 cells. Inflamm Bowel Dis 1999;5:285-94.
17. Inoue S, Matsumoto T, Iida M et al. Characterization of cytokine expression in the rectal mucosa of ulcerative colitis: correlation with disease activity. Am J Gastroenterol 199;94:2441-6.
18. Verstrepen L, Bekaert T, Chau TL et al. TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. Cell Mol Life Sci 2008;65:2964-78.
19. http://www.bu.edu/nf-kb/gene-resources/target-genes/
20. Kriegler M, Perez C, DeFay K et al. A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF. Cell 1988;53:45-53.

21. Tang P, Hung M-C, Klostergaard J. Human pro-tumor necrosis factor is a homotrimer. Biochemistry 1996;35:8216-25.
22. Black RA, Rauch CT, Kozlosky CJ et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 1997;385:729-33.
23. Nalejska E, Maczynska E, Lewandowska MA. Prognostic and Predictive Biomarkers: Tools in Personalized Oncology. Mol Diagn Ther 2014;18:273-84.
24. Aftimos PG, Barthelemy P, Awada A. Molecular biology in medical oncology: diagnosis, prognosis, and precision medicine. Discov Med 2014;17:81-91.
25. de Silva DG, Mendis LN, Sheron N et al. TNF alpha in stool as marker of intestinal inflammation. Lancet 1992;340:372.
26. Murch SH, Lamkin VA, Savage MO et al. Serum concentrations of tumour necrosis factor alpha in childhood chronic inflammatory bowel disease. Gut 1991;32:913-7.
27. Murch SH, Braegger CP, Walker-Smith JA et al. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. Gut 1993;34:1705-9.
28. Reinecker HC, Steffen M, Witthoeft T et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993;94:174-81.
29. D'Haens G. Infliximab (Remicade): the magic bullet for Crohn's disease? Dig Liver Dis 2000;32:653-6.
30. Knight DM, Trinh H, Le J et al. Construction and initial characterization of a mouse-human chimeric anti-TNF antibody. Mol Immunol 1993;30:1443-53.
31. Siegel SA, Shealy DJ, Nakada MT et al. The mouse/human chimeric monoclonal antibody cA2 neutralizes TNF in vitro and protects transgenic mice from cachexia and TNF lethality in vivo. Cytokine 1995;7:15-25.
32. Scallon BJ, Moore MA, Trinh H et al. Chimeric anti-TNF-alpha monoclonal antibody cA2 binds recombinant transmembrane TNF-alpha and activates immune effector functions. Cytokine 1995;7:251-9.
33. ten HT, van MC, Peppelenbosch MP et al. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. Gut 2002;50:206-11.
34. D'Haens G, Van DS, Van HR et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. Gastroenterology 1999;116:1029-34.
35. Baert FJ, D'Haens GR, Peeters M et al. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. Gastroenterology 1999;116:22-8.
36. Targan SR, Hanauer SB, van Deventer SJ et al. A short-term study of chimeric monoclonal antibody cA2 to tumor necrosis factor alpha for Crohn's disease. Crohn's Disease cA2 Study Group. N Engl J Med 1997;337:1029-35.
37. Gisbert JP, Gonzalez-Lama Y, Mate J. Systematic review: Infliximab therapy in ulcerative colitis. Aliment Pharmacol Ther 2007;25:19-37.
38. Present DH, Rutgeerts P, Targan S et al. Infliximab for the treatment of fistulas in patients with Crohn's disease. N Engl J Med 1999;340:1398-405.
39. Bruce E, Sands MD, Frank H et al. Infliximab maintenance therapy for fistulizing crohn's disease. The New England Journal of Medicine 2004;350:876-85.
40. Hanauer SB, Feagan BG, Lichtenstein GR et al. Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. Lancet 2002;359:1541-9.
41. Sands BE, Blank MA, Patel K et al. Long-term treatment of rectovaginal fistulas in Crohn's disease: response to infliximab in the ACCENT II Study. Clin Gastroenterol Hepatol 2004;10:912-20.
42. Dubinsky MC, Mei L, Friedman M et al. Genome wide association (GWA) predictors of anti-TNFalpha therapeutic responsiveness in pediatric inflammatory bowel disease. Inflamm Bowel Dis 2010;16:1357-66.
43. Dideberg V, Theatre E, Farnir F et al. The TNF/ADAM 17 system: implication of an ADAM 17 haplotype in the clinical response to infliximab in Crohn's disease. Pharmacogenet Genomics 2006;16:727-34.
44. Louis E, Vermeire S, Rutgeerts P et al. A positive response to infliximab in Crohn disease: association with a higher systemic inflammation before treatment but not with -308 TNF gene polymorphism. Scand J Gastroenterol 2002;7:818-24.
45. Papamichael K, Gazouli M, Karakoidas C et al. Association of TNF and FcgammaRIIIA gene polymorphisms with differential response to infliximab in a Greek cohort of Crohn's disease patients. Annals of Gastroenterology 2011;24:35-40.
46. Rutgeerts P, Sandborn WJ, Feagan BG et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 2005;353:2462-76.
47. Colombel JF, Sandborn WJ, Rutgeerts P et al. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. Gastroenterology 2007;132:52-65.
48. Hlavaty T, Pierik M, Henckaerts L et al. Polymorphisms in apoptosis genes predict response to infliximab therapy in luminal and fistulizing Crohn's disease. Aliment Pharmacol Ther 2005;22:613-26.
49. Louis E, El GZ, Vermeire S et al. Association between polymorphism in IgG Fc receptor IIIa coding gene and biological response to infliximab in Crohn's disease. Aliment Pharmacol Ther 2004;19:511-9.
50. Pierik M, Vermeire S, Steen KV et al. Tumour necrosis factor-alpha receptor 1 and 2 polymorphisms in inflammatory bowel disease and their association with response to infliximab. Aliment Pharmacol Ther 2004;20:303-10.
51. Sostegni R, Daperno M, Scaglione N et al. Review article: Crohn's disease: monitoring disease activity. Aliment Pharmacol Ther 2003;17:11-7.
52. Seiderer J, Brand S, Dambacher J et al. Adalimumab in patients with Crohn's disease--safety and efficacy in an open-label single centre study. Aliment Pharmacol Ther 2007;25:787-96.
53. Niess JH, Klaus J, Stephani J et al. NOD2 polymorphism predicts response to treatment in Crohn's disease--first steps to a personalized therapy. Dig Dis Sci 2012;57:879-86.
54. Caspersen S, Elkjaer M, Riis L et al. Infliximab for inflammatory bowel disease in Denmark 1999-2005: clinical outcome and follow-up evaluation of malignancy and mortality. Clin Gastroenterol Hepatol 2008;6:1212-7.
55. Ljung T, Karlen P, Schmidt D et al. Infliximab in inflammatory bowel disease: clinical outcome in a population based cohort from Stockholm County. Gut 2004;53:849-53.
56. Cohen RD, Tsang JF, Hanauer SB. Infliximab in Crohn's disease: first anniversary clinical experience. Am J Gastroenterol 2000;95:3469-77.

57. Keane J, Gershon S, Wise RP et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 2001;345:1098-104.
58. Schreiber S, Reinisch W, Colombel JF et al. Subgroup analysis of the placebo-controlled CHARM trial: increased remission rates through 3 years for adalimumab-treated patients with early Crohn's disease. J Crohns Colitis 2013;7:213-21.
59. Lionetti P, Bronzini F, Salvestrini C et al. Response to infliximab is related to disease duration in paediatric Crohn's disease. Aliment Pharmacol Ther 2003;18:425-31.
60. Hyams J, Crandall W, Kugathasan S et al. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. Gastroenterology 2007;132:863-73.
61. Vermeire S, Louis E, Carbonez A et al. Demographic and clinical parameters influencing the short-term outcome of anti-tumor necrosis factor (infliximab) treatment in Crohn's disease. Am J Gastroenterol 2002;97:2357-63.
62. Ferrante M, Vermeire S, Katsanos KH et al. Predictors of early response to infliximab in patients with ulcerative colitis. Inflamm Bowel Dis 2007;13:123-8.
63. Siegel CA, Melmed GY. Predicting response to Anti-TNF Agents for the treatment of crohn's disease. Therap Adv Gastroenterol 2009;2:245-51.
64. Parsi MA, Achkar JP, Richardson S et al. Predictors of response to infliximab in patients with Crohn's disease. Gastroenterology 2002;123:707-13.
65. Arnott ID, McNeill G, Satsangi J. An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease. Aliment Pharmacol Ther 2003;17:1451-7.
66. Fefferman DS, Lodhavia PJ, Alsahli M et al. Smoking and immunomodulators do not influence the response or duration of response to infliximab in Crohn's disease. Inflamm Bowel Dis 2004;10:346-51.
67. Weinberg A.M Rattan S, Lewis JD et al. Strictures and response to infliximab in Crohn's disease. The American journal of gastroenterology 2002;97:255.
68. Orlando A, Colombo E, Kohn A et al. Infliximab in the treatment of Crohn's disease: predictors of response in an Italian multicentric open study. Dig Liver Dis 2005;37:577-83.
69. Barreiro-de AM, Ouburg S, Morre SA et al. NOD2, CD14 and TLR4 mutations do not influence response to adalimumab in patients with Crohn's disease: a preliminary report. Rev Esp Enferm Dig 2010;102:591-5.
70. Louis EJ, Watier HE, Schreiber S et al. Polymorphism in IgG Fc receptor gene FCGR3A and response to infliximab in Crohn's disease: a subanalysis of the ACCENT I study. Pharmacogenet Genomics 2006;16:911-4.
71. Vermeire S, Louis E, Rutgeerts P et al. NOD2/CARD15 does not influence response to infliximab in Crohn's disease. Gastroenterology 2002;123:106-11.
72. Colombel JF, Sandborn WJ, Reinisch W et al. Infliximab, Azathioprine, or Combination Therapy for Crohn's Disease. The New England Journal of Medicine 2010;362:1383-95.
73. Willot S, Vermeire S, Ohresser M et al. No association between C-reactive protein gene polymorphisms and decrease of C-reactive protein serum concentration after infliximab treatment in Crohn's disease. Pharmacogenet Genomics 2006;16:37-42.
74. Marotte H, Miossec P. Biomarkers for prediction of TNFalpha blockers response in rheumatoid arthritis. Joint Bone Spine 2010;77:297-305.
75. Roozendaal C, Kallenberg CG. Are anti-neutrophil cytoplasmic antibodies (ANCA) clinically useful in inflammatory bowel disease (IBD)? Clin Exp Immunol 1999;116:206-13.
76. Taylor KD, Plevy SE, Yang H et al. ANCA pattern and LTA haplotype relationship to clinical responses to anti-TNF antibody treatment in Crohn's disease. Gastroenterology 2001;120:1347-55.
77. Esters N, Vermeire S, Joossens S et al. Serological markers for prediction of response to anti-tumor necrosis factor treatment in Crohn's disease. Am J Gastroenterol 2002;97:1458-62.
78. Lacruz-Guzman D, Torres-Moreno D, Pedrero F et al. Influence of polymorphisms and TNF and IL1beta serum concentration on the infliximab response in Crohn's disease and ulcerative colitis. Eur J Clin Pharmacol 2013;69:431-8.
79. Martinez-Borra J, Lopez-Larrea C, Gonzalez S et al. High serum tumor necrosis factor-alpha levels are associated with lack of response to infliximab in fistulizing Crohn's disease. Am J Gastroenterol 2002;97:2350-6.
80. Louis E, Vermeire S, Rutgeerts P et al. A positive response to infliximab in Crohn disease: association with a higher systemic inflammation before treatment but not with -308 TNF gene polymorphism. Scand J Gastroenterol 2002;37:818-24.
81. Marotte H, Maslinski W, Miossec P. Circulating tumour necrosis factor-alpha bioactivity in rheumatoid arthritis patients treated with infliximab: link to clinical response. Arthritis Res Ther 2005;7:R149-R155.
82. Marotte H, Arnaud B, Diasparra J et al. Association between the level of circulating bioactive tumor necrosis factor alpha and the tumor necrosis factor alpha gene polymorphism at -308 in patients with rheumatoid arthritis treated with a tumor necrosis factor alpha inhibitor. Arthritis Rheum 2008;58:1258-63.
83. Arijs I, Li K, Toedter G et al. Mucosal gene signatures to predict response to infliximab in patients with ulcerative colitis. Gut 2009;58:1612-9.
84. Ng PC, Levy S, Huang J et al. Genetic variation in an individual human exome. PLoS Genet 2008;4:e1000160.
85. Palmieri O, Latiano A, Valvano R et al. Multidrug resistance 1 gene polymorphisms are not associated with inflammatory bowel disease and response to therapy in Italian patients. Aliment Pharmacol Ther 2005;22:1129-38.
86. Matsukura H, Ikeda S, Yoshimura N et al. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1A and 1B affect responses to infliximab in Japanese patients with Crohn's disease. Aliment Pharmacol Ther 2008;27:765-70.
87. Mascheretti S, Hampe J, Kuhbacher T et al. Pharmacogenetic investigation of the TNF/TNF-receptor system in patients with chronic active Crohn's disease treated with infliximab. Pharmacogenomics J 2002;2:127-36.
88. Steenholdt C, Enevold C, Ainsworth MA et al. Genetic polymorphisms of tumour necrosis factor receptor superfamily 1b and fas ligand are associated with clinical efficacy and/or acute severe infusion reactions to infliximab in Crohn's disease. Aliment Pharmacol Ther 2012;36:650-9.
89. Moroi R, Endo K, Kinouchi Y et al. FCGR3A-158 polymorphism influences the biological response to infliximab in Crohn's disease through affecting the ADCC activity. Immunogenetics 2013;65:265-71.
90. Ozeki T, Furuya Y, Nagano C et al. Analysis of linkage between lymphotoxin alpha haplotype and polymorphisms in 5'-flanking region of tumor necrosis factor alpha gene asso-

ciated with efficacy of infliximab for Crohn's disease patients. Mutat Res 2006;602:170-4.
91. Tomita K, Chiba T, Sugai T et al. Association between tumor necrosis factor-alpha and Fc-gamma receptor polymorphisms with infliximab in Crohn's disease. Hepatogastroenterology 2010;57:535-9.
92. Papp M, Altorjay I, Dotan N et al. New serological markers for inflammatory bowel disease are associated with earlier age at onset, complicated disease behavior, risk for surgery, and NOD2/CARD15 genotype in a Hungarian IBD cohort. Am J Gastroenterol 2008;103:665-81.
93. Lu C, Waugh A, Bailey RJ et al. Crohn's disease genotypes of patients in remission vs relapses after infliximab discontinuation. World J Gastroenterol 2012;18:5058-64.
94. Urcelay E, Mendoza JL, Martinez A et al. IBD5 polymorphisms in inflammatory bowel disease: association with response to infliximab. World J Gastroenterol 2005;11:1187-92.
95. Mascheretti S, Hampe J, Croucher PJ et al. Response to infliximab treatment in Crohn's disease is not associated with mutations in the CARD15 (NOD2) gene: an analysis in 534 patients from two multicenter, prospective GCP-level trials. Pharmacogenetics 2002;12:509-15.
96. Csillag C, Borup R, Olsen J et al. Treatment response and colonic gene expression in patients with Crohn's disease. Scand J Gastroenterol 2007;42:834-40.
97. Dignass A, Van AG, Lindsay JO et al. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. J Crohns Colitis 2010;4:28-62.
98. Dignass A, Lindsay JO, Sturm A et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: current management. J Crohns Colitis 2012;6:991-1030.
99. http://pngu.mgh.harvard.edu/~purcell/gpc/
100. Gast A, Bermejo JL, Claus R et al. Association of inherited variation in Toll-like receptor genes with malignant melanoma susceptibility and survival. PLoS One 2011;6:e24370.
101. Bielinski SJ, Hall JL, Pankow JS et al. Genetic variants in TLR2 and TLR4 are associated with markers of monocyte activation: the Atherosclerosis Risk in Communities MRI Study. Hum Genet 2011;129:655-62.
102. Zhang F, Gao XD, Wu WW et al. Polymorphisms in toll-like receptors 2, 4 and 5 are associated with Legionella pneumophila infection. Infection 2013;41:941-8.
103. Hawn TR, Verbon A, Lettinga KD et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. J Exp Med 2003;198:1563-72.
104. Tao K, Fujii M, Tsukumo S et al. Genetic variations of Toll-like receptor 9 predispose to systemic lupus erythematosus in Japanese population. Ann Rheum Dis 2007;66:905-9.
105. Gu W, Shan YA, Zhou J et al. Functional significance of gene polymorphisms in the promoter of myeloid differentiation-2. Ann Surg 2007;246:151-8.
106. Baldini M, Lohman IC, Halonen M et al. A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am J Respir Cell Mol Biol 1999;20:976-83.
107. Mertens J, Bregadze R, Mansur A et al. Functional impact of endotoxin receptor CD14 polymorphisms on transcriptional activity. J Mol Med (Berl) 2009;87:815-24.
108. Thair SA, Walley KR, Nakada TA et al. A single nucleotide polymorphism in NF-kappaB inducing kinase is associated with mortality in septic shock. J Immunol 2011;186:2321-8.
109. Guo D, Li M, Zhang Y et al. A functional variant of SUMO4, a new I kappa B alpha modifier, is associated with type 1 diabetes. Nat Genet 2004;36:837-41.
110. Song S, Chen D, Lu J et al. NFkappaB1 and NFkappaBIA polymorphisms are associated with increased risk for sporadic colorectal cancer in a southern Chinese population. PLoS One 2011;6:e21726.
111. Wang S, Zhang M, Zeng Z et al. IkappaBalpha polymorphisms were associated with increased risk of gastric cancer in a southern Chinese population: a case-control study. Life Sci 2011;88:792-7.
112. Park JY, Farrance IK, Fenty NM et al. NFKB1 promoter variation implicates shear-induced NOS3 gene expression and endothelial function in prehypertensives and stage I hypertensives. Am J Physiol Heart Circ Physiol 2007;293:H2320-H2327.
113. Udalova IA, Richardson A, Denys A et al. Functional consequences of a polymorphism affecting NF-kappaB p50-p50 binding to the TNF promoter region. Mol Cell Biol 2000;20:9113-9.
114. Lv K, Chen R, Cai Q et al. Effects of a single nucleotide polymorphism on the expression of human tumor necrosis factor-alpha. Scand J Immunol 2006;64:164-9.
115. Karimi M, Goldie LC, Cruickshank MN et al. A critical assessment of the factors affecting reporter gene assays for promoter SNP function: a reassessment of -308 TNF polymorphism function using a novel integrated reporter system. Eur J Hum Genet 2009;17:1454-62.
116. Kaluza W, Reuss E, Grossmann S et al. Different transcriptional activity and in vitro TNF-alpha production in psoriasis patients carrying the TNF-alpha 238A promoter polymorphism. J Invest Dermatol 2000;114:1180-3.
117. Wang GB, Li CR, Yang J et al. A regulatory polymorphism in promoter region of TNFR1 gene is associated with Kawasaki disease in Chinese individuals. Hum Immunol 2011;72:451-7.
118. Elsby LM, Orozco G, Denton J et al. Functional evaluation of TNFAIP3 (A20) in rheumatoid arthritis. Clin Exp Rheumatol 2010;28:708-14.
119. Yoshida M, Shiroiwa K, Mouri K et al. Haplotypes in the expression quantitative trait locus of interleukin-1beta gene are associated with schizophrenia. Schizophr Res 2012;140:185-91.
120. Chen H, Wilkins LM, Aziz N et al. Single nucleotide polymorphisms in the human interleukin-1B gene affect transcription according to haplotype context. Hum Mol Genet 2006;15:519-29.
121. Wen AQ, Gu W, Wang J et al. Clinical relevance of IL-1beta promoter polymorphisms (-1470, -511, and -31) in patients with major trauma. Shock 2010;33:576-82.
122. Lind H, Haugen A, Zienolddiny S. Differential binding of proteins to the IL1B -31 T/C polymorphism in lung epithelial cells. Cytokine 2007;38:43-8.
123. Rafiq S, Stevens K, Hurst AJ et al. Common genetic variation in the gene encoding interleukin-1-receptor antagonist (IL-1RA) is associated with altered circulating IL-1RA levels. Genes Immun 2007;8:344-51.
124. Carrol ED, Payton A, Payne D et al. The IL1RN promoter rs4251961 correlates with IL-1 receptor antagonist concen-

trations in human infection and is differentially regulated by GATA-1. J Immunol 2011;186:2329-35.
125. Wallis SK, Cooney LA, Endres JL et al. A polymorphism in the interleukin-4 receptor affects the ability of interleukin-4 to regulate Th17 cells: a possible immunoregulatory mechanism for genetic control of the severity of rheumatoid arthritis. Arthritis Res Ther 2011;13:R15.
126. Smith AJ, D'Aiuto F, Palmen J et al. Association of serum interleukin-6 concentration with a functional IL6 -6331T>C polymorphism. Clin Chem 2008;54:841-50.
127. Rafiq S, Frayling TM, Murray A et al. A common variant of the interleukin 6 receptor (IL-6r) gene increases IL-6r and IL-6 levels, without other inflammatory effects. Genes Immun 2007;8:552-9.
128. Rees LE, Wood NA, Gillespie KM et al. The interleukin-10-1082 G/A polymorphism: allele frequency in different populations and functional significance. Cell Mol Life Sci 2002;59:560-9.
129. Andersen V, Ernst A, Christensen J et al. The polymorphism rs3024505 proximal to IL-10 is associated with risk of ulcerative colitis and Crohns disease in a Danish case-control study. BMC Med Genet 2010;11:82.
130. Espinoza JL, Takami A, Nakata K et al. A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. PLoS One 2011;6:e26229.
131. Oosting M, ter HH, van de Veerdonk FL et al. Role of interleukin-23 (IL-23) receptor signaling for IL-17 responses in human Lyme disease. Infect Immun 2011;79:4681-7.
132. Pravica V, Perrey C, Stevens A et al. A single nucleotide polymorphism in the first intron of the human IFN-gamma gene: absolute correlation with a polymorphic CA microsatellite marker of high IFN-gamma production. Hum Immunol 2000;61:863-6.
133. Shah R, Hurley CK, Posch PE. A molecular mechanism for the differential regulation of TGF-beta1 expression due to the common SNP -509C-T (c. -1347C > T). Hum Genet 2006;120:461-9.
134. Kariuki SN, Crow MK, Niewold TB. The PTPN22 C1858T polymorphism is associated with skewing of cytokine profiles toward high interferon-alpha activity and low tumor necrosis factor alpha levels in patients with lupus. Arthritis Rheum 2008;58:2818-23.
135. Aoyagi Y, Nagata S, Kudo T et al. Peroxisome proliferator-activated receptor gamma 2 mutation may cause a subset of ulcerative colitis. Pediatr Int 2010;52:729-34.
136. Hitomi Y, Ebisawa M, Tomikawa M et al. Associations of functional NLRP3 polymorphisms with susceptibility to food-induced anaphylaxis and aspirin-induced asthma. J Allergy Clin Immunol 2009;124:779-85.
137. Rutgeerts P, Sandborn WJ, Feagan BG et al. Infliximab for induction and maintenance therapy for ulcerative colitis. N Engl J Med 2005;353:2462-76.
138. Cohen RD, Tsang JF, Hanauer SB. Infliximab in Crohn's disease: first anniversary clinical experience. Am J Gastroenterol 2000;12:3469-77.
139. Danese S, Colombel JF, Peyrin-Biroulet L et al. Review article: the role of anti-TNF in the management of ulcerative colitis -- past, present and future. Aliment Pharmacol Ther 2013;37:855-66.
140. Jawaheer D, Olsen J, Hetland ML. Sex differences in response to anti-tumor necrosis factor therapy in early and established rheumatoid arthritis -- results from the DANBIO registry. J Rheumatol 2012;39:46-53.
141. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine. EPMA J 2013;4:7.
142. Mayeux R. Biomarkers: potential uses and limitations. NeuroRx 2004;1:182-8.
143. Arnott ID, McNeill G, Satsangi J. An analysis of factors influencing short-term and sustained response to infliximab treatment for Crohn's disease. Aliment Pharmacol Ther 2003;17:1451-7.
144. Salazar LA, Hirata MH, Cavalli SA et al. Optimized procedure for DNA isolation from fresh and cryopreserved clotted human blood useful in clinical molecular testing. Clin Chem 1998;44:1748-50.
145. Adkins KK, Strom DA, Jacobson TE et al. Utilizing genomic DNA purified from clotted blood samples for single nucleotide polymorphism genotyping. Arch Pathol Lab Med 2002;126:266-70.
146. Iovannisci DM, Ha TT, Shaw GM. Recovery of genomic DNA from residual frozen archival blood clots suitable for amplification and use in genotyping assays. Genet Test 2006;10:44-9.
147. Garg UC, Hanson NQ, Tsai MY et al. Simple and rapid method for extraction of DNA from fresh and cryopreserved clotted human blood. Clin Chem 1996;42:647-8.
148. Omar AH, Yasunami M, Yamazaki A et al. Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study. Malar J 2012;11:168.
149. Knight JC, Keating BJ, Kwiatkowski DP. Allele-specific repression of lymphotoxin-alpha by activated B cell factor-1. Nat Genet 2004;36:394-9.
150. Migita O, Noguchi E, Koga M et al. Haplotype analysis of a 100 kb region spanning TNF-LTA identifies a polymorphism in the LTA promoter region that is associated with atopic asthma susceptibility in Japan. Clin Exp Allergy 2005;35:790-6.
151. Tan W, Wu H, Zhao J et al. A functional RANKL polymorphism associated with younger age at onset of rheumatoid arthritis. Arthritis Rheum 2010;62:2864-75.