Crohn's disease
In the field of observation, chance favors the prepared mind
Louis Pasteur, 1854

To Olena Vasylenko
Yaroslava Zhulina

Crohn's disease
aspects of epidemiology, clinical course, and faecal calprotectin
Abstract


The overall aim of this thesis was to study epidemiological and clinical changes in the natural history of Crohn’s disease, its phenotype, the need for surgery and pharmacological therapy over time, as well as the role of faecal calprotectin as a biomarker of pathophysiology and disease course.

An increased incidence and prevalence of Crohn’s disease was seen in the period 1963-2010. The proportion of patients with non-stricturing, non-penetrating disease behaviour at diagnosis increased, suggesting that either patients with Crohn’s disease are diagnosed earlier in their disease course today or that the Crohn’s disease phenotype is changing.

A decrease in complicated disease behaviour, an increased use of immunomodulators, and a reduced frequency of surgical procedures five years after Crohn’s diagnosis was observed. The decrease in surgery at five years seemed to be explained mainly by a decrease in early surgery within three months from diagnosis, likely reflecting an increased proportion of patients with non-stricturing, non-penetrating disease. This suggests that the introduction of new treatment alternatives alone does not explain the reduction in surgery rates, and an increasing proportion of patients with uncomplicated disease at diagnosis may also play an important role.

Subclinical mucosal inflammation, mirrored by increased NFkB activity and increased neutrophil activity (i.e. FC and MPO expression), was observed in healthy twin siblings in both discordant monozygotic and discordant dizygotic twin pairs with IBD. These findings strongly support the hypothesis of an ongoing subclinical mucosal inflammation at the molecular level in healthy first-degree relatives of IBD patients.

Baseline FC as well as consecutive FC measurements predict relapse in IBD. The doubling of FC value increased the risk of relapse by 101% in the following three months. This increased risk attenuates with time by 20% for every three month period since the sample was obtained.

Keywords: Crohn’s disease, epidemiology, faecal calprotectin.

Yaroslava Zhulina, School of Health and Medical Sciences Örebro University, SE-70182, Sweden,
yaroslava.zhulina@regionorebrolan.se
# Table of Contents

LIST OF PUBLICATIONS .................................................................................................................. 9

ABBREVIATIONS ............................................................................................................................ 10

INTRODUCTION ............................................................................................................................ 11

Historical remarks .......................................................................................................................... 11

Epidemiology .................................................................................................................................. 11

  Incidence ..................................................................................................................................... 11

  Prevalence ................................................................................................................................... 12

  Patient characteristics .................................................................................................................. 12

  Clinical symptoms and disease course ....................................................................................... 13

  Risk of surgery ............................................................................................................................ 13

Etiology and pathogenesis .............................................................................................................. 14

  Genetics ...................................................................................................................................... 14

  Lifestyle and environmental factors ............................................................................................ 16

  Infection ..................................................................................................................................... 17

  Microflora- microbiome ............................................................................................................... 17

  Immunity ................................................................................................................................... 19

  Intestinal permeability ............................................................................................................... 19

Definitions and diagnosis .............................................................................................................. 19

  Diagnostic criteria ....................................................................................................................... 20

  Disease classification .................................................................................................................. 23

Treatment ........................................................................................................................................ 24

  Medical treatment ...................................................................................................................... 24

  Surgical treatment ...................................................................................................................... 24

Disease monitoring ........................................................................................................................ 26

  Disease activity scores ............................................................................................................... 26

  Mucosal healing ......................................................................................................................... 26

  Disease markers ........................................................................................................................ 27

  *Calprotectin* ............................................................................................................................ 28

  *MPO* ...................................................................................................................................... 30

  *NFκB* ..................................................................................................................................... 30

AIMS ................................................................................................................................................ 32

ETHICS .......................................................................................................................................... 32

MATERIAL AND METHODS ......................................................................................................... 33

Paper I and II ................................................................................................................................. 33
Strengths and limitations ................................................................. 64
GENERAL CONCLUSION ................................................................. 68
FUTURE PERSPECTIVES ................................................................. 69
ACKNOWLEDGEMENTS ................................................................. 70
POPULÄRVETENSKAPLIG SAMMANFATTNING .............................. 72
REFERENCES ..................................................................................... 73
List of publications

This thesis is based on the following studies, referred to in the text by their roman numerals.


Published papers have been reprinted with permission from the publisher.
Abbreviations

5ASA  5-amino salicylic acid
ASCA  Anti-Saccharomyces Cerevisiae Antibody
CD   Crohn’s disease
CDAI Crohn’s Disease Activity Index
CI   confidence interval
DNA  deoxyribonucleic acid
DZ   dizygotic
ELISA enzyme linked immunosorbent assay
EEA  equal environments assumption
GTP  guanosine triphosphate
GI   gastrointestinal
HCD  healthy twin to twin with Crohn’s disease
HUC  healthy twin to twin with ulcerative colitis
IBD inflammatory bowel disease
IBD-U inflammatory bowel disease unclassified
IL23R interleukin-23 receptor
IRGM immunity-related GTPase family M protein
IkB inhibitor of kappa B
IBSEN Inflammatory Bowel South-Eastern Norway cohort
ICD International Statistical Classification of Diseases and Related Health Problems
ICURE The Inflammatory Bowel Disease Cohort of the Uppsala Region
IR   incidence rate
IQR  interquartile range
MRP8 migration inhibitory factor-related protein 8
MRP14 migration inhibitory factor-related protein 14
MC   microscopic colitis
MZ   monozygotic
NOD2 nucleotide-binding oligomerization domain-containing protein 2
NSAID non-steroidal anti-inflammatory drugs
NFKB nuclear factor kappa B
PPD purified protein derivative
RAGE receptor for advanced glycation end-products
TNFα tumor necrosis factor alpha
UC   ulcerative colitis
Introduction

Crohn’s disease (CD), one of the major entities in the spectrum of inflammatory bowel diseases (IBD), is characterised by chronic inflammation of the digestive tract, causing abdominal pain and diarrhoea. The disease commonly affects young people and continues for all patients throughout their lives, causing impaired health and often a markedly reduced quality of life.

Historical remarks

Crohn’s disease is not a new disorder. Descriptions of a non-contagious, chronic, remitting and recurring, diarrhoeal disease with abdominal pain can be traced back to the sixteenth century.1–2 In the early twentieth century, several reports described distal ileum disease with inflammation and non-specific granuloma.3 The advent of purified protein derivative (PPD) later allowed Dr. Burrill Crohn to diagnose Crohn’s disease by distinguishing it from intestinal tuberculosis and chronic appendicitis.4,5

Epidemiology

By definition, epidemiology is the study of the occurrence and distribution of health-related events, states, and processes in specified populations, including the study of the determinants influencing such processes, and the application of this knowledge to control relevant health problems.6 There have been some important milestones along the way to the current understanding of IBD epidemiology. The importance of diagnostically well-defined and unselected patient cohorts was recognised during the 1960s.7 An international consensus on diagnostic criteria for CD and ulcerative colitis (UC) made possible the comparison and generalisation of epidemiological data on patients with IBD from different countries.8

The majority of epidemiological studies are derived from Western countries. Because these countries have the highest incidences, identification of populations of adequate size for such studies is easiest. Countries with free access to health care have an additional advantage in identifying all variants of the disease, including patients with milder symptoms.

Incidence

Incidence is the occurrence of new cases and is defined as the number of new cases divided by the population-at-risk over time. For CD, this is typically expressed as the number of new cases per year per 100 000
population. The incidence of CD varies greatly around the world with the highest figures in Northern Europe\cite{9} and North America.\cite{10,11}

Over time, incidences generally have increased in Western countries from less than 1 per 100 000 in the 1960s to 8-9 per 100 000 in 2010s.\cite{12,13}. This trend has accompanied urbanization and increases in the standard of living in these societies.\cite{14} The exact mechanisms involved in this correlation are still unclear, but it is apparent that countries in transition from developing to a Westernised lifestyle experience a sharp increase in CD incidences.\cite{15-17} More precise and accessible diagnostic methods, as well as IBD awareness, could have contributed to such a development, but these factors cannot account entirely for this trend.

The ratios of the incidence of UC to the incidence of CD (UC:CD) varies depending on the geographical region studied. In Scandinavia, the ratio is usually 2:1.\cite{18} France, Canada and New Zealand report ratios in the range 1.5:1 to 2.2:1.\cite{19-21} Given that the cause of CD and that of UC are still unknown, environmental factors together with different diagnostic traditions may contribute to this variance.

The geographical north–south gradient in CD incidence was first described in the literature in 1996\cite{22} and was recently confirmed for the USA\cite{23} and France.\cite{24} There have been some explanations suggested for this phenomena: the anti-inflammatory effect of vitamin D,\cite{23} an environmental trigger present in northern latitudes,\cite{25} dietary habits in colder climates, and agricultural differences.

**Prevalence**

Prevalence is a proportion of cases within a population and is defined as the number of cases divided by the population-at-risk.

An increasing temporal trend in the prevalence of CD has been observed during the second half of the twentieth century.\cite{26} This increase may have reflected changes in the incidence of CD, age distribution of the population, and survival of patients. The current overall prevalence of CD in Sweden is 190 per 100 000 population\cite{27} and in the USA 129 per 100 000.\cite{28}

**Patient characteristics**

The age at diagnosis is usually between 15 and 30 years,\cite{29} which is the largest peak. A second, much smaller peak occurs between 60 and 80 years.\cite{30} The median age of diagnosis increased from 25 years to 32 years between the 1960s and the 1980s due to an increase in the proportion of
patients diagnosed late in life. The female-to-male ratio in adults varied from 1.1:1 to 1.8:1 and in children from 1:1.6 to 1:4.0.

**Clinical symptoms and disease course**

The symptoms of Crohn's disease vary and are unspecific, often depending on which part of the digestive system is inflamed. Common symptoms include: recurring diarrhoea; abdominal pain and cramping, which is usually worse after eating; extreme tiredness; unintended weight loss; and blood and mucus in faeces. Patients may experience all or only one of the above, and some experience a completely asymptomatic disease course. Less common symptoms include: a fever of 38 °C or above; nausea; vomiting; joint pain and swelling; inflammation and irritation of the eyes (uveitis); areas of painful, red, and swollen skin – most often on the legs (erythema nodosum); and mouth ulcers.

Some patients may have long periods without any symptoms, since approximately 50% of all patients are in remission at any given time. Those in remission, however, will generally have recurrences, although some patients will spend years without a recurrence. Because periods of remission and recurrence vary so widely, it is difficult to predict how long a period of remission will be. During the initial five-year period after diagnosis, the indolent disease pattern accounted for 19% of patients, moderate disease activity for 56%, and aggressive disease for 25%. At the ten-year, follow-up study of the IBSEN cohort, 43% of patients had a decrease in the severity of bowel symptoms, 3% had an increase of symptoms, 19% had chronic continuous symptoms, and 32% had chronic relapsing symptoms.

**Risk of surgery**

The most common indications for intestinal resection in Crohn’s disease are the failure of medical management and complications such as intestinal obstruction and intestinal fistula. Less common indications for surgical intervention are: free perforation, uncontrollable bleeding, or cancer. Trends in surgical intervention for CD are inconsistent, with some studies reporting a reduction, and some reporting no significant change. The cumulative incidence of surgery varies between 37% and 62% at ten years depending on the time period and geographical population studied. The potential factors affecting surgery could include: age, initial disease phenotype, smoking, available medical therapy, and attitudes about surgery.
Surgical policies have varied over time and could well have affected surgery rates. For a long time, in the pre-immunomodulator era, CD was considered to be a surgical disease. The general perception was that the cure for CD was attainable by extensive surgery. Improved medical therapy opened the way for a change of treatment policies. Nowadays, surgery is often deferred until medical therapy has failed, thus surgical procedure rates act as a surrogate marker for the success or failure of medical therapy.

Younger age is associated with disease progression.44 Despite that, surgery rates in children have decreased,31 possibly due to better nutritional and medical treatments, especially the introduction of immunomodulator/biologic therapies serving as initial alternatives to surgery in patients not responding to conventional corticosteroid therapy.

Initial small bowel and ileocaecal presentation of CD has a strong impact on the probability of surgery45 in addition to presenting a more complicated disease behavior pattern at diagnosis.46

There is evidence that smoking influences the risk of surgery47, 48 as well as the risk for disease recurrence.49 Interestingly, while smoking influences several phenotypic characteristics, disease location rather than smoking per se may be the critical independent influence on complications and the need for surgery.50

**Etiology and pathogenesis**

Regarding the causes of Crohn’s disease, several theories exist, but none of them are finally proven. The prevailing hypothesis is that the mucosal immune system of people with CD reacts abnormally, treating commensal bacteria, food, and other beneficial intraluminal substances as foreign, harmful substances. During this reaction, white blood cells build up in the lining of the gut, initiating inflammation, which in turn leads to ulcerations and bowel injury. The pathophysiological basis of the disorder is still incompletely understood, but there is little doubt that inflammatory changes, selected immunological deficiencies, and genetic polymorphisms are involved.

**Genetics**

The familial nature of Crohn’s disease has long been recognised.51 As many as 12% of people with CD have a relative with the same disease.52, 53
Twin studies
In 1988, Tysk et al. published the first unbiased twin study showing a higher concordance rate in monozygotic (MZ) twin pairs with Crohn’s disease than in dizygotic (DZ) twin pairs with CD, 58% and 4% respectively, reflecting a pronounced genetic predisposition. Later, the proband concordance rates in monozygotic and dizygotic twins with CD were corrected to 38% and 2%. To set the heritability findings in perspective, one should mention that in type 2 diabetes, commonly considered to have strong life-style risk factors, the concordance rate in monozygotic twins is about 70%, whereas the concordance in dizygotic twins has been observed to be 20% to 30%, a much higher concordance than in CD.

Early linkage studies
The identification of over-proportional shared regions of the chromosomes in affected relative pairs led to the discovery of the IBD1 region on chromosome 16. Polymorphisms of the NOD2 gene located there were found to be associated with CD. Up to 40% prevalence rates of at least one of the polymorphisms associated with CD have been reported from both Europe and the USA. Furthermore, there is evidence that NOD2 mutations may be associated with the fibrostenosing ileal CD phenotype.

Genome Wide Association Studies
Comparing the allele frequency of a particular variant between unrelated cases and controls, the 71 susceptibility loci for Crohn’s disease were found, explaining 23.3% of the estimated heritability for CD. A substantial part of them are relevant for both CD and UC, suggesting that nearly all of the biological mechanisms involved in one disease play some role in the other. The exceptions are NOD2, the autophagy related genes ATG16L1 (encoding autophagy related 16-like 1 protein), and IRGM (encoding immunity-related GTPase family M), that are associated with innate immunity, autophagy and phagocytosis.

Intriguingly, seven of the eight susceptibility loci for infection with Mycobacterium leprae have also been associated with Crohn’s disease. Whether there is a causative role for mycobacteria in CD or merely convergent evolutionary adaptations to several pathogens remains to be elucidated.
Lifestyle and environmental factors

Smoking
It has been known that a higher percentage of smokers develop Crohn’s disease compared to non-smokers.\textsuperscript{64} In a meta-analysis, current smoking was associated with CD with an odds ratio of 1.76 (95% CI 1.40–2.22) compared to non-smoking.\textsuperscript{65} The course of Crohn’s disease is unfavourable for smokers, especially heavy smokers.\textsuperscript{48, 66} A two-fold increased risk of clinical recurrence, and a 2.5-fold increased risk of surgical recurrence at ten-year follow-up were reported in smokers compared to non-smokers.\textsuperscript{67}

Standard of living
On the societal level, the countries with higher levels of economic welfare have higher CD incidence rates. Countries that previously had very low incidence rates, such as Japan, are now observing the marked increase seen in Western countries four decades earlier, an observation that has been linked, correctly or incorrectly, to the Westernization of the developing countries.\textsuperscript{68} The same trend has been observed in the Baltic states\textsuperscript{17} and Hungary.\textsuperscript{15}

On the individual level, changes in the standard of living can be seen to have the same effect in migrant studies, where migrants from countries of low prevalence are tending to take on the prevalence rates of their adopted country.\textsuperscript{69-71} There are no observational or case-control studies that have evaluated the effect of decline in economic welfare on the occurrence of inflammatory bowel disease, as such studies would be methodologically difficult to design and conduct.

Diet and nutrition
The association of Crohn’s disease with Westernisation has implicated lifestyle factors in its pathogenesis, where diet is one of likely candidates. During the time period that CD has emerged as a major component of IBD and has been studied accordingly, the composition of foods has changed considerably. Not surprisingly, several dietary factors have been associated with CD including: quantity and quality of fat intake,\textsuperscript{72} fast food consumption,\textsuperscript{73} and total protein and energy intake.\textsuperscript{74} The positive association of refined sugar intake with CD is remarkably consistent regarding pre-illness, pre-diagnosis diet and current intake of sugars.\textsuperscript{75-77} The major difficulty here is the absence of a biologically plausible mechanism
for the relationship prior to the onset of disease, as well as a lack of evidence that specific changes in dietary habits or dietary intake affect disease prevention or disease course.

**Infection**

The best evidence for the role of bacteria in IBD comes from numerous genetically-engineered animal models that are susceptible to developing IBD only in the presence of luminal bacteria but fail to develop the disease in a germ-free environment. In humans, the evidence for an inflammatory trigger within the faecal stream comes from diversion studies, where the resection of Crohn’s ileocolitis with a proximal ileal diversion results in disease remission. Reanastomosis results in a clinical recurrence in less than a month, and reinfusion of the luminal contents results in inflammation in one week.

**Mycobacterium avium subspecies paratuberculosis**

There are striking similarities between Crohn’s disease and intestinal tuberculosis. The fact that anti-TNF therapy is good for CD but dangerously bad for tuberculosis strongly suggests that any association between mycobacteria and CD is unlikely to be causative. On the other hand, CD gene associations, such as NOD2 and IL23R, are also associated with increased susceptibility to mycobacterial disease. Moreover, there is now a consensus that a substantial majority of CD tissues show DNA evidence of the presence of Mycobacterium avium ssp. paratuberculosis (M. paratuberculosis). In addition, M. paratuberculosis possesses the mannan epitope for the anti-Saccharomyces cerevisiae antibody (ASCA) that is present in about two-thirds of Crohn’s disease sera. The M. paratuberculosis mannan-glycoconjugate is secreted and impairs macrophage killing of E. coli, so it is still possible that M. paratuberculosis might play an indirect role in pathogenesis.

**Microflora- microbiome**

Microbiota is “the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space”. Microbial diversity in humans increases from birth linearly with time over the first few years of life and, once established by around three years of age, remains remarkably constant over time. Microbial diversity can be negatively affected by inflammatory illness and by antibiotic use. Exercise tends to increase diversity, and diet can have either effect.
high intake of fruit and vegetables is associated with increased diversity.\textsuperscript{87} Conversely, the typical Westernised diet, rich in saturated fat and sugar, decreases bacterial diversity, especially beneficial Firmicutes, and leads to an expansion of the Proteobacteria phylum, including invasive, mucosal-adherent E. coli.\textsuperscript{89}

Generally, it seems that high bacterial diversity is beneficial, whereas low diversity has been linked with obesity, inflammatory bowel disease, and colorectal cancer.\textsuperscript{90} Short-term dietary changes, unless very major, tend to produce relatively more modest and less permanent changes, although severe energy restriction (by 35% for 6 weeks) has been shown to increase bacterial diversity, particularly among those who start from a low level of diversity.\textsuperscript{91} There is an association between an increase in faecal bacterial diversity and a reduction in serum sensitive C-reactive protein (CRP) as a marker for systemic inflammation.

In active IBD, both Crohn’s disease and ulcerative colitis, faecal microbiota commonly shows reduced diversity. In CD, it is associated with a reduction in obligate anaerobic bacteria belonging to the phylum Firmicutes, particularly a decrease in the probiotic F. prausnitzii. Conversely, there is an increase in facultative anaerobes or “microaerophilic” bacteria, such as the Proteobacteria, including E.coli.\textsuperscript{92-94} It seems likely that some, or even most, of the alterations in faecal microbiota seen in CD and UC may be, at least in part, secondary to inflammation. Reduced diversity accompanied by a reduction in F. prausnitzii has been shown, however, to be associated with an increased risk of relapse after cessation of anti-TNF therapy in CD.\textsuperscript{95} It has been shown that the presence of F. prausnitzii in the terminal ileum at the time of right hemicolecotomy is a very strong predictor of a low risk for subsequent relapse.\textsuperscript{96} F. prausnitzii has been shown to release a product that has a major anti-inflammatory effect.\textsuperscript{97}

A study of the mucosa-associated microbiota showed more dramatic differences between health and IBD than did a study of faecal microbiota, particularly with regard to CD. Mucosa-associated E. coli have been commonly found in mucosal biopsies from the ileum and colon of patients with CD.\textsuperscript{98-100} Many of these bacteria are present within the adherent mucus,\textsuperscript{101} although there is also evidence of intracellular E. coli from a study of gentamicin-treated and subsequently lysed mucosal samples.\textsuperscript{100} In CD tissue, E. coli has been identified within macrophages.\textsuperscript{102}
Immunity
The lack of bacterial clearance by macrophages and defective secretion of inflammatory cytokines cause a loss of tolerance to commensal flora by activating mucosal dendritic cells. This over-activation induces a strong differentiation of effector lymphocytes and other effector cells while abol-ishing production of regulatory cells. A strong shift towards the Th1/Th17-type immune response is present in CD. Anti-TNFα and thiopurins induce apoptosis of activated T cells; anti-IL12 antibodies inhibit IL2 and IFNγ, which in turn downplays Th1 response. Recent advances in autophagy studies demonstrate that physiological or pharmacological stimulation of autophagy pathways can increase bacterial clearance.

Intestinal permeability
Increased permeability of the intestinal epithelium to different molecular probes is not specific to Crohn’s disease. Various alterations of intestinal permeability are present in diabetes, celiac disease, multiple sclerosis, atopic dermatitis, ankylosing spondylitis, irritable bowel syndrome, as well as CD. In patients with CD, intestinal permeability clearly fluctuates with disease severity. The release of proinflammatory cytokines seems to increase the porosity of the epithelial barrier, even in the absence of overt epithelial damage or ulceration. Whether this process is simply a consequence of mucosal inflammation or is an early step in the pathogenesis of CD is not completely clear.

Definitions and diagnosis
Crohn’s disease cannot be determined solely on the basis of a blood test or an examination. The diagnosis is based instead on a combination of a medical history, morphological investigations, and tissue studies. Timing is also important, since the disease can be difficult to diagnose at the initial onset. There are several causes of inflammation in the small and large intestine that can mimic CD regarding: symptoms, laboratory tests, endoscopic findings, histological changes, and clinical progression. The diagnosis gains assurance if the disease shows a repeating pattern of remission and relapses.

Ideally, studies of the natural history of Crohn’s disease should take into account the availability of diagnostic methods over time. In the 1930s, the disease was diagnosed mainly by studying barium X-rays and surgical
resection specimens; meaning patients who did not develop complications were probably not diagnosed. With the advent of endoscopy, the diagnosis of CD became possible preoperatively, but patients without diarrhoea would probably not undergo colonoscopy. Computed tomography in the 1990s increased the availability of small bowel imaging; magnetic resonance imaging and double balloon enteroscopy made it possible to examine a larger part of the small intestine. Capsule endoscopy became available in the 2000s and is the most sensitive method for detecting lesions in the small intestine. Inception cohorts before the era of the Vienna and Montreal classifications, and before the introduction of capsule endoscopy, tend to underreport a small bowel disease. Population-based cohorts that include adult and pediatric cases tend to report a higher prevalence of pure ileal disease than do studies that include patients age 15 years and older.34

Diagnostic delay
The earliest documented patient with Crohn’s disease in Sweden was operated on in 1918 for suspected appendicitis.112 The surgeons found a narrowing in the ileum and performed an ileobypass. It was not until 1969 that the same patient presented with a perianal fistula. A resection was performed, and histological examination of the resected tissue (which included the bypassed segment of the ileum) confirmed the diagnosis of CD.112 This case demonstrates that there may be long periods before diagnosis. It also suggests that the second peak incidence, between 60-80 years of age, may actually represent missed or delayed diagnoses, rather than the result of the late onset of CD.

Diagnostic criteria
Definitions and diagnostic criteria for Crohn’s disease have been evolving over the years along with our understanding of the disease’s pathogenesis. The difference in diagnostic criteria used during the study period has different implications for prospective and retrospective studies. In a prospective study, patients who were misdiagnosed will be reclassified or excluded at some point after inclusion.113 In a retrospective study, there are factors that could result in selection bias. The exclusion of specific groups of patients, such as those with a spontaneous recovery after the first flare-up or those receiving antibiotic treatment proximate to the point of diagnosis, could introduce bias.
Garland diagnostic criteria

The Garland diagnostic criteria were first presented in 1981.114 In short, the criteria of Garland classify patients into three groups: definite, probable, and possible CD, depending on findings for histology, endoscopy, and radiology, along with the discharge diagnosis. The Garland criteria were used in a previous epidemiologic study in our area, where only definite and probable diagnoses were included.115

Copenhagen diagnostic criteria

The Copenhagen diagnostic criteria were published in 1982.116 These criteria were subsequently used in the Danish Crohn Colitis Database.12, 13, 34, 116

Lennard-Jones criteria

With the publication of the Lennard-Jones criteria,8 an international consensus on diagnostic criteria for Crohn’s disease and ulcerative colitis was reached, and the comparison and generalization of epidemiological data on patients with IBD from different countries became possible. Recently, the sensitivity of the Lennard-Jones criteria in the diagnosis of CD was assessed,117 showing that, for nearly half of the patients managed as long-standing CD cases in referral centers, the criteria would not have provided a diagnosis of CD from the initial examinations.
### Table 1. Comparative presentation of Garland, Copenhagen and Lennard-Jones diagnostic criteria of Crohn’s disease

<table>
<thead>
<tr>
<th>Garland diagnostic criteria&lt;sup&gt;114&lt;/sup&gt;</th>
<th>Copenhagen diagnostic criteria&lt;sup&gt;116&lt;/sup&gt; at least 2 of</th>
<th>Lennard Jones diagnostic criteria,&lt;sup&gt;8&lt;/sup&gt; at least 3 of following</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Definite</strong></td>
<td>A laparotomy report of characteristic naked-eye appearances of the small bowel but no specimen of gut resected for histology</td>
<td>Mouth to anus: Chronic granulomatous lesion of the lip or buccal mucosal (inspection, biopsy), Pyloroduodenal disease (radiology, endoscopy, biopsy), Small bowel disease (radiology, endoscopy, specimen), Chronic anal lesion (clinical examination, biopsy)</td>
</tr>
<tr>
<td><strong>Probable</strong></td>
<td>A medical record with discharge diagnosis of Crohn’s disease, regional enteritis, or granulomatous colitis: No findings, clinical or radiologic, inconsistent with the diagnosis; and an acceptable history</td>
<td></td>
</tr>
<tr>
<td><strong>Possible</strong></td>
<td>Case history of diarrhea, for more than 3 month</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Radiologic findings with typical stenosis and prestenotic dilatations in the small bowel or segmental findings with cobblestone appearance in the large bowel</td>
<td>Fibrosis: Stricture (to be distinguished from carcinoma or concentric muscular thickening in UC), which can be asymmetric and multiple (endoscopy, radiology, specimen)</td>
</tr>
<tr>
<td></td>
<td>A colonoscopic report compatible with Crohn’s disease and biopsy with features strongly suggestive of Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A radiologic examination strongly suggestive of intestinal or colonic inflammatory disease with obstructive or fistulous features.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>An equivocal histologic report from an operative or autopsy specimen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>An equivocal histologic report from an operative specimen with characteristic macroscopic features</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Histologic findings according to Morson&lt;sup&gt;118&lt;/sup&gt; with transmural lymphocytic infiltration or occurrence of epithelial granulomas with giant cells of Langhans type, or both</td>
<td>Lymphoid: Biopsy of small aphthoid ulcer or showing lymphoid aggregates</td>
</tr>
<tr>
<td></td>
<td>Occurrence of fistulas or abscesses, or both, in relation to intestinal lesion</td>
<td>Mucin: Retention of colonic mucin on biopsy in the presence of active inflammation (biopsy, specimen)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Granulomata: Not present in all cases of Crohn’s disease, distinguish from caseating granulomata of tuberculosis, foreign-body granulomata, or other causes (biopsy, specimen). When present, is regarded as diagnostic and may thus be given greater weight than other features.</td>
</tr>
</tbody>
</table>

<sup>114</sup> Garland diagnostic criteria

<sup>116</sup> Copenhagen diagnostic criteria

<sup>8</sup> Lennard Jones diagnostic criteria
Disease classification

The evolution of Crohn’s disease classifications was affected by the availability of diagnostic methods as well as the need to predict the course of the disease for the individual patient.

The initial report in 1932 by Crohn et al. described a disease limited to the ileum and sharply demarcated at the ileocaecal valve with no colonic involvement. Soon it was accepted that the disease could affect the jejunum and the colon as well.

Involvement of the duodenum was documented in 1937 followed by involvement of the stomach in 1949. The description of esophageal involvement in 1950 and the identification of oral lesions in 1969 completed the evidence that Crohn’s disease affects the entire gastrointestinal tract discontinuously from mouth to anus and is truly panenteric.

The first attempt to classify Crohn’s disease was done in 1975 by Farmer et al., who studied 615 consecutive patients at the Cleveland Clinic from 1966 to 1969. His classification grouped patients based on the site of initial anatomic involvement of CD: ileocolonic (41%), small intestine (28.6%), colon (27%), and anorectal (3.4%). These defined groups had distinct symptomatology, clinical course, and surgical outcomes.

In 1988, Greenstein et al. studied surgical indication in 770 patients at Mount Sinai Hospital in New York from 1960 to 1983. He classified CD based on surgical indications: perforating (48.7%) and non-perforating (51.3%). He found the indications for a second operation were closely dependent on the indications for a primary resection. This trend toward similarities in the surgical indications was maintained within each anatomical category of CD and even extended to a third operation.

The Rome classification for Crohn’s disease emerged in 1992. It was based on the following four entities: (1) anatomical location; gastroduodenitis, jejunitis, ileitis, colitis, and perianal; (2) disease extent; localized and diffuse; (3) disease behaviour modifiers; primary fibrostenotic, primary inflammatory, and primary fistulating; and (4) operative history; primary and recurrent.

The Rome classification was cumbersome to use clinically, allowing an opportunity for the Vienna classification to be proposed in 1998. This classification included the following three components: (1) age at diagnosis; A1 - less than 40 years, and A2 - 40 years or older; (2) disease location; L1 - ileum, L2 - colon, L3 - ileum and colon, and L4 - upper gastrointestinal; and (3) disease behaviour; B1 - inflammatory, B2 - stricturing, B3 - penetrating.
The update to the Vienna classification in 2005 was called the Montreal classification. It included a new category for age at diagnosis: A1 - less than 17 years. In the disease location category, L4 was changed into a modifier that can be added to L1, L2, and L3. In the disease behaviour category, a perianal disease modifier (p) could be added to B1, B2 and B3, removing perianal disease from the B3 group.129

The Paris classification was proposed in 2011 as a pediatric modification of the Montreal classification. The A1 group was subdivided into: A1a (0-10 years) and A1b (10-17 years); and the L4 group was subdivided into: L4a (proximal to ligament of Treitz) and L4b (above the distal ileum and distal to the ligament of Treitz). Stricturing disease and penetrating disease were allowed to be classified simultaneously as B2B3, and a new variable “growth failure” (G0/G1) was added.130

Treatment

Medical treatment
Initially, Crohn’s disease was regarded as an ileocaecal entity that could be cured by radical surgery. With time, however, corticosteroids became the first line therapy. Sulfasalazine became available in Sweden in 1945, but was successively replaced by 5-aminosalicylic acid (5-ASA) drugs in the 1990s. Treatment with azathioprine was initiated in selected cases in the early- to mid- 1980s and has been increasingly used since then, primarily for steroid-dependent or refractory disease. Methotrexate was occasionally used for thiopurine intolerance or failure. In 1995, budesonide was introduced, primarily for mild to moderate CD involving the ileum and/or ascending colon. The first anti-TNF agent, infliximab, was approved for Crohn’s disease in 1999. Initially, anti-TNFs were used for refractory disease only. However, the proportion of patients treated with anti-TNFs has increased over time, and the prescription strategies have changed from on-demand to continuous maintenance therapy.

Surgical treatment
Surgery nowadays is often deferred until medical therapy has failed in the treatment of Crohn’s disease. Surgical procedure rates act as a surrogate marker for the success or failure of medical therapy. Surgery can relieve symptoms that did not respond to medication and can also treat complications such as abscess, perforation, bleeding, and bowel obstruction.
Surgery practices underwent a substantial evolution over the years. Early investigators of granulomatous IBD reported that the prognosis of patients was poor unless surgery was performed. All patients operated on by Berg at Mount Sinai had undergone a two-stage procedure. At the first surgery, the ileum was divided proximal to the disease site, both bowel ends were closed, and a side-to-side ileotransverse colon anastomosis was performed. At the second stage, from three weeks to three months later, the excluded loop of ileum and caecum was resected. With time, Berg and his colleagues noticed that, at the second procedure, the diseased bowel seemed to have healed. They, therefore, began to perform only the ileocolostomy with exclusion. This procedure carried a far lower mortality than either the one-stage or two-stage resection. A bypass alone was not sufficient for many patients, since their disease did not always become quiescent. Resection became the established method of choice. Recurrent disease, however, was common, and radical surgery was advocated, meaning the resection of diseased bowel segments with wide margins. Studies, though, were unable to demonstrate the benefit of radical surgery. Disease recurrences were almost always located immediately proximal to the anastomosis, making repeat surgery necessary, and, in some patients, the short bowel syndrome developed. In Crohn’s colitis, proctocolectomy with stoma was the treatment of choice until the 1970s.

A change in attitudes towards more limited resection occurred in the 1970s and 1980s, when stricturoplasty was shown to be a feasible method for providing quick relief of obstructive symptoms. The procedure took hold and became one of the widespread surgical techniques, together with limited resection, for treating CD.

Endoscopic and interventional radiologic therapy

The advent of endoscopic balloon dilatation with the through-the-scope technique shifted the focus away from intestinal resection and stricturoplasty even further, since it is a safe and effective alternative for treating short, fibrotic, anastomotic strictures. The first dilation in our hospital was done in 1987. In the early period, dilations were repeated one to four times per year and were performed in patients with recurrent strictures but without clinical symptoms of bowel obstructions. It was thought that this might reduce the need for surgery. Today, only patients with obstructive symptoms are referred for dilation.

Development of interventional radiological techniques, such as computed tomography and ultrasound, employed with percutaneous-guided
drainage made it possible to precisely pinpoint abscesses and drain them percutaneously. This dramatically improved the management of patients with CD, postponing surgery even further.

**Disease monitoring**

Differences in the course of Crohn’s disease illustrate the need for monitoring disease activity, since it can vary widely from patient to patient. We could monitor each patient using colonoscopy. However, along with the expense and poor acceptance by patients, colonoscopy would not visualise the small bowel and would not provide information about the transmural nature of the inflammation. Cross-sectional imaging may be more appropriate, but the specific features associated with tissue healing have been less well described using this method.\(^{142}\) As we know, mucosal healing is associated with better outcomes.\(^{143-146}\) There is a poor correlation, however, between clinical activity and mucosal healing.\(^{147}\) This emphasises the continuing need to adjust treatment to optimise benefits and minimise cost and risk.

**Disease activity scores**

The definition of disease activity is a major challenge in Crohn’s disease. The clinical parameters most frequently used are daily bowel movements and the presence of abdominal pain and/or discomfort, whereas CRP and serum levels of orosomucoid are the classic laboratory tests.\(^{148}\) Ideally, a disease activity index should provide an assessment of the patient’s overall condition. It should take into account clinical measures, such as fever, diarrhea, abdominal pain, and gut inflammation, as well as reflect the patient’s general well-being and quality of life. The existing indices, such as the Crohn’s Disease Activity Index (CDAI) and the Harvey-Bradshaw Severity Index (HBSI), are invariably too complex to be applied in everyday clinical practice.\(^{149}\) Furthermore, the many indices indicate that, to date, none of them would appear to be adequately reliable and valid.

**Mucosal healing**

Mucosal healing has become an important endpoint of clinical trials.\(^{150}\) The term “mucosal healing” refers to a mucosa without visible signs of active inflammation\(^{151}\) as determined by endoscopic assessment. Unfortunately, there are no uniform, established, definitive criteria for mucosal healing. Ideally, the mucosa is restored, both macroscopically and histologically, to a state equivalent to that found in a healthy person.\(^{152}\)
The mucosal healing process is complex and includes not only suppression of inflammation, which can be achieved using corticosteroids, but repair and reconstruction of the damaged mucosa. Mucosal healing, as judged by endoscopy or imaging techniques, reflects long-term outcome better than remission/response based on the general clinical picture and patient symptoms. It is an important prerequisite for normalization of the patient’s general state of health and is closely correlated with an improvement in quality of life. It is evident that a fully healed mucosa with a restored barrier function is more resistant to potential triggers of new inflammation and is, therefore, essential for stable remission. Full mucosal healing also improves disease prognosis and reduces the need for surgical intervention. In a Norwegian population-based cohort study, 11% of CD patients with mucosa healing at one year needed a surgical resection compared to 20% of CD patients without mucosal healing. In contrast, when the mucosa is not fully healed, the risk of relapse increases significantly.

Endoscopic and histological examinations of the mucosa are relatively complex and expensive procedures, thus they cannot be used routinely to assess the progress of healing. It has been shown, however, that endoscopic and histological findings correlate closely with faecal inflammation markers such as calprotectin and lactoferrin. These markers are used primarily to determine whether symptoms are the result of inflammation, although these markers are not specific to inflammatory bowel disease, since elevated levels can also result from intestinal infections and neoplasia.

**Disease markers**

The ideal laboratory marker, one that would cover all aspects of the disease in one “gold standard”, is difficult to find. Such a disease marker should be disease-specific (able to distinguish IBD patients from other patients and UC from CD) and be able to do the following: identify individuals at risk for a given disease, monitor disease activity, monitor the effect of treatment, and provide prognostic information. In addition to these capabilities, the ideal disease marker should have the following characteristics: be easily applicable for screening purposes, employ minimally invasive sampling, use a fast and simple analysis procedure, and be inter- and intra-individually reproducible with regard to laboratories and patients. Figure 1 presents an overview of some serological and faecal bi-
Biomarkers of inflammation in IBD. Biomarkers, like faecal calprotectin, are attractive for the purpose of disease monitoring.

**Figure 1. Laboratory biomarkers in IBD.**

**Calprotectin**

Calprotectin was originally discovered as an antimicrobial protein that was present in the cytoplasm of neutrophil granulocytes. It consists of two subunits, an active lighter chain MRP-8 (8.3kDa) and a regulatory heavy chain MRP-14 (13.3kDa), which prevents early degradation of MRP8. The two subunits are non-covalently linked and belong to the S100 protein family, which is a subfamily of proteins with a Ca++-binding motif. The gene cluster of the S100 proteins is located on human chromosome 1q21, and the nomenclature of these proteins was established according to the organisation of these S-100 genes, namely S100A8 and S100A9. The binding of calcium induces conformational changes, where
heterodimeric complexes may tetramerize, allowing binding of other proteins.

Along with neutrophils, where calprotectin constitutes 30-60% of cytosolic proteins, it is found in monocytes, keratinocytes, muscle tissue, and tissue macrophages; it is also invariably found in endothelial and epithelial cells. Calprotectin is secreted extracellularly from stimulated neutrophils and monocytes, or it can be released into pus or abscess fluid as a result of cell disruption or death.

Immunohistochemical studies confirmed the presence of calprotectin not only in neutrophils and reactive tissue macrophages but also on the membrane of non-keratinizing squamous epithelia and kidney tubules. Some mucosal epithelial cells express calprotectin in the cytoplasm constitutively. The soluble form of calprotectin is found in plasma. (The reference range for healthy subjects is less than 2 mg/l.) It is also found in urine, body secretions, intestinal fluid, and faeces.

Membrane calprotectin inhibits the attachment of monocytes to collagen and fibronectin. Calprotectin takes part in the extravasation of leukocytes by the attachment to endothelial cells via the MPR14 subunit, interacting mainly with endothelial heparan sulphate proteoglycans. This process may also be induced by arachidonic acid. The putative receptors of calprotectin include CD36 and RAGE (receptor for advanced glycation end-products). There is a relationship between calprotectin expression and the higher capacity to release TNFa in human alveolar macrophages derived by bronchoalveolar lavage. The signalling pathways of calprotectin are not fully elucidated, but they involve MAP-kinase cascade activation.

Soluble calprotectin has antimicrobial and apoptosis-inducing activities, which are reversed by the addition of zinc. By sequestration of zinc, calprotectin affects the following: matrix metalloproteinases, zinc-dependent enzymes responsible for angiogenesis, embryonic development, wound healing, inflammation, and tissue destruction. By competition for zinc and iron, calprotectin inhibits bacterial growth. Calprotectin concentrations of 50-250 µg/ml inhibit the growth of Escherichia coli, Staphylococcus aureus, and Staphylococcus epidermidis; lower concentrations (4-32 µg/ml) are sufficient to inhibit the growth of Candida albicans. Cells expressing calprotectin are able to resist invasion of Listeria monocytogenes and Salmonella typhimurium. It is likely that calprotectin is a part of the defense mechanism protecting neutrophils and other expressing cells against invading microorganisms.
Faecal calprotectin is one of the established markers of inflammation in the GI tract. It is stable in faeces for up to seven days and correlates well with endoscopic activity. Intriguingly, faecal calprotectin is elevated in healthy relatives of CD and UC patients.

**MPO**
Myeloperoxidase (MPO) is an iron- and chlorine-containing enzyme that is stored in azurophilic granules in neutrophils and is released into the extracellular space during degranulation. The three major glycoisoforms of MPO (MPO I, II, and III) differ in charge, hydrophobicity, molecular mass of the heavy subunits, and subcellular localization. MPO is too large to freely cross membranes, therefore, chlorinating compounds are delivered first to the target surface. MPO converts the relatively harmless H$_2$O$_2$ into much more powerful antiseptics: hypochlorous acid, hypobromous acid and hypiodous acid. Hypochlorous acid reacts with amines to produce long-lived, bactericidal chloramines.

**NFκB**
Nuclear factor kappa B (NFκB) is a heterodimeric protein consisting of different combinations of Rel family transcription factors: NFκB1 (p50 and its precursor p105), NFκB2 (p52 and its precursor p100), RelA (p65), RelB, and cRel (Rel). NFκB is involved in the control of a large number of normal cellular processes, such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. These transcription factors are persistently active in a number of disease states including: chronic inflammation, arthritis, cancer, heart disease, and neurodegenerative diseases. NFκB dimers are sequestered in the cytosol of unstimulated cells via non-covalent interactions with a class of inhibitor proteins, called IκBs. Endotoxins or proinflammatory cytokines (like TNFa) cause the phosphorylation of IκBs; their dissociation and subsequent degradation, thereby, allow activation of the NFκB complex. Activated NFκB complex translocates into the nucleus and binds to DNA, inducing gene expression.

RelA (p65) is one of the most essential transcription factors under intensive study. RelA is expressed alongside p50 in various cell types, including epithelial/endothelial cells and neuronal tissues.

Immunohistological visualization of the RelA (p65) subunit incorporates the use of specific antibodies. The decision to use polyclonal or monoclonal antibodies depends on the intended use, the price, and whether or
not the antibodies are readily available from commercial suppliers or researchers. Polyclonal antibodies frequently have better specificity than monoclonal antibodies. They are produced by a large number of B-cell clones, each clone generating antibodies to a specific epitope, making polyclonal sera a composite of antibodies with unique specificities. Furthermore, the binding affinity of most antibodies is influenced by conformational determinants that may be altered by the following: association with other proteins, posttranslational modification, temperature, pH, salt concentration, and fixation. Polyclonal antibodies have an advantage since they recognise multiple epitopes, and conformational changes may not influence all epitopes to the same degree.190
Aims

The overall aim of this thesis was to study epidemiological and clinical changes in the natural history of Crohn’s disease, with examination of phenotype, changes in the need for surgery and pharmacological therapy over time, as well as the role of faecal calprotectin as a biomarker of IBD pathophysiology and disease course.

The specific aims of the enclosed studies herein were:

I. To study time trends in Crohn’s disease incidence, prevalence, and phenotype at diagnosis over time within Örebro University Hospital’s primary uptake area 1963-2010.

II. To study longitudinal changes in Crohn’s disease: its natural history, progression, the need for pharmacological and surgical treatment from diagnosis up to five years after diagnosis, and possible associations between therapy and CD phenotype within Örebro University hospital’s primary uptake area 1963-2005.

III. To explore whether there is a subclinical inflammation with increased neutrophil activity in healthy twin siblings of discordant twin pairs with IBD and to assess the genetic influence on the occurrence of elevated faecal calprotectin in a population of twins.

IV. To investigate whether repeated measurements of faecal calprotectin over time can be used as an instrument to predict disease relapse in clinical practice.

Ethics

Paper I and II: The Uppsala Regional Ethics Committee approved the study (Drn 2010/304)

Paper III: The Örebro County Ethical Committee approved the study (Drn 167/03)

Paper IV: The Ethics Committee of Uppsala University approved the study (Drn 2007/291)
Material and Methods

Paper I and II

Background population and study area
The primary uptake area of Örebro University hospital covers 3998 km² and includes five municipalities: Örebro (1620.6 km²), Kumla (205 km²), Askersund (1019.75 km²), Hallsberg (670.22 km²) and Lekeberg (481.63 km²). The number of inhabitants increased from 150 177 in 1963 to 189 603 in 2010 (26%), with an average 51.1% of the population being women. The age structure of the population changed during the study period, and median age increased from 36 years (IQR 18-57) in 1963 to 39 years (IQR 20-59) in 2010. Örebro University Hospital and 17 primary health care clinics, some private general practitioners, but no private gastroenterologists, serve the area. All colonoscopy procedures within the catchment area were performed at the Endoscopy unit, Örebro University hospital. Information about age and sex distributions of the general population in the uptake area was obtained from Statistics Sweden.191

Patient identification
Patients with the potential diagnosis of Crohn’s disease were identified retrospectively by evaluation of medical notes of all present and previous patients at the Colitis clinic, Department of internal medicine, Örebro University hospital. The county council’s patient registers (IMX), for both inpatients and outpatients, were searched to find additional patients using the ICD10 diagnosis codes: Crohn’s disease K500-K509, colitis undefined K528-K529, IBD associated arthropathy M074-M076, IBD-associated juvenile arthritis M091-M092. A similar search strategy was applied to the previous patient administration system (PAS), using the above-mentioned ICD10 codes: K500-K509, K528-K529, M074-M076, and
M091-M092. In addition the following ICD9 diagnosis codes were used: regional enteritis 555, ulcerative colitis 556, other and unspecified non-infectious gastroenteritis and colitis 558. Lastly, all histological reports were identified when tagged with any of the following search terms: Crohn’s disease; inflammation, fistula, or ulcer in gastric, small bowel, large bowel, or rectal mucosa within the histopathological records at the Department of Pathology, Örebro University hospital. The results of the search in the older PAS and pathology register were run together to identify unknown patients who underwent endoscopy and had a positive histology in the pathology database. We found 1026 patients and their medical notes were studied in detail.

![Flow chart illustrating the process of identifying cases with Crohn’s disease.](image)

**Data collection**

The medical notes of all patients with potential Crohn’s disease were reviewed by YZ to verify the diagnosis. The records for patients where the diagnosis was uncertain were jointly reviewed by YZ, CT and JH. Data extraction was performed using standardized questionnaires.

The key variables that apply to study I and II are:

- Date of birth.
Sex.
Clinical diagnosis. The diagnosis of Crohn’s disease was verified according to Lennard-Jones criteria.\textsuperscript{8} 
Date of diagnosis was defined as the date of the first examination consistent with Crohn’s disease. In patients where the diagnosis had been changed from ulcerative colitis or IBD-unclassified to Crohn’s disease, the date of the first IBD diagnosis was used. In any patient where a precise date of diagnosis could not be obtained retrospectively, the date was approximated to the fifteenth of the month where possible, otherwise to the thirtieth of June of the year of diagnosis.
Location and behaviour of disease at diagnosis were defined in all patients according to the Montreal classification.\textsuperscript{129} Data for patients diagnosed during the previously reported period, 1963-1987,\textsuperscript{115} were re-extracted for information on diagnosis and disease phenotype.
Location during follow-up.
Dates of change in location. Development of upper gastrointestinal (L4) disease was regarded as a modifier of disease locations and was not considered as a progression in location.
Behaviour during follow-up.
Dates of change in behaviour. Development of perianal disease was not considered as a change in behaviour.
Dates of moving in/out. Patients were included if they lived within the uptake area at any time during their disease course.
Patients who died or left the area were censored on the date of death or emigration.
Pharmacological treatments, commencement and termination dates.
Dates and types of surgical procedures from diagnosis and during follow-up.

Paper III

Twins
We invited twin pairs of the same sex, age less than 75 years, and living in the proximity of Örebro County (middle and southern parts of Sweden) to
undergo colonoscopy and to provide stool samples for faecal calprotectin assay. We excluded twins with previous, extensive, IBD-related resections, i.e., colectomy. This was the cohort of twins with IBD in Sweden earlier identified and studied by us. Twins living outside the vicinity of Örebro County, or those not willing to undergo colonoscopy, were invited to provide stool sample for the assessment of faecal calprotectin only.

Figure 3. Study groups of twins.

Controls
For faecal calprotectin analysis, a previously described control group was used. Control subjects, who were healthy lab personnel, filled in a health statement, according to which they were not suffering from any of the following maladies: thyroid disease, heart and vascular disease, tumors, joint disease, diabetes, liver disease, lung disease, allergic disease, dermatitis or eczema, food allergy, IBD or other gastrointestinal disease, frequent urinary infection, or other recent infection; nor were they receiving any anti-inflammatory treatment.

For immunohistochemistry analysis, biopsies from controls matched for age and sex, with macroscopically normal mucosa, were recruited from the biobank at the Department of Pathology, Örebro University Hospital. These individuals had undergone colonoscopy during the same period as the twins, 2006-2009, for investigation of one or more of the following: altered bowel habits, abdominal pain, unexplained rectal bleeding, or polyp surveillance, and had approved storage of their specimens in bi-
obank at the Department of Pathology according to The Swedish Act “Bi-
obanks in Medical Care” (SFS2002:297).

**Histology**

We used formalin-fixed, paraffin-embedded biopsies from the ascending colon and rectum. All biopsy sections were stained with hematoxylin and eosin and scored separately as non-inflamed or inflamed. The evaluator was blinded to diagnosis, faecal calprotectin levels, and immunohistochemistry results.

**Immunohistochemical analysis of NFκB and MPO**

We used NFκB as a general marker of inflammation and MPO as a specific marker for detecting neutrophils.

Biopsy sections were stained with the Envision technique (peroxidase) according to the manufacturer’s protocol. Antigen retrieval and deparaffinization was achieved by heating the samples in the EnVision Flex Target Retrieval Solution High and Low pH in a PT Link (Dakocytomation, Denmark) for 20 minutes. Staining was performed using a Dakos Autostainer Link 48 and DAB+ Envision Flex (Dakocytomation, Denmark). Polyclonal anti-NFκB ab16502 at 1:1000 (rabbit IgG, Abcam, USA) against p65 and polyclonal anti-MPO antibody (rabbit IgG Dept. of Medical Sciences, Clinical chemistry, Uppsala University, Sweden) at dilution 1:1600 were used. Slides were incubated with respective antibody for 30 minutes. All slides were stained simultaneously using a control slide exposed to the secondary antibody only. Sections were transferred through an ascending ethanol series and xylene before being mounted and evaluated under light microscopy.

Brown deposited granules in the cytoplasm and/or nucleus showed positive expression of NFκB p65. NFκB staining was assessed in a semi-quantitative way and graded into 4 categories: (0) absence of staining, (1) reduced staining, (2) moderate staining, and (3) strong staining.

The results for MPO staining were evaluated semi-quantitatively according to the percentage of positive cells in ten randomly selected fields under high-power microscopy (100 × magnification). The intensity of MPO staining was classified according to the percentage of stained cells: (0) 0% to 10%, (1) 10% to 50%, (2) 50% to 80%, and (3) more than 80%.
Figure 4. 1 Immunohistochemical staining of mucosal biopsies for NFkB and MPO. (1) Low grade NFkB, (2) High grade NFkB, (3) Low grade MPO, (4) High grade MPO.

Faecal calprotectin

Stool samples for faecal calprotectin analysis were collected in screw-capped plastic containers, sent the same day by mail to the laboratory, and were stored at −70 °C. Samples were thawed overnight, and the weight of each sample was measured prior to FC extraction. An extraction buffer was added and samples were homogenized in a multi-tube vortex mixer by vigorous shaking (highest speed) for 30 minutes. Homogenate were transferred into a 2 ml Eppendorf tube and centrifuged in a micro centrifuge for 5 minutes at 10 000 x g, then supernatants were taken into a fresh labelled tube. They were further diluted 1:50 in the incubation buffer provided by the manufacturer, and then calprotectin in faecal extracts were analysed with the calprotectin ELISA according to the manufacturer’s instructions (EK-CAL, Bühlmann Lab. AG, Switzerland). Calprotectin was
expressed as micrograms per gram of faeces, using <50 µg/g faeces as cut off.

**Paper IV**

**Patients**

We recruited patients 18 years and older, with a confirmed diagnosis of Crohn’s disease or ulcerative colitis, attending the outpatient Gastroenterology Clinic at Örebro University Hospital during the period from February 2009 to September 2012. Patients in clinical remission, based on the physician’s global assessment, were eligible for inclusion. Exclusion criteria were previous substantial surgical resections or any other systemic disease.

![Flow chart over the patient recruitment process](image)

*Figure 5. Flow chart over the patient recruitment process.*

**Faecal calprotectin**

Patients were asked to provide a faecal sample at baseline and every third month until the first clinical relapse or the end of the two-year follow-up period.
Faecal calprotectin was analysed with the calprotectin ELISA according to the manufacturer’s instructions (EK-CAL, Bühlmann Lab. AG, Switzerland). (See above study III)

**Questionnaire**

In conjunction with providing a faecal sample, patients were asked to fill in a questionnaire on clinical activity (number of stools/day, abdominal pain, blood and mucus in stool), present medication, dietary habits, use of antibiotics, use of NSAIDs, and patient defined remission at baseline and every third month until the first clinical relapse or the end of the two-year follow-up period.

**Definitions of remission and relapse**

Remission was defined as the absence of diarrhoea, abdominal pain, and malaise, with no need for hospital admission or surgery. Patients were instructed to contact the outpatient clinic in case of experiencing increased gastrointestinal symptoms in between any appointments during follow-up. Relapse was defined as the presence of diarrhoea, abdominal pain, and malaise with the need for escalation of medical therapy as judged by the responsible gastroenterologist.

**Statistical considerations in all papers**

Continuous variables are presented as median (range) or median (interquartile range [IQR]) where appropriate, and compared using the Mann-Whitney U test.

Categorical variables were presented as frequencies, and their distributions were compared with the chi-squared test.

For FC levels below the lowest detectable value of 10, 10/√2 served as a substitute (Study III and IV).  

A two-sided p value of <0.05 was considered statistically significant for all analyses.

**Paper I**

Incidence rates were calculated as crude and age standardised, and were aggregated over five-year periods by the direct method to allow for a changing population structure over time. The age and sex characteristics of the Örebro population for each year of the study period were used for standardization, while the Örebro population for 1999 was used as the standard comparison population. The 95% CIs of the incidence rates were
computed assuming a Poisson distribution. Point prevalence was calculated for the end of each five-year period.

Calculations were performed using the Stata statistical software (Version 12SE, Stata Corporation, College Station, TX).

Time trends in incidence were assessed using Poisson regression models. Possible temporal clusters in incidence rates were prospectively analysed based on discreet Poisson model analysis with a time aggregation length of five years, adjusted for age and sex, using SaTScan (v 9.3 for Windows, http://www.satscan.org).

**Paper II**

Patients were divided into three cohorts based on the year of diagnosis: 1963-1975, 1976-1990, and 1991-2005. In all analyses, patients were followed from the diagnosis of Crohn’s disease until the outcome of interest, death, emigration, or the end of follow-up (five-years from diagnosis), whichever occurred first. Our collected data was right censored, i.e. the exact survival time was incomplete due to the end of the study on 31 December 2010. To deal with this, we restricted the analysis to patients diagnosed 1963-2005 to allow for a complete follow-up period of five years for most of the study individuals.

Life tables using a Kaplan-Meier approach were used to estimate the cumulative probability of the following: first progression in disease location, initial complication (stricturing or penetrating disease whichever came first), receiving 5-aminosalicylic acids, immunomodulators, anti-TNFs, and undergoing surgery. Kaplan-Meier curves were compared using the log-rank test, comparing rates within three months, one year and five years after diagnosis. Non-parametric survival analysis, like the Kaplan and Meier method, compares time to the occurrence of the event and does not have to take into account assumptions about the distribution of the time to failure and the distribution of the effect of covariates.

Calculations were performed using Stata statistical software (Version 12SE, Stata Corporation, College Station, TX).

**Paper III**

Faecal Calprotectin was categorised with a predefined cut off at 50 µg/g. NFkB and MPO activities were dichotomized as low (grade 0-1) or high (grade 2-3). Study groups were compared with a logistic regression model adapted for small samples. ORs and corresponding 95% CI were calculated.
In addition, we analysed differences in the levels of FC as a continuous scale variable, using regression analysis of a mixed-model design with allowance for dependence within the twin pair and the clinical diagnosis as the explanatory variable. To comply with the statistical assumptions, the group comparisons were carried through on the logarithm of FC and the results transformed back afterwards to the original FC scale. The effect parameters for the defined study groups are ratios of geometric means and are given with corresponding 95% CI.

Calculations were performed in the statistical packages SAS (Version 9.2, www.sas.com) and LogXact (Version 8, www.cytel.com).

**Paper IV**
The predictive value of FC was assessed in the extended Cox regression model that allows for non-proportional hazards. The hazard ratios with corresponding 95%CI were calculated. Missing FC values were imputed with the “last observation carried forward” technique. Variables that potentially affected sampling completeness were assessed in the logistic regression model. To comply with the requirement for a normal distribution of continuous dependent variables, analyses were carried on logarithm of FC.

We performed a sensitivity analysis by including different covariates that might confound the effect of the FC in the Cox regression model. Model 1 included baseline FC, age and interaction term of FC×time. Model 2 included time-variant FC, age, sex and interaction term of FC×time. Model 3 included time-variant FC, age, sex, interaction term of FC×time, and adjustment for medical treatments (oral corticosteroids, local corticosteroids, oral sulfasalazine, oral 5-aminosalicylic acid, local 5-aminosalicylic acid, immunomodulators, and anti-TNF therapy).

The analysis of linear mixed models, with allowance for dependence of FC values within the study individual and diagnosis, was used to assess the influence of lifestyle factors, disease activity variables, and current treatments on the FC levels.

Calculations were performed using SPSS statistical software (Version 15.0, Chicago, SPSS Inc.)
Results

Paper I

Incidence rates by five-year periods

In total, 535 patients were diagnosed with Crohn’s disease within the study area between 1963 and 2010; 1963-1987 (n=225) and 1988-2010 (n=310). Crude and age-standardised incidence rates increased from 2.3 per 100 000 in 1963-1965 to 6.7 per 100 000 in 2006-2010.

<table>
<thead>
<tr>
<th>Period</th>
<th>Median age at diagnosis (years)</th>
<th>Number of CD cases</th>
<th>Crude IR (95% CI)</th>
<th>Age-standardized IR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1963-1965</td>
<td>20.5</td>
<td>8</td>
<td>2.3 (0.1 - 4.5)</td>
<td>2.1 (0.6 – 3.6)</td>
</tr>
<tr>
<td>1966-1970</td>
<td>23</td>
<td>36</td>
<td>5.0 (3.5-7.0)</td>
<td>4.6 (3.1 -6.1)</td>
</tr>
<tr>
<td>1971-1975</td>
<td>27</td>
<td>35</td>
<td>4.3 (3.0-6.0)</td>
<td>4.3 (2.9-5.8)</td>
</tr>
<tr>
<td>1976-1980</td>
<td>26</td>
<td>57</td>
<td>7.0 (5.3-9.0)</td>
<td>6.9 (5.1-8.7)</td>
</tr>
<tr>
<td>1981-1985</td>
<td>32</td>
<td>62</td>
<td>7.6 (5.8-9.7)</td>
<td>7.6 (5.7-9.5)</td>
</tr>
<tr>
<td>1986-1990</td>
<td>40.5</td>
<td>56</td>
<td>6.7 (5.1-8.8)</td>
<td>6.7 (4.9-8.5)</td>
</tr>
<tr>
<td>1991-1995</td>
<td>33</td>
<td>67</td>
<td>7.8 (6.0-9.9)</td>
<td>7.7 (5.8-9.6)</td>
</tr>
<tr>
<td>1996-2000</td>
<td>41.5</td>
<td>62</td>
<td>7.0 (5.4-9.0)</td>
<td>7.1 (5.3-8.8)</td>
</tr>
<tr>
<td>2001-2005</td>
<td>41.5</td>
<td>90</td>
<td>10.0 (8.1-12.3)</td>
<td>9.9 (7.8-11.9)</td>
</tr>
<tr>
<td>2006-2010</td>
<td>27</td>
<td>62</td>
<td>6.7 (5.1-8.5)</td>
<td>6.7 (5.0-8.3)</td>
</tr>
</tbody>
</table>

*Table 2. Crude and age standardized incidence rates of Crohn’s disease per 100 000 inhabitants with 95% CI aggregated over 5-year periods by direct method.*
The sex distribution over these five-year intervals was not different in the patient population, however the age distribution varied significantly (p<0.0001). Median age at diagnosis increased with time, and the incidence was generally increasing.

![Figure 6. Age-specific mean annual incidence rate of Crohn’s disease by periods 1963-1987 and 1988-2010.](image)

Trend analysis, using Poisson regression models, revealed statistically significant linear and quadratic trends with increasing incidence rate estimates over time, although the estimates successively levelled off. A SaTScan model revealed a statistically significant high incidence during 1991-2010 (p=0.0001).

**Disease phenotypes at diagnosis**

Information regarding disease location and behaviour at diagnosis was available in 534 (99.8%) and 532 (99.4%) patients, respectively. The proportion of patients with A3 category (≥40 years old) increased from 0% in 1963-1965 to 32.3% in 2005-2010 (p<0.0001), (Figure 7 panel a). Disease location at diagnosis in the five-year study intervals appears rather
stable during the 47-year period (p=0.086), especially during the later study period (Figure 7, panel b). The proportion of patients with non-stricturing, non-penetrating disease at diagnosis (B1) increased from 12.5% in 1963-1965 to 82.3% in 2006-2010 (p<0.0001) (Figure 7, panel c).

![Figure 7. Disease phenotype at diagnosis 1963-2010. A1; below ≤16 years old, A2; between 17 and 40 years old, A3; above ≥40 years old, L1; ileal, L2; colonic, L3; ileocolonic, L4; isolated upper gastrointestinal, B1; non-stricturing non-penetrating, B2; stricturing, B3; penetrating.](image)

**Point prevalence by five-year periods**
The point prevalence of Crohn’s disease gradually increased in the Örebro uptake area from 21 per 100 000 (95% CI, 14-32) on 31 December 1965 to 267 per 100 000 (244-291) on 31 December 2010. On 31 December 2010, 506 patients with Crohn’s disease lived in the area, and the corresponding point prevalence figures for men and women were 236 per 100 000 (206-269) and 297 per 100-000 (263-333), respectively.
Figure 8. Age standardised incidence rate and point prevalence with 95% confidence interval for the study period 1963-2010.

Paper II

Change in Crohn’s disease location and behaviour
The study was restricted to 472 patients within the primary uptake area of Örebro University Hospital diagnosed with Crohn’s disease during the period from 1 January 1963 to 31 December 2005 to allow for a complete follow-up period of five years for most of the study individuals. Information on disease location and behaviour at diagnosis and follow-up was available for 469 (99.4%) patients.

Among patients with ileal (L1) or colonic (L2) disease at diagnosis, 18.0% (95%CI, 10.1-30.9) had progressed to ileocolonic disease (L3) at the five-year follow-up in patients diagnosed 1963-1975; the percentage
was 3.9% (95%CI, 1.7-9.2) in patients diagnosed 1976-1990; and 6.1% (95%CI, 3.3-11.1) in patients diagnosed 1991-2005 (p=0.002).

The proportion of patients with complicated disease behaviour at five years decreased from 54.4% (95%CI, 43.9-65.6) in patients diagnosed 1963-1975 to 44.2% (95%CI, 37.2-51.9) and 33.3% (95%CI, 27.4-40.0) in patients diagnosed 1976-1990 and 1991-2005, respectively (p=0.002).

In patients with non-stricturing, non-penetrating (B1) disease at diagnosis, no difference in the proportion of patients who progressed to complicated disease behaviour (B2 and/or B3) was observed (p=0.435).

**Figure 9. Progression of disease behaviour in patients with non-stricturing non-penetrating disease at diagnosis in the cohorts.** (a) All patients irrespective of disease behaviour at diagnosis. (b) Patients with non-stricturing, non-penetrating disease at diagnosis.

**Medication**
The proportion of patients who received treatment with 5-aminosalicylic acids in the patients diagnosed 1963-2005 was 50.8% (95%CI, 46.4-55.5)
at five years after diagnosis. No notable difference in five-year cumulative probability of treatment with 5-aminosalicylic acids was observed between patients diagnosed 1963-1975, 1976-1990 and 1991-2005.

Long-term corticosteroid treatment, defined as any systemic corticosteroid therapy for one year or longer during which there was no more than eight weeks break before restarting, was used in 6.3% of patients diagnosed 1963-1975, in 8.0% of patients diagnosed 1976-1990, and in 5.9% of patients diagnosed 1991-2005 (p=0.710).

The proportion of patients exposed to immunomodulators at five years from diagnosis increased from 7.6% (95%CI, 3.5-16.1) and 7.2% (95%CI, 4.2-12.4) in patients diagnosed 1963-1975 and 1976-1990, respectively, to 37.7% (95%CI 31.6-44.7) in patients diagnosed 1991-2005 (p<0.001). The median time to initiation of immunomodulators in those treated with the drug within five years from diagnosis was 2.2 years (IQR 1.0-2.9), 2.0 years (IQR 1.2-3.3) and 0.6 years (IQR 0.1-1.7) in patients diagnosed 1963-1975, 1976-1990 and 1991-2005, respectively.

Figure 10. The proportion of patients treated with immunomodulators by cohort.
Among patients diagnosed 2000-2005 (n=104), the proportion treated with anti-TNF therapy was 2.0% (95% CI, 0.5-7.6) at one year and 8.4% (95% CI, 4.3-16.1) at five years after diagnosis.

**Surgery**

At five years after diagnosis, 196/472 (41.5%) of patients had undergone surgery related to Crohn’s disease. In 70 patients (14.8%), Crohn’s disease was diagnosed at the first surgical procedure. The proportion of patients undergoing Crohn’s disease related surgery within five years from diagnosis decreased from 65.5% (95% CI, 55.4-76.0) in patients diagnosed 1963-1975 to 41.4% (95% CI, 34.4-49.2) and 34.6% (95% CI, 28.6-41.5) in patients diagnosed 1976-1990 and 1991-2005, respectively. The decrease in surgery was due to a decrease in the proportion of ileocecal resections and other resections (primarily small-bowel resections and hemicolectomies), while the proportion of patients undergoing colectomy remained stable. The decrease in surgery was largely explained by a decrease in early surgery, since the cumulative probability of surgery at three months after diagnosis dropped from 34.2% (95% CI, 24.9-45.8) in patients diagnosed in 1963-1975 to 24.5% (95% CI, 18.9-31.7) and 14.7% (95% CI, 10.6-20.1) in patients diagnosed in 1976-1990 and 1991-2005, respectively (p=0.001).
Figure 11. The proportion of patients who underwent Crohn’s disease related surgery in the cohorts.

Paper III

Twins
In both the colonoscopy and FC-based studies, 45 twin pairs participated, and an additional 34 twins participated in the FC-based study only. In total, 124 stool samples were analysed for FC.

Histology
In the study, 23 of 28 twins (82%) with Crohn’s disease and 17 of 18 twins (94%) with ulcerative colitis were in histological remission. In addition, 17 of 18 healthy twin siblings (94%) in discordant pairs with Crohn’s disease, 16 of 16 healthy twin siblings (100%) in discordant pairs with ulcerative colitis, and all the controls were histologically normal.
**NFκB and MPO status**

Increased NFκB and MPO activity was more often detected in healthy twin siblings in twin pairs discordant for Crohn’s disease than in healthy controls. In twin pairs discordant for ulcerative colitis, healthy twin siblings had increased NFκB activity, but the MPO staining did not differ significantly.

The differences in NFκB activity between healthy twin siblings and controls remained when monozygotic and dizygotic twins were analysed separately. NFκB was increased in 6 of 10 healthy twin siblings in discordant monozygotic and in 7 of 8 discordant dizygotic twin pairs with Crohn’s disease, [OR 6.7 (1.2-40.2)] and [OR 29.5 (3.2-1496)], respectively. Similarly, NFκB was increased in 5 of 9 healthy monozygotic and in 7 of 7 healthy dizygotic twin siblings in discordant pairs with ulcerative colitis, [OR 5.5 (1.0-35.0)] and [OR 38.1 (5.1-∞)], respectively.

The increased activity of MPO in healthy twin siblings in discordant twin pairs with Crohn’s disease appeared to be independent of zygosity as well, since MPO seemed to be increased in both healthy monozygotic and healthy dizygotic twin siblings to twins with Crohn’s disease, [OR 11.7 (1.9-129.1)] and [OR 3.0 (0.9-1730)], respectively. MPO in healthy twin siblings in discordant monozygotic and discordant dizygotic twin pairs with ulcerative colitis did not significantly differ from controls, [OR 3.8 (0.7-22.6)] and [OR 0.5 (0.0-5.1)], respectively.

NFκB and MPO status in the rectum were correlated to their status in the ascending colon (Spearman’s rho, 0.941, P<0.0001) and (0.325, P<0.0001), respectively.
<table>
<thead>
<tr>
<th>Groups compared</th>
<th>NFκB Low grade, n (%)</th>
<th>NFκB High grade, n (%)</th>
<th>OR (95% CI)</th>
<th>MPO Low grade, n (%)</th>
<th>MPO High grade, n (%)</th>
<th>OR (95% CI)</th>
<th>FC&lt;50 n(%)</th>
<th>FC≥50 n(%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>37 (82)</td>
<td>8 (18)</td>
<td>1.0 (reference)</td>
<td>34 (76)</td>
<td>11 (24)</td>
<td>1.0 (reference)</td>
<td>25 (81)</td>
<td>6 (19)</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>CD</td>
<td>5 (18)</td>
<td>23 (82)</td>
<td>20.0 (5.5-89.9)</td>
<td>13 (46)</td>
<td>15 (54)</td>
<td>3.5 (1.2-11.0)</td>
<td>8 (17)</td>
<td>39 (83)</td>
<td>19.2 (5.6-78.4)</td>
</tr>
<tr>
<td>UC</td>
<td>1 (6)</td>
<td>17 (94)</td>
<td>71.2 (9.0-3369)</td>
<td>8 (44)</td>
<td>10 (56)</td>
<td>3.8 (1.0-14.3)</td>
<td>7 (29)</td>
<td>17 (71)</td>
<td>9.6 (2.5-43.0)</td>
</tr>
<tr>
<td>HCD</td>
<td>5 (28)</td>
<td>13 (73)</td>
<td>11.4 (2.9-54.0)</td>
<td>6 (33)</td>
<td>12 (67)</td>
<td>6.0 (1.6-24.6)</td>
<td>20 (63)</td>
<td>12 (37)</td>
<td>2.7 (0.8-10.2)</td>
</tr>
<tr>
<td>HUC</td>
<td>4 (25)</td>
<td>12 (75)</td>
<td>13.1 (3.0-71.2)</td>
<td>10 (62)</td>
<td>6 (38)</td>
<td>1.8 (0.4-7.3)</td>
<td>7 (33)</td>
<td>14 (67)</td>
<td>7.9 (2.0-36.3)</td>
</tr>
<tr>
<td>MZ HCD</td>
<td>4 (40)</td>
<td>6 (60)</td>
<td>6.7 (1.2-40.2)</td>
<td>2 (20)</td>
<td>8 (80)</td>
<td>11.7 (1.9-129.1)</td>
<td>8 (53)</td>
<td>7 (47)</td>
<td>3.5 (0.8-17.3)</td>
</tr>
<tr>
<td>DZ HCD</td>
<td>1 (12)</td>
<td>7 (88)</td>
<td>29.5 (3.2-1495)</td>
<td>4 (50)</td>
<td>4 (50)</td>
<td>3.0 (0.9-1730)</td>
<td>12 (71)</td>
<td>5 (29)</td>
<td>1.7 (0.3-8.3)</td>
</tr>
<tr>
<td>MZ HUC</td>
<td>4 (44)</td>
<td>5 (56)</td>
<td>5.5 (1.0-35.0)</td>
<td>4 (44)</td>
<td>5 (56)</td>
<td>3.8 (0.7-22.6)</td>
<td>4 (36)</td>
<td>7 (64)</td>
<td>6.9 (1.3-44.1)</td>
</tr>
<tr>
<td>DZ HUC</td>
<td>0 (0)</td>
<td>7 (100)</td>
<td>38.1 (5.1-∞)</td>
<td>6 (86)</td>
<td>1 (14)</td>
<td>0.5 (0.0-5.1)</td>
<td>3 (30)</td>
<td>7 (70)</td>
<td>9.0 (1.5-71.2)</td>
</tr>
</tbody>
</table>

Table 3. NFκB and MPO activity in ascending colon, and FC levels in faeces, OR and corresponding 95% CI from logistic regression models adapted for small samples. CD, twin siblings with Crohn’s disease; UC, twin siblings with ulcerative colitis; HCD, healthy twin siblings in discordant twin pairs with Crohn’s disease; HUC, healthy twin siblings in discordant twin pairs with ulcerative colitis;
Faecal calprotectin status and levels

Faecal calprotectin was assessed in the different study groups and compared to the controls. The median concentration of FC was higher in healthy twin siblings in discordant twin pairs with ulcerative colitis compared to controls, 72.0 (<10-317) and 28.0 (<10-225) µg/g, respectively. The median concentration of FC in healthy twin siblings in discordant twin pairs with Crohn’s disease (37.0 (<10-278) µg/g) did not differ significantly from the controls.

A statistically significant increase in FC was observed in 14 of 21 (67%) healthy twin siblings in discordant twin pairs with ulcerative colitis [OR 7.9 (2.0-36.3)]. The observed increase in FC remained when healthy twin siblings in monozygotic and dizygotic twin pairs were analyzed separately. FC was increased in 7 of 11 (64%) healthy twin siblings in discordant monozygotic twin pairs with ulcerative colitis and in 7 of 10 (70%) healthy twin siblings in discordant dizygotic twin pairs with ulcerative colitis, [OR 6.9 (1.3-44.1)] and [OR 9.0 (1.5-71.2)], respectively. FC tended to increase in 12 of 32 (37%) healthy twin siblings in discordant twin pairs with Crohn’s disease, but compared to controls this increase did not reach statistical significance, [OR 2.7 (0.8-10.2)].

The same results were obtained in the regression analysis of mixed-models design with allowance for dependence within the twin pair. The OR for having higher FC in healthy twin siblings compared to controls are elevated and elevated significantly in both monozygotic and dizygotic twins to twins with ulcerative colitis.

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Ratio of means</th>
<th>95% CI for ratio</th>
<th>P-value for testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ HCD</td>
<td>1.45</td>
<td>0.75-2.81</td>
<td>0.27</td>
</tr>
<tr>
<td>DZ HCD</td>
<td>1.04</td>
<td>0.51-2.10</td>
<td>0.91</td>
</tr>
<tr>
<td>MZ HUC</td>
<td>2.36</td>
<td>1.13-4.95</td>
<td>0.02</td>
</tr>
<tr>
<td>DZ HUC</td>
<td>2.68</td>
<td>1.15-6.26</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table 4. Ratio of geometric means for FC between selected groups of twins, MZ and DZ pairs analyzed separately. Estimations from mixed model with type of diagnosis as the only explanatory factor.
Paper IV

Patients
In total, 169 patients were screened, 17 patients declined to participate, and 22 were excluded due to active disease. Of the remaining 130 patients, 26 patients did not provide a faecal sample and were excluded from the analyses. Thus 104 patients entered the analyses and provided 525 faecal samples during the follow-up, 6 samples of which were discarded due to inadequate packaging.

Predictive value of baseline FC measurement
During the 24-month follow-up, 37 (35.6%) patients relapsed. The risk of relapse was increased by 47% (HR: 1.47; 95% CI: 1.09-1.99, p=0.012) per unit increase in log2-transformed baseline FC (model 1). In other words, a doubling of the concentration of FC at baseline resulted in a 47% increased risk of clinical relapse during follow-up. Inclusion of age as a predictor of relapse in the model did not change the results. A significant interaction between time until relapse and baseline FC was observed (p=0.017), which indicates that the proportional hazard assumption does not hold. In other words, the hazard ratio of the baseline FC changed across time.

Predictive value of the consecutive FC measurement over time
Serial estimations of FC every third month revealed stable concentrations in the patients that remained in remission throughout the follow-up (Figure 12). In patients with a clinical relapse, the concentration of FC at the time of relapse (t=0) was significantly elevated (513 µg/g (231-1173)) compared to those in sustained remission (162 µg/g (53-264); p<0.0001). At three months prior to relapse, the concentration of FC was 305 µg/g (72-827) compared to 197 µg/g (51-468) in patients in remission; but the difference was not statistically significant (p=0.12).
Figure 12. Serial estimations of FC in patients with Crohn’s disease or ulcerative colitis: time 0 refers to the end of study period or time of relapse. Data are shown as boxes (median and IQR), separately for patients with relapse and for patients with sustained clinical remission during the study period.

To investigate the effect of time-variant FC, we treated FC as a segmented time-variant variable in the extended Cox regression model (model 2). A 101% increased risk of relapse was observed per unit increase of log2-transformed FC (HR: 2.01; 95% CI: 1.53-2.65; p<0.001). There was an interaction between FC and time (HR: 0.80; 95% CI: 0.75-0.86; p<0.001) corresponding to a 20% decreased risk of relapse in relation to concentration of FC per unit of time, that is, per three-month period since the sample was obtained. In other words, the most recent FC had the most predictive value. The risk of relapse and the time interaction remained significant when the analysis was performed for CD and UC patients separately, (HR: 
2.37; 95% CI: 1.46-3.87; p<0.001) and (HR: 1.57; 95% CI: 1.11-2.23; p=0.012), respectively.

To explore whether FC levels were systematically confounded by any additional variables other than those included in model 2 (i.e. IBD therapy, patient characteristics, patient reported outcomes, diet, and the use of antibiotics or NSAIDs), we used analysis of mixed-models design to allow for dependence of FC within a diagnosis and an individual. We found that male sex and poor general well-being were associated with higher FC levels, (p=0.03) and (p<0.001), respectively.

We decided to validate our results further by adding the treatment options (i.e. oral or topical 5-ASA, oral or topical corticosteroid treatment, immunomodulators or anti-TNF) as covariates to the extended Cox regression model (model 3). As expected, the associations remained significant and very similar HRs were observed when the model was adjusted for treatments.
Table 5. Extended Cox regression of predictors of clinical relapse.

<table>
<thead>
<tr>
<th>Model 1</th>
<th>Predictors</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline FC</td>
<td>1.47</td>
<td>1.09-1.99</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.99</td>
<td>0.97-1.02</td>
<td>0.66</td>
</tr>
<tr>
<td></td>
<td>Baseline FC×Time (in 3 months intervals)</td>
<td>0.001</td>
<td>0.0-0.19</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model 2</th>
<th>Predictors</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>2.01</td>
<td>1.53-2.65</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>1.01</td>
<td>0.98-1.04</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1.40</td>
<td>0.56-3.47</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>Calprotectin×Time (in 3 months intervals)</td>
<td>0.80</td>
<td>0.75-0.86</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Model 3</th>
<th>Predictors</th>
<th>HR</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>2.17</td>
<td>1.57-2.99</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>1.01</td>
<td>0.97-1.01</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>Sex</td>
<td>1.41</td>
<td>0.52-3.78</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Oral steroids</td>
<td>1.16</td>
<td>0.40-3.33</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>Topical steroids</td>
<td>2.61</td>
<td>0.26-26.01</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Oral 5-ASA</td>
<td>1.48</td>
<td>0.60-3.69</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Oral sulfasalazine</td>
<td>1.54</td>
<td>0.49-4.84</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>Topical 5-ASA</td>
<td>0.82</td>
<td>0.08-8.11</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>Immunomodulator</td>
<td>1.70</td>
<td>0.70-4.14</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Anti-TNF therapy</td>
<td>0.98</td>
<td>0.08-12.50</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Calprotectin×Time (in 3 months intervals)</td>
<td>0.79</td>
<td>0.73-0.85</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
General discussion

Epidemiology of Crohn’s disease

Epidemiologic studies of Crohn’s disease are important in several aspects. They provide a clinician with knowledge of the natural history of the disease. They can also provide insight into its cause and prevention, enhance understanding of disease pathophysiology, and help to find an effective treatment. On a population level, epidemiology provides knowledge of the disease burden to society and produces grounds for rational resource allocation.

The reason for the increasing incidence of IBD and Crohn’s disease, particularly in the Western world, was thought to be the interplay of genetic susceptibility and environmental factors, mainly due to increased economic welfare in a short period. In Study I, the epidemiological study of Crohn’s disease in Örebro 1963-2010, we found that disease incidence seemed to increase. The most probable explanation for this finding is that trigger(s) for Crohn’s disease may still be active despite a stable genetic susceptibility profile and a quite stable level of economic welfare. This interpretation has some possible pitfalls though. Increased disease awareness, which definitely has occurred during this long study period, might have led to earlier diagnoses. However, information on the date of onset of symptoms was incomplete, particularly for patients diagnosed during the earlier years, due to the retrospective nature of the study, thus diagnostic delay could not be analysed. Improved diagnostic methods, like colonoscopy, magnetic resonance imaging, computed tomography, bowel ultrasound, and capsule enteroscopy have been introduced and replaced barium X-ray of the large and small bowel. In general, radiological examinations underestimate mild disease activity compared with endoscopy and might have led to a general underestimation of disease incidence in the earlier years. However, if the effect of new diagnostic modalities on disease diagnostics were substantial, it would have influenced assessment of disease location as well, and this was not seen in our study. Furthermore, the general perception of health might have changed over time and people might seek help at an earlier stage for milder symptoms today than in the past.

The disease incidence shows a possible plateau in 2006-2010, which might portend a possible future decline in incidence figures or could simply be due to the still substantial diagnosis delay. Increased incidence fol-
allowed by a plateau was reported before\textsuperscript{18, 196-199} as well as in the previous study in our uptake area,\textsuperscript{115} pointing out that this phenomenon is not unique. Some proportion of patients diagnosed with IBD-U and UC in the last cohort would be reclassified in the future, increasing the incidence in 2006-2010.\textsuperscript{113, 200} In the prospective Inflammatory Bowel Disease Cohort of the Uppsala region (ICURE) the incidence leveling-off was not observed.\textsuperscript{201}

The proportion of patients with non-stricturing, non-penetrating phenotype (B1) at diagnosis showed an increasing trend in our study. If it can be assumed that non-stricturing, non-penetrating disease is a plausible marker of early disease,\textsuperscript{202, 203} then the increasing proportion of B1 at diagnosis simply reflects the proportion of patients getting their diagnosis earlier in the disease course. The other explanation for our finding would be the changing phenotype of Crohn’s disease. It is possible that Crohn’s disease actually serves as an umbrella term for a number of different diseases\textsuperscript{204, 205} with different trigger factors affecting the incidence of respective disease. The speculated water pathogen\textsuperscript{206} or infection\textsuperscript{207} could be active triggers for stricturing or/and penetrating phenotype, whereas specific dietary patterns (“Westernised diet”) drives more subtle gut microbiome changes, making the microbiome generally more unstable and susceptible to a number of unfavourable changes.\textsuperscript{208} A diseased microbiome, when persistent, may predispose to inflammation in the mucosa, either subclinical or overtly symptomatic. This, in turn, could result predominantly in a diagnosis of non-stricturing, non-penetrating disease as seen in our study. “Westernised diet”, as such, is poorly defined and might include several aspects: an increased quantity of foods consumed (due to an increased level of economic welfare), a change in foods quality (a higher proportion of processed foods and additives consumed), or a different pattern of meal times (foods consumed ad libitum, a higher proportion of individuals skipping breakfast, or increased consumption of snacks and late meals). All those aspects can potentially affect not only the gut microbiome but the status of the gut immune system as well, primarily by halting the autophagy mechanisms.\textsuperscript{209, 210}

We found that range of age at diagnosis is getting wider, and is expanding towards older ages. Keeping in mind that the population structure is getting older, one can suspect that a presumptive disease trigger is probably extrinsic, with all ages susceptible to it.

We found an increasing Crohn’s disease prevalence over time. This was to be expected, given the high incidence, the chronicity of the disease, the
absence of a cure, and long life expectancy. Our figures are higher than recently reported in the register-based study, \(^{27}\) 267 per 100,000 vs. 194 per 100,000, respectively, which might be explained by better case ascertainment in our study. We did not study specifically the societal burden of disease, but in the above-mentioned study the prevalence of actively treated patients with Crohn’s disease (defined as two or more IBD-related visits, of which one occurred in 2010, plus at least one dispensed prescription of IBD-related drugs in 2010) was 79 per 100,000, which gives a proportion of 40.7%.

**Natural history of Crohn’s disease**

At the time of diagnosis, patients and their clinicians need to discuss the future clinical course of the disease. The long-term natural course of the disease in its pure form is difficult to study, as both the medical profession and patients have continuously interfered with it, trying to treat and relieve the consequences of the disease. Gradually, the meaning of “natural course” has changed to “the course of the disease when treated in accordance with established and well-defined treatment policies”. \(^{211}\) This extends the task of clinical epidemiology from simple estimation of the incidence and prevalence to the long-term follow up of cohorts of patients, in order to reveal prognostic evidence that cannot be obtained in any other way. The Örebro University Hospital uptake area is well-suited for this type of study. Its long-term history of referral of all patients with possible IBD to secondary care, the absence of private gastroenterologists, its use of centralized endoscopic and histopathological assessment, and the almost complete inclusion and secured follow-up with a minimum of untraced patients all contribute to this suitability.

We decided to report trends over time rather than associations between specific medications and outcomes, like Crohn’s disease-related surgery or progression in disease behaviour. Such analyses are prone to be influenced by indication bias, where patients with the most severe disease course are those treated most aggressively.

In Study II, we found a decrease over time in the proportion of patients undergoing CD-related surgery within five years from diagnosis. In parallel with the drop in surgery rates, a reduction in the proportion of patients with complicated disease behaviour during follow-up was observed. This reduction could reflect a more aggressive medical approach at diagnosis. However, the difference in surgery rates over time seemed to be driven by a decrease in early surgery (within three months), possibly reflecting the
increasing proportion of patients with non-stricturing non-penetrating disease at diagnosis.\textsuperscript{212} The reduction in surgery preceded the widespread use of immunomodulators; the latter did not happen until the last cohort. In contrast, the five-year cumulative risk of surgery had dropped already in the cohort of patients diagnosed 1976-1990 and was not explained by increased long-term corticosteroid use, because the five-year cumulative probability of long-term corticosteroid treatment remained low during the study period. These findings suggest that the introduction of new treatments, including anti-TNFs, alone do not explain the decreasing surgical rates in Crohn’s disease and that difference in the phenotypic presentation over time may have played an important role.

Interestingly, the relative proportion of patients with non-stricturing, non-penetrating disease at diagnosis who progressed to complicated behaviour was stable during our study period. Complicated disease behaviour is intimately associated with risk of surgery.\textsuperscript{126, 213} Therefore, it seems likely that the observed increasing proportions of patients with non-stricturing, non-penetrating disease at diagnosis explain the decrease in surgery rates and, specifically, the decrease in early surgery. These findings probably reflect the fact that patients are diagnosed earlier in their disease course today than in the past, and that this improves treatment outcomes in terms of surgery at five years after diagnosis. The previously reported association between diagnostic delay and increased risk of surgery supports this hypothesis.\textsuperscript{214} Increased access to specialist care within one year from diagnosis has also been associated with decreasing surgery rates over time\textsuperscript{215} and might also have contributed to the decrease in surgery rates, since the number of specialists increased during the study period (data not shown). Older age at diagnosis has also been associated with a milder disease and less need for surgery.\textsuperscript{216} Therefore, the increase in non-stricturing, non-penetrating disease at diagnosis and the observed decrease in surgery within five years from diagnosis might partially be related to an aging population structure.

Differences in disease course illustrate the need for monitoring disease activity, since it varies from patient to patient. Faecal calprotectin (FC) is one of established markers of inflammation in the gastrointestinal tract.

**Biological perspective of faecal calprotectin**

The Swedish twin cohort with IBD has been studied earlier concerning various clinical and pathophysiologic aspects of IBD.\textsuperscript{54, 192, 217-230} In Study III, we analysed FC in this population of concordant or discordant,
monozygotic or dizygotic twins of the same sex with Crohn’s disease or ulcerative colitis. We complemented FC analysis by histological and immunohistochemical studies (NFκB and MPO) of colon biopsies. Despite the fact that all healthy twin siblings to twins with IBD were clinically healthy and almost all were histologically non-inflamed, we found subclinical inflammation shown by analysis of NFκB, FC, and MPO.

Increased NFκB activity was observed in 74% of these healthy twin siblings. The observed increased NFκB activity remained when monozygotic and dizygotic pairs were analysed separately. Increased NFκB activity was observed in healthy twin siblings in both discordant monozygotic (60%) and discordant dizygotic pairs with CD (88%). Correspondingly, 56% of healthy twin siblings in discordant monozygotic and 100% of discordant dizygotic twin pairs with UC had increased NFκB activity.

Consistently, FC was increased in both monozygotic and dizygotic healthy twin siblings in discordant twin pairs with UC. The difference was significant irrespective of whether FC was analysed as a categorical parameter or as a continuous parameter.

Increased neutrophil activity, as reflected by MPO, was also observed in healthy twin siblings in discordant twin pairs with CD and appeared to be independent of zygosity, since MPO seemed to be increased in both monozygotic (80%) and dizygotic (100%) discordant pairs.

Similarly, the earlier reported increased proportion of immunoglobulin G1 producing immunocytes in rectal biopsies of discordant monozygotic twins pairs with UC,219 and the altered mucin glycoprotein profiles in discordant monozygotic pairs with UC217 and CD,224 further support the hypothesis of an ongoing subclinical mucosal inflammation at the molecular level in healthy first-degree relatives of patients with IBD.

The influence of shared environment during childhood and adolescence is equal in monozygotic and dizygotic twin pairs in contrast to the influence of genetics, which is more pronounced in monozygotic pairs. Subclinical inflammation with neutrophil activity was observed in healthy twin siblings in both discordant monozygotic and discordant dizygotic pairs. Thus, these findings point toward the importance of environmental factors rather than genetics alone in the aetiology of this subclinical inflammation. In general, twins in each twin pair were separated around the age of 20 years. Thus, environmental factors driving the subclinical inflammation seem to interact early in life, during childhood or adolescence. The data raise the very important clinical question whether the degree of subclinical
inflammation in first-degree relatives is associated with future risk of manifest IBD.

**Faecal calprotectin for the prediction of IBD relapse**

Increased FC and associated subclinical inflammation in healthy unaffected relatives might reflect non-genetic influences of environment on the occurrence of inflammatory response in the GI tract. We turned to the clinical aspect of FC and decided to investigate whether FC as a marker of subclinical mucosal inflammation can be used to monitor patients with IBD in remission and whether FC can be useful in predicting relapse.

In Study IV, a 101% increased risk of clinical relapse in the following three months was observed per unit increase of log2-transformed consecutive FC measurements. An interaction between FC and time was also observed, corresponding to a 20% decreased risk of clinical relapse per each three-month period after the sample was obtained. The associations between risk of relapse and concentration of FC, as well as the duration since obtaining of the faecal sample, remained significant when Crohn’s disease and ulcerative colitis patients were analysed separately.

The variability of FC differs depending on disease activity, stool consistency, and time between bowel movements. There is a significant positive relationship between FC and increasing age, obesity, and physical inactivity, in addition to an inverse relationship with fiber intake and vegetable consumption. Furthermore, the predictive value of FC might differ in patients maintained on immunosuppressive therapy from those on anti-TNF. Therefore, we explored the influence of patient characteristics, patient reported outcome measures, use of antibiotics or NSAIDs, as well as dietary effects on FC in our dataset. Increased concentrations of FC were observed in patients with poor daily rating of quality of life. However, independent associations with other dimensions of patient reported outcomes, like the number of bowel movements or blood in stool, could not be statistically confirmed for the entire IBD population.

The longitudinal monitoring of FC levels is practical and feasible despite the unpleasantness of sample collection and the difficulties that some patients experienced in keeping up submitting samples, which were more pronounced for younger patients. Recently, Lasson et al. found that FC levels could be used to identify patients with ulcerative colitis at risk for a flare, and that a dose escalation of their 5ASA agent is a therapeutic option for these patients.
Our data suggest that longitudinal monitoring of FC is informative to predict relapse in IBD. By consecutive measurement of FC every third month, we quantified the risk of relapse related to FC change and observed attenuation of the risk across time.

**Strengths and limitations**

**Paper I and II**

This study was retrospective in its design but was based on prospectively gathered data. The methodology of prospective and retrospective cohort studies is fundamentally the same, but the retrospective study is performed post hoc, as the cohort is followed retrospectively. The huge advantage of retrospective study is that the time it takes to complete is only as long as it takes to collect and interpret the data. Unfortunately, confounding and bias are more common in a retrospective study. We cannot control exposure or outcome assessment but instead need to rely on others for accurate record keeping; moreover, temporal relationships are frequently difficult to assess. Information bias (misclassification) results from systematic differences in the way data on exposure or outcome is obtained from the various study groups. Treatments might be less accurately documented in earlier medical notes. Prescription dates do not necessarily reflect drug intake; therefore, start and termination dates might reflect approximate dates. Changes in disease extent may not be accurate, especially if repeat assessment through objective measures, such as imaging and endoscopy, was not performed routinely for all subjects.

Reporting bias is present where an individual with severe disease tends to have complete records and, therefore, more complete information about exposures and a greater association is found. Selection bias occurs if subjects in one of the exposure groups are more or less likely to be selected if they had the outcome of interest. All diagnoses were reassessed to reduce the risk of bias due to historical differences in diagnostic assessment. However, differential bias by period might still have occurred, since the perception of symptoms qualifying for referral to the Department of Gastroenterology might have differed over time. Completeness in registration of patients with Crohn’s disease may differ between the cohorts because a computerized search of records has only been available since 1988.

We could not identify individual patients from the previous study (raw data are no longer available). Furthermore, different diagnostic criteria were applied during the older study, Garland vs. Lennard-Jones. In short,
the criteria of Garland classify patients into definite, probable, and possible Crohn's disease: depending on findings for histology, endoscopy, and radiology, as well as the discharge diagnosis; where only definite and probable diagnoses were included in our previous study. To our knowledge, there are no direct comparisons between the criteria of Garland and the Lennard-Jones criteria. However, definite and probable Crohn's disease according to the criteria of Garland seem to depend more on histological and surgical findings, paying less attention to the overall clinical picture, compared to the Lennard–Jones criteria. Therefore, we re-evaluated all the diagnoses for the whole study period.

The strengths of our study include the strict population-based design, allowing for almost complete follow-up, and the thorough scrutiny of each patient’s medical notes, providing information on disease phenotype, as well as medical and surgical history at an individual level.

**Paper III**

There were a limited number of enrolled twin pairs, resulting in a wide 95%CI associated with results in our study. Even though twins were recruited through linkage of national population-based registries, only 45 IBD twin pairs were enrolled and underwent colonoscopy. A substantial number of pairs were excluded due to previous extensive IBD-related surgical resections in the diseased twins. The overall participation in the FC-based study was 60% (124/208), probably due to the unpleasantness of collecting the sample.

We had to carry on with different control groups for the FC study and for colonoscopy-based analysis. For FC controls, we used healthy volunteers (lab personnel) and for colonoscopy-based analysis we used age and sex matched endoscopy department referrals (functional GI disorders, polyp screening).

There is no guarantee that our FC control group of healthy volunteers did not have undiagnosed GI disorders, which might have given us some abnormal FC values in that group. That could affect the statistical significance of some results.

**Internal validity**

Internal validity is a property of scientific studies that reflects the extent to which a study minimises systematic error (bias).
As our twin study is non-interventional, the internal validity is not affected by a response or recall bias. The selection bias that might apply here is already reduced by measures applied at the time of establishing the Swedish twin cohort with IBD (i.e. running two registries together as opposed to recruitment by advertisement). Zygosity determination in the Swedish twin registry was based on questions asked of the twins about their level of physical similarity and about how often people confused them when they were growing up. While a biological assay is the gold standard for determining zygosity, the validity of zygosity measures based on survey questions is high. Furthermore, since zygosity appears to be unrelated to any confounding variables, twin studies seem to have high internal validity.

**External validity**

External validity is the extent to which the results of a study can be generalised to other situations and to other people.

We can be confident that twin studies reveal the extent to which variation in twin outcome can be attributed to variation in genetics. But there is some uncertainty about the extent to which the results of twin studies can be extrapolated to the non-twin population.

According to the equal environments assumption (EEA), the environments of MZ twins are not more similar than the environments of DZ twins in any way that causes MZ twins to turn out to be more similar to each other than DZ twins turn out. Some twin researchers have argued that they do not need to control for environmental similarity because previous studies have found evidence supporting the EEA.

Nonetheless, identical twins receive more similar treatment than non-identical twins. Moreover, identical twins spend more time together than non-identical twins, especially in the formative early years. These facts do not necessarily pose a problem for twin studies, but may seriously undercut the results of twin studies if the higher levels of similarity observed between identical twins is partly the result of similarity in socialization.

It is recommended that researchers should not conduct twin studies without including control variables for environmental similarity. However, the findings also indicate that many of the results of twin studies are robust with these controls. In Study III, the statistical analysis was not adjusted for environmental similarity, due to variables that were not originally collected and the limited number of twins.
Paper IV

The strength of our study is a prospective and consecutive recruitment of patients in a real-time outpatient setting. The study population was unselected, strengthening the external validity of our results. The presence of formal scheduling of FC monitoring and clinical decisions blinded to FC levels reduced the relapse/outcome detection bias. The observation that the results remained significant when adjusting for possible confounders like IBD therapy, patient characteristics, diet, and use of antibiotics or NSAIDs, further strengthen this study.

The analyses of FC as a continuous variable instead of a categorical cut-off variable constitute a more natural approach to the behaviour of this biomarker. The FC distribution is right-skewed, as most of the blood tests, and statistical analysis had to be carried out on normalised data, which we achieve by log2-transformation. By avoiding dichotomization of FC we kept the power of analysis and accounted for the between-individual variation of FC at the same time. The results are easily applied into clinical practice, as an increase of FC in the same individual two times the previous value translates into an increased risk of relapse by 101% within the following three months. Should an individual not develop a relapse within this period, the increased risk during the subsequent three-month period is 81%, a 20% smaller increase in the risk of relapse.

The weakness of the study is that we did not perform endoscopies to verify relapse, and the definition of relapse is purely clinical. Another weakness is that we did not use a formal definition of disease activity, like a generally accepted disease activity index. The definition of remission is still a matter of debate. It can be defined as mucosal healing or as an endoscopy score. We did not confirm whether our patients were in endoscopic remission at the time of enrolment, and we did not perform a subsample analysis for validation purposes. Another weakness of the study is that the variable reflecting the duration of remission before inclusion was not collected, since the time in remission is associated with time to relapse/flare.

Our patient group is heterogeneous, comprised of Crohn’s disease and ulcerative colitis patients together, regardless of localization, behaviour, and duration, which might have somewhat attenuated our results. Despite that, the predictive value of FC remained statistically significant even when the data had been analysed for Crohn’s disease and ulcerative colitis separately.
General conclusion

An increased incidence and prevalence of Crohn’s disease was seen in the period 1963-2010. Age at diagnosis increased as well in the later study period 1988-2010. The proportion of patients with non-stricturing, non-penetrating disease behaviour at diagnosis increased. This suggests that patients with Crohn’s disease are either diagnosed earlier in their disease course today or that the Crohn’s disease phenotype is changing.

We observed a decrease in complicated disease behaviour, an increased use of immunomodulators, and a reduced frequency of surgical procedures five years after Crohn’s diagnosis. The decrease in surgery at five years seemed to be explained mainly by a decrease in early surgery within three months from diagnosis, likely reflecting an increased proportion of patients with non-stricturing, non-penetrating disease. This suggests that the introduction of new treatment alternatives alone does not explain the reduction in surgery rates, and an increasing proportion of patients with uncomplicated disease at diagnosis may also play an important role.

Subclinical mucosal inflammation, mirrored by increased NFkB activity and increased neutrophil activity (i.e. FC and MPO expression), was observed in healthy twin siblings in both discordant monozygotic and discordant dizygotic twin pairs with IBD. These findings strongly support the hypothesis of an ongoing subclinical mucosal inflammation at the molecular level in healthy first-degree relatives of IBD patients.

Baseline FC as well as consecutive FC measurements predict relapse in IBD. The doubling of FC value increased the risk of relapse by 101% in the following three months. This increased risk attenuates with time by 20% for every three month period since the sample was obtained.
Future perspectives

Further long-term, retrospective studies on ulcerative colitis and IBD-U will elucidate the whole panorama of IBD in our uptake area, and such studies are currently underway.

It is important to follow-up the current retrospective cohort with Crohn’s disease to find out if the levelling of incidence figures during the later study period holds true or is just an effect of patient-doctor delay.

The long-term follow-up in our cohort will allow the study of cancer incidence and death causes in a Crohn’s disease population over time, as well as the safety of Crohn’s disease medications.

The question of the effect of early immunomodulation on disease outcomes in terms of surgery in Crohn’s disease currently could not be answered, but a different study design, with avoidance of confounding by indication, and a larger study group comprising Crohn’s disease and ulcerative colitis patients might be feasible soon.

Studies of health rather than disease constitute a different approach to the field of epidemiology. As some patients with Crohn’s disease go into long-term, spontaneous remission, this urges us to study the possible factors that contribute to that.

IBD is caused by the interplay of a susceptible genetic profile and an unfavorable environment. While some biomarkers in IBD are mostly environmentally determined, others are not. Further studies on biomarkers in IBD are vital to finally disentangle the complex disease aetiology and pathogenesis.

Healthy twins with elevated FC in our cohort were disease free for a median of 23 years since diagnosis in the diseased twin. This supports the prospect that these healthy twins probably will not develop manifest IBD. Whether they acquired some protective factors or avoided environmental factors remains to be shown.

The biologic functions and pathophysiologic role of FC need to be investigated further, especially factors that trigger FC production, since an elevation of FC is observed months before a disease relapse. Here is where studies of microbiota changes in connection to FC elevation would be the next step.

To translate our findings from study IV into a patient benefit, an open, randomized study in an unselected population of Crohn’s disease and ulcerative colitis patients in remission needs to be done.
Acknowledgements

There are many people who helped and supported me during the years of doctoral studies.

I would like to give wholehearted thanks to my supervisor, Doc. Jonas Halfvarson, and co-supervisor, Prof. Curt Tysk. Without your dedication and continuous involvement, this project would not have been possible. I am very grateful for your professional guidance and supportive attitude that helped me grow into a researcher. I admire you for being an endless source of inspiration, for your encouragement and patience, for being flexible, and for always finding time. Many PhD graduations to come!

I am indebted to Ruzan Udumyan, Yang Cao, Scott Montgomery and Anders Magnusson of the Department of Epidemiology and Biostatistics for sharing your statistical and methodological knowledge and for all the help and guidance during this time. Without your guidance the woods of statistics would be too dark to find a way.

I wish to thank Birgitta Borjesson and Linda Götberg for their enormous help with ordering articles and necessary books.

Thank you to Mia Svantesson, research administrator at Karlskoga Hospital, for her supervision and practical consultations during the initial years of this project.

My sincere thanks go to the secretaries at the Gastroenterology Department at Örebro University Hospital: Åsa Ekblom, Birgitta Meijer, and Ann-Britt Löv for their practical help with the retrieval of paper medical notes during data collection in the Colitis archive; much thanks to Mia Lagerroos, Anders Dahl, Michael Lundberg and Mats Granberg for their help with database searches; much thanks to Gun-May Lignell, Carina Emilsson, Pia Gustavsson, Ann-Christin Gustavsson, Kerstin Eriksson, Ulla-Britt Widén, Kristina Holmberg, Gun Einarsson, Ulla Johansson, Anette Wendt, Margaretha Berglund, Christina Figaro and Åsa Johansson for being helpful and supportive during tedious hours in the Colitis archive.

I thank colleagues at the Internal Medicine and Geriatrics clinic at the Karlskoga hospital: Karin Nyborg, Tobias Franz, Julio Loayza, Ahmed Hamza, Martin Ferletta, Gustavo Gutierres, Dia Saleh, Katarina Palm, Jacob Bäckman, Lina Rosenberg, Adina Raicu, Ioannis Vergenelakis and others who have all taken care of the patients in my absence and never complained about my studies.
My warmest gratitude goes to nurses at the Internal Medicine Outpatients Clinic in Karlskoga hospital: Grethe Eriksson, Eveline Weman, Marie Lagerberg, Ingela Ågren, Sofie Nilsson, Lenita Sandström, Carina Svanberg, Ann Johansson, Gerd Skogman and Inga-Lill Molander for your encouragement, optimism, and simply being helpful and positive.

I wish to thank the clinical administrator, Elisabeth Andersson, and the head of the Internal Medicine and Geriatrics clinic, Håkan Lindvall, for their flexibility and for making my work schedule reconcile with the research; and to Lena Adolfsson, director of Karlskoga hospital, for believing in me and this project.

I wish to thank Mr. David Anderson for kindly revising English language of this thesis.

From the bottom of my heart I would like to express gratitude to my nearest family, my husband Tomas and my children Joanna and Björn. Thanks for putting up with me during these years, and for your love and patience.
Populärvetenskaplig sammanfattning

Det övergripande syftet med denna avhandling var att studera förändringar i naturlsförloppet av Crohns sjukdom, fenotyp, behov av farmakologisk terapi och kirurgi över tid; liksom betydelsen av fekal kalprotektin som biomarkör av patofysiologi och sjukdomsförlopp.

En ökad incidens och prevalens av Crohns sjukdom sågs under perioden 1963-2010. Andelen patienter med icke-strikturerande, icke-penetrerande sjukdom vid diagnos har ökat, vilket tyder på att patienter med Crohns sjukdom diagnostiseras tidigare idag än förr; alternativt att fenotyp av Crohns sjukdom förändras med tiden.

I studien observerades en minskning av komplicerad sjukdomsfenotyp, en ökad användning av immunmodulerande läkemedel, och en minskad frekvens av kirurgiska ingrepp fem år efter diagnos. Minskningen av kirurgiska ingrepp vid fem år efter diagnos verkade förklaras huvudsakligen av en minskad tidig kirurgi (inom tre månader från diagnos), som troligen återspeglar en ökad andel av patienter med icke-strikturerande, icke-penetrerande sjukdom. Detta tyder på att införandet av nya behandlingsalternativ inte ensamt kan förklara minskningen av kirurgi. Ökande andel av patienter med icke-komplicerad sjukdom vid diagnos kan också spela en viktig roll.

Subklinisk slemhinneinflammation, som speglas av ökad NFkB aktivitet och ökad neutrofil aktivitet, dvs FC och MPO uttryck, observerades i friska tvillingssykon i både diskordanta monozygota och diskordanta dizygota tvillingpar med inflammatorisk tarmsjukdom. Detta fynd stöder starkt hypotesen om att det finns pågående subklinisk slemhinneinflammation på molekylär nivå hos friska förstagradssläktingar till IBD-patienter, som ter sig ej vara orsakat av enbart genetisk predisposition utan snarare av gemensamma miljöfaktorer under uppväxten.

Genom att i följd måta FC var tredje månad, kvantifierades risken för skov av IBD associerad med förhöjning av FC värde. Samtidigt observerades en minskning av risken över tiden. Ett fördubblat FC värde var associerat med en 101 % ökad risk för skov av IBD inom kommande tremånadersperiod. En interaktion mellan FC och tid observerades också, vilket indikerar att associationen mellan ökningen av FC värde och skovet av IBD avtar över tid. Denna risk dämpas med tiden med 20 % per tremånadersperiod sedan provet erhölls.
References


YAROSLAVA ZHULINA  Crohn's disease


the number of confirmed Crohn's disease susceptibility loci. Nat Genet 2010;42(12):1118-25.


152. Lichtenstein GR, Rutgeerts P. Importance of mucosal healing in ulcerative colitis. Inflamm Bowel Dis 2010;16(2):338-46.


173. Eue I, Sorg C. Arachidonic acid specifically regulates binding of S100A8/9, a heterodimer complex of the S100 class of calcium binding proteins, to human microvascular endothelial cells. Atherosclerosis 2001;154(2):505-8.


Publications in the series
Örebro Studies in Medicine


35. Söderqvist, Fredrik (2009). Health symptoms and potential effects on the blood-brain and blood-cerebrospinal fluid barriers associated with use of wireless telephones.


41. Gustafsson, Sanna Aila (2010). The importance of being thin – Perceived expectations from self and others and the effect on self-evaluation in girls with disordered eating.

42. Johansson, Bengt (2010). Long-term outcome research on PDR brachytherapy with focus on breast, base of tongue and lip cancer.

43. Tina, Elisabet (2010). Biological markers in breast cancer and acute leukaemia with focus on drug resistance.


46. de Leon, Alex (2010). *Effects of Anesthesia on Esophageal Sphincters in Obese Patients.*


52. Loiske, Karin (2011). *Echocardiographic measurements of the heart. With focus on the right ventricle.*


64. Nordin Olsson, Inger (2012). Rational drug treatment in the elderly: "To treat or not to treat”.


67. Thuresson, Marie (2012). The Initial Phase of an Acute Coronary Syndrome. Symptoms, patients’ response to symptoms and opportunity to reduce time to seek care and to increase ambulance use.


75. Gustavsson, Anders (2012): Therapy in Inflammatory Bowel Disease.


83. Lönn, Johanna (2013): The role of periodontitis and hepatocyte growth factor in systemic inflammation.


96. Sundh, Josefin (2013): Quality of life, mortality and exacerbations in COPD.


98. Palmetun Ekbäck, Maria (2013): Hirsutism and Quality of Life with Aspects on Social Support, Anxiety and Depression.


109. Törös, Bianca (2014): Genome-based characterization of Neisseria meningitidis with focus on the emergent serogroup Y disease


120. Pelto-Piri, Veikko (2015): Ethical considerations in psychiatric inpatient care. The ethical landscape in everyday practice as described by staff.


139. Elwin Marie (2016): *Description and measurement of sensory symptoms in autism spectrum.*

140. Östlund Lagerström, Lina (2016): "The gut matters" - an interdisciplinary approach to health and gut function in older adults.