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PATHOPHYSIOLOGICAL AND CLINICAL STUDIES ON CROHN´S DISEASE IN CHILDREN

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"It was only a sunny smile, and little it cost in the giving,
but like morning light it scattered the night
and made the day worth living."

F. Scott Fitzgerald

To my family

ABSTRACT

Crohn´s disease (CD) is often diagnosed in late childhood and early adulthood. Clinical findings include abdominal pain, diarrhea, rectal bleeding, perianal lesions, weight loss and growth retardation. Extra-intestinal manifestations, strictures and fistulas may complicate the disease. The disease course is characterized by a chronic relapsing pattern and disease severity varies between individuals. Environmental, genetic and immunological factors influence the development of CD. The exact mechanisms are not clear, but there are probably individual combinations of aberrations that contribute to the heterogeneity of the disease phenotype. *NOD2/CARD15* gene polymorphisms are reported in up to 60% of CD patients and are associated with ileal disease, more complicated disease behaviour and younger age at onset. The diagnosis of CD is based on clinical, endoscopic and radiological findings. Presence of epithelioid cell granulomas in intestinal biopsies is one, non-compulsory, criterion of CD, but the reason why some patients have granulomas and not others is elusive. The treatment regimen of pediatric CD is moving towards an early, more aggressive approach, aiming at preventing disease progression and complications, such as growth retardation. However, reliable prognostic markers are lacking and it is difficult to predict which patient would benefit most from aggressive treatment.

The aim of this thesis is to broaden the knowledge of genetic, histopathological and clinical factors that may be related to the disease course of CD in children.

In **paper I** the prevalence of and influence from the main *NOD2/CARD15* polymorphisms in Swedish pediatric CD patients is described. *NOD2/CARD15* polymorphisms are uncommon; a single allele variant is present in only 8.6% of the patients and genotype-phenotype correlations are difficult to establish.
In **paper II** the correlation between the location of the disease and the age at onset of CD in children is investigated. Ileal involvement before the age of nine is rare and the probability of ileal disease increases with age until adulthood. Isolated ileitis is seldom found in pediatric CD and, consequently, ileal involvement is most often associated with colonic disease.
In **paper III** the effects of interleukin (IL)-6 and variants of the *IL6* gene on growth is studied in an animal colitis model and in pediatric CD patients. In rats with induced colitis, IL-6 causes growth suppression. Blocking IL-6 with antibodies results in increased IGF-I levels and enhanced growth, but without improvement of the intestinal inflammation or increased food intake among the rats. The children studied with the *IL6* -174 GG genotype, which previously has been associated with higher IL-6 levels, are more growth-retarded at diagnosis compared to other genotypes. This indicates a negative influence on growth as a consequence of a higher expression of IL-6.
In **paper IV** the significance of granuloma findings in biopsies in pediatric CD patients is evaluated. A description of clinical characteristics and growth of a pediatric CD cohort followed into adulthood is also performed. Granulomas are found in half of the patients at onset and are associated with both upper gastrointestinal involvement and a shorter time to initiating immune modulating agents (i.e. thiopurines, methotrexate or anti-TNF-α), suggesting an association with a more aggressive disease. The follow-up (median 12.3 years) shows that this Swedish group of childhood onset CD has more colonic disease and less ileal involvement, and that growth impairment is infrequent both at onset and at follow-up.

This thesis adds to the growing knowledge of the etiological and prognostic factors in CD in children. Due to the complex pathophysiology of CD and the heterogeneity of the phenotype, large-scale, multicenter trials, in which genetic, clinical, serologic and environmental information is combined, would be valuable to accurately determine the risks and prognosis for each afflicted individual. The ultimate goal is to develop personalized therapy strategies, with the aim of improving prognosis and quality of life of patients with this disease.

Keywords: age, colitis, Crohn´s disease, granuloma, growth, ileitis, inflammatory bowel disease, interleukin 6, NOD2, pediatrics, prognosis

LIST OF PUBLICATIONS

This thesis is based on the following publications. The papers will be referred to in the text by their Roman numerals:

I. **Ideström M**, Rubio C, Granath F, Finkel Y, Hugot JP.
CARD15 mutations are rare in Swedish pediatric Crohn disease.
J Pediatr Gastroenterol Nutr. 2005 Apr;40(4):456-60

II. Meinzer U, **Ideström M**, Alberti C, Peuchmaur M, Belarbi N, Bellaiche M, Mougenot JF, Cezard JP, Finkel Y, Hugot JP.
Ileal involvement is age dependent in pediatric Crohn's disease.
Inflamm Bowel Dis. 2005 Jul;11(7):639-44

III. Sawczenko A, Azooz O, Paraszczuk J, **Idestrom M**, Croft NM, Savage MO, Ballinger AB, Sanderson IR.
Intestinal inflammation-induced growth retardation acts through IL-6 in rats and depends on the -174 IL-6 G/C polymorphism in children.
Proc Natl Acad Sci U S A. 2005 Sep 13;102(37):13260-5

IV. **Ideström M**, Rubio C, Onelöv E, Henter JI, Fagerberg UL, Finkel Y.
Pediatric Crohn´s disease from onset to adulthood: granulomas may predict a more aggressive disease but growth is not impaired.
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

5-ASA	5-Aminosalicylates
ATG16L1	Autophagy related 16-like 1
BMI	Body mass index
CARD	C-terminal caspase recruitment domain
CD	Crohn´s disease
CRP	C-reactive protein
DAMP	Damage associated molecular pattern
DNA	Deoxyribonucleic acid
EEN	Exclusive enteral nutrition
ESPGHAN	European Society for Paediatric Gastroenterology, Hepatology and Nutrition
GALT	Gut associated lymphoid tissue
GH	Growth hormone
GI	Gastrointestinal
IBD	Inflammatory bowel disease
IBDU	IBD unclassified
IC	Indeterminate colitis
IFN	Interferon
IGFBP-3	IGF-I-binding protein-3
IGF-I	Insuline-like growth factor-I
IL	Interleukin
IRGM	Immunity-related GTPase family M
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
MHC	Major histocompatibility complex
MUC	Mucine
NF-κB	Nuclear factor kappa beta
NOD	Nucleotide oligomerization binding domain
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen associated molecular pattern
PP	Peyer´s patches
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SDS	Standard deviation score
SNP	Single nucleotide polymorphism
TGF	Transforming growth factor
Th	T helper cell
TLR	Toll like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumor necrosis factor
TNFRSF	Tumor necrosis factor receptor super family
TNFSF	Tumor necrosis factor super family
UC	Ulcerative colitis

FOREWORD

I have more than once during my years working as a pediatrician been fascinated by the seemingly inexhaustible strength and healing ability among the children, and I have also been hit by their positive attitude and spontaneity. On the other hand, I have many times been concerned about the situations and conditions their diseases have put them in. Unfortunately, I have also been brutally reminded of the vulnerability of the small and developing child.

In the field of pediatric gastroenterology and nutrition I meet children and adolescents in all ages from new-born babies up to the age of 18. It is thrilling and demanding but also an enormous privilege to follow these young people up to adulthood.

Crohn´s disease, a chronic inflammatory bowel disease, is a life-long illness, but not necessarily with life-long symptoms, which can be difficult and disabling for those who are affected. It often strikes them in a very sensitive age, physically, socially and psychologically. The fact that the pathogenesis is unclear and the course unpredictable, as well as the nature of the symptoms (with a frequent need of a bathroom), the relapsing course of the disease and the uncertainty of when the next relapse will appear, offers difficulties to caregivers, children and their parents. Moreover, it may be time consuming and distressing for both children and parents to have frequent doctor visits and blood sampling as well as continuous medication, which itself may be associated with side effects and long term risks. For me, as a doctor, it is frustrating not being able to provide more information about the future, and not knowing who would benefit from aggressive treatment right from the start.

My idea and wish when I started working on this thesis on Crohn´s disease in children was to find out more about the disease, the factors that influence the disease course and, if possible, also find early markers for predicting the prognosis since this could help both me and my colleagues as well as the children.

STOCKHOLM, MARCH 2012

Maja Ideström

1 INTRODUCTION

The Nobel Prize in Physiology or Medicine 2011 was recently awarded with one half equally to Jules A. Hoffmann and Bruce A. Beutler for their discoveries concerning the activation of innate immunity and the other half to Ralph M. Steinman for his discovery of the dendritic cell and its role in adaptive immunity.[1] They have revealed how the innate and adaptive immune responses are activated and thereby provided novel insights into disease mechanisms.[2,3,4] Thanks to their work, it has been possible to do subsequent work on the development of therapy against infections, cancer, and inflammatory diseases.

An immune system is necessary for defending ourselves against intruders and pathogens, and the response itself, causes an inflammatory process. With an immune system that is dysfunctioning we can develop a variety of diseases.[5] A total non-functioning immune system is not associated with life, malfunction gives problems with repeated difficult infections, and if the system is not properly configured or balanced, there will be reactions towards "self" as in autoimmune diseases or result in other chronic inflammatory disorders.

The intestine is the largest immune organ in the body, and as such is the location for 70– 80% of the body's immune cells.[6,7] The epithelium of the bowel is constantly exposed to pathogens and foreign material from ingested food and the colon is colonized with microorganisms since just after we were born. There is a continuous interaction between the epithelium, underlying immune active tissues and the microflora in the intestines, balanced by the immune system to maintain the homeostasis in health.[8]

When this homeostasis in the gut is disturbed, as in an infectious inflammation, we may develop symptoms. The immune system battles the intruder and when the pathogen is defeated, the inflammation is suppressed and homeostasis is restored. If the immune system reacts on something normally harmless, fails in eliminating the pathogens or is not able to restore the homeostasis, we develop chronic inflammation. There are several chronic inflammatory diseases of the gastrointestinal tract and inflammatory bowel disease, including Crohn´s disease and Ulcerative colitis, is one. This thesis will mainly focus on aspects of Crohn´s disease in children.

2 BACKGROUND

Crohn´s disease is named after Dr Burrill Crohn (1884-1984) who was an American gastroenterologist, that made the first description of "regional ileitis" in 1932, based on 14 cases.[9] Even though several case-reports had preceded this paper, it is often regarded as the original description of the disease. Initially the condition was believed to be limited to the ileum only, but in 1960 Lockhart-Mummery and Morson defined colonic Crohn´s disease, which probably had been included within the entity ulcerative colitis previously.[10]

2.1 DEFINITIONS

Inflammatory bowel disease (IBD) is divided into:

- **Crohn's disease** (CD) is a chronic inflammation that can occur throughout the gastrointestinal tract. Inflammation is often segmental, discontinuous and transmural (involving all intestinal wall layers). Inflammation can be accompanied with complications such as perianal abscesses, fistulas and fibrotic stenoses.
- **Ulcerative colitis** (UC) is a chronic inflammation that occurs in the rectum and can spread in oral direction to cover part of, or the entire, colon. The inflammation is continuous and usually involves only the mucosa.
- **Indeterminate colitis** (IC) or **IBD unclassified** (IBDU) is a colitis which, despite extensive investigations, cannot be distinguished between CD and UC.

2.2 EPIDEMIOLOGY

Inflammatory bowel disease is often diagnosed in late childhood and early adulthood, in the ages between 15 and 30 years, and up to 25% of the patients have their onset during childhood.[11] The mean age at onset of pediatric CD is between 11 and 13 years of age.[11]

The incidence of pediatric IBD has increased in Sweden in recent decades.[12-15] Other countries in Europe and North America have also reported a sharp increase, particularly in the incidence of pediatric CD, during the same period.[16-19] In a study from northern Stockholm during the years 1990-2001 there was a marked increase in the number of new cases of pediatric CD and a clear predominance of CD compared with UC.[13] A subsequent study from northern Stockholm 2002-2007 noted that the incidence in pediatric IBD has reached a plateau at 12.8 new IBD cases per 100 000 children per year and the incidence of CD (9.2/100 000) remains at a high level in international comparison (Malmborg P., personal communication).[20] It is estimated that 150-200 children below 16 years of age will develop IBD each year in Sweden. Two out of three of these will be diagnosed with CD.

This increase of the incidence has also been reported in adult IBD during the last 10-15 years,[21] but there are large differences in incidence worldwide, varying from as low as 0.3 cases per 100 000 individuals in China to as high as 20.2 in Canada.[21] Even though

comparison of the results from different epidemiologic studies is problematic as the diagnostic criteria, time periods, design, population size and age groups differ between studies, it is obvious that the incidence varies globally. The highest incidence numbers are reported from centers in developed, industrial and urbanized countries with high socioeconomic status in the population[21] and there is an increasing incidence in developing areas worldwide, thus changes in lifestyle seem to play an etiologic role.

On the other hand, epidemiological studies have revealed familial and ethnic gathering of CD, suggesting a role of genetic factors in the etiology of the disease.[22,23] The prevalence of IBD is high among Jews, with the highest numbers in Ashkenazi Jews and there is different prevalence in various ethnical groups living in the same country.[24] However, in UK there is a significant difference in incidence between the first-generation immigrants from Asia (low incidence) compared to the second-generation (high incidence), indicating that exposure to environmental factors uncovers the susceptibility for the disease.[25]

2.3 ETIOLOGY AND PATHOGENESIS

Crohn´s disease is a multifactorial immune mediated disease and although the etiology and pathogenesis is not fully clear, many putative causes have been studied and suggested. Environmental, genetic and immunological factors all contribute and interact in the development of CD. The progress in the field of genetics the last decade have led to increased knowledge of the pathogenesis of CD by identifying alterations in susceptibility genes involved in the inflammatory response.

2.3.1 Genetics

2.3.1.1 Introduction to genetics and genetic studies

The mass of genetic information, which is necessary for development and maintenance of life, is located in our chromosomes as a chemical code within the DNA molecules. Humans have 23 pairs of chromosomes; one copy of each chromosome is inherited from the mother and the other from the father. Each chromosome contains several genes, which are the functional units of the DNA that encodes a protein. Of all genetic material, the genome, only about 1.5% codes for proteins.[26] There are between 20,000 and 25,000 protein-coding genes within that small piece of the genome, the rest consists of non-coding DNA.[27] Each gene has its specific chromosomal location, called a “locus”.

Although any two people share about 99.5% of their DNA sequence, there are a number of differences in their genome. The differences can be larger variations such as copy number variations (deletions and duplications) and insertions or small variations such as a single nucleotide exchange. An allele is one of two or more versions of a gene and an individual has two alleles for each gene (one from each parent). If the two alleles are the same, the individual is homozygous for that gene, whereas if the two alleles differ she is heterozygous.

A change in the genetic code can either represent a mutation or a polymorphism. Mutations are changes in the DNA sequence that may cause or contribute to a disease. An allelic variant that occurs at a frequency greater than 1/100 within a population is referred to as a polymorphism. Polymorphisms may contribute to disease susceptibility in complex diseases.

The genotype refers to the genetic code and the phenotype is the characteristics that a person or a disease has. A specific genetic alteration may result in different phenotypes and a specific phenotype may be the result of (several) different genes, referred to as genetic heterogeneity.

Far from all of the variants associated with disease are positioned in the protein coding regions of the DNA. Instead, they are often located in the large non-coding regions on the chromosome between genes, or in the intron sequences that are edited out of the DNA sequence when proteins are processed. These sequences may instead play a role in the expression of the genes. Epigenetics is the variety of the human genome that does not include variations of the nucleotide sequence in the DNA, for example chromatin packaging, histone modifications and DNA methylation. These variations are important in regulating gene expression, genome replication and other cellular processes.

In genetic linkage studies, chromosomal regions (loci or alleles) inherited together with the disease in families are identified. These regions (candidate regions) are likely to harbor susceptibility genes. Alleles that are physically close to one another tend to stay together during meiosis and are therefore genetically linked. Linkage disequilibrium is the association of alleles at two or more loci, inherited together more often than expected. For studying complex diseases, large multiplex families with multiple affected individuals are preferable but hard to find. Sib-pair analysis in a large number of families is an alternative.

In genetic association studies, the frequency of an allele of a genetic variant is found more often than expected in individuals with the disease compared to healthy controls. This method is better than linkage studies at detecting weaker genetic associations, as in complex diseases. Fine mapping is used when the approximate chromosomal location of an association is known, to narrow the region until the candidate gene and its alterations are found.

Inheritance studies of genetic linkage in families were the primary method around the year 2000, prior to the introduction of genome-wide association studies. In a genome-wide association study, where high-density single nucleotide polymorphism (SNP) array technology is used, hundreds or thousands of SNPs and common genetic variants can be studied at the same time to find associations with a specific disease. The associated SNPs are then considered to mark regions of the genome which influence the risk of the disease. Parallel with development of new technologies and the ability to analyze a higher number of SNPs the costs have gradually decreased and the number of studies using this technique increases rapidly. One of these technologies is whole-

exome sequencing, which focuses on all exons (the coding regions of the genome) and therefore is less extensive than studies of the whole genome but on the other hand also much less expensive.

2.3.1.2 Genetics in Crohn´s disease

Epidemiological studies, as described above, support the importance of genetic influence in the etiology of CD and the mode of inheritance suggests that CD not is a simple monogenic Mendelian disorder but a genetically complex disease, where multiple susceptibility genes interact with environmental factors.

Familial aggregation in CD has been reported since many years and up to 1/5 of CD patients have a first-degree relative with IBD.[23,28-30] The risk for CD among offsprings of patients with CD is 13 times higher than the risk within the general population.[31] There are studies suggesting concordance in disease location and extra-intestinal manifestations between relatives,[29,30,32,33] and an association between positive family history and younger age at diagnosis.[22,30] There are conflicting opinions weather genetic anticipation, i.e. lower age at diagnosis and a greater extent of disease in the offspring, occurs in CD or not.[34,35]

Studying twin pairs regarding the concordance of a disease is a way to find out more about the relative contribution of genetic and environmental factors to the etiology. Monozygotic twins are genetically identical and have, to a high extent, been exposed to the same environmental factors early in life. Dizygotic twins also have shared the same environment but only half of the genes, in average. The first study on twins with IBD, published 1988, showed that monozygotic twins with CD had a much higher concordance rate than dizygotic twins with CD and monozygotic twins with UC, stating that CD has a strong genetic component and that this is more pronounced in CD than in UC.[36] Repeated twin studies in Europe have found CD concordance rates for monozygotic twins between 33% and 50%, and for dizygotic twins between 3% and 10%.[36-39]

During the 1990s the evidence for heritability in IBD, and not least in CD, was growing strongly, leading to an intense search for linkage regions and susceptibility genes. In 1996 the first two genome scans using linkage strategy in IBD were published.[40,41] In 2001 four loci with significant linkage to IBD had been reported on chromosome 16q (IBD1), 12q (IBD2), 6p (IBD3) and 14q (IBD4) with the strongest linkage to CD in the IBD1 region. The same year, two independent groups identified the first CD susceptibility gene, the nucleotide-binding oligomerization domain 2 (*NOD2*), within the linkage region on chromosome 16.[42,43] Hugot *et al* succeeded in their identification of *NOD2* using a positional cloning strategy, and Ogura *et al* used both a positional and functional gene approach based on the location and the structural similarities to NOD1. Hugot *et al* found three different polymorphisms within the gene, independently associated to CD; two single nucleotide polymorphisms (SNPs) (Arg 702Trp / R702W and Gly908Arg / G908R) and one frameshift mutation (Leu1007fs / 3020insC). The *NOD2* gene was suggested by the HUGO gene nomenclature committee to be renamed

as C-terminal caspase recruitment domain 15 (*CARD15*) a few years after Hugot´s discovery. Today *NOD2* and *CARD15* are used synonymously, however *NOD2* is the official name.[44]

In the last decade several genome-wide association studies have discovered an increasing number of susceptibility genes and SNPs associated with CD, more than 10 such studies have been performed in adult IBD[45] and two in pediatric IBD.[46,47] The most recent meta-analysis, published 2010, based on 6 genome-wide association studies, including both adult and early onset disease, sum up to 71 significant susceptibility loci for CD.[48]

Many of the identified CD loci have previously been described in other chronic inflammatory disorders that can be seen clustered within families and individuals with CD, suggesting shared genetic risk factors.[45] One example is anchylosing spondylitis which, like CD, is associated with variants of interleukin (IL) 23 receptor, tumor necrosis factor (ligand) super family (*TNFSF) 8* and *TNFSF15* genes.[48] More than 20 of the identified CD associated loci are also associated with ulcerative colitis.

The precise functional alleles or the causative genes have not been defined in most of these loci, and many loci contain multiple genes and some contain no genes, but with supposed regulatory functions. Altogether, these 71 loci are suggested to explain not more than 23% of the total genetic risk or heritability[48] and regardless of all achievements in genetic research there are still an enormous number of unexplained or unidentified genetic factors, the so called "missing heritability".[49] Nevertheless, all studies performed this far have provided important clues into genetic susceptibility and pathogenesis of CD and the majority of the identified polymorphisms seems to be involved in the immune responses related to the development of chronic inflammation.

2.3.1.3 Polymorphisms associated to Crohn´s disease

The susceptibility genes that have been found associated to CD can be grouped according to their suggested role in the immune system, as shown in **Table 1.**
(Note that all 71 loci and genes not are presented in the table). A few of the genetic aberrations will be described further below.

Table 1. Examples of susceptibility genes associated with Crohn´s disease.

Genes involved in microbe recognition by innate immune system	Genes involved in autophagy	Genes involved in lymphocyte activation, differentiation, survival and growth
NOD2/CARD15	ATG16L1	IL23R, IL27 and IL10
RIPK2	IRGM	IL12B (IL12 and IL23 p40 subunit)
TNFSF15	LRRK2	IL18RAP, PTPN 2, PTPN 22
CARD9	MTMR3	TNF,TNFSF15, TNFRSF6B
TLR4		STAT3, JAK2, TYK2
		SULT1A1, SULT1A2, ICOSLG
		NOD2/CARD15, CCR6
		HLA class I and II

Genes involved in various functions	
MUC1, MUC19	constituent of mucus (mucin)
SMAD3, ZMIZI	modulation of TGF-β and immune tolerance
CCL2, CCL7, CCL20	leukocyte recruitment and migration
PSMG1	degradation of intracellular components
ORMDL1	endoplasmic reticulum function and stress response
SLC22A4 and SLC22A5 (=OCTN1 and OCTN2)	mucosal permeability
TNFSF11 (=RANKL)	osteoclast activity, proliferation of naïve T cells

2.3.1.3.1 NOD2/CARD15

Even though many other CD related genetic variants have been identified since the discovery of *NOD2/CARD15*, this is still one of the individually most important genetic variants associated to CD. Up to 50% of adult[50] and as much as 60% of pediatric CD patients[51] carry at least one of the three main NOD2/CARD15 coding variants; Arg 702Trp, Gly702Arg and Leu1007fs. As more studies have been published, it has become clear that the prevalence of these variants varies widely; the allele frequency vary from zero (not present) in Asian cohorts[52,53] to between 7% and 42% in European and North American cohorts.[42,54-61] In a meta-analysis of case-control studies involving Europeans it was stated that carriage of one or more alleles of the *NOD2/CARD15* variants is associated with a two to 17-fold greater risk of having CD, with the highest risk for homozygotes or compound heterozygotes.[62] The prevalence of the three main variants among healthy individuals is rather high with an allele frequency in Caucasian controls of in average 8%, with large geographical variance between 3.5% and 11.5%. Despite the increased risk for CD in carriers the disease penetrance is limited, confirming that *NOD2/CARD15* acts in interaction with other risk factors.[63,64]

There are several studies on the clinical implications of carriage of one or more *NOD2/CARD15* variants. The genotype-phenotype data are quite consistent on association with ileal disease rather than colonic disease,[50,51,55,57,61,65-68] younger age at

onset,[50,57,66,69,70] and more complicated (stricturing and/or fistulizing) disease behaviour.[50,55,56,66,68,71,72] Association to young age at onset is suggestive of *NOD2/CARD15* polymorphisms being more frequent in pediatric CD.

The *NOD2/CARD15* gene is located on chromosome 16q12 and consists of two amino-terminal caspase recruitment domains (CARDs), a centrally located nucleotide binding domain and multiple leucine rich repeats (LRRs) at its carboxy-terminal end. The main three polymorphisms are all located in or near the LRR region. The gene encodes the NOD2 protein, which is a cytoplasmic pattern recognition receptor, involved in bacterial recognition and expressed by monocytes, dendritic and epithelial cells, such as Paneth cells.[73,74] The LRR region of the protein recognizes a fragment of peptidoglucan, muramyl dipeptide, found on the surfaces of both Gram-negative and Gram-positive bacteria. This recognition leads to activation of nuclear factor kappa beta, which regulates the expression of several proinflammatory cytokines that are central in the pathogenesis of CD, i.e. tumor necrosis factor (TNF), IL-1, IL-6, IL-8 and IL-18. A defect in the *NOD2/CARD15* gene gives a deficit in nuclear factor kappa beta activation in response to bacterial components.[73,75]

Lately it has been shown that NOD2 is involved also in several other pathways in the immune response.[76] One is the muramyl dipeptide mediated activation of NOD2 inducing the epithelial cells to release defensins, which are antimicrobial peptides that protect the mucosa against adherent or invasive microorganisms.[77] Another pathway is the bacteria clearance mechanism autophagy, that is partly mediated by NOD2 by involving the autophagy regulator ATG16L1 (autophagy related 16-like 1).[78] Autophagy has been shown to be impaired in dentritic cells from CD patients with the *NOD2/CARD15* gene variant.[79] Alterations in the *ATG16L1* gene have also been found to be associated to CD[80] and a combination of disease-associated alleles of *ATG16L1* and *NOD2/CARD15* synergistically increase susceptibility for CD.[81]

Moreover, NOD2 has been shown to interact with DUOX2, a ROS (reactive oxygen species)-generating enzyme in epithelial cells,[82] important for bacterial killing. The functional implications of this interaction in CD patients carrying *NOD2* genetic variants are not clear.

It is also suggested that NOD2 is involved in promoting the production of IL-17; NOD2 senses muramyl dipeptide and activates dendritic cells, which elevate their IL-23 and IL-1 production, and in turn this promotes the development of human Th17 cells and IL-17 production. In dendritic cells from CD patients carrying a *NOD2/CARD15* variant, the muramyl dipeptide mediated induction of the IL-17 production was impaired, and there was no up-regulation of the IL-23 and the IL-1 expression.[83]

Although the mechanism is unclear, another interesting reported effect of carriage of *NOD2/CARD15* gene variants (especially the frameshift mutation Leu1007fs) is evidence of increased intestinal permeability, not only in the CD patients but also in their non-affected relatives who carry a gene variant.[84] Finally, the NOD2 protein has

recently been shown to activate antiviral innate immune responses involving interferon beta production.[85]

2.3.1.3.2 Genes involved in autophagy

ATG16L1

The protein encoded by *ATG16L1* gene is part of a large protein complex that is necessary for autophagy, the major process by which intracellular components (including microorganisms) are targeted to lysosomes for degradation. The gene is expressed in a variety of epithelial and immune cells, most highly expressed in T helper cells, cytotoxic T cells and B cells. Defects in this gene are associated to susceptibility to both UC and CD,[48] especially T300A variants are associated to CD[48,80] and certain genotypes have been found associated to ileal disease and younger age.[81] The exact functional consequences of *ATG16L1* gene alterations are not known, but they may have roles in both epithelial and immunological aspects of CD pathogenesis.

IRGM

Expression of the immunity-related GTPase family M (*IRGM*) gene regulates cellular autophagy of internalized bacteria. CD is associated with SNPs around *IRGM*, but not in the coding-sequence of the gene. A common deletion polymorphism upstream of *IRGM* that causes alteration in IRGM regulation and thereby affects the efficacy of autophagy, is likely the causal variant.[86]

2.3.1.3.3 Genes involved in lymphocyte activity and life

IL23R

IL-23 receptor variants were first described in 2006 and are inversely correlated to CD.[87] The prevalence of the "protective" IL-23 receptor variant in two pediatric studies were 5.5% and 6% in controls and 3% and 2% in CD patients[88,89] but no convincing phenotype correlations have been found.[90] The discovery of this gene as a susceptibility gene for CD has added support to the many studies describing the central role of the Th17 cell in chronic immune mediated disorders like CD. The relationship between IL-23 and Th17 will be described further in a coming section.

TNFRSF6B

Two SNPs associated to CD are located within a region containing several genes including the tumor necrosis factor receptor super family member 6B gene (*TNFRSF6B*).[46] The gene encodes the decoy receptor 3 and a variant is suggested to regulate receptor load, lymphocyte signaling, serum cytokine levels and to induce resistance to apoptosis in T cells and intestinal epithelial cells.[91,92] Expression of TNFRSF6B is increased in intestinal biopsies from IBD patients,[46] DCR3 levels are elevated in serum from CD patients[92] and the effects of the SNPs are most pronounced in exclusively colonic CD or pancolitis in UC.[91]

TNF-α

Tumor necrosis factor (*TNF*)-α polymorphisms have been found to be associated to susceptibility for CD, in Asian and European cohorts.[48,93,94] Some polymorphisms

increase the circulating TNF-α level, whereas others decrease the TNF-α transcription levels. Levine *et al* found that the two polymorphisms believed to decrease TNF-α levels either were associated to pediatric onset CD and isolated colonic disease (i.e. TNF-863C/A polymorphism)[95] or were associated with higher mean heights thus having a protective effect on height retardation (i.e. TNF-238G/A polymorphism).[96] No association to milder disease was found with these two polymorphisms.

2.3.2 Environmental factors

It is known since many years that the presence of microflora in the gut is required for intestinal inflammation to occur; in a murine model with certain knock-out mice, the animals develop colitis in the presence of normal microflora but not in a sterile germfree environment.[97] In patients with CD, remission can be induced by diversion of the fecal stream[98,99] and antibiotics can both induce and maintain remission.[100,101] A number of different microorganisms have been suggested to be involved in CD pathogenesis, and several triggers have been identified, especially agents and pathogens that break the mucosal barrier. These include nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics, virus and bacteria (e.g. Mycobacterium avium paratuberculosis, non-pylori Helicobacter, adherent-invasive Escherichia coli, Campylobacter species, among others)[102] and there is today no evidence that a single factor is responsible for the development of CD.

Many chronic inflammatory disorders started to increase in Europe in the mid-nineteenth century (e.g. allergies, inflammatory bowel diseases, autoimmunity, type 1 diabetes and multiple sclerosis) and all show evidence of defective immunoregulation.[103] The importance of changes in environment, lifestyle, diet and hygiene conditions during this period and the influence on developing diseases has been described in "the hygiene hypothesis",[104] later also called "the Old Friends hypothesis" by some.[105] Rooks *et al* suggest that in the urban environment there is a reduction of organisms that earlier followed man during evolution.[105] Some of these organisms were essential intestinal microbiota, establishing soon after birth that came to play crucial roles in the priming and optimal functioning of immunoregulatory pathways that are involved in tolerance of luminal antigens. Others, like helminths, had to be tolerated and the immune system was therefore down-regulated.[105] The inflammatory diseases today may partly be explained by the lack of these microorganisms and a dysregulated immune response, so that certain genotypes (which have not changed as much the last century), in the presence of certain triggers, can develop these diseases.[105] A subcomponent of this hypothesis is that a delayed exposure to viruses in infancy, and perhaps other early events related to infections or antibiotic treatment, will exacerbate the immunoregulatory deficit.

The change in the commensal flora not only means a regulatory impairment, but also an actual lack of certain species that holds anti-inflammatory properties. In rural areas in developing countries, inflammatory bowel disease is still unusual. When comparing feces from children from rural Africa and Europe, the European children have less short-chain fatty acids (SCFAs),[106] which have a protective anti-inflammatory role in

the gut[107] and also significantly less microbial richness and biodiversity.[106] Decreased biodiversity has been observed repeatedly in CD patients.[108-110] Faecalibacterium prausnitzii, an anti-inflammatory commensal bacterium that also generates SCFA, was present in the microbiota of the African children[106] but has often been lacking in European CD patients.[110,111] Another example of the symbiosis with the commensal flora is the regulatory effect of polysaccharide A, produced by Bacteroides fragilis. In an animal model, polysaccharide A is shown to suppress proinflammatory IL-17 production via anti-inflammatory IL-10 producing T cells.[112] Last, analyses of the available data conclude that exposure to helminths is one of the environmental factors that is most convincingly associated with a low risk of IBD.[113,114]

Changes in diet not only alter the flora, but may also increase intestinal permeability due to the increased consumption of foods containing saponins, lectins, gliadin and capsaicin.[115] There is some evidence that a very fatty diet can increase permeability and that a heavy input of sugars can cause overgrowth of inappropriate intestinal flora.[116]

Other suggested environmental factors that can interact with the immune system, apart from lack of microbiotic and dietary changes, are vitamin-D deficiency, pollution and smoking.[117] Smoking is repeatedly found as a risk factor for Crohn´s disease.[118]

2.3.3 Immune defence

The two basic components of the immune response are the innate and the adaptive response. The innate immunity lacks specificity and memory, and is responsible for the early initial immune response; to identify and remove the foreign substance[119] as well as to recruit and activate lymphocytes. It is represented primarily by monocytes, macrophages, dendritic cells and neutrophils. The adaptive immunity is characterized by clonal expansion of cells, specificity and memory and is mediated by lymphocytes, T and B cells, which express antigen receptors on their surface.[120] It enhances the response upon reinfection and helps the innate immune system when there is an intense pathogen load. Although often described separately, there is a frequent crosstalk between the adaptive and innate immune systems through the antigen presenting cells, cytokines and chemokines, which orchestrate the responses. In CD there is evidence of impairment in all levels of defence; the mucosal barrier, the innate immunity and the adaptive immunity.

2.3.3.1 Bowel mucosa barrier

Under normal conditions the gastrointestinal epithelial cells constitute a relatively impermeable physical barrier, connected with tight junctions that impede paracellular transport. On the luminal side of the epithelial cells, there is a thick layer of mucus, composed by mucin glucoproteins, containing defensins, immunoglobulins and other molecules with additional anti-microbial and defensive functions. Mucus and its content is secreted by enterocytes, lymphocytes and goblet cells, and in the small bowel also by Paneth cells. Defects in the mucus layer and its protective functions has been shown in CD patients with polymorphisms in the *MUC19* gene,[121] or with other alterations in expression and modifications of mucins,[122] and impaired production of

defensins has been found in CD patients with *CARD15/NOD2* gene variants.[77] In CD increased intestinal permeability has not only been found during active disease, but also in inactive disease[123] and in family members of CD patients[124] suggesting that this could be a primary initiating factor rather than secondary to the inflammation. There are other possible reasons for mucosal damage such as viral and bacterial infections, trauma, NSAIDs and hypoxia, but an underlying increased permeability or impaired barrier function most likely make the mucosa more susceptible to damage.

2.3.3.2 Gut associated lymphoid tissue (GALT)

The gastrointestinal tract contains the largest mass of lymphoid tissue in the human body, called GALT. GALT is made up of several types of lymphoid tissue that store immune cells. Peyer's patches (PP) are aggregates of lymphoid tissue that are spread at intervals just beneath the gut epithelium in the small intestine, especially in the distal part of the ileum. They consist of B cell follicles located under specialized areas, or 'domes,' of the epithelium known as follicular-associated epithelium, with T cell zones in the areas between the follicles. In the follicular-associated epithelium there are multi-fenestrated M cells (micro fold cells) whose function is to transport luminal antigen into the area of the follicle. Between the epithelial cells there are tight junctions, which allow the selective entry of fluids, nutrients, and microorganisms. The number of PPs that occurs in the intestines seems to be age related and peaks at ages between 15 and 25 years.[125-127] In the colon, instead of PPs, there are isolated lymphoid follicles[128] and the number, the diameter and the density of these are increasing in inflammatory conditions and are important not only for immune surveillance, but also for the mucosal regeneration and repair.[129] The isolated lymphoid follicles in the colon do not vary with age in the same extent as PPs. GALT is of great importance in the development of oral tolerance and immunologic homeostasis, accepting the presence of some microorganisms (the commensal flora) but not others (the pathogens). When there are defects in the epithelial barrier, GALT is exposed to either an excess of, or harmful, luminal antigens, resulting in an immune response and mucosal inflammation. The lymphoid tissue of the gut, especially the PPs, is suggested to be the potential site of onset of CD.[130]

2.3.3.3 Homeostasis and tolerance

The healthy mucosal immune system is unique in its ability to protect against invasion by pathogens, yet not respond to the commensal bacterial flora or dietary antigens. Dendritic cells are antigen presenting cells in the intestinal mucosa that act as guards that send signals to the surrounding cells and assist in the assessment of the commensal and pathogenic bacteria. Invasive and non-invasive enteric pathogens, but usually not commensal flora, trigger the inflammatory cascade through activation of receptors (as described below). The toll like receptor (TLR) signalling is kept under control by certain inhibitors, such as the Toll-inhibitory protein (Tollip). Prolonged exposure to lipopolysaccharides (LPS), which most commensal organisms contain in their cell wall, leads to elevated expression of inhibitors which in turn makes the intestinal epithelial cells less responsive to TLR-mediated response to commensal microflora. The antigen presenting cells also stimulate the activity of regulatory T cells, which in turn produce

IL-10, suppress adaptive immune response and also regulate the activity of the innate immune system. The mucosal regulation also includes IL-2 and transforming growth factor (TGF) β, and altogether this negative regulation of the responses to the commensal flora is crucial for maintaining gut homeostasis and oral tolerance.[131,132]

2.3.3.4 Innate immunity in Crohn´s disease

In the intestines the first line defence includes the epithelial barrier as described above. The intruding microorganisms typically bear certain pattern of molecular structures, called PAMPs (pathogen associated molecular patterns). Some of the PAMPs include complex macromolecules such as LPS, peptidoglycans, polypeptides (e.i. flagellin), and nucleic acids. Receptors of the PAMPs are the pattern recognition receptors, such as TLR and NOD proteins (NOD1 and NOD2) and these are expressed by antigen-presenting cells (i.e. monocytes, dendritic cells and macrophages) and epithelial cells. When PAMPs bind to pattern recognition receptors the cells usually respond by expressing and/or secreting a characteristic pattern of cytokines (IL-1β, TNF, IL-6, IL-8 and IL-12), adhesion molecules, and major histocompatibility complex (MHC) class II. A major convergent pathway in this response is through MyD88 (myeloid differentiation primary response protein), which via IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) activates kinases that degrade inhibitory molecules, leading to the nuclear factor kappa beta (NF-κB) translocation into the nucleus. NF-κB activates genes for the inflammatory response.[131]

The secretion of proinflammatory molecules leads to the recruitment of a wide variety of effector cells, including neutrophils, monocytes, lymphocytes and eosinophils to the infected or damaged area. Neutrophils, tissue macrophages and newly recruited monocytes, which differentiate into macrophages, kill microorganisms through phagocytosis. The granules of the neutrophils and the lysosomes of the macrophages contain cationic proteins, defensins, cathepsin, proteolytic enzymes, lysozyme, myeloperoxidase, toxic oxygen products and lactoferrin that kill the pathogen. Natural killer cells can nonspecifically kill virus infected and tumor cells, and eosinophils have granules which contents are effective in killing certain parasites, but take part also in the unspecific response. Opsonization by antibodies or complement factors, for phagocytosis or lysis of the pathogens, facilitates the killing. All these mechanisms of defence also contribute to the tissue damage.

As mentioned before, there is evidence that defects in the innate immune response in CD result in both high levels of proinflammatory cytokines and impaired clearance of pathogens. Associations to polymorphisms in genes involved in pathogen recognition, such as *NOD2/CARD15* and *TLR4*, as well as in genes coding important cytokines, such as TNF and IL-10, have been confirmed in large genome-wide association studies.[48] A number of polymorphisms associated to CD susceptibility are located in or near genes involved in autophagy (*ATG16L1, IRGM, LRRK2, MTMR3*).[48] Autophagy is an essential, homeostatic process by which cells degrade their own components (e.g. unwanted organelles). Originally this pathway was known as cell adaptation to starvation, but it is now also known as a process of lysosomal degradation of other

intracellular components (including microorganisms), as well as antigen presentation via MHC class II molecules, thus playing a role in both innate and adaptive immune response.[133] The importance of autophagy is emphasized by the large number of immune-related signalling molecules that regulate autophagy such as different PAMPs and damage-associated molecular patterns (DAMPs) (e.g. adenosine-5'-triphosphate (ATP), ROS, misfolded proteins, heat-shock proteins, high-mobility group box (HMGB) proteins, pathogen receptors, IFN-γ, TNF-α, inhibitor of NF-κB and NF-κB).[134]

The importance of the dendritic cells in CD has also been studied and it is suggested that dentritic cells falsely recognize commensal bacteria, induce a proinflammatory immune response, activate the adaptive immunity and prolong the survival of activated T cells, thereby maintaining the inflammation. The mechanisms are not fully known, but exaggerated or dysregulated receptor responses on PAMP recognition as described above, with known genetic aberrations, may be part of the explanation. CD patients have shown to have increased number of activated mucosal dendritic cells and even in remission states those dendritic cells secrete more TNF-α and IL-8, and express more TLR4, which may suggest an abnormal LPS response and/or a regulatory dysfunction.[135]

A primary failure in the acute inflammation due to macrophage dysfunction has been suggested as the reason for CD by Segal and his group. They showed impaired accumulation of neutrophil granulocytes to traumatized bowel mucosa in CD patients as well as to a "skin window" model, and reduced local levels of IL-8 and IL-1β.[136,137] The underlying reason for this was a disordered macrophage cytokine secretion due to degradation of the cytokines in the cell lysosomes, resulting in a much lower clearance of bacteria.[138]

The aberrant response to microorganisms and/or impaired clearance of pathogens which follow various defects in the innate immune response in CD, may lead to granuloma formation (that will be described further on) and an overactivated or dysregulated secondary, adaptive immune response.

2.3.3.5 Adaptive immunity in Crohn´s disease

The recognition by the adaptive immune system of antigens from the commensal flora, or other microorganisms presented by innate immune system, in combination with the stimulation that the innate immune system provides, play an important role in the pathogenesis of IBD. Cells from both the innate and adaptive immune systems have the ability to kill and remove the invaders and they also enhance each other´s actions,[120] partly through the release of various cytokines.

The adaptive immune system consists of B and T lymphocytes, which through a highly diverse antigen receptor repertoire have the ability to recognize all possible antigens. B lymphocytes can differentiate into plasma cells and produce specific antibodies.

T lymphocytes are divided into T helper (Th) cells, which are marked by the co-receptor CD4 on the cell surface and cytotoxic T cells, which express CD8. These cells

recognize antigenic peptides bound to MHC class II and class I molecules, respectively. Depending on the cytokine milieu, the T helper cells will differentiate or mature into subsets of helper cells as shown in **Figure 1.** (Adapted from Brand [139] with permission).

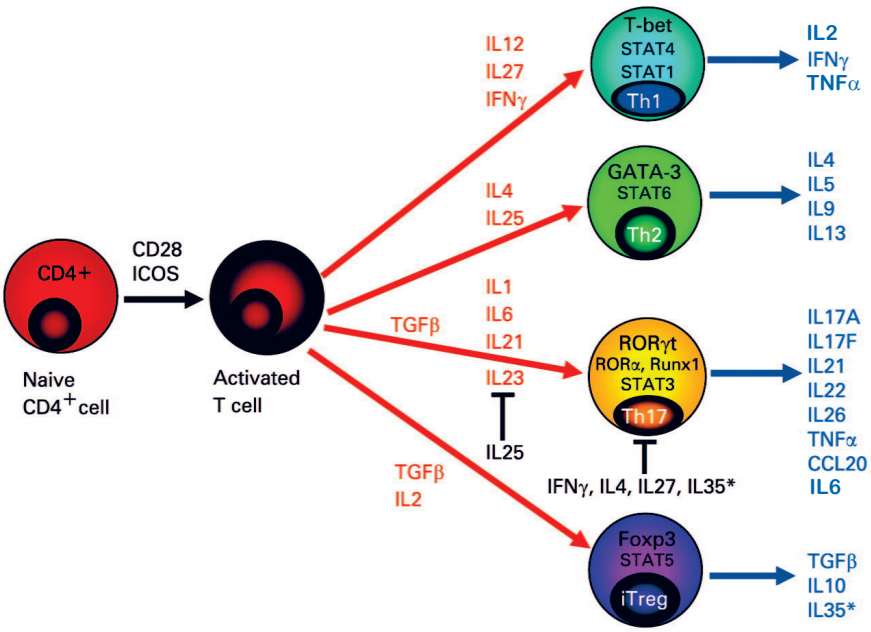


Figure 1. The cytokines involved in the development of Th1, Th2, Th17 and Treg cells from naïve CD4+ cells (marked in red) and the main effector cytokines of these Th subtypes (marked in blue). Cytokines which inhibit the Th17 cells are marked in black. * IL35 is only produced by murine Tregs. FOXP3 =forkhead box P3; STAT =signal transducer and activator of transcription; T-bet =T box expressed in T cells.

The regulatory T cells (FoxP3+, T regs) are active regulators of immune responses and play an important role not only in keeping the gut homeostasis, but also in the termination of immune responses, by the ability to suppress activated T cells and by regulating the activity of the innate immune system. The mechanisms for their regulatory activity can be cytokine-independent but also cytokine-mediated by TGF-β and IL-10 from the innate immune system. In CD the homeostasis is obviously not maintained, and the activation of the immune response is not terminated as it should, therefore the function of the T regs has been suggested as an important part of CD pathogenesis.[132]

Both Th1 and Th2 cells have been shown to cause chronic gut inflammation. CD has a predominately Th1 cytokine profile; the proinflammatory IL-12 promotes the development of Th1 effector cells, which produce INF–γ, TNF-α and IL-2.

The recent years it has become clear that IL-23, a member of the IL-12 family, has a central role in the inflammatory response. IL-23 consists of the p19 and the p40 subunits, and IL-12 is composed of the p35 and the p40 subunits, thus are they sharing the p40 subunit. IL-12 and IL-23 are primarily secreted by activated dendritic cells, monocytes and macrophages. As the IL-23 receptor is also expressed by dendritic cells and macrophages, it has been proposed that IL-23 secretion can amplify local expression of cytokines like IL-1β and TNF-α, which in turn stimulates release of additional proinflammatory mediators by stromal, epithelial, and endothelial cells. The fact that a genetic variant of the IL-23 receptor gene is known to be associated with decreased susceptibility to CD has stressed the importance of this pathway in CD pathogenesis.

IL-23 is not only important for the acute and intensive cytokine release, but also for promoting the chronic intestinal inflammation[87,140] through its ability to support the development of a novel subset of Th cells known as Th17 cells.[141] The discovery of the IL-23 / Th17 immune axis has lead to a paradigm shift; CD has changed from being regarded as a Th1 to a Th17 mediated disease.

Th17 cells are characterized by their production of IL-17, IL-22, IL-6, and TNF-α and have been associated with chronic inflammation in several diseases (e.g. multiple sclerosis, rheumatoid arthritis and psoriasis), as well as CD.[142] In murine models the Th17 cell lineage differentiation is driven by TGF-β and IL-6, while IL-23 is necessary for maintaining the Th17 populations. In humans, TGF-β, IL-1, IL-6, IL-21 and IL-23 all, separately, have been suggested as responsible for the differentiation of precursors into Th17 cells. On the other hand, the Th17 cells may be redirected away from IL-17 production towards a Th1 phenotype (and INF-γ production) in the absence of IL-23, suggesting that Th17 cells may be either unstable or in a non-terminally differentiated state.[143,144]

IL-17, which is the main cytokine released from the Th17 cells, also promotes expansion and recruitment of innate immune cells, such as neutrophils. Its receptor, IL-17RA, is expressed nearly everywhere; on hematopoietic cells as well as many non-immune cell types such as osteoblasts, fibroblasts, endothelial cells and epithelial cells, reflecting the importance of IL-17. In CD, high levels of IL-17 have been found in both sera and intestinal biopsies.[145-148]

In addition to the cytokines mentioned this far there are many more, interacting in a complex network, and new discoveries are reported continuously. Many of the cytokines that have a large potential in the inflammatory process (e.g. IL-17) are also of great importance in the homeostatic state (steady state) of the gut.[149]

Even though the IL-23/IL-17 immune axis is now accepted as dominating, it does not exclude other parallel immune pathways from being involved in the development of CD. This means that therapeutic targeting of one axis may not be effective and could in fact accelerate another.[131] There might be a variation of the expression of Th1 and Th17

cytokines along the intestine within the same patient, indicating specific local regulation mechanisms.[150] Whether it is a Th1 or Th17 response is probably also dependent on the duration of the inflammatory process. Kugathasan *et al* noticed a Th1 response in the early stage of pediatric CD and a shift to a Th17 response in late disease.[151] The relative importance of Th1 and Th17 response to inflammation has been discussed and there are studies showing that Th1 response is actually quantitatively greater and thus more likely to be the driving force of the inflammation.[152]

To summarize, the constant activity of both the innate and the adaptive immune systems, in combination with lack of, or impaired, down regulation leads to continuous production of proinflammatory cytokines, recruitment of effector cells and tissue damage. It has not yet been possible to point out exactly what has failed in the immune response in CD, due to the multilayered and complex nature of the response. There are probably individual combinations of aberrations, which contribute to the heterogeneity of the disease phenotype.

2.4 CLINICAL PRESENTATION

The clinical presentation of CD depends on the site, extent and severity of the inflammation. See **Table 2**. The main symptoms are abdominal pain, diarrhea (+/- bloody stools), weight loss, and perianal lesions. In children growth retardation and delayed puberty might be present.[153,154] Concomitant fever, anemia, nausea and fatigue can also be seen. Symptoms may be rather discrete even with pronounced intestinal inflammation, especially in small bowel disease and in younger children. Extra-intestinal manifestations may be present at onset, or occur later during the disease course and involve joints, skin, eyes, liver, bile ducts, pancreas or blood vessels (i.e. vasculitis or thromboembolism). Up to 30% of the patients have fibrostenotic strictures or fistulas at onset, and many others develop these complications further on.[155-157] The disease course is characterized by a chronic relapsing pattern, with exacerbations (flare ups) and spontaneous or treatment induced remissions. The duration of the remission periods and the intensity of the relapses vary between and within the individuals, and the disease course is therefore hard to predict.

Table 2. Symptoms and signs at presentation of pediatric IBD.

Symptoms or signs at presentation	Crohn´s disease	Ulcerative colitis
Diarrhea	++	+++
Rectal bleeding	++	+++
Mucus in stool	+	++
Abdominal pain	+++	+
Tenesmus	+	++
Fever	++	(+)
Perianal symptoms	++	(+)
Oral ulcers	+	-
Fistulas	++	-
Strictures	++	-
Palpable mass (in RLQ)	++	-
Weight loss	+++	++
Deviation in height	++	+
Delayed puberty	++	+

+++ = typical, ++ = common, + = may occur, (+) = unusual, - = do not occur

RLQ = right lower quadrant of the abdomen

2.5 DIAGNOSTIC CRITERIA

There is no internationally accepted "golden standard" that defines exactly what criteria that must be fulfilled for the diagnosis of CD or UC. However, as detailed below, a patient may be considered to have CD or UC if she has a clinical picture compatible with the disease and the work-up, including endoscopy, histology, radiology and biochemistry, reveals disease typical findings according to the Porto criteria 2005 (described more thoroughly below) with the caution that possible differential diagnoses have been excluded.

During the 1990s and in the beginning of the last decade, various criteria were used for IBD diagnosis. In the Lennard-Jones criteria from 1989,[158] CD diagnosis was defined as the presence of three of the inclusion features, found in any of the available methods; clinical examination, radiology, endoscopy or histology from biopsy or surgical specimen. The inclusion features were: a) Mouth to anus, b) Discontinous, c) Transmural, d) Fibrosis/strictures, e) Lymphoid aggregates/small aphthoid ulcers, f) Mucin retention, and g) Granuloma (which itself was regarded as diagnostic). Infections, ischemia, irradiation and lymphoma/carcinoma had to be excluded. Basically, those criteria were the same as those in use today but they lacked a definition of basic or complete work-up. The Porto criteria were introduced as the result of a consensus reached by the inflammatory bowel disease working group of ESPGHAN (European Society for Paediatric Gastroenterology, Hepatology and Nutrition) in 2005, aiming towards uniformity in the work-up and criteria used for pediatric IBD diagnosis.[159] Diagnostic findings are summarized in **Table 3**.

Table 3. Endoscopy and histology in IBD (The Porto criteria).

	Crohn´s disease	Ulcerative colitis
Endoscopy (and visualization of oral and perianal regions)	Ulcers (aphthous, linear, or stellate) Cobblestoning Skip lesions Strictures Fistulas Abnormalities in oral region # Abnormalities in perianal region ¤ Segmental distribution	Ulcers Erythema Loss of vascular pattern Granularity Friability Spontaneous bleedings Pseudopolyps Continuous (proximal extension from rectum)
Histology	Submucosal or transmural involvement Ulcers, crypt distortion Crypt abscesses Granulomas Focal changes (within biopsy) Patchy distribution (between biopsies)	Mucosal involvement Crypt distortion Crypt abscesses Goblet cell depletion Continuous distribution

CD in oral region; lip swelling, gingival hyperplasia and aphthous ulcers

¤ CD in perianal region; deep fissures, fistulas and abscesses

(Adapted from [159] with permission)

2.6 DIAGNOSTIC WORK-UP

Since the diagnosis of IBD is based on the combination of clinical and endoscopic findings (macroscopic and microscopic picture) as well as visualization of the small intestine and the exclusion of a number of possible differential diagnoses, it is important to strive for a complete work-up in every child. In addition to the laboratory tests and the diagnostic procedures, the work-up involves history taking (including questions on family history of IBD and parental height and weight), physical examination (including inspection of the perianal region and assessment of pubertal development) and measurements of height and weight.

2.6.1 Laboratory tests

Screening blood tests include full blood count, erythrocyte sedimentation rate (SR), C-reactive protein (CRP), serum levels of urea and creatinine, serum albumin, immunoelectrophoresis, liver function tests and screening for celiac disease (tissue transglutaminas antibody, tTGA). However, a considerable number of the children with mild CD may have normal values for the majority of those markers.[160] Fecal calprotectin is an unspecific marker for intestinal inflammation, which may guide the need for invasive investigation such as endoscopy, and distinguish from functional gastrointestinal (GI) disorders.[161] Normal levels of fecal calprotectin (<50 microgram/gram feces) make active disease in the lower GI tract unlikely.[162,163] Infectious causes of enteritis or colitis should be excluded by stool cultures (for Salmonella, Shigella, Yersinia, Campylobacter and Clostridium difficile), stool direct microscopy (for Giardia lamblia and Entamoeba histolytica) and stool tests for Clostridium difficile toxins A and B. However, identification of a pathogen does not

necessarily exclude IBD, as the first episode of IBD may present after an enteric infection.

2.6.2 Endoscopy

Endoscopy is considered the golden standard for diagnosing IBD. The last couple of decades there have been improvements in the technology and feasibility regarding endoscopic examinations in children. The routines have changed from partial colonoscopies (rectosigmoidoscopies) in the 1980s, through increasing ileum intubation rates during the 1990s (22%-66%),[164] towards complete endoscopic examinations, including ileocolonoscopy and esophago-gastro-duodenoscopy, in the recent 10 years, in agree with the Porto criteria.[159,165] All endoscopic procedures in children are performed under deep sedation, most often general anesthesia.

Colonoscopy including intubation of the terminal ileum and multiple biopsies for histology obtained from all segments of the lower intestinal tract (ileum, cecum, ascending colon, transverse colon, descending colon, sigmoid and rectum) is the most important investigation to differentiate between CD and UC, and it identifies localization and extent of inflammatory disease. Intubation of the terminal ileum with terminal ileum biopsies should always be attempted, as isolated ileal inflammation may occur in the presence of a normal colon in up to 9% of children with CD.[154] In addition, visualization of the ileum and ileal biopsies are crucial in the differential diagnosis of patients with pancolitis.[166] Upper endoscopy with multiple biopsies is also included in a full work-up in children, since histology of the upper GI tract may confirm a CD diagnosis that would otherwise have been missed in up to one out of four cases.[167-169]

The macroscopic findings in CD vary and depend on the severity of the disease. The features and differences compared to UC are listed in **Table 3.** Continuous inflammation can be seen in CD when there is extent disease, but patchy inflammation is more Crohn specific, as well as involvement of multiple sites in the GI tract, other than colon.

2.6.3 Radiology

Imaging of the small bowel is of great importance when doing a work-up for suspected IBD and a part of the diagnostic program according to the Porto criteria. The small bowel may be abnormal even though the terminal ileum is normal, thus the examination might be crucial for a correct diagnosis of CD. The imaging may also give information on both extent and possible complications of small bowel involvement including stenoses and internal fistulas. The most widely used method for this purpose is the small bowel follow through or enema. The advantages are low costs and good accessibility, and the disadvantages are the radiation exposure and the discomfort for the child. Leukocyte scintigraphy was used for small bowel assessment up in the 1990s, but has been found to have insufficient diagnostic sensitivity.[170] Magnetic resonance imaging is probably a more sensitive and specific method for visualizing the small intestines (as well as fistula formations),[171-173] and has lately replaced the small bowel follow through in many cases.[165] In a recent ESPGHAN-endorsed ECCO (European

Crohn´s and Colitis Organisation) guideline on pediatric CD, magnetic resonance imaging is recommended as primary investigation for small bowel imaging in children with IBD.[174] Transabdominal ultrasound is noninvasive and may reveal intestinal or colonic wall thickening or infiltrate, but does not show subtle inflammatory changes, and can be used in follow-up of local bowel changes such as ileocecal involvement. Ultrasound can also be performed transanally for the diagnosis of fistulas and abscesses.

2.6.4 Histopathology

Histology is important for correct diagnosis, for assessing the extent and intensity of the inflammation. In both CD and UC acute and chronic inflammation with architectural changes, loss of glands and branching of crypts are found. In CD submucosal (if biopsy includes sufficient submucosal tissue) or transmural involvement (in surgical specimen), ulcers, crypt distortion, crypt abscesses, focal changes, patchy distribution and granulomas are typical. See **Table 3.**

Findings in upper endoscopy should be interpreted with caution. A specific lesion, such as an aphthoid ulcer or an epitelioid cell granuloma, is typically correlated to CD. Focal active gastritis is indeed more frequently seen in CD than in UC but does not reliable distinguish between them.[175] Nonspecific inflammation in the upper GI tract should not be interpreted as CD related, as it may be present in up to 70% of children with UC.[168,176]

2.6.4.1 Granulomas

A granuloma, which is "the hallmark" in CD, is of noncaseating and nonforeign body type and located away from crypts. If it is found near a crypt, it may be confused with granulomas that are associated with epithelial destruction and crypt damage and those may also be present in UC.[177] The CD related epithelioid cell granuloma is a well-circumscribed, discrete, cluster of epithelioid cells (activated histiocytes/ macrophages with homogenous eosinophilic cytoplasm), surrounded by lymphocytes and with, or without, well-formed multinucleated giant cells.[177,178] Both the innate and the adaptive immune systems are involved in the initiation, formation and maintenance of granulomas. The development of a granuloma is usually a response to antigenic stimulation and is orchestrated through chemokines and cytokines. Evidence for the role of microorganisms, and suggested impaired clearance of pathogens, are findings of adherent-invasive Escherichia coli DNA within granulomas[179] and the ability of the same bacteria to induce granuloma formation in a research model.[180] An exaggerated inflammatory immune response with increased levels of proinflammatory interleukins (such as IL-1, IL-6, IL-12, TNF-α and IFN-γ) is necessary for the formation of granulomas. Since granulomas are dynamic structures they need continuous stimulation from its activated macrophages and/or cytokines from T cells. In line with this the macrophages and epitelioid cells within the granuloma have been found to express and produce both IL-12 and INF-γ.[181] It has also been shown that the granuloma cells express class II and co-stimulatory molecules, thus functioning as antigen presenting cells[182] attracting and interacting with the surrounding T lymphocytes.[183]

There are a number of other granulomatous disorders (e.g. tuberculosis, sarcoidosis, chronic granulomatous disease and Langerhans cell histiocytosis) which may give GI symptoms accompanied with findings of granulomas in biopsies.[184,185] Although there are overlapping features, the overall presentation with differences in symptoms and signs, normally makes it possible to distinguish between the diseases.

2.7 CLASSIFICATION

Crohn´s disease is a clinically heterogeneous disorder with a variety of demographic, clinical and phenotypic features. Classification of different phenotypes is necessary to be able to compare disease characteristics over time and place. It also could improve the possibilities to find genotype-phenotype associations and possibly give strength to prognostic statements that may influence the choice and timing of future medical and surgical treatment. A classification system for CD, regarding age at onset, disease location and behaviour (i.e. inflammatory, stricturing and/or penetrating), was introduced 1998 at the World Congress of Gastroenterology in Vienna. "The Vienna classification" was refined by The Working Party of the 2005 Montreal World Congress of Gastroenterology, thereafter called "The Montreal classification".[186,187] In 2010 a pediatric evidence based modification of the Montreal classification was proposed; "The Paris classification".[188] In this classification children are subgrouped according to age and growth failure, and upper GI/small bowel involvement is better defined. The Vienna, Montreal and Paris classifications are presented in **Table 4.** Note that in the Paris classification it is stressed that the definition of extent and location of the inflammation is based on (endoscopic) macroscopically identified mucosal ulcerations (or erosions) or bowel wall thickening on radiography[188] and therefore not based on the combination with microscopic findings, as was done earlier. Regarding the definition of IC, it was suggested in the Montreal classification that the IC diagnosis could only be used after colectomy and the term IBDU could be used in those who could not be classified into CD or UC despite an accurate clinical work-up (and had not undergone colectomy).[186,187]

Table 4. Vienna, Montreal and Paris classifications of Crohn's disease.

	Vienna (1998)	Montreal (2006)	Paris (2010)
Age at diagnosis	A1: below 40 y A2: above 40 y	A1: below 17 y A2: 17 - 40 y A3: above 40 y	A1a: 0 - <10 y A1b: 10 - <17 y A2: 17 - 40 y A3: >40 y
Location	L1: ileal L2: colonic L3: ileocolonic L4: upper	L1: terminal ileal ± limited cecal disease L2: colonic L3: ileocolonic L4: isolated upper disease*	L1: distal 1/3 ileum ± limited cecal disease L2: colonic L3: ileocolonic L4a: upper disease proximal to ligament of Treitz* L4b: upper disease distal to ligament of Treitz and proximal to distal 1/3 ileum*
Behaviour	B1: non-stricturing non-penetrating B2: stricturing B3: penetrating	B1: non-stricturing non-penetrating B2: stricturing B3: penetrating p: perianal disease modifier	B1: non-stricturing non-penetrating B2: stricturing B3: penetrating B2B3: both penetrating and stricturing disease p: perianal disease modifier
Growth			G_0: no evidence of growth delay G_1: growth delay

* may coexist with L1, L2 and L3, respectively
B1 –uncomplicated inflammatory disease without evidence of stricturing or penetrating disease.
B2 - the occurrence of constant luminal narrowing demonstrated by radiologic, endoscopic, or surgical examination combined with prestenotic dilation and/or obstructive signs or symptoms but without evidence of penetrating disease.
B3 - the occurrence of bowel perforation, intraabdominal fistulas, inflammatory masses and/or abscesses at any time in the course of the disease (excluding secondary postoperative intra-abdominal complications and isolated perianal fistulas).
B2B3 – the presence of both B2 and B3 phenotypes in the same patient, either at the same moment in time or separately over a period of time.

2.8 TREATMENT

The ultimate goal of treatment is to achieve and maintain clinical, endoscopic and histological remission, normal growth and puberty and good quality of life. The treatment of children and adolescents with CD needs to be individualized depending on a number of factors such as location and severity of the inflammation, the nutritional status, medication side effects and age-dependent factors, such as their ability to take pills. CD treatment consists of pharmacotherapy, nutritional therapy, surgical treatment, and psychosocial support. The responsiveness to treatments varies widely among children, as well as adults, with CD.

2.8.1 Pharmacological

The general strategy for treatment is the "step up" model; treatment is started with agents that are well known, have few or mild side effects and is suitable for most patients. If the first level is insufficient for inducing or maintaining remission, the intensity of the treatment is increased to the next step. A combination of two or more medications is often used.[189,190] The model and treatment options are shown in **Figure 2**.

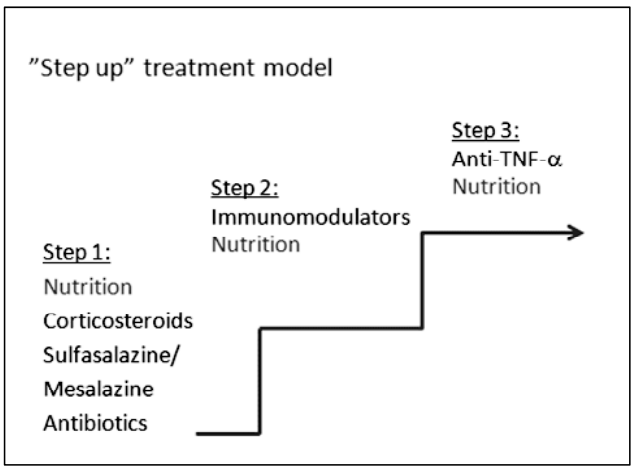


Figure 2. Treatment of pediatric Crohn´s disease according to the "Step up" model.

Enteral nutrition for induction and maintenance of remission
Sulfasalazine and mesalazine for induction and maintenance of remission
Corticosteroids = Predniso(lo)ne or budesonide for induction of remission
Antibiotics = Metronidazole or ciprofloxacin for induction of remission and for fistulizing disease
Immunomodulators = Azathioprine, 6-mercaptopurine or methotrexate for maintenance
Anti-TNF-α (tumor necrosis factor-alpha antibodies) = Infliximab or adalimumab for induction and maintenance of remission in severe, refractory disease and fistulizing disease

The treatment regimen of pediatric CD is shifting towards a more aggressive approach already at the presentation of the disease. Immunomodulators are introduced early in selected patients, aiming at preventing disease progression and complications. The indications for early immunomodulating treatment are extensive or severe disease at onset.[174]

2.8.2 Surgical

Surgical treatment may be considered in children at all stages of treatment for specific indications, such as fibrostenosis, localized inflammation or severe pancolitis and in children with disease resistant to medical therapies, especially in pre-pubertal or early pubertal children with growth failure and localized CD. Surgical interventions may also be needed for fistulizing and perianal disease.

2.9 SPECIFICITIES IN PEDIATRIC CROHN´S DISEASE

Compared to CD in adults, pediatric CD has distinct characteristics. These are related to gender, disease location, extension and treatment, and thereto severity and complications. Growth is naturally one significant difference between adult and pediatric CD.

2.9.1 Epidemiology

The increasing incidence of IBD, especially of CD, follows the same pattern in both pediatric and adult populations and this is reported in a previous section. However, there are epidemiologic differences related to age. The male to female ratio of CD differs in multiple studies when comparing pediatric and adult disease. In adult CD there is either an equal ratio, or a slight female dominance, whereas there is a male predominance in pediatric CD, especially in prepubertal period. The male to female ratio is reported to be approximately 1.5 in children younger than 15 years, [13,155,156] suggesting an effect of puberty and sex hormones on disease pathogenesis, although the mechanisms are not yet clear.

2.9.2 Environment and genetics

There is some evidence supporting the hypothesis that CD starting earlier in life is more likely to be influenced by genetic factors than adult onset disease, because of less time for impacts by environmental factors.[191-194] Genome-wide association studies on pediatric cohorts have revealed pediatric risk loci.[46] However, those loci have later been found significant also in adult CD and the most recent pediatric genome-wide association study, published in 2009,[47] pointed towards clear genetic similarities between pediatric and adult disease. These results, however, do not exclude the possibility that pediatric CD is more influenced by genetic factors than adult CD. They may all have the same genetic background but the patients with early onset disease might have inherited a larger dose of genetic risk factors, or there might be genetic factors lying outside the common CD pathways or rare genetic variants modulating age at onset, which are difficult to detect. Further studies involving larger pediatric onset CD cohorts may reveal new insights.

2.9.3 Disease characteristics

2.9.3.1 Histopathology and granuloma

The frequency of granulomas found at histopathologic assessment of biopsies from endoscopy or surgical resections is higher in children than in adults and is correlated with younger age.[195-197] The reported frequency in children varies from 24 to 61%.[195,196,198-200] The incidence of granulomas has been found to be reduced after the second year of illness under the influence of treatment and after the 16th year of life,[201] and similarly granulomas are more frequent in untreated than in treated patients.[196] The reason why some patients with CD have granulomas at onset (and/or later during disease course) and not others is elusive, as well as the clinical significance of the granuloma findings.

Nevertheless, histopathological assessment is of great importance when diagnosing inflammatory bowel diseases and a granuloma finding may be the only finding that discriminates between the diagnoses. This is especially relevant in children given that pancolitis is commonly seen in both pediatric UC and CD, and not least in the youngest age group where diagnostics often is more difficult.

2.9.3.2 *Disease location and extension*

Disease location differs, for unclear reasons, in pediatric CD compared to adult CD. A majority of the children have greater extension of the disease at onset with involvement of both ileum and colon or colon only, whereas adults more often have terminal ileum disease without involvement of colon.[155] Multiple pediatric studies have reported colonic involvement, with or without ileal disease, in 60-90% of children.[11,154-156,202] The reported frequency of isolated colonic disease in all children with CD varies between 7% and 38%.[11,154,155,157,253] Among children in the youngest age group, with disease onset before the age of 5 or 6, isolated colonic disease seems to be more common (30-76%)[193,203,204] than in children with onset later during childhood (20-26%).[193,204] Upper GI involvement is also more frequently reported in children than in adults, but this may be due to the fact that, unlike in adults, routine upper endoscopy is performed in children at onset.

2.9.3.3 Growth and growth failure

Growth failure is a unique complication of pediatric inflammatory bowel disease, especially in CD. Studies from the 1990s, dealing with growth in children diagnosed and treated during the 1980s and 1990s, report impaired growth in various frequencies up to 65% of the children.[153,205,206] However, comparing studies is difficult due to different definitions of growth impairment and different study populations. In many children decreased growth is present before diagnosis and some do not achieve their expected final height. Severity of disease, location of disease (i.e. small bowel involvement) and delay (i.e. time from onset of symptoms to CD diagnosis) have all been correlated to growth impairment.[154,207,208] Hopefully, better awareness of the presence and understanding of the pathogenesis of growth impairment, as well as the introduction of more effective treatment alternatives, will lead to enhanced growth in this group of patients. Indeed, Griffiths studied how the frequencies of linear growth impairment (i.e. <-2 standard deviation score [SDS]) have changed over time and she noted that the frequency has decreased from fully 20% during the years 1980-86 and 1990-96 to 7% during 2001-2006.[209]

Growth retardation may be the only symptom at diagnosis.[154] This emphasizes the important fact that IBD should be suspected in all children with growth retardation where no cause has been found (irrespective of the presence or absence of gastrointestinal symptoms) and it is essential to assess growth parameters at onset as well as during follow-up in every child. To obtain as much information as possible from this assessment, it should include not only height, weight, and body mass index (BMI) (preferably transferred into SDS based on appropriate local reference values), but also pre-illness data of height and weight and calculation of the growth velocity.

Parental height and weight are needed to calculate the target height of the patient.[210] Pubertal delay is often seen and pubertal development should be assessed at onset and regularly thereafter.[211] Many factors may contribute to growth failure. These are summarized in **Table 5** (adapted from Griffiths[209] with permission from S. Karger AG, Basel).

Table 5. Factors contributing to growth impairment in children with Crohn´s disease.

Factor	Explanation
Decreased or insufficient food intake	Loss of appetite (cytokine mediated) or fear of worsening of symptoms
Increased stool losses	Mucosal inflammation, protein loss
Increased nutritional needs	Ongoing inflammation, fever
Pro-inflammatory cytokines	Inhibition of IGF-I and direct negative effect on bone growth
Corticosteroid treatment	Interfering with growth hormone and IGF-I

Normal growth is dependent on the growth hormone (GH)/insulin-like growth factor-I (IGF-I) axis, as well as thyroxine, cortisol and the sex steroids for growth maintenance. IGF-I is produced under the influence of GH and mediates the GH effects at the growth plate of bones. Caloric and protein restriction can cause a reduction in the levels of IGF-I-binding protein-3 (IGFBP-3), resulting in decreased availability of IGF-I in the circulation. The pubertal growth spurt is primarily induced by estrogen, which increase the activity of the GH/IGF-I axis. The sex steroids, especially the androgens, appear to stimulate growth by a direct effect on growth plate chondrocytes.[209]

An association between low IGF-I levels and impaired linear growth in children with chronic inflammatory conditions, including IBD, is well recognized. However, GH production has been shown to be normal, suggesting a degree of hepatic GH insensitivity. Mainly undernutrition and inflammatory cytokines are responsible for this resistance, but chronic corticosteroid therapy can contribute.[212] It is well accepted that the use of glucocorticoids contributes to the slowing of growth, although growth impairment has also been observed in patients with other chronic inflammatory diseases (e.g. systemic and polyarticular juvenile idiopathic arthritis) who have never received corticosteroid treatment.[213] In CD there is often a nutritional deficit contributing to the decreased growth. However, according to studies on pair-fed rats in a colitis model, undernutrition accounted for about 60% of the growth impairment, whereas the remaining loss in growth resulted from the inflammatory process itself and was correlated to a decrease in IGF-I.[214]

There is a growing mass of evidence that the inflammatory process with its proinflammatory cytokines has a direct growth inhibitory effect. The major upregulated proinflammatory cytokines found in tissues in CD are, as mentioned previously, IL-6,

IL-1β and TNF-α. In animal models they affect longitudinal bone growth by disrupting the GH/IGF-I axis.[212] The underlying cause of the IL-6 mediated decrease in IGF-I production is suggested to be a primary reduction in IGFBP-3 levels, which in turn are caused by reduced production and/or increased proteolysis of this binding protein.[215] IL-6 antibodies increase both plasma concentrations of IGF-I and linear growth, whereas antibodies against TNF-α increase linear growth but have no effect on plasma concentrations of IGF-I.[214] Instead, cytokines can act locally on the growth plate level where IL-1β and TNF-α in synergy suppress longitudinal growth and this effect can be partially reversed by IGF-I.[216] In further studies on metatarsal bones from fetal rats, normal growth was achieved by TNF-α inhibitor (etanercept) and IL-1β receptor antagonist (anakinra) treatment in a dose-dependent way, and combining the treatment with IGF-I further improved growth.[217] In a review by Walters and Griffiths it is stated that chronic IL-6 exposure, in a process independent of IGF-I, promotes osteoclast maturation and activation, influence osteoblasts, is associated with osteoclast and osteoblast "uncoupling" and results in thinning of the growth plate, but the underlying mechanism is yet not known.[218] Additionally, cytokines may alter the secretion of gonadotropin-releasing hormone and impair end-organ responsiveness to circulating testosterone, which may contribute to delayed progress of puberty.[219]

When a child with CD fails to grow, treatment for their underlying inflammation is likely to be inadequate. The treatment should be intensified, adequate intake of calories ensured and long-term corticosteroid therapy should be avoided. Exclusive enteral nutrition is an appealing alternative in those cases, nocturnal or cyclic supplemental enteral nutrition may also improve growth and resection of localized CD should be considered when present. Moreover, anti-TNF-α treatment has, in both observational and clinical trials data, demonstrated a clear beneficial effect on linear growth (as long as treatment is undertaken prior to, or during, puberty) and should be considered in these patients in time.[209]

2.9.4 Disease course and complications

2.9.4.1 Treatment

In pediatric patients, who may face lifelong chronic disease, the choice of treatment (nutritional, medical, or surgical) is of particular concern. It does not only depend on the location and extent of the disease, but also on other factors related to the age of the child, such as suitable drug preparations, growth and pubertal stage, non-compliance (especially in adolescents) and drug safety issues, due to lack of evidence or safety studies in younger children and unknown long-term effects of treatments. The general treatment strategies are described in the previous section and here the special considerations related to the treatment of children will be reviewed.

Regarding growth there are no specific treatment modalities that have been shown to be superior in improving growth, thus focus should be put on controlling disease activity and avoid long term use of corticosteroids. In the national guidelines for pediatric IBD in Sweden it is stated that exclusive enteral nutrition (EEN, i.e. liquid formula by mouth or via tube) is the treatment of choice for pediatric CD, in particular for young

individuals in puberty with impaired growth. EEN in children is as effective as corticosteroids for induction of remission irrespective of disease activity or location,[220] although there is evidence that children with colonic disease show a better response to EEN if there is also ileal involvement.[221] EEN has no side effects in contrast to steroids and promotes growth. The growth stimulating effect is not only a result of better nutrition since anti-inflammatory and growth-stimulating effects actually precede restored nutritional status.[222] Additionally, EEN but not corticosteroids causes mucosal healing.[220] It has been shown that sole enteral nutrition is better than partial (i.e. 50% of the daily energy intake).[223]

According to a recent Cochrane analysis, corticosteroid therapy is more effective than EEN for inducing remission of CD in adults[224] and in adult CD, EEN is therefore mostly used when pharmacological treatment is unsuitable or as a supplement to pharmacological treatment. There is evidence suggesting that supplementary enteral nutrition may be effective for maintenance of remission in CD.[225]

Unlike the situation in adult CD, 5-Aminosalicylates (5-ASA) are often used in pediatric CD. The advantages are that the agents are well tolerated and have few side effects. The rational for using them despite lack of evidence for induction or maintenance of remission, is that 5-ASAs probably have most therapeutic efficacy in colonic disease (regardless of whether the diagnosis is CD or UC) and pediatric CD consists of more colonic disease than adult CD.

Biologic therapy, such as TNF-α blockers have proven to be effective in many children with moderate to severe CD. Compared to adults, a shorter interval between infusions may be beneficial in pediatric CD.[226] There is, however, an overall increased risk of lymphoma in patients with IBD who have been exposed to biologic or immune modulating therapy and a rare fatal form of lymphoma, hepatosplenic T cell lymphoma, is linked only to children and young adults with IBD,[227] with a predominance in young males. For this reason, combination therapy with immunomodulators (i.e. azathioprine, 6-mercaptopurine and methotrexate) and TNF-α blockers is normally avoided.[228]

2.9.4.2 Disease severity

The severity of the disease can be assessed in various ways. Regarding disease behaviour, the majority of patients with both adult and pediatric onset disease has an inflammatory, non-stricturing, non-penetrating disease phenotype at onset, and develops these complications over time.[155-157] Stricturing and penetrating disease behaviour is most often associated to small bowel disease.[155,229] As disease location differ between adult and pediatric onset disease, comparing these two groups according to complications is somewhat difficult. To overcome this problem Pigneur *et al* studied differences in severity in a childhood onset CD cohort and an adult onset CD cohort matched according to disease location (15 years of follow-up) and found that patients with childhood onset disease were more likely to have severe disease; the childhood onset CD patients had lower final adult height, more active disease, higher frequencies of immune modulating and anti-TNF-α therapy but there was no difference regarding

the need for surgery (>50% needed excisional surgery in both groups) or frequency of complications (i.e. stricture, perforation or perianal disease).[230] Adding the suggestion from Farmer *et al* that location is predictive for disease behaviour and prognosis, in sense that ileocolonic, rather than purely ileal, involvement predicts a more severe and treatment resistant disease course,[231] the conclusion might be that pediatric CD is more severe than adult CD.

2.9.4.3 Surgery

Pediatric studies of CD have reported various frequencies of surgery; 28-60% after >10 years of follow-up.[11,230,232,233] The frequency of surgery has been found to be associated to stricturing and penetrating disease behaviour, which in turn is associated to the disease location (i.e. small bowel) as mentioned above. Similarly, disease limited to the colon is associated with less surgery.[234]

2.9.4.4 Psychosocial complications

Chronic disease presenting in childhood and adolescence may be associated with marked psychological morbidity which has impact on education, relationships, psychosexual development and adherence to therapy. There is limited data available with various results, but adolescents with IBD might be more depressed and anxious and have worse health related quality of life scores than their healthy peers.[174] Therefore, psychosocial support of children and adolescents with CD is important, especially in certain vulnerable subgroups.[235] Examples of such could be patients with severe disease, children with single parents[236] (or other family related psychosocial factors) or adolescents with growth impairment and accompanying pubertal delay. Body image issues including height and weight are among the concerns most frequently cited by adolescents with CD in a quality-of-life measure.[237]

2.10 PROGNOSTIC FACTORS

In general there is a lack of relevant prognostic factors. Low age *per se* seems to be a negative prognostic factor. There are correlations between disease characteristics and complications as described above, although far from all patients with a specific phenotype actually develop the associated complications. Small bowel involvement is a risk factor for growth impairment, stricturing disease and surgery. Extensive disease and upper GI tract involvement at onset have also been reported to be associated to severe disease course and early need for immune modulating treatment.[238] Current smoking is a well known negative prognostic factor for disease recurrence and surgery[239] although not relevant among the youngest. Genetic alterations, serological or immunological markers,[240] specific histopathological features (e.g. granulomas), response to treatment and remission within a certain time span are examples of possible prognostic markers that need to be elucidated further.

3 AIMS

3.1 GENERAL AIMS

The overall aim of this thesis was to increase the knowledge of CD in children through studying genetic, histopathological and clinical factors that may be related to the disease course. By correlating these markers to disease characteristics in different ages and stages of the disease we may learn to better predict the needs of the individual patient. The ultimate aim is to improve the prognosis and the quality of life in the affected individual by providing an early individualised anti-inflammatory treatment.

3.2 SPECIFIC AIMS

The specific aims were:

- to describe the prevalence of the main *NOD2/CARD15* polymorphisms in a Swedish pediatric CD population
- to find out the correlation between genotype (regarding *NOD2/CARD15* polymorphisms) and phenotype (characteristics of the disease) in pediatric CD
- to investigate if and how age at onset correlates to ileal location of the disease in children with CD
- to evaluate the effect of IL-6 on the IGF-I system and linear growth in an animal colitis model
- to analyze whether growth impairment in pediatric CD is correlated to variants of the *IL6* gene
- to evaluate the clinical significance of granuloma findings in intestinal biopsies in pediatric CD
- to describe a Swedish pediatric CD cohort followed into adulthood regarding clinical characteristics and growth

4 MATERIAL AND METHODS

4.1 STUDY SUBJECTS

All participating children in **papers I and IV** (n=58 and n=45, respectively) were attending the pediatric gastroenterology unit at Astrid Lindgren Children´s Hospital, Stockholm, Sweden, when recruited in 2001 and all were younger than 17 years when diagnosed with CD between 1992 and 2001. In **paper I** the parents (n=106) and siblings (>8 years of age, n=44) of the participants were also included for genotypic analyses.

In **paper II** the patients were younger than 16 years of age at CD diagnosis and followed at Robert Debre´ Hospital in Paris, France, between 1999 and 2003 (n=136) or attending Astrid Lindgren Children´s Hospital in 2002, diagnosed between 1992 and 2001 (n=55).

The patients included in **paper III** attended Astrid Lindgren Children's Hospital (n=43), the Royal London and St Bartholomew's hospitals in London, the Chelsea and Westminster, Northwick Park, Bristol Children's, Lewisham, and Bury St. Edmond's hospitals in England (n=110) and were younger than 16 years when diagnosed with CD. Control samples for DNA analyses in **paper III** were donated from English teenagers (n=204) and from adult Swedish blood donors (n=147). For the animal studies in **paper III**, 25-day-old prepubertal Wistar rats from Charles River Laboratories were used (n=37).

A presentation of how many patients in **papers I-IV** were also included in the other papers in this thesis is shown in **Table 6.**

Table 6. The number of patients included in multiple papers of this thesis. (In all, 315 patients were included).

	Paper I (n=58)	Paper II (n=191)	Paper III (n=153)	Paper IV (n=45)
Paper I (n=58)	-	55	32	45
Paper II (n=191)	55	-	32	43
Paper III (n=153)	32	32	-	28
Paper IV (n=45)	45	43	28	-

4.2 METHODS

In **papers I-IV** the diagnosis of CD was established according to international criteria, corresponding to Lennard-Jones,[158] based on clinical, histological, endoscopic, and radiological data. The location was determined by findings of macroscopic lesions during endoscopy and/or microscopic lesions on biopsies and/or radiologic images compatible with CD lesions. In **paper IV** the disease was classified according to the Montreal classification, with the addition of the Paris classification regarding age at diagnosis. In **paper III** only patients with Northern European, Caucasian origin were included, because the *IL6* -174 polymorphism was previously reported to vary with ethnicity.

Clinical data for **papers I-IV** was retrospectively retrieved from medical records and blood samples for genotypic analysis for **papers I and III** were collected at the same time as routine sampling took place. A specialized gastrointestinal pathologist, blinded to the clinical status of the children, reviewed the slides prepared from biopsies taken at the diagnosis in the majority of the patients in **papers I, II and IV** regarding location and severity of inflammation, and presence of granulomas and their anatomic location in the GI tract.

For **papers III and IV** data on height and weight of the patients at onset were collected from medical records and were defined as a measurement within one month of the diagnostic endoscopy. Most parental heights were measured for **paper III**, and in those cases measured values were not available for **papers III and IV**, reported measured parental heights were used. At follow-up for **paper IV** the participants were asked to report their present measured height and the measured heights of their parents. Height (**papers III and IV**), weight and BMI (**paper IV**) were expressed as standard deviation scores (SDS) and height at diagnosis and final height was adjusted to target height [210] in **paper IV**. The patients in **paper IV** were considered to have reached their final height when the difference in height was less than 0.5 cm/year.

*Genotyping of NOD2/CARD15 (**paper I**)*
Genomic DNA was isolated from peripheral blood according to standard procedures. The three main *CARD15* polymorphisms associated with CD (R702W, G908R and 1007fs) were searched for using primers and methods developed by Lesage *et al*, as described previously.[50] For detection of the R702W polymorphism an allele-specific polymerase chain reaction assay was used. The G908R variant was detected by a restriction enzyme digestion of polymerase chain reaction-amplified DNA (the sequence variation create a novel restriction enzyme site). Finally, the detection of the 1-basepair insertion variant (1007fs) was based on sizing of a labeled polymerase chain reaction product after electrophoresis in an acrylamide gel with fluorescently labeled primers and automated sequencers. The analyses were performed in the laboratory of Fondation Jean Dausset CEPH / INSERM U434 in Paris (France).

*Genotyping for IL6 promoter haplotypes (**paper III**)*
Genotyping was performed at the Research Centre for Gastroenterology, Institute of Cell and Molecular Science, University of London in United Kingdom. Genomic DNA was extracted from fresh blood samples by using a salting-out process, from frozen samples by using the Quantikine kit (Amersham Biosciences Corp., Piscataway, NJ, USA), and from dried blood spots by using the Chelex technique. High-throughput analysis of the *IL6* -174 polymorphism was undertaken by using TaqMan 5′ endonuclease assay on a 7900 HT sequence-detection system with SDS software (Applied Biosystems, Forster City, CA, USA). Details regarding primers can be found in the original paper.

*Studies on TNBS colitis and control rats (**paper III**)*
The rats were housed individually in a room temperature of 22°C, with light and dark cycles of 12 hours and with free access to standard laboratory chow (RMI cubed; Special Diet Services, Witham, U.K.) and tap water. Trinitrobenzene sulfonic acid (TNBS) induced colitis was used as a model for intestinal inflammation since it has similarities to human disease regarding T cell activation and cytokine profiles (i.e. increased IL-6, TNF-α, IL-1, and IFN-γ) and has been shown to inhibit linear growth, with a decrease in IGF-I.[241]

The animals were divided into three groups matched for sex, weight, and body length: healthy free-feeding controls (n=13), a TNBS colitis group treated with IL-6 antibodies (n=11) and a TNBS colitis group treated with nonspecific sheep IgG antibodies (n=13). Under anesthesia (Hypnorm, Janssen Pharmaceutics, Belgium), TNBS (8 mg per 100 g of body weight) was inserted 5 cm proximal to the anus in the TNBS colitis groups, and in the healthy control group a plastic catheter was passed into the colon and removed after one minute. The TBNS colitis rats received either 10–15 mg/kg IgG polyclonal antibodies to IL-6 (National Institute for Biological Standards and Control, Potters Bar, U.K.) or nonspecific IgG antibodies subcutaneously at induction of colitis and two days later.

Daily measurements of body weight, food and water intake were performed. Body length (as the mean of two measurements of the distance from nose to tail base) was measured at induction of colitis and 5 days later. The animals were killed after 5 days by an overdose under anesthetic, trunk blood was collected and the liver and the colon were removed (by a midline laparotomy). The severity of the colonic inflammation was assessed macroscopically by using a previously validated scoring system (i.e. assessment of number and size of ulcers and presence or absence of diarrhea and adhesions)[242] as well as by measuring the thickness of the colonic wall. A section of the colon was assessed for myeloperoxidase activity. Intestinal edema was determined by measuring wet and dry weights of the remaining left colon. Plasma and liver concentrations of total IGF-I and plasma levels of IGFBP-3 were measured by using an ELISA method (Diagnostic Systems Laboratories, Webster, TX, USA). Hepatic IGF-I mRNA was measured using specific primers and RT-PCR, see original paper for details.

4.3 STATISTICAL ANALYSES

Descriptive data are presented as mean or median with ranges (minimum to maximum in **papers I and IV** and first to third quartiles in **papers II and III**) according to the distribution of data of each variable. In **paper III** the Kolmogorov-Smirnov test was used for examining data and the results are given as SEM (standard error of the mean) or median. Differences between groups were assessed by using the Fisher's exact test, students T-test, Chi-Square and linear regression analyses (**papers I-IV**). 95% confidence intervals were calculated for qualitative variables presented as frequencies e.g. polymorphism and allele frequencies in **paper I** and probability for ileal disease in **paper II**. The Mann–Whitney U test / Wilcoxon rank test were used for comparing the median times of occurrence of CD depending on the initial localization and the carriage of one or more *CARD15/NOD2* polymorphisms in **paper II**, for determining differences in CRP and macroscopic scores of colitis in **paper III** and for comparing medians in **paper IV**. Kaplan-Meier curves with log-rank test are presented for comparing the time to event in different subgroups in **papers II and IV**. Differences were generally considered as statistically significant when the p value was less than 0.05.

SAS 8.02 (SAS, Cary, N.C., USA) and Splus 6.2 (MathSoft, Seattle, WA., USA) software packages for PC were used in paper II. The SPSS (version 20.0, SPSS, Inc., Chicago, USA) system software and the KIGS Auxology calculator software (version 1.1, Pfizer, Inc. Sollentuna, Sweden) were used in paper IV.

4.4 ETHICS

Ethical approvals were obtained from the Regional Research Ethics Committee at Karolinska Institutet, Karolinska University Hospital, Stockholm for **papers I, II and IV**. For **paper III** the human studies committee relevant to each participating hospital (including the Regional Research Ethics Committee at Karolinska Institutet, Karolinska University Hospital) approved the study. All animal experiments in **paper III** were carried out in London in accordance with the United Kingdom Animal Scientific Procedures Act of 1986.

5 RESULTS

5.1 PAPER I

CARD15 mutations are rare in Swedish pediatric Crohn disease

In this study, 58 children, more boys (62%) than girls, participated. Before diagnosis they had symptoms for up to 54 months, median duration was 3.6 months and they were diagnosed at a median age of 10.9 years (range 2.8 to 16.9 years). Seven children (12%) had a first-degree relative with CD. Altogether 21% had a positive family history of IBD.

Nearly all patients (97%) had colonic involvement at onset and/or later during the disease course and only 57% of the patients had ileal lesions at any time during follow-up (median 4.2 years). Fifteen % had perianal disease and granulomas were found in the initial biopsies in 50% of the children.

The genotyping regarding polymorphisms in the *NOD2/CARD15* gene revealed polymorphisms in only five patients (8.6%) and all were heterozygous. The distribution of the different polymorphisms, as well as the allele frequencies in the children and their relatives, are shown in **Table 7**.

Table 7. Allele frequencies of *NOD2/CARD15* polymorphisms in patients and relatives.

Polymorphism	Patients n=58 (allele %)	Relatives n=150 (allele %)
R702W	3 (2.6 %)	5 (1.7%)
1007fsinsC	2 (1.7 %)	4 (1.3%)
G908R	0	0
Sum	5 (4.3 %)	9 (3 %)
[95% CI] for allele frequency	[1.4-9.8]	[1.4-5.6]

CI = confidence interval

In the five CD patients carrying a polymorphism, we looked for the parent transmission and found the polymorphism in the healthy mother but not in the father in four cases. In the fifth family the mother carried another polymorphism than the child, so the polymorphism the child had must have been transferred from the father, although we did not have DNA to confirm this. This observation can suggest an excess of transmission from the mother. Interestingly, none of the five patients had a positive family history of CD and all of the relatives with polymorphisms were healthy.

The low prevalence of children having one or more of the three *NOD2/CARD15* polymorphisms in this study (8.6%) made testing for a genotype-phenotype relationship very difficult. We did note a weak trend towards an excess of granuloma findings in the first biopsies in patients with a polymorphism, compared to those with wild type *NOD2/CARD15* gene, 80% and 43% respectively (p =0.17).

5.2 PAPER II

Ileal involvement is age dependent in pediatric Crohn´s disease

This study comprised children with CD recruited in Stockholm (n=55) and Paris (n=136). The cohorts differed in some aspects regarding sex ratio, disease location at diagnosis and *NOD2/CARD15* genotypes as shown in **Table 8**.

Table 8. Comparison between the pediatric CD cohorts from Paris and Stockholm.

	Paris	Stockholm
Number of patients	136	55
Sex ratio (M/F)	1.0	1.5
Age at diagnosis (median), months	12.0	11.8
Diagnostic delay (median), months	6.0	3.0
Isolated ileal disease, % of patients	6%	7%
Isolated colonic disease, % of patients	31%	56%
Ileocolonic disease, % of patients	63%	36%
NOD2/CARD15 polymorphism carriage	24/44 (55%)	4/55 (7%)

The patients were divided into groups and because of the small number of patients with isolated ileal disease (only 12 patients), ileal and ileocolonic disease were merged into one group and isolated colonic disease formed the other group. The diagnostic delay did not differ between those two groups of patients, 7.0 [inter quartile range 3.0-16.0] months and 5.0 [2.0-8.0] months, respectively.

We analyzed the age at diagnosis according to initial localization of the disease for each geographic group and for the pooled cohort and found that those with isolated colonic disease were younger than those with ileal disease. For the pooled cohort, median age at diagnosis was 11 [8-13] years in the group without ileal involvement and 12 [10-14] years in the group with ileal disease. The Kaplan Meier curves in **Figure 3** show the occurrence of isolated colonic and ileal disease in relation to age and the difference between the curves was highly significant (p <0.0001). Colonic location occurred as early as the age of one year, whereas the youngest child with an ileal location was five years old. In both Paris and Stockholm most of the youngest patients show an involvement of the large bowel only while small bowel involvement occurred mainly in children older than 9 years. This finding was most apparent in the Swedish cohort as shown in **Figure 3 B**.

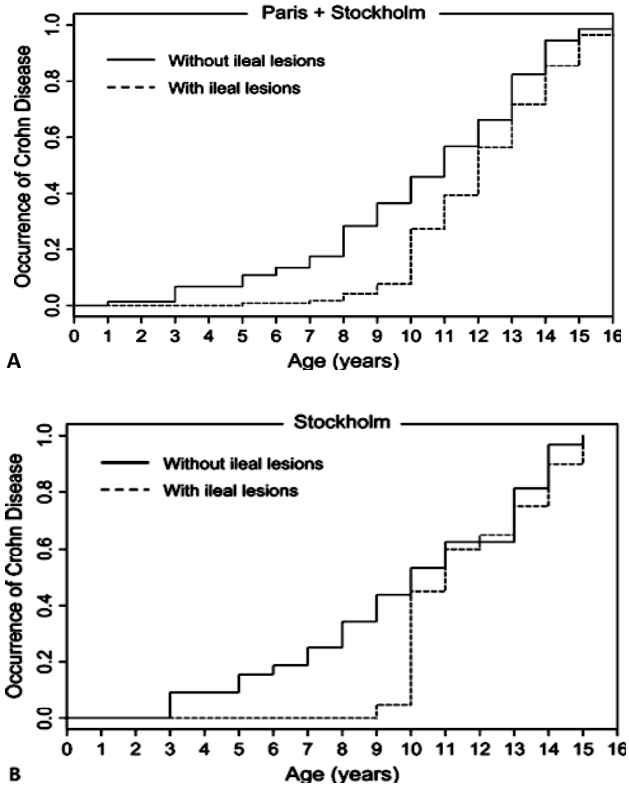


Figure 3. Occurrence of isolated colonic and ileal disease in relation to age.
Kaplan-Meier curves showing the occurrence of colon only (solid line) and ileal (dashed line) CD in relation to age in pediatric patients from Sweden and France (**A**) and from Sweden only (**B**).

Additionally, we calculated the cumulative probability (95% confidence interval) of initial ileal lesions dependent on the age at diagnosis. This probability increased from 0.0 (0.0-0.48) at the age of 5 years to 0.61 (0.54-0.68) at the age of 16 years, with a striking change between the age of 9 years (0.25 [0.12-0.42]) and 12 years (0.57 [0.48-0.66]). When adding data on carriage of one or more of the main three *NOD2/CARD15* polymorphisms, we found that such carriage was associated with a slightly higher probability for ileal involvement at 16 years (0.75 [0.55-0.89]) compared to the probability in wild-type patients (0.46 [0.34-0.58]) and the Kaplan-Meier curves were significantly different between patients with or without polymorphisms ($p < 0.02$) (shown in the original paper).

5.3 PAPER III

Intestinal inflammation-induced growth retardation acts through IL-6 in rats and depends on the -174 IL-6 G/C polymorphism in children

Anti IL-6 antibodies, IGF-I and growth in TNBS colitis in rats

The rectal TNBS-ethanol administration to the rats resulted in colitis, extending from the splenic flexure to the anus, with bowel wall thickening, edema, skip-lesions, areas of ulceration and higher levels of myeloperoxidase in colonic tissue, parameters which were assessed when the animals were sacrificed (5 days after induction of colitis). The rats with colitis ate less ($p < 0.001$) and had significantly reduced linear growth ($p < 0.001$) compared with healthy controls (**Figure 4c**). Treatment with IL-6 antibodies had no significant effect on the severity of colitis, nor did it alter the food intake ($p > 0.05$). However, IL-6 antibodies increased linear growth significantly in rats with TNBS colitis ($p = 0.023$), but because of the decreased nutrient intake, growth in the IL-6 antibody-treated rats with TNBS colitis remained less than the healthy controls (**Figure 4c**).

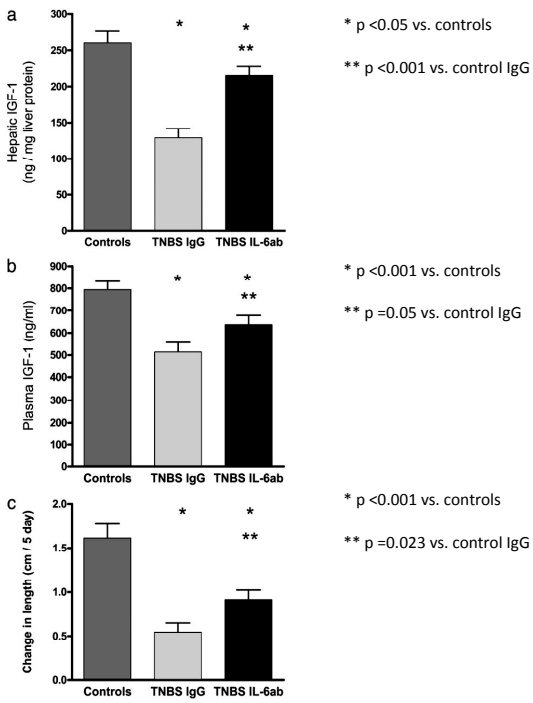


Figure 4. The effects of colitis and IL-6 antibodies on hepatic IGF-I, plasma IGF-I and growth in TNBS colitis rats. IL-6 antibodies increase IGF-I concentrations in the liver (a), IGF-1 plasma levels (b) and linear growth (c) in rats with TNBS colitis. Growth in the IL-6 antibody-treated rats with TNBS colitis remained less than the healthy controls because of the decreased nutrient intake (c).

To evaluate the effect of IL-6 on the IGF-I system, plasma and liver IGF-I levels as well as IGF-I expression were analyzed. Administration of IL-6 antibodies significantly increased liver IGF-I mRNA and elevated liver and plasma IGF-I concentrations in rats with colitis and thus impaired linear growth in TNBS colitic rats was associated with a reduction in hepatic and plasma concentrations of IGF-I (**Figure 4a and 4b**). To determine whether a decrease in the binding protein IGFBP-3 contributed to the low circulating concentration of IGF-I, IGFBP-3 was also measured. However, the concentrations were similar in the rats with colitis and healthy controls indicating that the reduction in IGF-I was not caused by a decrease in IGFBP-3.

-174 IL6 G/C haplotype and growth in children with Crohn´s disease

Previous studies have shown that IL-6 expression is increased in CD and circulating levels parallel disease activity. IL-6 induces CRP, which is used clinically to monitor disease activity. As described above in our studies on rats, inflammation-related growth retardation and decreased levels of IGF-I can be reversed with IL-6 blocking antibodies, suggesting that IL-6 levels might be important for growth impairment associated to intestinal inflammation also in humans.

IL-6 expression is primarily regulated by alterations in gene transcription. A functional G->C SNP at position -174 of the *IL6* promoter has been described[243] and a certain haplotype has been found associated with greater induction of IL-6. We hypothesized that patients with the GG genotype express higher levels of IL-6 and therefore they would be more growth impaired than patients with other genotypes. To study this hypothesis we examined the *IL6* -174 genotype in 153 patients with CD from Sweden and England. All were diagnosed in the childhood with a mean age at diagnosis of 11.5 (SD, 2.7) years and 65% of the patients were male. There was no difference in genotype distribution between the patients and the 351 controls (p =0.7), between the 110 English and 43 Swedish children with CD (p =0.8), or between the 204 English and 147 Swedish control samples (p =0.8).

At diagnosis the 153 CD children were significantly growth impaired, with a mean height SDS of -0.26 (SEM 0.10, p =0.012). The reduced height was regarded as a result of their untreated CD, because their parents' heights were normal; maternal mean height SDS was 0.09 (SD 1.09), and paternal mean height SDS was 0.08 (SD 0.89). When analyzing growth impairment in relation to the *IL6* -174 genotypes, we found that children with the *IL6* -174 GG genotype were significantly more growth-retarded compared with the combined GC/CC group, as shown in **Figure 5.**

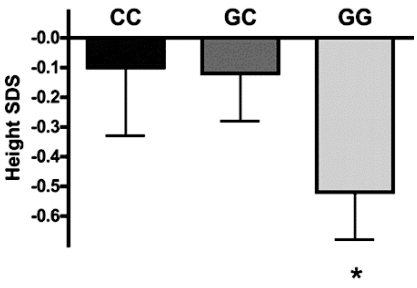


Figure 5. Height at diagnosis related to the *IL6* -174 genotype in children with CD.
Height SDS at diagnosis was significantly more reduced in the *IL6* -174 GG genotype compared with the combined GC/CC group, mean height SDS -0.51 vs. -0.1 (SEM 0.16 vs. 0.13), * p =0.031.

We also wanted to test whether the *IL6* genotype is contributory during active inflammation, or during both active and inactive inflammation. CRP, which is an acute-phase respondent that is induced by circulating IL-6, is recorded routinely and in this study there were 73 children who had CRP measured both before and after their initial therapeutic course. Before therapy, CRP was significantly higher in children with the *IL6* -174 GG genotype than in those with the GC/CC genotypes (median, 36 vs. 18 mg/dL, p =0.028). After 6 weeks of treatment, CRP levels were significantly reduced from a median of 21.5 to 9.5 mg/dL for all groups (p <0.001). However, after treatment, patients with the *IL6* -174 GG genotype no longer had higher CRP levels compared with the GC/CC group (p =0.68). This means that the *IL6* -174 GG genotype is associated to higher CRP levels only during active disease.

5.4 PAPER IV

Pediatric Crohn´s disease from onset to adulthood: granulomas may predict a more aggressive disease but growth is not impaired

Altogether 45 of the 58 participants in study I participated in this follow-up study. All patients were diagnosed with CD during childhood; median age at diagnosis was 10.3 years and the median duration of symptoms before diagnosis (delay) was 3.4 months. The children were followed into adulthood with a median follow-up period of 12.3 years (range 9.3-18 years). All but one had reached final height.

The diagnostic endoscopic procedures in this group of patients comprised of ileocolonoscopy in 20 children, colonoscopy in 24 children and one patient had an emergency surgical procedure at onset. Upper endoscopic examination at diagnosis was performed in 26 children.

We found granulomas in 49% (22/45) of the patients in the histopathological examination at onset. There was no association between findings of granuloma at diagnosis and the age, gender or diagnostic delay. At diagnosis the granulomas were found in biopsies from the colon (22/45) and additional granulomas were found in the

upper GI tract in two (8%) out of the 26 patients who were examined with upper endoscopy and in the ileum in three (15%) of the 20 patients who underwent ileocolonoscopy at diagnosis. We found no significant difference regarding number of performed endoscopies from diagnosis to the latest follow-up between the patients with granuloma findings at onset and the patients without granulomas (mean number 3.6 and 3.3, respectively, p =0.5), although children with granulomas at onset had a shorter follow-up period compared to those without findings of granulomas, 11.7 and 13.2 years, respectively (p=0.02). As concluded in paper I, colon was the dominating site of the disease and the same distribution of disease involvement was found when we extracted data on children who had undergone a full ileocolonoscopy at diagnosis, as shown in **Table 9.**

Table 9. Location of the disease at onset and at follow-up (median 12.3 years) in patients with childhood onset CD in Stockholm.

Location (Montreal classification)	At onset (n=45) (%)	At onset, ileo-colonoscopy* (n=20) (%)	At follow-up (n=45) (%)
L1: terminal ileum (+/- limited cecal disease)	2 (4.4)	1 (5)	2 (4.4)
L2: colonic	23 (51.1)	10 (50)	19 (42.2)
L3: ileocolonic	20 (44.4)	9 (45)	24 (53.3)

***** Data on children who had a full ileocolonoscopy at diagnosis

Upper GI disease (L4) was found in 24.4% of the children at diagnosis and 44.4% at follow-up. In total, eleven patients (24%) had increased the extension of their disease at follow-up, but only four patients (9%) had a shift in disease extent from only colonic (L2) to ileocolonic (L3) during the course of the disease and the rest had additional disease extension to the upper GI tract during follow-up.

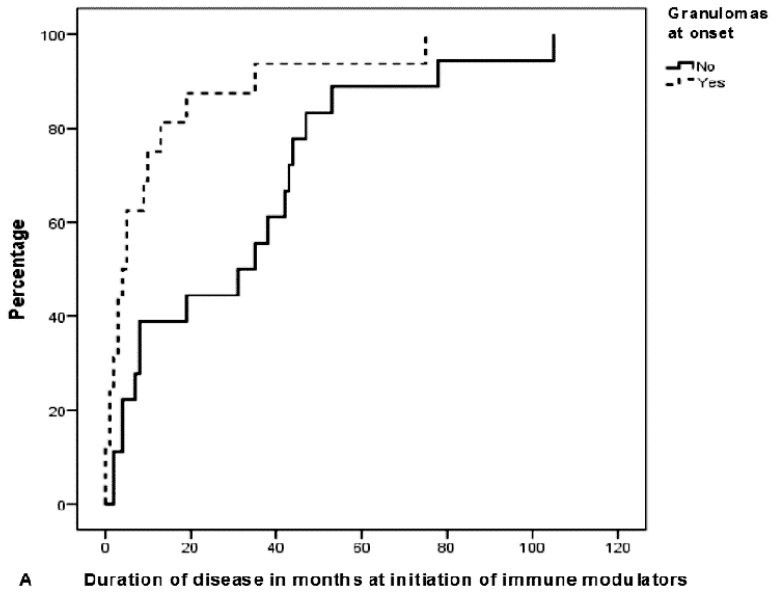
Findings of granulomas correlated to upper GI involvement in that nine of eleven patients (82%) with inflammation of the upper GI tract at onset had granulomas at onset (p =0.017). During follow-up, granulomas were found in 17 of 20 patients (85%) with upper GI involvement (p =0.006). No significant correlations were found to either colonic or ileal disease, nor to developing more extensive disease.

The majority (80%) of the patients (n=36) had non-stricturing, non-penetrating disease and there was no correlation between disease behaviour (B1/B2/B3) and findings of granulomas at diagnosis (p =0.45). We found a trend towards more granulomas in colonic biopsies at onset in children who presented with or developed perianal disease (8/11; 73%) as compared to the patients without perianal disease (14/34; 41%), but the difference did not reach statistical significance (p =0.07).

During follow-up, ten of the 45 (22%) patients underwent a total of 14 surgical interventions (six ileocecal resections, three colectomies, three colon resections, and two small bowel resections) and the median duration of disease before the first surgical intervention was 6.9 years (range 0-12.3). We found no significant differences between the group of patients with granulomas at diagnosis and those without regarding incidence of surgery or total number of surgical interventions, nor regarding time interval from diagnosis to the first surgery, although the granuloma positive group seemed to have longer interval compared to the granuloma negative group (median 2.4 years and 7.8 years, respectively, $p = 0.29$).

Data on immune modulating treatment was used as a proxy for severe disease in this study and we therefore also examined whether such therapy was correlated to findings of granulomas. Altogether 34 of the total of 45 patients (76%) were treated with immune modulating agents, 94% of these were younger than 18 years at start of therapy and 20/34 (59%) started immune modulating treatment within one year after diagnosis. Interestingly, children treated with immune modulating therapy in the granuloma positive group (16/22) had a significantly shorter time from diagnosis to initiating immune modulating treatment (median 4.5 months, range 0-75), as compared to the children treated with immune modulating therapy in the granuloma negative group (18/23) (median 33 months, range 2-105; $p = 0.01$). This finding is shown in the Kaplan-Meier curve in **Figure 6A**. The same trend, although not statistically significant, was found regarding treatment with TNF-α blocking drugs (i.e. infliximab or adalimumab). See **Figure 6B.**

Altogether 15/45 patients (33%) were treated with TNF-α blocking drugs, 41 % of the granuloma positive patients and 26% of the granuloma negative patients ($p = 0.29$), and the disease duration prior to starting TNF-α blocking treatment was 45 months (median) in patients having granulomas at onset and 78 months (median) in those without granulomas ($p = 0.09$). Additionally, we found that the patients with immune modulating or biological treatment had a longer time until the first surgical intervention as compared to those without such treatment ($p < 0.05$).



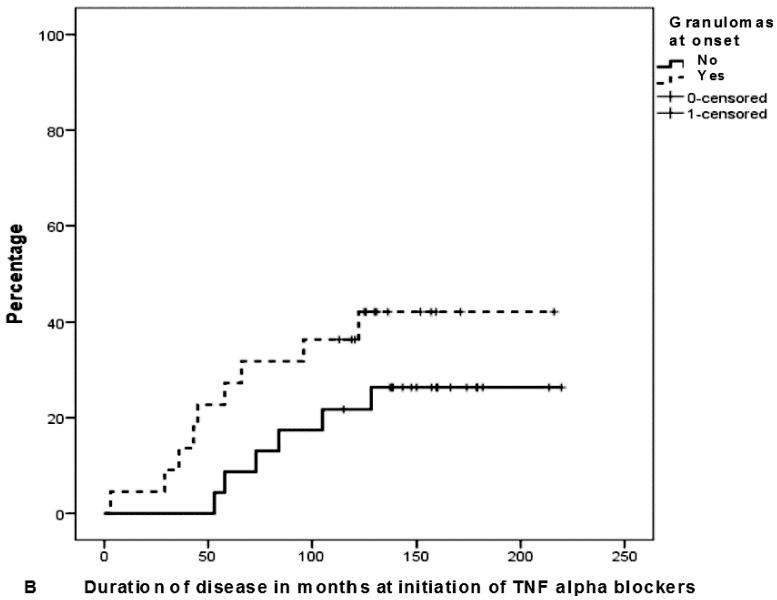


Figure 6. Duration of disease at initiation of immune modulating and TNF-α blocking treatment.

Kaplan-Meier (one minus cumulative survival) curves on the duration of disease at initiation of **A)** immune modulating treatment in patients with such treatment, with and without granuloma findings at onset (p =0.01), cumulative frequency 73% and 78% respectively (NS) and **B)** TNF-α blockers in all patients with and without granuloma findings at onset (p =0.09), cumulative frequency 41% and 26% respectively (p =0.29).

We also set out to use growth impairment as a proxy for severe disease. Weight, BMI, height and height adjusted for target height at onset (n=42) as well as final height and final height adjusted for target height (n=44) were analyzed and related to Swedish national standards. Parental heights were normal, i.e. they did not significantly differ from 0 SDS (-0.11 SDS, p =0.33). To our surprise we did not find any significant growth impairment in this cohort of pediatric CD neither at onset, nor at follow-up. Only one child had a weight less than -2SDS at diagnosis and none had height, height adjusted for target height or BMI less than -2 SDS at diagnosis. Among the 44 patients that had reached final adult height only one had a final height below -2 SDS, but after adjusting final height for target height none was growth retarded.

To rule out the impact of puberty on the results we grouped the children according to age and age below 10 was considered as before puberty. In the pre-pubertal group (n=18) the median height, weight and BMI at diagnosis were not negatively affected. In the group of older children the median height, weight and BMI at diagnosis were below 0 SDS (-0.19 SDS, -0.42 SDS and -0.46 SDS, respectively), but when adjusting height for target height it was not affected (+0.14 SDS).

We also analyzed the possible correlation between growth parameters and the findings of granulomas in histopathology of biopsies from the diagnostic endoscopic examination. There was no correlation between granulomas at diagnosis and growth impairment. Instead the median height SDS (adjusted for target height) at diagnosis in the granuloma positive children appeared to be higher than in the granuloma negative children (p =0.002).

6 DISCUSSION

We have learnt that genetic, environmental and immunologic factors are of importance for the development of CD. Such factors also influence the disease course, the ability to respond to different treatment regimens, the complication frequency and the prognosis. This thesis has contributed to the growing knowledge of the etiological and prognostic factors in CD in children.

6.1 GENETICS AND EPIDEMIOLOGY

Paper I was the first description of *NOD2/CARD15* polymorphisms in a cohort of Swedish pediatric patients with Crohn´s disease. Prior to our study other groups had reported earlier onset of disease in the presence of polymorphisms,[50,57,66] which was suggestive of *NOD2/CARD15* polymorphisms being more frequent in pediatric forms of CD. At the same time, studies on children from the United States and Germany showed similar results as the adult studies with carriage prevalence of 29% and 60%, respectively.[51,244] Thus, it was unexpected to observe such a low percentage of polymorphisms as 8.6% in our cohort, strikingly lower than the previous reports from other European or North American countries.

Consistent with our findings were reports from other Scandinavian countries, such as Finland where 15.5% of the CD patients carried at least one polymorphism[55] and Norway where the frequency was only 7%.[71] Since then, multiple studies have confirmed the variation in prevalence of *NOD2/CARD15* polymorphisms in CD in the general population in different ethnic groups and in different geographic areas.[24,63,64,245] There is no support for the hypothesis that *NOD2/CARD15* genetic variants are more frequent in children with CD as compared to adults.[64] Subsequent studies from Sweden have confirmed our finding of a low prevalence of *NOD2/CARD15* polymorphisms; Halfvarson *et al* reported *NOD2/CARD15* polymorphisms in 13% of Swedish twins with CD[60] and Törqvist *et al* reported a prevalence of 15.2% in a Swedish adult CD cohort.[61] The prevalence in Swedish healthy controls was 5.2% and 4.2% in respective study and no combined heterozygotes or homozygotes were found among the controls.[60,61] Recently, *NOD2/CARD15* polymorphisms were reported in 14.2% of CD patients in Denmark[64] and in 23% of pediatric CD patients in Norway.[200]

The prevalence of each of the 3 different polymorphisms also varies geographically, as well as the prevalence within the healthy population. In a pooled European cohort, 82% of the general population carried the wild-type alleles of *NOD2/CARD15* and homozygosity for polymorphisms is unusual.[63] Even though the penetrance of the disease is relatively low, there are odds ratios for having CD in heterozygote carriers of any polymorphism is between 1.2 and 4.1, and for compound heterozygote or homozygote carriers between 3.3 and 17.1, compared to non carriers.[62,64]

The results regarding the prevalence of *NOD2/CARD15* polymorphisms suggest a south-north gradient of the distribution. The large geographic heterogeneity is likely

related to population history, genetic drift, admixture, and migrations. General observations of such gradients for genetic polymorphisms through Europe are compatible with a spread of Neolithic farmers from the Levant 10,000 yr ago.[246]

Contrary to what one might expect, there is an inverse geographic relationship between the prevalence of *NOD2/CARD15* polymorphism and the CD incidence. Hugot *et al* compared the prevalence of *NOD2/CARD15* polymorphisms and the incidence of CD in the populations studied, and found a low frequency of *NOD2/CARD15* polymorphisms in Finland where the CD incidence was relatively high while the opposite was found in Italy,[63] suggesting that *NOD2/CARD15* polymorphism frequency in the general population does not directly predict a high CD incidence. Similarly, Riis *et al* found significantly lower prevalence of polymorphisms in Denmark and Norway, in combination with a high CD incidence, compared to other southern countries in a non selected European cohort.[245]

Regarding the incidence of CD, a north-south gradient has been described in Europe, with higher incidences in the northern countries and lower incidences in the southern countries (year 1991-1993).[247] The same north-south gradient has been reported within countries, such as Scotland[248] and France.[249] A recent study from the US also reported that the incidence of CD increased significantly with increasing latitude.[250] The reason for these observations may of course be different genetic contributions from ancestral genes from founder populations settled in these areas, but another suggested reason is a relative vitamin-D deficiency in northern (and less sunny) countries or latitudes, which could affect the immune system negatively.[117,249,251] The increasing incidence of CD in some areas[252] and a stabilized incidence in others (e.g. Sweden[20] and Denmark[253]) may result in a diminished gradient.

The low prevalence of *NOD2/CARD15* variants in Sweden opens for speculations on other genetic variants important for CD susceptibility in this geographic region. Torkvist *et al* made an analysis of the CD risk loci reported in a previous genome-wide association study meta-analysis[121] in Swedish IBD patients and the results were only partially concordant with previous results. Associations to CD were found for SNPs in or near *IL23R, IRGM, ZNF365, LRRK2* and *C13orf31,* best nominal significance were found for *TNFSF18, IL18RAP, JAK2, CUL2* and *NKX2-3* whereas other loci repeatedly found in other populations were of small or no relevance (i.e. *ATG16L1, NOD2, TNFSF15, C5orf56* and *PTPN2*).[254] In an earlier study by Torkvist *et al*, the IBD5 locus was associated with CD in the Swedish population with the strongest association with a SNP marker near *SLC22A4* and *SLC22A5* and a trend towards a more severe disease phenotype was observed,[255] which was consistent with other published data in Northern European populations.[256,257] Contrary to the others, Torok *et al* reported associations with colonic disease, non-fistulizing non-fibrostenotic disease and reduced need for surgery.[258] This illustrates the difficulty in identification of the causal variants due to the strong linkage disequilibrium across the IBD5 region, which includes a number of other candidate genes coding for proteins that are involved in immune processes (e.g.

IRF1 (interferon regulatory factor-1), IL-3, IL-4, IL-5 and CSF2 (colony stimulating factor 2).

In paper I we set out to study genotype-phenotype correlations regarding *NOD2/CARD15* polymorphisms and CD in children. Due to the low prevalence this was not possible, but a number of studies have confirmed the association of these polymorphisms with ileal, stricturing and penetrating disease, especially in compound heterozygotes or homozygotes.[50,55,66,68] Ogura *et al* reported that *NOD2/CARD15* linked to ileal disease, through its high expression in Paneth cells in the ileum and its weak expression in the colon.[74] We noted a trend towards granulomas being more frequently found in *NOD2/CARD15* positive patients (p =0.17), and larger studies have reported such association with statistical significance.[50,68,259] This would be in line with the hypothesis that granulomas are formed as a result of defect clearance of microorganisms[179,180]and the suggested negative effects on the immune defense from *NOD2/CARD15* aberrations.

6.2 DISEASE LOCATION

Interestingly, in Sweden the CD patients have a low prevalence of the *NOD2/CARD15* polymorphisms but also, according to the results in papers I, II and IV, relatively low frequency of ileal disease, especially isolated ileal disease, and rather high frequency of isolated colonic disease. An increase in pediatric colonic CD in Stockholm was noted already in a paper in 2003 by Hildebrand *et al*.[13] The disease distribution at onset in pediatric CD in a number of reference papers are shown in **Table 10**. Colonic involvement was present in 58-92%, ileal involvement in 62-90%, isolated ileal disease in 6-37%, and isolated colonic disease in 7-38% of the patients.

Comparison between studies regarding the distribution of the disease at onset is hard, because the results depend on how the patients were investigated (colonoscopy with or without ileum intubation), whether the assessment was made on macroscopic and/or microscopic findings, whether radiology was included, what type of center that was performing the study and how the selection of patients was performed. One can argue that the relatively low frequency of ileal involvement reported in our studies depends on the lack of a full work-up (i.e. endoscopy including ileum intubation and biopsies). To overcome this we extracted the patients in paper IV who had an endoscopic ileum investigation at diagnosis and found the same distribution of the disease; 5% had isolated ileal disease, 50% had isolated colonic disease and 45% had ileocolonic disease.

Table 10. Disease distribution at diagnosis in pediatric Crohn´s disease.

Study	Country	Age at diagnosis	L1	L2	L3
Freeman, 2004 [233]	Canada	<20 y	21%	21%	57%
Griffiths, 2004 [11]	Canada	<17 y	36%	20%	42%
Mamula *et al,* 2002 [203]	USA	<5 y	11%	30%	59%
Heyman *et al,* 2005 [193]	USA	<6 y		32%	
Heyman *et al,* 2005 [193]	USA	6-12 y 13-17 y		20% 24%	
Paul *et al,* 2006 [204]	USA	<5 y		76%	24%
Paul *et al,* 2006 [204]	USA	<16 y	24%	29%	47%
Kugathasan *et al,* 2003 [157]	USA	< 18 y	25%	32%	29%
Levine *et al,* 2007 [262]	USA, Italy, Israel	<17y	21%	25%	54%
Levine *et al,* 2007 [262]	USA, Italy, Israel	<10 y		*NOD2* neg. 48% *NOD2* pos. 20%	
Levine *et al,* 2007 [262]	USA, Italy, Israel	>10 y		*NOD2* neg. 25% *NOD2* pos. 14%	
Sawczenko *et al,* 2003 [154]	UK	<16 y	9%	7%	>80%
Van Limbergen *et al*, 2008 [155]	Scottland	<17 y	6%	36%	51%
Auvin *et al,* 2005 [202]	France	<17 y	19%	10%	71%
Vernier-Massouille *et al*, 2008 [156]	France	<17 y	14%	17%	69%
Pigneur *et al*, 2010 [230]	France	<16 y	37%	26%	36%
Perminow *et al,* 2009 [200]	Norway	<18 y	8%	23%	69%
Jakobsen *et al,* 2011 [253]	Denmark	<15 y	21%	38%	41%
Pediatric CD, range (subgroups excluded)			6-37%	7-38%	29%-80%
Ideström *et al*, Paper IV	Sweden	<17 y	5%	50%	45%

Disease distribution; L1= isolated ileal, L2= isolated colonic and L3= ileocolonic disease y =years

One can speculate whether colonic disease is more common also in adult CD in northern countries as both a Swedish and a Danish population-based study on adult CD found an increased colonic phenotype (>50%)[260,261] and, in line, a recent population-based Danish study found isolated colonic localization in 48% of adults with CD.[253] Riis *et al* compared disease characteristics in adult CD between North and South European centers, but found no statistically significant difference regarding gender, age, disease location or behaviour at time of diagnosis, although isolated colonic disease was most frequent in Norway (41.9%), Denmark (41.4%) and the Netherlands (41.5%).[245]

In paper II we showed that ileal disease is age dependent and that it occurs rarely before the age of 9. When ileal disease is present, it is most often associated with colonic disease. We also showed that the probability of ileal disease increases with age until puberty and/or adulthood. These findings in paper II, as well as findings regarding disease distribution and *NOD2/CARD15* polymorphisms in papers I and IV, are consistent with other reports on less ileal disease and more colonic disease in patients younger than 5-6 years[193,203,204] and an association between isolated colitis in CD at young age (<10 years) and absence of *NOD2/CARD15* polymorphisms[262] (also shown in **Table 10**). Our finding of age- dependent ileal involvement supports the hypothesis that the Peyer´s patches (PPs) and the isolated lymphoid follicles are potential sites for CD lesions. While the colonic isolated lymphoid follicles do not vary with age, the ileal PPs develop early in fetal life, grow in size and number until puberty, and then undergo involution.[125,126] As a result, the ratio ileitis/colitis should be lower in young children, as shown in our study, and in seniors, which is supported by reports on less ileal involvement in seniors than in young adults.[30]

6.3 GROWTH

Growth impairment is regarded as a prominent feature of CD in children, although studies in pediatric CD report a great variety on the prevalence of growth impairment. Not all have used the same definition of growth impairment, or considered the influence of the patients´ age, pubertal stage or target height. Target height is considered the best predictor for final height[263] and it is, therefore, advisable to evaluate final height as well as height at diagnosis after adjusting for target height. In paper IV we did adjustments of both height at diagnosis and final height to target height, and we also extracted a subgroup of children younger than 10 years to rule out the influence of puberty. Interestingly, the patients in paper IV were not growth impaired, neither at onset nor as adults. The absence of growth impairment is congruent with the current trend towards lower frequencies of growth impaired children with CD[209] but differs from other studies reporting impaired final height.[208,264]

The normal growth and attainment of expected final height among our patients during the course of their disease may be explained by fact that the children were not growth impaired at diagnosis, but an early use of enteral nutrition, lesser use of corticosteroids, a frequent use and early start of immune modulating agents and TNF-α blockers might also be contributing reasons. The absence of growth impairment at the time of

diagnosis seems more difficult to explain, since that is dependent on factors unrelated to treatment modalities. We speculate that a general awareness among referring colleagues and an easily accessible health care may have contributed to the short duration of symptoms before diagnosis (median 3.4 months). Since colonic and not small bowel disease prevailed among the children, the prominent symptoms caused by colitis may have contributed to symptoms prompting to seek medical care with less delay. Both longer delay and small bowel disease are known from other studies to be associated to growth impairment.[96,154,265]

Normal growth is dependent on hormones, of which growth hormone acting through IGF-I is of great importance. The actions of IGF-I are modulated by IGF binding proteins, mainly IGFBP-3, which affects the availability of IGF-I in the circulation. Undernutrition depresses IGF-I and growth failure in children with CD has therefore been attributed to poor nutrient intake. However, animal work (on TNBS colitis) has shown that the inflammatory process depressed growth and IGF-I partly independently of nutrition and that it was characterized by elevated IL-6 levels.[241]

The severity of growth impairment in individual patients does not always correlate to the level of intestinal inflammation as assessed by symptoms, raising the speculation that genetic polymorphisms might influence growth patterns. However, the difficulty and lack of consistency in defining growth impairment, makes analyses of correlation of growth impairment with genotype almost impossible in large scale studies. In paper III we have strengthened the hypothesis that common genetic polymorphisms which alter cytokine expression may contribute to growth impairment, but not influence the overall susceptibility to CD. In the animal studies in paper III, using the TNBS colitis model, we demonstrated that blocking the effect of IL-6 enhances growth, not through reduced intestinal inflammation or improved nutrient intake, but directly through the decrease in circulating IL-6. With the finding that IL-6 may regulate growth in inflammatory diseases, we hypothesized that genetic variations in IL-6 expression would influence the consequences of disease activity on growth retardation. We studied the *IL6* -174 polymorphism because variations in this region of the *IL6* promoter are associated with variations in the IL-6 response[243] and found that the *IL6* -174 genotype determines growth retardation at diagnosis in childhood onset CD. There was no difference in genotype distribution between the children with CD and the controls indicating that the *IL6* -174 genotype was not associated with the disease itself, which is in agreement with previous and later reports.[48,266,267] Other studies have also shown that there is no correlation between haplotype and short stature among healthy people in whom the promoter would have been inactive.[268] However, the *IL6* -174 promoter has been shown to be important as a disease modifying gene, influencing not only growth but also disease location (i.e. the GG haplotype associated with ileocolonic disease and the CC haplotype associated with isolated colonic disease)[269] and age at onset (i.e. younger age at onset in males with the GG haplotype).[267]

Similarly to our data regarding the *IL6* promoter polymorphisms, a study of Israeli patients suggests that relatively common variations in the promoter region of the TNF-

α gene may have an independent effect on linear growth outcomes.[96] *NOD2/CARD15* gene polymorphisms have not been associated with growth retardation or growth failure.[207] Lower weight and a trend towards reduced height was found by Tomer *et al*, although this finding may rather be secondary to the association between *NOD2/CARD15* gene variants and ileal disease.[244]

Growth sparing treatment should be a priority in children with the *IL6* -174 GG genotype, as well as in all children with growth failure. One example of such treatment is EEN, which is already used as primary treatment for CD in children.[174,190] EEN results in a rapid fall in IL-6 and an increase in circulating IGF-I.[222] IL-6 blocking treatment has not been studied in children with growth or height as an endpoint, but results in a trial on adult CD (n=36) showed some clinical effects, but there were no endoscopic or histological differences compared with placebo.[270]

6.4 COMPLICATIONS AND SURGERY

According to previous long-time follow-up studies, disease behaviour rather than location show progression with time in pediatric and adult CD.[155,156,229] In paper IV we followed a cohort of 45 pediatric CD patients for 9 to 18 years, median 12.3 years, which is ample time to observe changes in disease behaviour, for complications to appear, and to monitor medical and surgical treatments. The majority (80%) of the patients (n=36) still had non-stricturing, non-penetrating disease at follow-up but 20% had developed strictures. Vernier-Massouille *et al* reported that only 41% of the children had non-stricturing, non-penetrating disease at 10 years follow-up and 44% had actually developed stricturing disease.[156] Pigneur *et al* reported that 25% of the pediatric patients had stricturing disease after 10 years,[230] and Freeman *et al* found strictures in 29% after median follow-up of 12.2 years.[233] The differences in complication rates may reflect differences in disease location as stricturing disease behaviour has been found associated to ileal and jejunal disease.[155,229] Risk for surgery is also associated with stricturing and/or penetrating disease as well as ileal or ileocolonic, rather than colonic disease.[233,271] Strictures, abscesses/fistulas and surgery are also associated with older age within pediatric CD (>13 years),[232,271] which may be an effect of the age influencing the location. The relatively low incidence of small bowel involvement in our studies (paper I, II and IV) might be a factor influencing the low number of complications in terms of internal fistulas, strictures and the frequency of surgery. We found that surgery had been performed in 22% of the patients in total and in 9% within the first 5 years (paper IV), which is somewhat lower compared to other pediatric studies of CD that has reported surgery in 28-60% after >10 years of follow-up[230,232,233] and 14-34% after 5 years of disease.[156,232,253,271] Our findings are in line with the results of Picco *et al* which indicate that disease limited to colon is associated with less surgery[234] and the results of Schaefer *et al* which show a decreased risk of surgery when colon was involved.[271] Of course, reluctance to surgery in young CD patients and a different treatment policy in our unit compared to others may also have played a role for the relatively low number of patients that had undergone surgery.

6.5 TREATMENT AND DISEASE MODIFICATION

Although the treatment goal, to achieve and maintain remission, normal growth and puberty as well as a good quality of life, is well accepted regarding pediatric CD, the choice of strategy to reach the goal may be discussed. On one hand the treatment needs to be safe and cause as little negative side-effects as possible, on the other hand it must be effective and if possible protect the child with CD from complications. We generally use a step up treatment strategy, but there is an increasing awareness of the need of early immune modulating treatment in subgroups of patients.[174]

In paper IV we noticed that a large proportion of the patients (76%) were treated with immune modulating agents and almost half of them were started on such treatment during the first year of illness. It has been shown that surgical treatment is less frequent among patients treated with immune modulators or TNF-α blockers, compared to those without such treatment[232,234,272] and the time to initiation of immune modulators was shorter in a non-surgery group compared to patients who underwent surgical treatment.[156] In our data in paper IV we could only trace a similar trend, as the time to the first surgical procedure was longer in patients who were treated with immune modulators or TNF-α blockers compared to those who did not receive such treatment, although the low number of patients in the surgery group makes these results unreliable.

The effects of specific treatments on the rate of surgery often become speculative due to limitations of the design of the previous studies. More aggressive treatment may reflect a worse disease course, which makes it difficult to interpret the results. Even though the use of immune modulating agents (i.e. thiopurins or methotrexate) has increased over a period of time and is initiated earlier during the disease course, Cosnes *et al* found that the frequency of stricturing and perforating complications, as well as the need for intestinal resections, have remained stable over 25 years.[273] However, the limitations of that study are important to consider. It was a retrospective study from a tertiary referral center (generally treating patients with severe CD) and very few (8.4%) of the surgical patients had azathioprine prior to surgery, which may be the reason for the need of surgery and probably azathioprine was initiated too late in their disease course to prevent stricture or fistula formation. Benchimol *et al* showed that treatment changes in children with CD between 1994-2007 (including increased immunomodulator use and increased outpatient care by pediatric gastroenterologists) were associated with reduced surgical rates[274] and Ramadas *et al* also found decreasing surgical rates associated with increased and earlier thiopurine use over time (1998 to 2003).[272]

The potential for disease modification in CD, by various therapeutic interventions is discussed further in a recent review by Van Assche *et al.*[275] It was concluded that one surrogate marker of disease modification is the healing of CD lesions, since the mucosal lesions reflect ongoing inflammation and tissue damage, which in turn lead to fistulas, abscesses, adhesions and strictures and thereby also surgery. Nevertheless, no prospective long-term trial has studied the value of mucosal healing to predict a

decreased need for surgery or hospitalization as the primary outcome, and it has not yet been convincingly demonstrated that mucosal healing also means a turn off of deeper inflammation.

Both thiopurines and anti-TNF-α agents have shown potential of inducing mucosal healing. The time point at which the initiation of therapy takes place is of great importance, as the longer the disease is left untreated the more irreversible the damage will be. There is evidence from pediatric IBD that more aggressive treatment early in the disease course is associated with improved response and remission rates.[276-279] Indirect evidence that early aggressive treatment may induce profound remission are the results from a study on step up verses top down treatment strategy on newly diagnosed CD by D'Haens *et al* where the top down approach included induction therapy with infliximab and azathioprine. After two years, complete mucosal healing was observed in 75% of patients in the top down group compared with only 21% of patients in the step up treatment group. The top down group continued on episodic infliximab treatment with a median of 16 weeks between infusions, as compared to 8 weeks in the step up group.[280]

Anti-TNF-α agents are proven to be an effective maintenance therapy for pediatric CD, but a problem is that a substantial number of patients lose initial response, and adjustments in the treatment schedule (i.e. dose escalation, reduction in dose interval, or both) are frequently needed to maintain clinical remission.[281,282] In adult CD, a combination of azathioprine and inflixmab has been shown to be more effective than monotherapy of either agent.[283] Studies on the potential benefit of combination therapy in children are limited and due to a possibly increased risk of toxicity in children on combination therapy, we presently convert from combination therapy to monotherapy within 6 months (or combine the anti-TNF-α agent with methotrexate).

Accordingly, more prospective intervention studies in newly diagnosed patients, preferably large randomized controlled studies, will be needed to evaluate the effects on disease course by different agents and strategies.

It is of great importance to weigh the probable benefit of more and early aggressive therapy against the disadvantages and potential risks (i.e. mainly increased rates of infections and malignancies) associated with such treatment, especially in children who have many years on medication ahead. Patients with IBD have an increased risk of opportunistic infections and especially patients on a combination of more than one immunomodulator and those with malnutrition.[284] Regarding anti-TNF-α treatment in children, pooling of pediatric IBD studies shows serious or unusual infections in approximately 3% of patients treated with infliximab (n=1483) or adalimumab (n=237) (e.g. sepsis, Listeria monocytogenes meningitis, herpes zoster or varicella infections, reactivation of Epstein–Barr virus, pneumonia, abscesses, cutaneous tinea infections, opportunistic fungal infection, osteomyelitis, cellulitis, Pseudomonas infection, pseudomembranous colitis, gastroenteritis, appendicitis and pancreatitis).[285] Other rare events in CD are hemophagocytic lymphohistiocytosis (HLH),

histoplasmosis and disseminated cytomegalovirus. Studies in adult IBD on the risk of developing malignancies when treated with thiopurines and/or TNF-α blockers have shown conflicting results. However, a meta-analysis showed a fourfold increased risk of lymphoma in IBD patients treated with thiopurines[286] and another meta-analysis on adult CD patients demonstrated that use of anti-TNF-α drugs in addition with immunomodulators was associated with a threefold increased risk of non-Hodgkin's lymphoma.[287] In young IBD patients treated with anti-TNF-α and/or thiopurines, a serious and extremely aggressive type of lymphoma, hepatosplenic T cell lymphoma (HSTCL), has been reported[227] and the rates for malignancies in anti-TNF-α treated children (with various diagnoses) in USA were higher than in the general US pediatric population.[288] It is currently not known whether the development of malignancies, or the other rare events previously mentioned, is related to the severity and chronicity of the inflammation, to the long term use of immunosuppressants, or to a combination of both.

Finally, mucosal healing in a larger proportion of patients could of course have additional advantages in terms of quality-of-life of our patients and cost for society, assuming that surgery, hospitalization and open clinic consultations for flare-ups are expensive, and thereto added the costs of sick leave and disability, but a possible downside of for example earlier anti-TNF-α therapy may be the increased cost of the drug, added to the risks of infections and malignancies. We do not yet know who would benefit the most from early aggressive (top down) treatment because of lack of reliable predictors, and one should keep in mind that a large proportion of the patients in fact have a disease remission state over time,[239,289] which means that having anti-TNF-α agents as the first-line therapy would correspond to an overtreatment for many CD patients.

6.6 MARKERS FOR PREDICTING DISEASE COURSE

Several previous studies on both adult and pediatric CD have investigated which clinical variables are associated to complicated disease course, most often defined as need for surgery, progression towards non-inflammatory disease behaviour, relapse frequency and steroid dependency. In paper IV we used growth impairment, immune modulating (including thiopurines and methotrexate) and TNF-α blocking treatment, as well as surgical interventions as surrogate markers for severe disease.

Disease location as a risk factor has already been discussed; small bowel disease is associated to higher risk of complications such as strictures, fistulas, surgical procedures and growth impairment, and since age influences the disease location (paper II), older age within the pediatric population may in that sense be regarded as a risk factor. Involvement of upper GI tract has been found associated to higher surgery risk[239] and is a predictive factor for disease recurrence.[289] Lower age at diagnosis and coexisting upper GI tract involvement have been found significantly associated with steroid dependency in children with CD.[290] Lack of mucosal healing after induction therapy has also been proposed to be a predictor of aggressive disease course including

greater disease activity and an increased need for medical treatment.[291,292] Current smoking has already been mentioned as a risk factor for developing CD and is associated with higher recurrence rates and increased risk for surgery.

In paper IV we studied the significance of granuloma findings and conclude that presence of granulomas at onset may be a marker for a more severe disease. Why some patients, preferably children, form granulomas, and others do not, probably has to do with the function of the immune system and the possible underlying genetic aberrations. Perminov *et al* found an increased frequency of mucosal macrophages, together with an elevated activation level (e.g. CD40 expression), especially in the colon of untreated CD children, which might explain the colonic phenotype and the increased numbers of granulomas in pediatric CD.[293] The vast majority of the granuloma findings in paper IV were done in biopsies from colon in agreement with previous studies stating that the frequency of granulomas gradually becomes higher more distally along the GI tract.[201,294,295] One could suspect that the formation of granulomas is associated with variants in *ATG16L1* and *IRGM* genes, which are believed to affect the autophagy pathways and give rise to impaired pathogen clearance in the cell, but variants in these genes have not shown any significant association with granulomas.[296] The possible association between granulomas and *NOD2/CARD15* polymorphisms is discussed above, whereas studies on known genetic variants in *TLR4* and *TNF*-α genes did not show associations with granulomas.[295,297]

Granuloma findings at diagnosis were found to be associated to upper GI involvement and earlier initiation of immune modulating agents in children with CD in paper IV. We interpreted this as these children were having more extensive disease and a need of more aggressive treatment and thus having a more severe disease. Other studies have indeed shown associations between granulomas and complicated or severe disease.[195-199,298-300] Markowith *et al* noted that pediatric patients with granulomas in the initial rectosigmoid biopsies more often had extensive disease, perianal complications and surgical interventions.[299] Molnar *et al*, as well as Freeman *et al,* found associations between granulomas and complicated disease behaviour, especially penetrating and extensive disease with upper GI localization, also requiring more surgical interventions, hospitalizations and immunosuppressive treatment.[195,199] De Matos *et al* found an association between granulomas in initial biopsies in children and perianal disease, gastritis and infliximab treatment.[196] Recurrence after surgery is the most studied outcome and a recent meta-analysis on studies mainly in adults showed significantly higher rates of recurrences and reoperations in the granulomatous group compared to the non-granulomatous group.[300] On the other hand, there are other clinical studies that did not confirm an association between granulomas and a more complicated disease phenotype.[294,301,302] Gupta *et al* even found that granulomas were inversely associated with risk for surgery.[232]

In a recent review, Kaser *et al* speculate that the subset of patients which exhibit granulomas, seen as the pathologic hallmark of IFN-γ expression, may benefit from

INF-γ blockade,[303] although such blockade has showed limited efficacy in human IBD (compared to a positive effect in murine experimental colitis).[304]

The rapid development in the area of genetics is of course promising when it comes to the ability to subgroup the patients regarding their genotypes and further on hopefully foresee the phenotypes and prognosis of the disease. Today we can generally only state what clinical features that are statistically associated to different genetic alterations, and not forecast which patients that will develop a specific complication.

The prognostic power of *NOD2/CARD15* alterations has been studied in a recent meta-analysis.[305] The presence of a single *NOD2/CARD15* variant had poor predictive ability for disease phenotype in CD. However, the presence of two variant alleles in *NOD2/CARD15* (either homozygous or complex heterozygous) was associated with a relative risk of 1.44, with a high degree of specificity (98%) for aggressive disease phenotype, although the sensitivity was poor (11%). The presence of homozygosity for Leu1007fs had an even higher degree of specificity for aggressive disease phenotype, although the sensitivity remained poor.[305] Thus testing for double dose variant alleles could identify a particularly high-risk group, where targeted early-intensive therapy might be beneficial, although it is a very insensitive prognostic test and would likely miss many patients with aggressive disease. In populations with low prevalence of the CD associated *NOD2/CARD15* variants, as in Sweden, such testing would rarely add helpful information.

The *IL6* -174 genotype mediates growth failure in children with CD. As suggested in paper III, growth promoting treatment should be a priority in children with the *IL6* -174 GG genotype. Since IL-6 is central in many inflammatory conditions, our findings could be relevant for growth impairment in other such diseases in childhood. The *IL6* genotype may also influence other clinical aspects of the disease, as discussed above, but whether different haplotypes of the *IL6* gene hold prognostic power for disease phenotype has not yet been evaluated.

Several immune-mediated antibodies against microbial antigens have been described in IBD and the most studied antibodies are perinuclear antineutrophil cytoplasmic antibody (pANCA), anti–Saccharomyces cerevisiae antibody (ASCA), antibody to the outer membrane porin of Escherichia coli (anti-OmpC), antibody against flagellin expressed by Clostridial phylum (anti-CBir1), anti–chitobioside carbohydrate antibody (ACCA), anti–laminaribioside carbohydrate antibody (ALCA), and anti–mannobioside carbohydrate antibody (AMCA).[306] Evidence suggests that these serologic markers can help to establish a diagnosis of IBD and to differentiate CD from UC, particularly when used in combination.[307] and in CD, multiple studies have linked the presence of antibodies to more complicated disease, including fibrostenosis, internal penetrating disease, as well as increased need for surgical interventions involving the small bowel.[306] Moreover, it seems that having multiple positive serologies or high titers predicts poor outcome. Dubinsky *et al* examined how the degree of immune response to ASCA, anti-OmpC, and anti-CBir1 correlated with internal penetrating and

stricturing disease and the need for surgery in a large pediatric CD cohort, and they showed that the risk for the development of complicated CD was higher among patients positive for three of these antibodies compared to those positive only for two or less.[308]

Fecal calprotectin has been suggested to be useful marker for predicting the disease course in a shorter perspective, i.e. predicting disease relapse within the coming months or years (depending on the study) in both adult and pediatric IBD. Cut-off values of 250-400 μg/g have been proposed to predict a coming relapse in patients in clinical remission.[309-311] A recent meta-analysis stated that fecal calprotectin is useful to predict relapse in quiescent IBD patients and the pooled sensitivity and specificity of predicting relapse was 78% and 73%, respectively.[312] Fecal calprotectin has also been studied as a prognostic marker for outcome after induction treatment with TNF-α blocking agents in adult luminal IBD, and a cutoff concentration of 139 μg/g had a sensitivity of 72% and a specificity of 80% to predict a risk of relapse within 1 year, and a normal fecal calprotectin after induction therapy with a TNF-α blocker predicts sustained clinical remission in the majority of patients on scheduled therapy.[313] Fecal calprotectin has been shown to reflect the degree of microscopic inflammation,[163] thus these results are all in line with the advantages of mucosal healing discussed above.

Due to the complex pathophysiology of CD and the heterogeneity of the phenotype, it is important to combine genetic, clinical and serologic information into predictive models for disease course and treatment response. Panels that incorporate multiple genetic loci and/or multiple serologic markers have been presented and more will likely develop further over the next few years.[314-317] Future studies dealing with prognostic markers will require large-scale, multicenter trials to find sufficient clinical data to accurately determine the cumulative risk of clinical, serologic, genetic, and environmental factors. The importance of uniformed diagnostic work-ups, diagnostic criteria and phenotypic classifications cannot be stressed too much and accordingly ESPGHAN has defined the criteria for pediatric IBD[159] and now the EUROKIDS registry collects prospective data on pediatric IBD from 17 European countries.[165] It would also be of interest to use well defined sub-phenotypes, such as treatment responders/non-responders and defined geographic areas, to understand possible differences between CD in various countries as well as ethnical groups. When additional predictive factors are identified and validated, predictive models will hopefully become powerful tools that can be used in the clinical setting and then we will be better able to study targeted therapies in high-risk patients. The ultimate goal is to treat the individual patient with the most suitable agent, with the optimal timing and with minimized risks, to improve prognosis and quality of life.

7 CONCLUSIONS

The following conclusions can be drawn from the studies presented:

- Pediatric Crohn´s disease in our patient cohort is dominated by more colonic disease and less ileal involvement (paper I and IV)

- Growth impairment is infrequent in our patient cohort of pediatric CD, both at onset and at follow-up (paper IV)

- *NOD2/CARD15* polymorphisms are uncommon in Swedish pediatric CD (paper I)

- Ileal involvement seldom occurs before the age of nine years and the probability of ileal disease increases with age until adulthood (paper II)

- Isolated ileitis is rarely found in pediatric CD and ileal involvement is often associated with colonic disease (paper II)

- IL-6 causes growth suppression in rats with TNBS colitis and by blocking IL-6 with antibodies the IGF-I levels are increased and growth is enhanced, without improving the intestinal inflammation or increasing the food intake (paper III)

- Children with CD that have the *IL6* -174 GG genotype have higher CRP levels during active disease and are more growth-retarded at diagnosis, indicating a negative influence from a higher expression of IL-6 in patients with the GG haplotype (paper III)

- Epithelioid cell granulomas are found in half of the pediatric CD patients at onset and are associated with both upper GI involvement and a shorter time to initiating immune modulating drugs, suggesting an association with a more aggressive disease (paper IV)

- Final length is not negatively affected by findings of granulomas at CD diagnosis (paper IV)

8 FUTURE PERSPECTIVES

The advances in the fields of genetics and immunology have enhanced our knowledge and understanding of the pathophysiology and etiology of CD. For many of the genetic loci that are associated with CD potentially causal genes have been identified.[48] For confirmation of their role, detailed fine mapping as well as expression and functional studies are necessary. An important future goal is to find evidence of sub-phenotype associations, both related to disease behaviour to predict disease course, and to response to various treatment options. A successful example from another area is the identification of a genetic variant in the *IL28B* gene that is associated with response to anti-hepatitis C virus treatment and the natural clearance of a hepatitis C virus.[318,319]

To be able to find associations with genetic variants with lower allele frequencies and/or in smaller phenotypic subgroups, the studies have to be large. This is especially challenging when studying individuals from certain geographical areas or with specific ethnic background, as well as children, since these study populations are smaller.

A strategy to obtain large-scale fine mapping studies is to use custom array-based technologies. Underway is the Immunochip project of the International IBD Genetics Consortium where a custom designed chip with 200 000 markers is to be studied on 15 000 CD patients, 12 000 UC patients, 21 000 healthy controls and 100 000 samples from other immune-mediated conditions.[320]

As discussed above, it is of great importance to have uniform diagnostic work-ups, diagnostic criteria and detailed phenotypic classifications, as well as to combine genetic, clinical and serologic information to be able to predict disease course and treatment response. The influence from the environment must also be taken into account and in the future an integration of epigenetic data obtained from micro-RNA profiles will also be useful. (Epigenetics refers to modifications of the genome that have functional effects, but does not involve changes in the nucleotide sequence of the DNA).

The growing field of transcriptomics, referred to as expression profiling, may hold potential in terms of refining the diagnostic and prognostic information in IBD. Tools that allow measurements of genome-wide RNA from the peripheral blood and tissue of patients can result in gene expression profiles that may predict prognosis. For example, a recent study identified a gene expression profile from peripheral blood T cells that was associated with a more severe disease course of IBD[321] and another study demonstrated the role of gene expression profiles (in biopsies) in predicting response to TNF-α therapy in colonic CD.[322]

Several times in this thesis it has been stated that relevant prognostic markers would be very useful when selecting patients for early aggressive treatment, to avoid both over-treatment accompanied with the risk of unnecessary side-effects and under-treatment

with the risk of irreversible complications of the disease. Of importance is of course to know not only at what time point a certain treatment should be initiated but also which treatment is the most suitable for each individual. Further genotyping may also give the potential for sub-classification of the disease regarding the dominating disease causing alterations and impairments of the immune response in the individual, and this would give us the possibility to use more targeted therapy. One example could be the autophagy dysfunction, which is thought to be an important mechanism in the pathogenesis in CD following the alterations in several genes involved in autophagy (e.g. *ATG16L1* and *IRGM*) that have been found to be associated with CD. Since there are agents already known to either upregulate autophagy (rapamycin, lithium) or inhibit it (quinine, valproate) this could theoretically be a treatment option for selected individuals.[323]

Most of the therapeutic agents used in CD today are aiming at dampening the immune response. The individuals in whom future screening may reveal an impaired innate immune defense as the dominating underlying cause would perhaps instead benefit from immunostimulating treatment. One example of such treatment is the granulocyte-macrophage colony stimulating factor which has shown therapeutic effect in (congenital) phagocyte disorders. Clinical trials of recombinant granulocyte-macrophage colony stimulating factor (Sargramostim) have been performed in CD patients, with positive effects in some patients,[324-327] although large, randomized trials have failed to reach primary clinical end points,[325] suggesting that alterations in granulocyte-macrophage colony stimulating factor function may be linked only to subsets of IBD.[328] This is supported by studies that have reported neutralizing autoantibodies to granulocyte-macrophage colony stimulating factor in subsets of pediatric and adult CD patients[329] and defective expression and function of the granulocyte-macrophage colony-stimulating factor receptor among IBD patients.[330]

Several new biologic agents have been tested or are under development (e.g. anti-IL-12/23, anti-IL-17, IL-6 inhibitors, anti-IFN-γ and antibodies blocking adhesion molecules or chemokines) and even though none have yet proved superior to anti-TNF-α[331] they might have to be re-evaluated when using specific sub-populations,[332] higher doses or different ways of drug administration.

Another way of targeting the treatment would be to deliver potent therapeutic agents directly to the sites of inflammation in the GI tract. Trials with engineered enteric bacteria, which produce and secrete the anti-inflammatory cytokine IL-10, have been performed in CD, but the efficacy is unclear.[333] Using a nonreplicating adenoviral vector for IL-10 expression in colon has shown positive effects in IL-10 knock-out mice[334] but there are safety concerns regarding the use of viral vectors in humans. Instead, a nonviral method of gene transfer would be preferred. For example, nonoparticles can be designed to concentrate in the target tissue and deliver the therapeutic agents.[335] Hopefully, the increasing knowledge in the field of nanomedicine will lead to progress regarding treatment of IBD in humans.[336]

Stem cell therapy for intestinal diseases is also an emerging area. Although clinical remission of IBD has been reported in patients who were treated with autologous (i.e. originating from the recipient) or allogeneic (i.e. originating from a donor) hematopoietic stem cell transplantation for coincidental malignancies, the risks accompanied with the long term immunosuppressive treatment and risk of graft versus host disease, are seldom outweighed by the benefits. However, there are reports on autologous stem cell therapy as salvage therapy for IBD and a multicenter, prospective, randomized, phase III trial (ASTIC trial) is supposed to report the initial analyses within a year.[337,338] There are also sporadic reports on successful allogeneic bone marrow stem cell transplantation in cases with well defined underlying genetic mutations, such as in the gene encoding the IL-10 receptor,[339] and in which autologous stem cell transplant would not alter the underlying genetic polymorphism.

Mesenchymal stem cells have clear advantages due to their immunosuppressive capacity and they have been tested in several preclinical models for the treatment of IBD and, subsequently, in a few clinical trials. Injected mesenchymal stem cells are believed to home to areas of injury where they have reparative functions and/or act as a source of secreted factors that stimulate repair and inhibit inflammation. These multipotent progenitor cells can be isolated from a variety of tissues and can differentiate into many different cell types.[340] They were earlier thought to be 'non-immunogenic', however, it is now known that these cells are detected by the immune system of the recipient which results in the clearance of the injected cells.[341] One small study on autologous mesenchymal stem cells, given to patients with active and treatment refractory CD, reported clinical improvement in 3/7 patients.[342] A larger, randomized, placebo controlled, double-blind Phase III study on a allogeneic mesenchymal stem cell product derived from healthy donors (Prochymal brand of remestemcel-L) for CD was begun in 2007 and is still ongoing.[343,344] Local injections of autologous mesenchymal stem cells have shown efficacy in fistulizing CD.[345-347] Further research is needed in this area to answer a number of questions regarding safety, efficacy, mechanism, the optimal dose and way of administration (systemic, local injection or topical application) and usage (as inductive and/or maintenance therapy) of mesenchymal stem cell therapy in CD.

There is exciting and challenging future research to be done to further identify the etiology of CD and its pathophysiology. The heterogeneity in disease genotype and phenotype, as well as disease outcome and prognosis, has to be especially addressed when aiming towards individualized treatment, which hopefully may direct the disease course in the desired way.

9 SAMMANFATTNING PÅ SVENSKA

Förkortningen IBD (efter engelskans ”inflammatory bowel disease”) används som samlingsnamn för huvudsakligen två kroniska inflammatoriska tarmsjukdomar; ulcerös kolit och Crohns sjukdom. Till skillnad från ulcerös kolit som endast involverar tjocktarmen, kan Crohns sjukdom vara lokaliserad var som helst längs mag-tarmkanalen, från munhålan till anus. Sjukdomsstart sker oftast i tonåren eller i ung vuxen ålder, men även små barn kan drabbas. I Sverige insjuknar upp emot 200 barn (under 16 år) varje år i IBD, 2/3 av dessa får diagnosen Crohns sjukdom. Vanliga symtom är buksmärtor, diarré, blod och slem i avföringen, viktnedgång och försämrad längdtillväxt. Vissa har, eller får, komplicerande s.k. fistlar eller bölder kring anus, förträngningar på tarmen (stenoser) eller påverkan på andra organ så som lever, gallvägar, leder, hud eller ögon. Sjukdomen går i skov, dvs. besvären blossar upp i perioder, vilka är svåra att förutse. Hur svår sjukdomen kommer att bli för den enskilde individen är mycket varierande och det saknas idag pålitliga markörer för att förutspå förloppet. Orsaken till Crohns sjukdom är inte helt känd, men flera faktorer samverkar, så som genetiska avvikelser, miljöfaktorer och förändringar i immunförsvarets funktion.

Denna avhandling omfattar fyra olika studier av Crohns sjukdom hos barn. Dessa studier syftar till att öka kunskapen kring sjukdomen och vad som påverkar dess förlopp och att därigenom bättre kunna förutse behoven hos den enskilde patienten.

Deltagarna i studierna är barn och ungdomar med diagnosticerad Crohns sjukdom som kontrolleras på barngastroenterologiska enheter i huvudsakligen Stockholm, Paris och London. I studie 3 ingår även en delstudie på råttor.

I **studie 1** undersöks förekomst och betydelse av varianter av den s.k. NOD2/CARD15-genen som finns hos upp till 60 % av personer med Crohns sjukdom i södra Europa och i Nordamerika. Det visar sig att i gruppen med svenska barn med Crohns sjukdom (58 st) är det endast 8,6 % som är bärare av någon variant i denna gen. På grund av den låga förekomsten av dessa genvarianter hittas inga associationer till en särskild sjukdomsbild, men ett samband mellan NOD2/CARD15-varianter och sjukdom lokaliserad i tunntarmen har rapporterats av andra forskare. Fynden i studie 1 är intressanta eftersom det tycks som om gruppen med svenska barn med Crohns sjukdom inte bara har lägre förekomst av just dessa genvarianter, utan också har mer tjocktarms- än tunntarmslokalisation av sin sjukdom. Lägre förekomst av NOD2/CARD15-varianter har på senare tid även rapporterats från grupper av vuxna Crohn-patienter i Sverige och i andra nordiska länder.

I **studie 2** undersöks 191 barn med Crohns sjukdom med avseende på om det finns något samband mellan ålder och lokalisation av inflammationen vid insjuknandet. Det visar sig att inflammation i nedersta delen av tunntarmen (ileum) är ovanligt hos barn som är diagnosticerade före 9 års ålder, och att sannolikheten för sådan lokalisation sedan ökar med stigande ålder upp till vuxen ålder. Således är det vanligast att de yngsta barnen med Crohns sjukdom har inflammation i tjocktarmen. Att de allra yngsta

har mer tjocktarmslokalisation har bekräftats i flera andra studier. En förklaring till detta samband skulle kunna vara att den s.k. lymfoida vävnaden, som föreslagits som inflammationens startplats, förekommer oberoende av ålder i tjocktarmen, medans den i tunntarmen utvecklas med stigande ålder upp till vuxen, och sedan tillbakabildas.

I **studie 3** undersöks om reducerad längdtillväxt hos barn med Crohns sjukdom korrelerar till någon särskild variant av den gen som styr produktionen av ett inflammationsbefrämjande ämne som heter interleukin (IL)-6. Vid tidpunkt för Crohn-diagnos hos de 153 undersökta barnen, visar det sig att barnen med en specifik variant av genen är mer tillväxthämmade jämfört med de barn som har andra genvarianter. Detta, och resultatet från en parallell studie på råttor, talar för att IL-6 och en specifik variant av IL6-genen bidrar till hämmad längdtillväxt vid aktiv Crohns sjukdom. Sannolikt finns det på liknande sätt olika varianter även av andra gener som påverkar t.ex. risken för tillväxthämning eller mer komplicerad sjukdom.

I **studie 4** undersöks betydelsen av att s.k. granulom hittas i de biopsier (små bitar av tarmslemhinnan) som tas i samband med utredning av Crohns sjukdom hos barn. Granulom består av en ansamling av särskilda celler från immunförsvaret som ger en specifik bild vid mikroskopisk granskning av biopsierna. Hos ungefär hälften av de 45 studerade barnen hittas granulom och förekomsten visar sig kunna vara relaterad till ett svårare sjukdomsförlopp. Tecken på aggressivare sjukdom är i denna studie förekomst av inflammation i övre mag-tarm-kanalen (matstrupe, magsäck eller tolvfingertarm) och att behandling med mer potenta läkemedel (s.k. immunmodulerare) påbörjas kortare tid efter sjukdomsstart.

Studie 4 är samtidigt en långtidsuppföljning, mellan 9 och 18 år efter insjuknandet, av dem som fått Crohn-diagnosen som barn eller tonåring och som nu blivit vuxna. I den svenska gruppen med barn med Crohns sjukdom är det ovanligt med tillväxthämning vid såväl sjukdomsstart som vid vuxen ålder då slutlängd uppnåtts. Det tycks också som om Crohn-relaterade komplikationer (t.ex. fistlar eller stenoser) och kirurgi är något mindre vanligt i denna grupp, jämfört med vad som rapporterats från andra delar av Europa och Nordamerika. Man kan spekulera i om en av förklaringarna till detta skulle kunna vara skillnader i sjukdomslokalisationen eftersom andra studier har visat att både kirurgi och tillväxthämning är vanligare vid sjukdomsutbredning i tunntarm och tunntarmsutbredning tycks mindre vanligt i gruppen som studerats i studie 1 och 4.

Utvecklingen av Crohns sjukdom påverkas av en komplicerad kombination av genetiska, miljömässiga och immunologiska faktorer och dessa faktorer har även betydelse för sjukdomsförlopp, hur individens sjukdom svarar på olika behandlingar, komplikationer, sjukdomsprognos etc. Studierna i den här avhandlingen har bidragit till den hela tiden växande kunskapen om hur olika faktorer har betydelse för sjukdomsuttryck och förlopp av Crohns sjukdom hos barn. Exakt hur dessa faktorer samverkar får framtida forskning avslöja. Storskaliga multicenterstudier, där befintlig information om genetik, klinik, serologi och miljö kombineras, kommer sannolikt att krävas för att kunna fastställa framtida risker och behov av behandling hos den enskilda patienten. Förhoppningen är att då kunna utveckla individuella terapistrategier som syftar till förbättrad prognos och livskvalitet för patienten.

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"How did it get so late so soon?
Its night before its afternoon.
December is here before its June.
My goodness how the time has flewn.
How did it get so late so soon?"

Dr. Seuss

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