UMEÅ UNIVERSITY

LIFESTYLE, BIOMARKERS AND THE RISK OF DEVELOPING INFLAMMATORY BOWEL DISEASE

Lovisa Widbom

Department of Medical Biosciences, Clinical Chemistry
Umeå, 2023

This work is protected by the Swedish Copyright Legislation (Act 1960:729)
Dissertation for PhD
ISBN: 978-91-8070-034-4 (print)
ISBN: 978-91-8070-035-1 (pdf)
ISSN: 0346-6612, New Series Number: 2247
Cover design by: Hans Karlsson, Marlene Lahti
Electronic version available at: http://umu.diva-portal.org/
Printed by: Cityprint i Norr AB
Umeå, Sverige 2023

To family and friends

I solemnly swear that I am up to no good.

– H. Potter

Table of Contents

Abstract iii

Abbreviations v

Publications and manuscripts vi

Enkel sammanfattning på svenska vii
Introduktion till inflammatorisk tarmsjukdom vii
Metod och syfte vii
Resultat och diskussion ix
Rökning och kotinin ix
Järnstatus ix
B-vitaminer och kynureniner x

Introduction/Background 1
Inflammatory bowel disease 1
Epidemiology 3
Late-onset disease 4
Ulcerative colitis 5
Crohn's disease 5
Risk factors 6
Environmental risk factors 6
Genetic risk factors 8
Pathogenesis 10
Microbiota 10
Epithelial barrier and innate immunity 15
Adaptive immunity and innate lymphoid cells (ILCs) 20
Concluding remarks on pathogenesis 24
Diagnosis 25
Endoscopic findings 25
Histological findings 25
Radiological findings 26
Biomarkers 26
Cotinine 27
Iron status 28
B-vitamins 30
The kynurenine pathway 31

Aims of Papers 1-4 34

Materials and Methods 35
Study population 35
The Västerbotten intervention programme 35
The mammography screening project 35
Cohorts in papers 1-4 35
Sample collection and preparation 36
Matching of cases and controls 36
Biochemical analyses 37
Iron status 37
B vitamins, tryptophan metabolites and cotinine 38
Statistical analyses 40

Ethical considerations 41

Results 42
Demographics 42
Paper 1: 44
Paper 2 47
Paper 3 49
Paper 4 52

Discussion 57
Main findings 57
Results contradicting prior findings 57
Role in IBD pathogenesis 57
Smoking and cotinine 58
Iron deficiency 59
Vitamin B6 and the kynurenine pathway 61
Strengths and limitations 64
Conclusions and future perspectives 65

Funding 67

Acknowledgements 68

References 70

Abstract

Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a chronic disease causing inflammation in the gut mucosa. The pathogenesis involves alteration in gut microbiota and in the intestinal barrier due to genetic factors, environmental exposure and dysregulation of the immune response. Several environmental risk factors and risk genes have been identified, but still, the pathogenesis is not fully understood.

Methods

Included papers are all case-control studies based on previously collected data stored with the biobank in Umeå, Sweden. Cases are individuals that participated in the Northern Sweden Health and Disease Study (NSHDS) at least one year before developing IBD. Information was available for all cases regarding age, time and place for inclusion in NSHDS, height and weight, sex and tobacco use. Part of the cases also had available data from a detailed food-frequency questionnaire. For each available case, controls matched for age, sex and time and place were selected. Analysed factors included tobacco use, with smoking and snuff use analysed separately), cotinine (a metabolite of nicotine), iron status (including ferritin, iron, transferrin and transferrin saturation), B-vitamins and tryptophan metabolites.

Results

Smoking was associated with an increased risk of developing IBD both based on questionnaire data and using cotinine as a marker for exposure. Snuff use was not associated with risk for developing IBD. A lower ferritin was associated with an increased risk of developing IBD, whereas no association was seen for other iron status analytes. When analysing iron deficiency based on ferritin and c-reactive protein, it was shown that iron deficiency was more common among men before onset of IBD, whereas no difference was seen for women.

Active vitamin B6 was lower among cases compared to controls, as well as an index indicating functional B6 deficiency. Kynurenic acid and xanthurenic acid, both tryptophan metabolites with immunomodulatory

properties, were lower among cases than controls. For CD only, picolinic acid was lower among cases later developing IBD.

Discussion

Smoking increases the risk of developing both UC and CD. Snuff use did not increase the risk for IBD, indicating that tobacco exposure is not the reason for increased IBD risk. Low ferritin indicates an early pathological process affecting iron storage unrelated to inflammation. Changes in vitamin B6 and tryptophan metabolites might indicate early pathological processes possibly related to gut microbiota changes.

To conclude, multiple differences between individuals later developing IBD and controls can be seen years before IBD diagnosis. Some of which give insight into early pathophysiology in IBD.

Abbreviations

AHR – Aryl hydrocarbon receptor

BMI – Body mass index

CD – Crohn's disease

CRP – C-reactive protein

EIM – Extraintestinal manifestation

GC-MS/MS – Gas chromatography, tandem mass spectrometry

GWAS – Genome-wide association study

HKr – Hydroxykynurenine ratio

IBD – Inflammatory bowel disease

ILC – Innate lymphoid cells

KTR – Kynurenine, tryptophan ratio

LC-MS/MS – Liquid chromatography, tandem mass spectrometry

PAr – Pyridoxal acid ratio

SCFA – Short-chain fatty acid

SNP – single nucleotide polymorphism

UC – Ulcerative colitis

Publications and manuscripts

Salih, A., Widbom, L., Hultdin, J., Karling, P. (2018). "Smoking is associated with risk for developing inflammatory bowel disease including late onset ulcerative colitis: a prospective study." Scand J Gastroenterol 53(2): 173-178.

Widbom, L., Schneede, J., Midttun, Ø., Ueland, P-M., Karling, P., Hultdin, J. (2020). "Elevated plasma cotinine is associated with an increased risk of developing IBD, especially among users of combusted tobacco." PLoS One 15(7): e0235536.

Widbom, L., Ekblom, K., Karling, P., Hultdin, J. (2020). "Patients developing inflammatory bowel disease have iron deficiency and lower plasma ferritin years before diagnosis: a nested case-control study." Eur J Gastroenterol Hepatol 32(9): 1147-1153.

Widbom, L., Ekblom, K., Stefansson, K., Schneede, J., McCann, A., Midttun, Ø., Ulvik, A., Ueland, P-M., Karling, P., Hultdin, J. "Tryptophan metabolism and Vitamin B6 status are associated with the risk of later developing Inflammatory Bowel Diseases in a prospective study." (Manuscript)

Enkel sammanfattning på svenska

Introduktion till inflammatorisk tarmsjukdom

Inflammatorisk tarmsjukdom är ett samlingsnamn för flera sjukdomar karaktariserade av en immunreaktion mot slemhinnan i tarmen. Den här avhandlingen fokuserar på ulcerös kolit och Crohn's sjukdom, båda två väldefinierade sjukdomar med en bred forskningsbas. Ulcerös kolit ger en ytlig inflammation i slemhinnan i tarmen och drabbar bara tjocktarmen. Inflammationen börjar mest aboralt (i ändtarmen) och sprider sig kontinuerligt mer oralt. Crohn's sjukdom karakteriseras istället av en djupare inflammation som kan gå igenom hela tarmens tjocklek och kan drabba hela mag-tarmkanalen från munnen till ändtarmen med ibland flera inflammationshärdar med frisk vävnad emellan.

Inflammationen i tarmen ger upphov till symtom som är gemensamma för båda sjukdomarna så som buksmära, diarréer med eller utan blodtillblandning och kraftiga trängningar till defekation. Crohn's sjukdom kan även ge upphov till besvär relaterat till den djupa inflammationen som kan orsaka strikturer och fistlar. Ulcerös kolit är i högre utsträckning associerad med ökad risk för tjocktarmscancer orsakad av inflammationen i tjocktarmen.

Symtomen ger upphov till mycket lidande, och sjukdomen är vanlig i västländer. I Sverige är ungefär en halv procent av befolkningen drabbad, och när man väl utvecklat sjukdom är den livslång och ingen botande behandling finns.

I nuläget finns ingen möjlighet att förhindra att någon ska utveckla sjukdom eftersom man inte vet vad som gör att någon drabbas. Denna avhandling fokuserar på påverkbara livsstilsfaktorer som kan bidra till att någon utvecklar ulcerös kolit eller Crohn's sjukdom. Om påverkbara faktorer kan identifieras så är förhoppningen att sjukdomens debut kan fördröjas eller helt undvikas.

Metod och syfte

Enheten för biobanksforskning i Västerbotten har sedan många år tillbaka sparat befolkningsdata från enkäter och prover för att använda i

forskning. Via Västerbottens hälsoundersökningar och mammografiscreeningsprojektet som samarbetar med enheten för biobanksforskning har vi fått tillgång till enkäter och blodprover från personer i västerbotten som är insamlade innan de utvecklat inflammatorisk tarmsjukdom. För varje person som sedan utvecklat inflammatorisk tarmsjukdom har 2-4 friska personer valts ut från samma kohort, med samma ålder, kön, samt tid och plats för inhämtning av prover och enkäter.

Blodproverna har analyserats för kotinin, järnstatus, B-vitaminer och kynureniner.

Kotinin är en metabolit av nikotin och används som en objektiv markör på tobaksanvändning. Rökning har visats öka risken för Crohn's sjukdom, och att sluta röka har assocoierats med debut av ulcerös kolit, men forskningen sedan tidigare är baserad på självrapporterad data.

Järnstatus inneftattar järn, transferrin, transferrinmättnad och ferritin. Järnstatus kan spegla malabsorption, men är också en markör för inflammation. Vid aktiv sjukdom påverkas järnstatus av båda mekanismerna, men prediagnostiskt är detta sedan tidigare ostuderat.

B-vitaminer både påverkar och påverkas av inflammation, och även malabsorption eller restriktiv kost kan påverka nivåerna. Låga prediagnostiska nivåer av vissa B-vitaminer skulle kunna leda till minskad antiinflammatorisk effekt och predisponera för utveckling av inflammatorisk tarmsjukdom.

Kynureniner är metaboliter av aminosyran tryptofan med viktiga immunomodulerande egenskaper. Man har sett associationer mellan kynureniner och autoimmuna sjukdomar som exempelvis reumatiod artrit, men gällande prediagnostisk inflammatorisk tarmsjukdom finns inga studier publicerade.

Från enkäter har vi fått information om livsstilsfaktorer: kost, rökning, snus, alkohol, fysisk aktivitet, vitaminsupplement, BMI, utbildningsnivå, civilstånd och ålder.

Vissa av faktorerna har använts till att undersöka om de ökar risken att utveckla inflammatorisk tarmsjukdom. Andra faktorer har använts för att justera för i olika statisktiska modeller. Detta då de i sig kan vara associerade med både sjukdomsutveckling och riskfaktorn vi primärt vill undersöka och därmed påverka resultatet.

Resultat och diskussion

Rökning och kotinin

Enkätdata visade att rökning ökar risken för både ulcerös kolit och Crohn's sjukdom. Det skiljer sig från tidigare uppfattning att rökning skulle vara skyddande mot att utveckla ulcerös kolit då man sett att personer ofta insjuknar då de slutar röka. En förklaring är att man måste ha rökt tidigare för att kunna sluta röka. På grund av den prospektiva designen i den här studien fångas fler rökare som kommer sluta röka och därefter utveckla sjukdom, därmed faller rökning ut som en riskfaktor.

För att verifiera fynden från enkätdatan analyserades kotinin som en objektiv markör för tobaksbruk. Detta bekräftade tidigare fynd att rökning, och kotinin, är en riskfaktor för att utveckla både ulcerös kolit och crohn's sjukdom. Vidare sågs att snusning, som ger högre nikotinnivåer och därmed även högre kotininnivåer än rökning, inte var associerat med ökad risk. Detta implicerar att det är själva rökningen, och inte nikotinet, som ger upphov till den ökade risken. Det kan bero på antingen administreringsvägen via lungorna eller att själva förbränningen ger upphov till andra ämnen som ökar risken för sjukdom.

Järnstatus

Lägre ferritin var associerat med en ökad risk att utveckla inflammatorisk tarmsjukdom. Övriga ingående parametrar i järnstatus var inte påverkade. Ferritin ökar vid inflammation och minskar vid järnbrist. Då inflammatorisk tarmsjukdom karaktäriseras av inflammation kan man vänta sig ett högre ferritin jämfört med friska kontroller. Dock sågs motsatsen i det här materialet. Det tyder på att det föreligger järnbrist hos personer innan man utvecklar symtom på inflammatorisk tarmsjukdom. Detta kan vara ett tecken på tidiga förändringar i tarmen som påverkar upptaget av järn. Det kan också bero på förändrad kost orsakad av undvikande av vissa födoämnden relaterat till begynnande subkliniska tarmsymtom. Alternativt kan järnbrist i sig leda till ökad risk för utveckling av inflammatorisk tarmsjukdom.

B-vitaminer och kynureniner

Aktivt vitamin B6 var lägre hos de som senare kom att utveckla inflammatorisk tarmsjukdom. Flera kynureninmetaboliter som analyserades är beroende av vitamin B6 för att kunna bildas, och tre av dessa var påverkade, kynurensyra, xanturensyra och pikolinsyra. Kvoter baserade på kynureninmetaboliter, möjligen indikerande en funktionell B6-brist, var förhöjda hos de som senare utvecklade sjukdom. Övriga B-vitaminer var inte påverkade.

Kynurensyra och xanturensyra var lägre hos individer som senare utveckalde ulcerös kolit. Pikolinsyranivåerna var lägre hos individer som senare utvecklade Crohn's sjukdom. Kynurensyra binder till två receptorer, G-proteinkopplad receptor 35 och arylkolvätereceptorn, och har via dessa receptorer immunmodulerande effekter. Xanturensyra binder till och aktiverar arylkolvätereceptorn, men har en svagare aktivering än kynurensyra. Pikolinsyra aktiverar produktion av inflammatoriska protein i makrofager så som CCL3 och CCL4. Samtliga dessa uppvisar en tydlig koppling till immunförsvar och inflammation och kan bidra till dysregleringen av imunförsvaret man ser vid inflammatorisk tarmsjukdom.

Introduction/Background

Inflammatory bowel disease

Inflammatory bowel disease, or IBD, is a group of chronic inflammatory diseases with an immune response against the gut mucosa. Traditionally the term includes two separate diseases, Crohn's disease (CD) and ulcerative colitis (UC). More recently, microscopic colitis is also classified as an inflammatory bowel disease according to international consensus guidelines.[1] This thesis will cover CD and UC.

CD is characterised by inflammation that can affect any part of the gastrointestinal tract and by non-contiguous inflammatory patches with macroscopically unaffected tissue in between. The inflammation is transmural and can thus lead to stenosing and perforating complications.[2] Anatomical distribution of CD can be classified using the Montreal classification, dividing into ileal (16-36%), colonic (21-49%), ileocolonic (24-54%) and isolated upper gastrointestinal location (~1%). Colonic presentation is more common in elderly-onset, and ileal and ileocolonic are more common in pediatric-onset disease. Common presenting symptoms in order of frequency include abdominal pain, diarrhoea, weight loss, rectal bleeding and fever.[3-6]

UC is, in contrast to CD, restricted to the colon. The inflammation is continuous and affects only the mucosa and submucosa, starting in the rectum and progressing orally to a varying degree. The muscularis propria and serosa are unaffected, and therefore stenosis or fistulation does not occur.[2] Like for CD, anatomical distribution can be classified using the Montreal classification, which for UC includes proctitis (19-37%), left-sided colitis (32-52%) and pancolitis (19-31%)[3,7]. Disease extent in an individual can vary over time, and disease severity is not solely determined by disease extent. Symptoms in order of frequency include rectal bleeding, urgency, incomplete evacuation, tenesmus and abdominal pain.[6,8]

Both diseases follow a remitting-relapsing course. Treatment can be divided into medical and surgical approaches. Most patients require maintenance therapy and more intensive treatment during flare-ups, whereas for some individuals, it is sufficient only to treat the flare-ups. Surgical treatment is required for strictures and fistulas in CD, and in UC for toxic megacolon, a severe manifestation requiring colectomy. Other

indications for surgery relevant to both diseases are unacceptable treatment-related complications, inability to taper medication due to repeated recurrence of symptoms, or unacceptable symptom load despite maximal medical therapy. No curative treatment exists except for total colectomy, which is curative in UC.[2]

Another common feature of IBD is the presence of several extraintestinal manifestations (EIMs). EIMs can present before or after IBD diagnosis, and patients can present with multiple EIMs. Depending on the strictness of the defining criteria, the prevalence of EIMs wary between 6-47%.[9] Prevalences of common EIMs are presented in Table 1. Other EIMs include metastatic CD, Sweet syndrome, pulmonary manifestations and psoriasis. Extraintestinal complications caused by chronic inflammation, malabsorption and treatment also occur.

Table 1. Prevalences for extraintestinal manifestations in inflammatory bowel disease[2,9-12]

Extraintestinal manifestation	Crohn's disease	Ulcerative colitis
Peripheral arthritis	10-20%	4-14%
Erytema nodosum	5-15%	2-10%
Oral aphthous ulcers	5-50%	10%
Uveitis	5-12%	3,5-4,1%
Anchylosing spondylitis	1-12%	
Primary sclerosing cholangitis	0.4%	2-7%
Pyoderma gangrenosum	0.4-2,6%	
Tromboembolic events	1-2%	
Episcleritis and scleritis	1%	

Microscopic colitis, in contrast to UC and CD, does not cause macroscopic changes in the gastrointestinal tract. The dominating

symptom is diarrhoea, which is most common among older women.[13] Since the epidemiology, clinical presentation, and known risk factors differ from UC and CD, this will not be covered further here.

Epidemiology

The global incidence of IBD shows considerable geographic variability, both for UC and CD. In Europe, UC is more common than CD, whereas, in Australia, the opposite is true. In North America, there is no apparent difference in incidence between UC and CD.[14,15] Figure 1 shows the global distribution of IBD incidence. A higher incidence is seen in Western countries, with Sweden having one of the highest incidences globally (28.3-40.0/100 000 per year[16]).[15] The prevalence of IBD in Sweden is 540/100 000, 350/100 000 for UC and 190/100 000 for CD. [17]

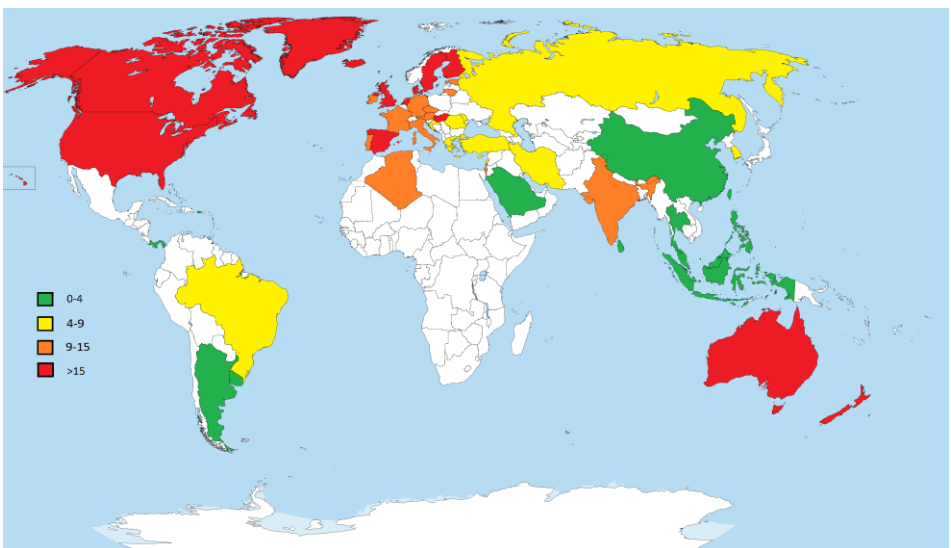

Figure 1. The combined incidence of UC and CD per 100 000 per year, adapted from Ng et al. 2017. Based on studies with study-periods from the year 2000 and later.

The incidence of IBD is increasing globally, with the highest increase in countries in Asia, South America and the Middle East following rapid industrialisation. There is a west-to-east gradient in incidence in Europe, with a higher incidence in the west, despite the similar availability of colonoscopy. This gradient is weakening as living conditions equalise. The incidence remains low in migrants and first-generation offspring

migrating from low to high-incidence countries but approaches the host country in second and third generations.[14]

The age distribution of the IBD incidence has been described in a Swedish cohort, showing one distinct incidence peak at 10-29 years of age and one smaller peak at the ages 60-79.[4,18] Among 15-20% of patients, the disease develops after the age of 60.[19]

Late-onset disease

It has been shown that inflammatory bowel disease differs considerably regarding clinical presentation, risk factors and prognosis depending on age at disease onset.

Table 2. Differences between pediatric- and elderly onset IBD.[19,20]

	Pediatric onset disease, ≤16 years	Elderly onset disease, ≥60 years
UC onset	Sudden, severe	Progressive, discrete
UC disease location	Usually pan colonic	Often left-sided
CD, strictures and fistulas	Common	Less common
CD, phenotype change	Common	Typically none
Genetic risk factors	More important	Less important
Environmental risk factors and gut microbiota	Less important	More important
Medication considerations	Need for individualised dosing related to age and weight	Multi-drug interactions, susceptibility to side effects
Need for surgery	High	Low

Younger age at onset is associated with more aggressive disease and poorer prognosis, whereas older age is associated with the opposite. Also, genetic risk factors play a more prominent role in early-onset disease, and environmental factors play a more prominent role in late-onset disease.[20] Some differences between pediatric and elderly onset IBD are summarised in Table 2.

Ulcerative colitis

Early debuting UC typically presents with pancolitis; if presenting with more limited colitis, progression to pancolitis is very common. The risk for colectomy is significantly increased among patients debuting with pancolitis or with early progress to pancolitis. Atypical phenotypes are more common, with rectal spearing present in up to 5 % of patients.

Late-onset UC usually presents with proctitis or left-sided colitis, while extensive colitis is less common. The disease location is usually stable and does not progress proximally. Relapses of disease are less likely.

Crohn's disease

In pediatric-onset CD, the typical presentation involves both the colon and small intestine, and upper gastrointestinal involvement is common. Among the youngest pediatric patients, isolated colonic involvement is often seen. Progression with increased anatomic distribution is seen in 39% of patients in the first two years after diagnosis. A complicated disease behaviour is more likely among pediatric-onset CD, often requiring biological therapies.

Late-onset CD has a high frequency of isolated colonic involvement. Fistulation and structuring disease is uncommon, as is anatomical disease progression. Among late-onset CD, abdominal pain, fever, weight loss, and EIMs at diagnosis is less common, but rectal bleeding is often seen. Perianal disease at diagnosis is equally common throughout different age groups, but with time, it becomes more common among pediatric-onset patients.[19,20]

Risk factors

Environmental risk factors

Environmental factors affecting the risk of inflammatory bowel disease are presented in Figure 2. There are few established risk factors, and the mechanisms are largely unknown. Interventional studies focusing on known risk factors are hard to perform, as many risk factors are lifestyle associated with exposure over time. Dietary intervention studies are challenging to perform, and the incidence of IBD is relatively low in relation to the effect size of known risk factors. Most known risk factors are based on case-control studies or prospective cohort studies.[21,22] The majority of studies are also performed on already diagnosed IBD patients. Few studies have access to pre-diagnostic biomarkers, and attempts to gather pre-diagnostic survey data at diagnosis are prone to recall bias. Disease-related symptoms may influence dietary data collected at or after diagnosis.

Factors associated with an increased risk for both UC and CD are urban living, caesarian delivery, oral contraceptive use, and vitamin D deficiency.[21,23] Tonsillectomy and antibiotic use increase the risk only for CD and do not affect UC.[21,24] Appendectomy increases the risk for CD but decreases the risk for UC.[25,26] Smoking influences UC and CD risk differently depending on if the comparison is between current, former, ever or never smokers. Those who have never smoked are at lower risk for developing CD, while no such correlation is seen for UC. Current smokers are at increased risk of CD, while active smoking decreases the risk of UC. Smoking cessation is associated with an increased risk of developing UC.[27]

Specific dietary factors are seen in Figure 2. Increased intake of fruit and vegetables is associated with decreased risk for both UC and CD. Soft drinks increase the risk for both UC and CD. Fibre and fish reduce the risk for CD but do not affect UC. Coffee intake lowers the risk for UC but does not affect CD.[21]

Other risk factors include campylobacter enteritis and IgA deficiency.[28]

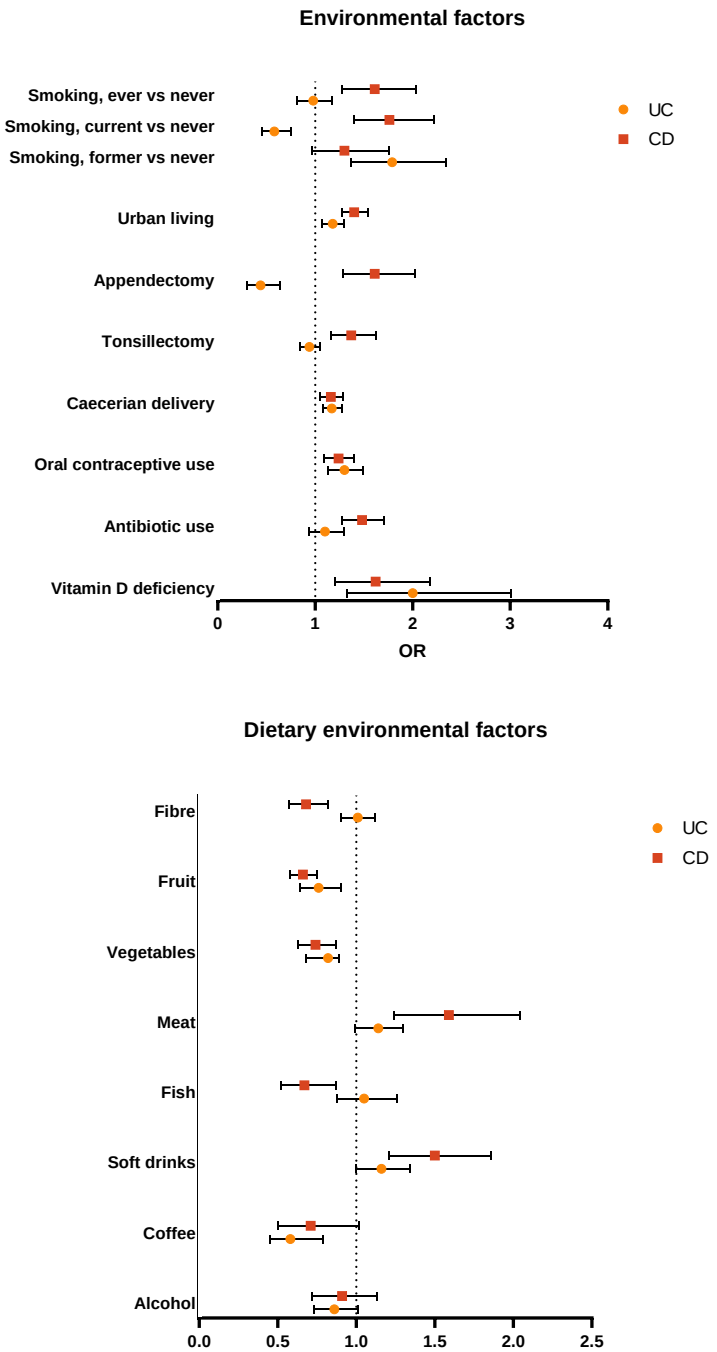

Figure 2. Risk factors for IBD identified in epidemiological studies.

Genetic risk factors

Genetic factors play a significant role in developing inflammatory bowel disease, especially early onset.[19] The strongest risk factor for IBD is family history; around 10% of patients have a first-degree relative with the disease.[29,30] Studies have shown higher concordance in monozygotic twins compared to dizygotic twins with CD.[29,31]

Table 3. Example-genes associated with inflammatory bowel disease.

Gene	Gene function
Epithelial barrier	
ITLN1	Expressed in goblet and paneth cells, binds carbohydrate moieties.[32,33]
HNF4A	Transcription factor in enterocytes, regulates epithelial cell structure, cell junctions and polarity.[34]
C1orf106	Regulates E-cadherin surface levels and the integrity of the intestinal epithelial barrier.[35]
GSDMB	Epithelial repair and wound closure by cell proliferation, migration and adhesion.[36]
RNF186	Needed for EBFN-induced autophagy in colonic epithelial cells. Regulates basal xenophagy/ autophagy.[37]
GPR35	G-protein coupled receptor. Orphan receptor, activated by kynurenic acid in supraphysiological concentrations. Promotes wound healing through increased migration by fibronectin expression and ERK phosphorylation.[38] Activates Rho and PYK2 through $G\alpha_{13}$.[39]
CDH1	E-cadherin, protein making up adherence junctions in the intestinal epithelium.[40]
Adaptive immunity	
FCGR2A	IgG-receptor expressed in macrophages. Binds IgG against commensal bacteria in UC, causing IL-1 secretion.[41]
MHCII	Antigen presentation. Multiple associated alleles, HLA-DRB1*01:03 strongest association both in UC and CD.[42]

Cytokines	
IL10RB	Subunit in IL10 receptor. Restricting IL-23 secretion from lamina propria in macrophages. Hampers Th17 recruitment and IL-22 production.[43] Also subunit in IL-22 receptor.[44,45]
IL23R	Receptor for IL-23, activation of Th17 cells.[46]
RORC	Induced by TGFβ and IL6. Transcription factor for Th17 cells, required for IL-17 expression.[45,47]
IL6ST	Common subunit for IL-6 family receptors. Activates JAK-STAT pathway.[45,48]
TYK2	JAK family tyrosine kinase. Expressed in various immune cells and intestinal epithelial cells. Activates STATs.[49]
STAT3	Transcription factor regulating Th17 cell differentiation.[45,48]
IL18RAP	Accessory subunit of IL18 receptor. Expressed in CD4+ T-cells, including regulatory T cells. Inhibits Th17 cell differentiation and activates some T_{reg} cell effector molecules.[50]
IL1RL1	Receptor for IL-33. Activates NFκB and MAPK. Stimulates Th2 response, Tregs and ILC2.[51]
Microbe sensing	
NOD2	Senses muramyl dipeptide. Activates NFκB, Caspase 1 and MAPK. Results in IL1β, TNFα, IL6, IL8 and secretion of antimicrobial peptides.[52]
CARD9	Activated by pattern recognition receptor. Activates NFκB and MAPK. Results in IL1β, TNFα and IL6.[53]
CLEC7A	Encodes pattern recognition receptor Dectin-1, sensing β-glucans from, for example, fungi.[54]
Microbicidal mechanisms	
ATG16L1	Part of autophagosome complex. Lipidates LC3. Interacts with C3 and NOD2, homes autophagosomes to bacteria and viruses. Negative regulator of NOD2, IFN-1 production, IL1β activation and IL18 activation.[55]
GPR65	G-protein coupled receptor. Controls lysosomal acidification, affecting autophagy and xenophagy.[56]

Initial genome-wide association studies (GWAS) have identified 163 loci associated with increased risk.[45] In 2015, this was expanded to 201 loci.[57] Since then, several additional loci have been identified.[58-60]

Genes identified by GWAS studies are implicated in microbe sensing, adaptive immunity, microbicidal mechanisms, epithelial barrier function and integrity, and immune signalling. Well-characterised genes with a strong association in GWAS studies or a particular relevance based on biomarkers used in this thesis are presented in Table 3.

Pathogenesis

IBD develops due to a maladaptive immune response in the gut mucosa. This immune response is often initiated against the microbiota and is self-stimulating, causing further dysbiosis. The microbiota, in conjunction with the immune response, causes damage to the epithelial barrier, which is a part of the innate immune system. The damaged epithelial barrier exposes the lamina propria to pathogen-associated molecular patterns, antigens from the microbiota, and damage-associated molecular patterns due to the damage itself. This exposure leads to further immune activation creating a self-stimulating feedback loop sustaining the disease.

External environmental factors can affect the microbiota and the immune system. Diet and antibiotics are known to alter the gut microbiota[61,62] and are known risk factors. Examples of environmental risk factors affecting the immune system are tonsillectomy or appendectomy.

Genetic factors that can cause dysfunction of the epithelial barrier and dysregulation of the immune response, including inter- and intra-cellular signalling and autophagy have been identified. The microbiota is also affected by genetic factors, including dysfunctional microbial sensing and microbicidal mechanisms.

Microbiota

The gut microbiota describes the microorganisms in the gastrointestinal tract, comprised of bacteria, viruses, fungi and archaea. Two extensive studies have described the human gut microbiota.[63,64]

The microbial biomass and composition vary throughout the gastrointestinal tract. Bacteria are the main constituent of the microbial biomass, ranging from 10^{2-3} cells per millilitre of luminal content in the stomach to 10^{11} in the colon.[65] In stool samples from healthy individuals, the dominating species are Bacteroidetes and Firmicutes constituting 90% of the communities. Actinobacteria and Proteobacteria comprise another 9%, although interindividual variations are substantial. Analyses of metabolic pathways show high stability despite differences in bacterial phyla.[63,64]

The composition of the microbiota is not stable over a lifetime. At birth, the infant is colonised by its mother's microbiota. During the first 2-3 years of life, it undergoes significant changes caused by a changing diet and an evolving immune system.[66] In adults, the constitution of the microbiota is shown to be relatively stable over time, with around 60% of microbial strains being unchanged over a five-year period.[67] Ageing has been associated with decreased diversity of microbial species and higher inter-individual variations. Relative proportions change with age, with Bacteroidetes being more and Firmicutes less abundant in older compared to younger individuals.[68]

The gut microbiota in faeces from IBD patients differs from healthy individuals. The proportion of Bacteroidetes and Firmicutes is decreased, whereas Actinobacteria and Proteobacteria are increased.[69] Over time, the microbiota composition in IBD patients is more variable compared to healthy individuals.[70] In twins discordant for IBD, the composition of the gut microbiota differs.[71] The correlation between changes in gut microbiota in IBD patients does not necessarily imply causation. For example, an inflammatory environment high in oxygen facilitates conditions for Actinobacteria and Proteobacteria that are aerotolerant. Inflammation may also increase the production of terminal electron acceptors giving an advantage to facultative anaerobes. E. coli, a facultative anaerobe, has been found in increased numbers in patients with IBD and has been suggested to act as a pathobiont, a commensal bacteria that, under some circumstances, can cause disease.[72]

Environmental reasons for microbiota changes

Multiple known environmental risk factors for IBD may act through interaction with the microbiota. Dietary fibre is fermented to short-chain fatty acids (SCFA) by bacteria in the colonic microbiota.[73] SCFA:s have been shown to inhibit histone deacetylases promoting Treg generation, suppressing proinflammatory macrophages and inhibiting dendritic cell

differentiation. SCFA activates multiple G-protein coupled receptors:s, and GPR43 and GPR109A have been shown to activate the NLRP3 inflammasome.[74] The NLRP3 inflammasome is a protein complex inducing inflammatory functions by activating caspase-1. Caspase-1 cause cleaving pro-IL-1β to active IL-1β , and Gasdermin-D, causing pyroptosis.[75]

Antibiotics cause dysbiosis that often persists many months after cessation of antibiotic treatment. Positive associations between developing IBD and the usage of most antibiotics have been shown, with the notable exception of penicillins, which are known to have minor effects on the microbiota.[76] Faecal transplants from healthy mice ameliorate DSS-induced colitis in mice, but faecal transplants from mice pre-treated with Streptomycin or Vancomycin do not.[77]

Cecerian delivery is a known risk factor for IBD. It is known that cesarian delivery alters the infant's gut microbiota. However, since the difference in gut microbiota composition related to birth mode disappears after around 100 days, it is unlikely that the increased IBD risk is related to gut microbiota variations.[78] Appendectomy is associated with gut microbiota changes, including bacterial and fungal communities.[79] Urban and rural living cause differences in microbiota composition with higher diversity and several unique phyla among rural dwellers. No direct connections to IBD are shown, and many differences seem to be caused by differences in diet.[80] Studies on urban and rural living are typically performed on extreme rural dwellers, including, for example, Amazonians from French Guiana or Congolese Pygmies.[80]

Genetic reasons for microbiota changes

IBD susceptibility genes can cause changes in the gut microbiota. NOD2 encodes an intracellular pattern recognition receptor that senses muramyl-dipeptide, a component in bacterial peptidoglycans. Activation of the receptor by commensal bacteria causes secretion of antimicrobial peptides. IBD-associated variants in NOD2 lead to gut microbiota dysbiosis through altered microbicidal secretions in the intestine.[81,82] IBD-associated mutations in ATG16L1, encoding a protein involved in autophagosome formation, are associated with decreased mucin secretion from goblet cells, causing dysbiosis of microbial communities in the epithelial mucus layer in mice.[83] CARD9 encodes a protein downstream pattern recognition receptors and is important, especially in fungal immune responses. In CARD9 knockout mice, dysbiosis was seen for both the bacterial and fungal gut microbiota.[84]

How do changes in microbiota cause disease?

Several microbiota-derived metabolites can affect the immune system. Bile acid signalling via activation of the farnesoid X receptor is protective in colitis models by inhibiting NF-κB signalling.[85,86] Intestinal bacteria hydrolyse bile acids, altering affinity for the farnesoid X receptor. Changes in the gut microbiota composition can result in variations in the hydrolysis of bile acids, altering the activation of the farnesoid X receptor.[71] The gut microbiota synthesise several tryptophan metabolites, including indole, indole-3-acetate, and tryptamine, all ligands for the aryl hydrocarbon receptor (AHR). Activation of the AHR affects cell adhesion, antimicrobial peptide production, release of cytokines and epithelial permeability.[87,88] Activation of the AHR is associated with the improvement of colitis in mouse models.[89] The gut microbiota also synthesise essential B vitamins, most importantly vitamin B6, where over 85% of the required daily amount can come from microbial synthesis.[90] Vitamin B3, B9 and B12 can also, to a significant extent, originate from the gut microbiota.[90] Vitamin B6 is an essential cofactor for enzymes that produce several compounds relevant in IBD pathogenesis, such as AHR agonists,[89] spingolipids[91] and hydrogen sulfide.[92]

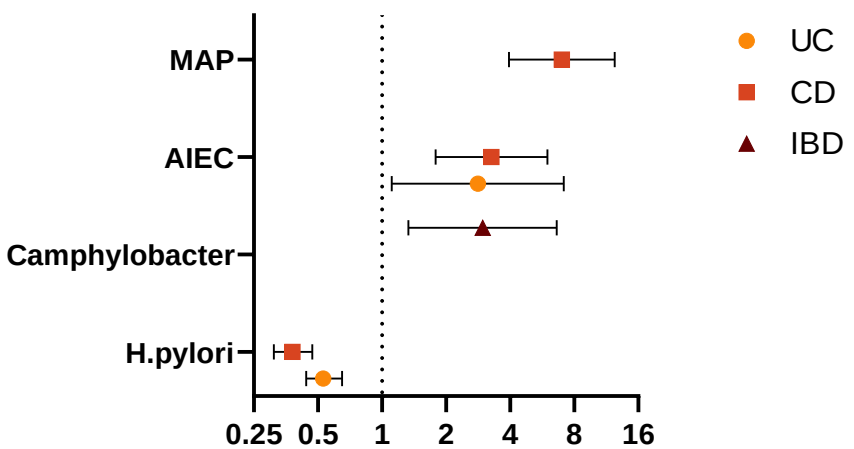

Figure 3. Odds ratios and 95% confidence interval for developing IBD for different bacterial infections. MAP – Mycobacterium avium subspecies paratuberculosis, AIEC – Adhesion-invasive E. coli, [93-95]

Overgrowth of specific pathobionts may be partly causative in IBD. Adhesion-invasive *E. coli* is increased in number among CD patients compared to healthy controls. Macrophages in CD patients have decreased ability to restrict intracellular Adhesion-invasive E.coli compared to those in healthy individuals, and epithelial surface proteins crucial for adhesion are more abundant in CD patients. Adhesion-invasive *E. coli* overgrowth causes cytokine release, recruitment of immune cells and increased epithelial permeability and can thus contribute to IBD pathogenesis.[96]

Mycobacterium avium subspecies *paratuberculosis* has been suggested as a possible etiological agent for CD based upon similarity with Johne´s disease, a chronic granulomatous inflammation in cattle and sheep caused by *Mycobacterium avium* subspecies *paratuberculosis*. *Mycobacterium avium* subspecies *paratuberculosis* is enriched in intestinal biopsies from CD patients compared to controls.[93] Several mycobacterial susceptibility loci have been identified as IBD susceptibility loci in GWAS studies.[45] Antibiotic treatment of *Mycobacterium avium* subspecies *paratuberculosis* has not had any effect on IBD in randomized controlled trials.[93]

Toxin-producing *Bacteroides fragilis* have also been proposed as a disease-causing pathobiont. The toxin interacts with GPR35, which in GWAS studies has been associated with IBD.[45,97]

Campylobacter species have been implicated in IBD, with particularly increased prevalences of *Campylobacter concisus* and *Campylobacter showae* among IBD patients.[95] ([98])

Helicobacter pylori, in contrast, is proposed as protective against IBD, with evidence of this on both epidemiological and molecular levels.[95]

Mycobiome and virobiome in IBD

The mycobiome in IBD is less studied compared to the bacterial gut microbiota. *Candida Albicans* are more abundant among IBD patients compared to controls.[99] *Candida Albicans* in CD patients are present as hyphae to a greater extent, indicating a more infectious state and secreted IgA against its virulence factors are lower among CD patients.[100] Antibodies against *Saccharomyces Cerevisiae*, with a widely shared antigenic structure among fungal taxa, are associated with CD. Higher levels have also been shown in prediagnostic studies. *Malassezia* spp from colonic washing samples were associated with CD when compared

to healthy controls. The same has been shown in surgical mucosal samples for CD, but no association was seen for UC.[99]

For the viral microbiota, an increase in *Caudovirales* richness and a decrease in the richness of *Microviridae* have been observed among IBD patients. The increase in *Caudovirales* richness was associated with a reduction of bacterial diversity.[101]

Epithelial barrier and innate immunity

The gut epithelial barrier consists of the epithelial layer, the mucus layer and antimicrobial peptides. The barrier functions as a selective membrane, allowing nutrient passage while preventing microbial access. Another essential function is immune education, regulating the immune response to the luminal microbiota and environmental antigens, thus creating and maintaining immune tolerance.[102]

Permeability and barrier integrity

In the epithelial layer, epithelial cells adhere to each other through tight junctions, adherence junctions and desmosomes. The tight junctions regulate the paracellular permeability and consist of claudins and occludins. Adherence junctions are made up of E-cadherins and connected to the intracellular actin skeleton, contributing to cell-cell adhesion, paracellular permeability and signalling. Desmosomes contribute to cell-cell adhesion by linking the intermediate filaments of adjacent epithelial cells.[103]

In IBD, structural differences in tight junction proteins have been observed, together with upregulation of pore-forming claudin-2 and downregulation of other claudins with barrier-enhancing properties.[102] Myosin light chain kinase is upregulated in intestinal biopsies from IBD patients and correlates with disease activity.[104] Myosin light chain kinase is a regulator of several proteins essential for tight junction and adherence junction functions and causes an increase in paracellular permeability.[103] Endoscopic confocal laser endomicroscopy has shown an increase in intestinal permeability in IBD patients with ongoing symptoms.[105]

C1orf106 is a risk gene for IBD identified in GWAS studies that regulate the surface levels of E-cadherins, proteins that make up the adherence junctions in the epithelium. In intestinal epithelial cells and colonic

organoids, knockout of C1orf106 was associated with internalisation and decreased surface levels of E-cadherins. This was associated with increased permeability of smaller molecules and increased susceptibility to bacterial translocation in mice *Citrobacter rodentium* models.[35]

Increased paracellular permeability allows environmental antigens such as microorganisms and food particles to come in contact with the intestinal immune system to a greater extent, with resulting immune activation.[102]

Mucosal healing, cell proliferation and migration

Mucosal healing is essential in IBD and a desired endpoint in IBD treatment. Treatment to symptom remission only does not induce as long symptom-free remission as treatment to endoscopic mucosal remission.[106,107] Several proteins associated with mucosal healing have been identified in GWAS studies on IBD.[45]

Wound healing is dependent on intestinal epithelial cell proliferation. Mice lacking STAT3 in intestinal epithelial cells show reduced capacity to close mucosal lesions. Deletion of IL-22, a potent inducer of STAT3 in intestinal epithelial cells, produces similar results. As both STAT3 and IL-22 are known risk genes for IBD, this indicates the importance of mucosal wound healing in the pathogenesis of IBD. Furthermore, IL-23 is a potent inducer of IL-22 in the intestinal mucosa, and the IL-23 receptor has been associated with IBD in GWAS studies.[45,108,109]

Knockout of the risk gene GSDMB results in impaired wound healing in monolayers of colonic epithelial cells and decreased expression of proteins associated with cell migration, proliferation and adhesion. Rescue of GSDMB knockout cells with GSDMB mutants found to increase the risk of IBD shows defects in the same areas as are impacted in the knockout models.[36]

Impaired wound healing results in increased exposure of antigens to the lamina propria and prolonged inflammation, which might help sustain the dysregulated immune response found in IBD.

The mucus layer

The intestinal mucus layer, composed of mucins, and containing secreted antimicrobial peptides, is the first layer of defence against the commensal microbiota, pathogens and environmental antigens. The

mucin layer in the stomach and small intestine is composed of a single adherent layer of mucin, while the colonic mucin layer is divided into an inner adherent layer, usually free of microbes and a free-flowing outer layer where commensal microbes exist. The major secreted mucin coating intestinal epithelial cells in the bowel is MUC2.[110]

Several alterations in the mucus layer have been described in IBD, including reduced MUC2 secretion and thinner mucin layers in UC and reduced glycosylation of mucins in both UC and CD. Accumulation of misfolded mucins in goblet cells of UC patients causing endoplasmatic reticulum stress has been shown. Endoplasmatic reticulum stress is linked to autophagy, the impairment of which is implicated in IBD pathogenesis. Knockout mice for MUC2 develop spontaneous colitis.[38] Mucin production is induced by SCFA and AHR ligands, both known to be affected in IBD.[21,89] Several known risk genes for IBD are also involved in mucin production and secretion, including NOD2, STAT3 and IL-22.[110]

Defects in the mucus layer expose the epithelium to bacterial colonization and luminal antigens, leading to epithelial damage and increased antigen exposure to immune cells.

Antimicrobial peptides

Antimicrobial peptides secreted in the gastrointestinal tract include pore-forming peptides, metal-chelating proteins, bacterial protease inhibitors and bacterial cell wall degrading enzymes. Secretion can be both constitutional or induced through various PAMPs or cytokines. Many antimicrobial peptides are secreted from intestinal epithelial cells, but specialized paneth cells secret a unique subset of antimicrobial peptides to protect the intestinal crypts and stem cells.[111]

Alterations in antimicrobial peptides in IBD patients include human beta-defensin 2, which is highly expressed in inflamed colonic mucosa compared to normal colonic mucosa. In UC, an increase in expression is also seen for human beta-defensin 3 and 4. The expression of cathelicidin mRNA in the colon of UC patients is increased compared to healthy controls. Secondary bile acids (bile acids metabolised by the commensal microbiota) and SCFAs, both microbiota-derived metabolites involved in IBD pathogenesis, are known inducers of cathelicidins.[112]

In mouse models of colitis, beneficial effects of antimicrobial peptides, including alpha- and beta-defensins, cathelicidins, elafin and lactoferrin, have been demonstrated. The effects are partly attributed to antimicrobial effects, but multiple antimicrobial peptides also serve as immunomodulators. For example, cathelicidin directly increases mucin gene expression, and lactoferrin modulates cytokine secretion, reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines.[111]

Constitutional secretion of antimicrobial peptides shapes the commensal microbiota; in return, the commensal microbiota is required for adequate antimicrobial peptide production. Bacterial products such as lipopolysaccharide, muramyl dipeptide and SCFAs have been shown to induce antimicrobial peptide secretion.[111] The IBD risk gene NOD2 is an intracellular sensor for muramyl dipeptide, and NOD2-deficient mice have increased ileal bacterial loads caused by paneth cell dysfunction.[52]

Altered antimicrobial peptide production may allow overgrowth of pathobionts, change the composition of the commensal microbiota to more proinflammatory and dysregulate immune signalling.

Autophagy

Autophagy is a process of degrading intracellular material in a cell. The process can either be unspecific, as in starvation conditions, where degradation is required to access building material, or specific, as in xenophagy, the process of degrading intracellular pathogens, or mitophagy, the process of degrading mitochondria.[113]

In IBD, many identified risk genes are genes involved in autophagy. One of the most studied is ATG16l1, encoding part of the autophagosome complex. ATG16l1 lipidates LC3 analogues and initiates the formation of autophagosomes. ATG16l1 also regulates cytokine production and cellular trafficking by non-autophagy-related mechanisms.[55] Other GWAS-associated genes involved in autophagy include LRRK2, associated with IBD, and RIPK2 and NOD2, associated with CD.[45]

Autophagy imparement can cause changes in the gut microbiota. When comparing CD patients homozygous for genetic variants in ATG16l1 with those homozygous for wildtype ATG16l1 higher abundance of pathobiont groups such as *Enterobacteriaceae* and *Bacteroidaceae* have been shown. Defects in several autophagy genes related to IBD are associated with a decrease in *Roseburia species*. *Roseburia species* are important

producers of SCFAs.[114] Impaired autophagy also interferes with the intestinal epithelial cells' ability to clear intracellular pathogens such as AIEC through xenophagy, causing excessive inflammatory activation.[115] In addition to the ability to efficiently capture intracellular bacteria in autophagosomes and deliver them to lysosomes, lysosomal acidification is required for intracellular pathogen clearance. GPR65 is an H^+-sensing receptor regulating acidity in lysosomes. IBD patients with a missense variant of GPR65 cannot efficiently clear intracellular bacteria.[56]

Defect autophagy is associated with impaired epithelial barrier function with increased permeability. The pore-forming tight junction protein claudin-2 is degraded through autophagy, and defects in autophagy or reduction of autophagy through inflammatory signalling increase claudin-2 levels in intestinal epithelial cells.[116]

Paneth cells are significant producers of antimicrobial peptides in the intestinal epithelium. The autophagic system is involved in the secretion of antimicrobial peptides from paneth cells through lysosomal sorting and transduction of secretion-stimulating signals from the commensal microbiota.[114]

Activation of the NLRP3 inflammasome is inhibited by autophagy. The CD risk-gene PTPN22 dephosphorylates the NLRP3 inflammasome causing autophagic degradation. Loss of function mutations in PTPN22 increases CD risk while gain of function mutations are protective for CD.[117]

Autophagy also has functions in the adaptive immune system. In T cell-specific knockout of ATG16l1 in mice, reduced numbers of T_{reg} cells in the intestine have been shown, along with T_H2 immune reactions against food and commensal microbial antigens that caused spontaneous colitis in mice.[118]

In summary, functional autophagy is essential for many intestinal processes impaired in IBD patients, including gut microbiota regulation and composition, epithelial barrier function, antimicrobial peptide secretion and other innate and adaptive immune responses.

Microbe sensing

Sensing of both commensal and pathogenic microorganisms by the innate immune system depends on preserved molecules such as lipopolysaccharide from bacterial membranes or flagellin from bacterial

flagella. These pathogen-associated molecular patterns (PAMPs) and endo- or exogenic molecules associated with tissue damage, damage-associated molecular patterns (DAMPs) are sensed by preserved families of receptors, including the toll-like receptors (TLR), NLRP-family proteins and NOD2.[119,120]

TLR4 senses lipopolysaccharide and is expressed on intestinal cells of the innate immune system and apically on intestinal epithelial cells. The commensal microbiota in intestinal epithelial cells induces constitutive low-level TLR4 signalling. In IBD, TLR4 expression is significantly increased.[121] Polymorphisms in TLR4 have been associated with the risk of IBD, and polymorphisms in other TLRs have been associated with disease severity. Mice deficient for TLR2/3/4/5/9 display reduced tissue repair response after DSS challenge, as luminal agonists that gain access to the lamina propria after damage induces a tissue repair response, which is impaired with TLR deletion.[119]

The earliest identified risk gene for IBD was NOD2, an intracellular sensor for the bacterial cell wall component muramyl dipeptide. It has been shown that up to 40% of CD patients have mutated NOD2.[122] Some of the associated risk is possibly related to its microbe sensing functions, but as other functions in autophagy and signalling have been described and shown to be relevant in IBD, the risk associated with each function remains to be determined.

Microbe sensing is another highly involved immune system function with probable importance in IBD pathogenesis, with connections to genetic variations and gut microbiota interactions.

Adaptive immunity and innate lymphoid cells (ILCs)

Intestinal adaptive immunity involves a variety of lymphocytes, including b-lymphocytes, several subtypes of T-lymphocytes and their interaction with antigen-presenting cells. Intestinal lymphoid cells are located mainly in the submucosa, but subsets of T-lymphocytes are located in the epithelium, and, primarily in the ileum, specific lymphoid tissues known as Peyer's patches exist.[2]

$CD4^+$ T cells commonly form infiltrates in the mucosa of IBD patients. The infiltrates primarily consist of T_H1 and T_H17 cells, with a less pronounced increase in T_H2 cells. The ratio of immune-modulating T_{regs} and Tr1 to inflammatory T cells is decreased. In CD, the populations of

ILCs are skewed, with increased numbers of ILC1 cells and decreased numbers of ILC3 cells.[123]

T_H1

The main function of T_H1 cells includes fighting infections caused by viruses and intracellular bacteria. They are induced by the cytokine IL12, and upon activation, they release IFNγ and TNFα. In IBD, increased activation of T_H1 cells has been shown and increased levels of its inducing cytokine IL12. In the mucosa of CD patients, higher levels of the T_H1 cell-derived effector cytokine IFNγ have been shown.[124] Increased TNFα facilitates epithelial apoptosis and inflammatory signalling.[124] Through activation of myosin light chain kinase, epithelial permeability is increased by TNFα signalling.[125] IFNγ initiates the recruitment of innate immune cells, such as monocytes and neutrophils, to the mucosa.[124] The efficacy of anti-TNFα treatment in IBD further strengthens the notion that T_H1 cells play an essential role in IBD pathogenesis.[126]

T_H17

T_H17 cells induce immune responses toward bacterial and fungal pathogens at mucosal surfaces. The induction of T_H17 cells is dependent on IL6, IL23 and IL1β, but a subpopulation of T_H17 cells with less pro-inflammatory functions has been described and is induced by TGFβ and IL6. The transcription factors regulating T_H17 differentiation are known IBD risk genes, RORC and STAT3, and the receptor for IL23 (IL23R) that supports T_H17 differentiation is also associated with IBD in GWAS studies. The main effector cytokine is IL17, but significant amounts of IL21, IL22, GM-CSF, IFNγ and TNFα are also produced.[124]

In IBD patients, T_H17 cells are increased in peripheral blood and levels of the typical T_H17 cytokines are increased. The main source of IL23 in IBD patients is CD14+ macrophages in the mucosa, maintaining the intestinal T_H17 cell population. Antibody therapies targeting IL12/23 have been shown to be effective in treating IBD.[127]

IL17, produced by T_H17 cells, mainly affects non-hematopoietic cells and induces secretion of chemokines, including IL-8, which attracts neutrophils. In epithelium, IL17 induces secretion of antimicrobial peptides and strengthens tight junctions. Major cytokines induced by IL17 are IL6 and G-CSF, both supporting myeloid cell proliferation.[128] Although IL17 is involved in a proinflammatory response in IBD,

therapies directly targeting IL17 have not been effective and prone to cause exacerbations. This may be caused by the permeability-reducing effects of IL17 on intestinal epithelium being lost with a blockade.

IL22 is secreted from T_H17 cells in response to inflammatory stimuli in conjunction with obligatory AHR stimulation.[129] Receptor expression for IL22 is restricted to the epithelium.[130] Effects of IL22 on intestinal epithelium are pleiotropic and include effects that can be assumed to be protective in IBD, such as increased cell proliferation, antimicrobial peptide secretion and mucin secretion. Possibly deleterious effects include increased claudin-2 expression that increases epithelial permeability. Results from animal models are mixed, and a clinical phase 2 trial of an IL22-IgG4 fusion protein (UTTR1147A) is ongoing.[130]

T_{reg}

The primary function of T_{reg} cells is to limit immune responses to endogenous antigens and exogenous harmless antigens. The primary inducing cytokines of T_{reg} cells are IL2 and TGFβ. They are characterized by the transcription factor FoxP3 and the cytokines they secrete, IL10, IL35 and TGFβ. Depending on the origin of T_{reg} cells, they can be divided into natural T_{reg} and peripheral T_{reg}. Natural T_{reg} cells are identified as autoreactive during development in the thymus and steered towards an immune dampening profile, while other $CD4^+$ cells that do not react to Autoantigens can continue their maturation towards $CD4^+$ T cells. Peripheral T_{reg} cells relevant to the gut are generated in mesenteric lymph nodes and provide tolerogenic responses towards antigens from the commensal microflora and food antigens.[124] Recently, a new antigen-presenting cell with characteristics of both thymic epithelial cells and dendritic cells has been described, the Thetis cell. The numbers of Thetis cells increase during the window of tolerance development in neonates. Substantial defects are shown in peripheral T_{reg} generation with disturbing antigen presentation or co-stimulation in Thetis cells, making them an attractive explanation for the development of commensal tolerance.[131]

Knockout of IL10, specifically in T_{reg} cells, induces spontaneous colitis in mice.[132] Recombinant IL10 has been effective in suppressing T-cell transfer colitis.[133] In GWAS studies, the interleukin 10 receptor beta chain and its downstream effector, STAT3, have been associated with IBD risk.[134] T_{reg} are important intestinal repressors of T_H17 cells. TGFβ inhibits proliferation of effector T-cells and inhibits T_H1 differentiation in mouse colitis models. Trough downregulating the T_H1 and T_H17

transcription factors, T-bet and RORC, recombinant IL35 ameliorate symptoms in chemical mouse colitis models. T_{reg} cells also express an IL2 receptor with higher affinity than T effector cells and can scavenge IL2 to starve T effector cells.[134]

Innate lymphoid cells

ILCs are cells derived from the same progenitors as lymphocytes but lack the T-cell receptors or immunoglobulins generated by somatic recombination. As they lack the ability to develop specific antigenic responses, they are considered members of the innate immune system. They are capable of self-replication and do not need to be replenished from circulation, and they secrete cytokines at a steady level.[123]

ILCs are divided into three subgroups that share similarities with subgroups of the adaptive T cells. ILC1s ILC2s and ILC3s are similar to T_H1, T_H2 and T_H17 cells, respectively. Transcription factor profiles and cytokines secreted are usually alike. In IBD, ILC1 and ILC3 populations are particularly interesting as they are present in the gut at significant levels. The amount of ILC3s increases along the GI canal, with the highest levels in the terminal ileum and colon. ILC3s are linked to the gut microbiota as SCFA, and stimulation of AHR promotes differentiation to ILC3 cells. ILC3s express several microbe-sensing receptors, including a variety of toll-like receptors.[123]

ILC3s influence adaptive immunity by producing GM-CSF and IL2 in response to the microbiota, promoting T_{reg} differentiation.[123] ICL3s express MHCII, an antigen-presenting, cell-surface-bound molecule. Antigen presentation on ILC3s promotes apoptosis in T effector cells reacting against the commensal flora.[135]

Patients with IBD have increased levels of ILC1s and a subsequent increase in INFγ. Levels of an IL17-producing subpopulation of ILC3 are increased, and an IL22-producing subpopulation is instead decreased in IBD patients.[123]

B-Lymphocytes and autoantibodies

Plasmacytes are a component of inflammatory infiltrates in the mucosa of IBD patients. Increased IgG-positive B- and plasma-cell levels have been shown in both UC and CD. The produced IgG is microbiota reactive, and anti-saccharomyces and anti-flagellin have been shown in CD patients years before the onset of clinical disease. The FCGR2A gene

encoding FcγR, a receptor for IgG, is implicated as a risk gene for UC. Autoantibodies against tropomyosin-5 and epithelial cells have been associated with UC, and anti-GM-CSF has been associated with severe CD. Although this evidence points to the involvement of B-cells in IBD, treatment attempts with the B-cell-depleting biological Rituximab have not shown any effect.[136]

CD8+ T cells

CD8+ T cells are T cells interacting with MHC class I, compared to MCH class II for CD4+ T cells. They have more direct cytotoxic effects, thus they are commonly known as cytotoxic T cells. CD8+ T cells are less studied in IBD compared to CD4+ T cells. IBD patients have been shown to have increased numbers of activated INFγ positive T_C1 cells and T_C17 cells in peripheral blood, and CD8+ T_{reg} in inflamed mucosa in IBD patients. The role of CD8+ T cells in IBD pathogenesis is unclear and further research is needed.[137]

Other Lymphocytes

Other adaptive immune cells that may be involved in IBD include T_H2 cells, which, for example, release IL33, known to be elevated in UC patients, and administration of IL33 leads to worsened colitis in mouse-models.[124] T_H9 cells, which are increased in the mucosa of UC patients, are a source of IL9 and IL10. IL9 is linked to the expression of tight junction proteins in the intestinal epithelium.[138] In inflamed mucosa of UC patients, levels of IL9 correlated with inflammatory score.[139] T_H22 cells might be of importance related to the secretion of IL22. The AHR promotes the development of T_H22 cells and is involved in IL22 secretion.[140] In contrast to T_H17 cells, T_H22 cells secrete only IL22, and not IFN-γ or IL-17.[141]

Concluding remarks on pathogenesis

The pathogenesis of IBD includes an intricate covariation between genetic factors, gut microbiota factors, epithelial barrier defects and reactions related to adaptive immunity. There is no single reason for disease development; several of the described mechanisms above occur simultaneously and affect each other. The involved mechanisms also vary between individuals, making understanding the pathophysiology challenging.

Diagnosis

Diagnosis of IBD is suspected based on medical history and clinical presentation, and confirmed by endoscopic, histological and radiological findings.

Endoscopic findings

Endoscopy is a diagnostic modality where a tube with a camera is passed through the lumen of the gastrointestinal tract to directly visualise the mucosa and allow the possibility to obtain tissue samples. Endoscopic signs of inflammation include erythema, loss of vascularisation, oedema, ulcerations, mucosal friability, pseudopolyps and mucosal granularity. In UC, the inflammation typically starts in the rectum and progresses continuously proximally, and the mucosa gradually transitions from inflamed to normal. Therefore, colonoscopy or sigmoidoscopy are ideal methods of diagnosing UC. In CD, specific colonoscopy findings include strictures, cobblestoning, longitudinal, and aphthous ulcers. Lesions can be found anywhere in the gastrointestinal tract, often in the form of "skip lesions", indicating patches of inflammation surrounded by unaffected mucosa. Some individuals with CD or suspected CD may have symptoms from the upper gastrointestinal tract, where esophagogastroduodenal endoscopy is used to visualise lesions.[142,143]

Another form of endoscopic examination is capsule endoscopy, where the patient swallows a capsule with a camera collecting pictures as it passes through the gastrointestinal canal. The method is sensitive in finding mucosal lesions in the small intestine. This method does not allow for collecting tissue samples for histological examination and cannot be used when there is a suspected stricture.[142]

Histological findings

During endoscopy, generally, several biopsies from different areas are collected. Histological evaluation of the mucosa in IBD is used both in diagnosing IBD and to monitor the disease course, where the term histological healing is frequently used.

UC is histologically characterised by inflammatory infiltrates of lymphocytes, plasma cells and neutrophils in the mucosa and, in severe

cases, the most superficial layers of the submucosa. Other characteristic findings are distortion, atrophy and branching of crypts.

In CD, the inflammatory infiltrates are typically composed of T-lymphocytes and macrophages and often involve the submucosa. A typical histological finding in CD is granulomas made up of epithelioid histiocytes, although this is not required for diagnosis. Changes in crypt architecture, as in UC, are also seen.[144]

Radiological findings

For CD, not all lesions can be found by esophagogastroduodenal endoscopy or colonoscopy since lesions might be somewhere in the small intestine that is inaccessible. A radiological alternative is magnetic resonance imaging of the small intestine. Typical findings in CD with small intestinal involvement include mural hyperenhancement, wall thickening, intramural oedema, strictures and ulcerations.[145]

Biomarkers

A biomarker is a substance objectively measured from tissues or biofluids of the patient. Commonly used biomarkers in IBD include faecal calprotectin, c-reactive protein (CRP) and erythrocyte sedimentation rate. No single biomarker is enough to diagnose IBD on its own. Thus biomarkers are instead used to exclude IBD, evaluate the severity of the disease or assess response to treatment.[146]

Faecal calprotectin is elevated in IBD patients with active disease. Calprotectin is an antimicrobial protein in neutrophils. When activated, the neutrophils release calprotectin which chelates zinc and manganese in order to lower the availability of these minerals and thereby hinder bacterial growth. In IBD, inflammation and neutrophil infiltration are confined to the gastrointestinal tract, and therefore, calprotectin levels are high in faeces. It is important to note that IBD is not the only disease which causes high calprotectin since it is merely a marker of inflammation. Thus the results need to be interpreted in relation to other findings.[146,147] CRP and erythrocyte sedimentation rate are both measured in samples from the blood stream and indicate systemic inflammation and are thus even more unspecific for IBD than calprotectin and are used as prognostic markers in IBD patients to determine the severity of disease and not as an aid in diagnosing the disease.[146]

Biomarkers can be used to diagnose diseases, follow a disease development, or asses the risk for a disease. It can also be used to further the understanding of the pathophysiology of a disease. In the following paragraphs, biomarkers analysed in plasma in studies 2-4 will be presented.

Cotinine

Cotinine is a metabolite of the more commonly known substance nicotine. The problem with using nicotine as a smoking or snuff use marker is its short half-life of only about 2 hours.[148] This implies that it shows recent use but is inaccurate regarding use over time and is, therefore, an inadequate marker of tobacco exposure. Conversely, cotinine, on the other hand, has a half-life of around 16 hours, making it a suitable marker for tobacco use over time.[148] Cotinine can also be used to quantify cigarette use. Measurements in urine, saliva, serum and plasma have all been associated with the intensity of tobacco use.[149,150] Cotinine is also considered a better smoking marker than self-reported data.[151] Table 4 shows how tobacco exposure can be categorised by cotinine in plasma.[152]

Table 4. Categories of tobacco use based on cotinine

Category	Cotinine, nmol/L
Non-tobacco user	<5
Passive tobacco user	5 – 85
Tobacco user	>85 – 1700
Heavy tobacco user	>1700

Based on Kim et al. 2016.[152]

In Sweden, one common way of using nicotine products is snuff use. This is important since cotinine, a metabolite of nicotine, is elevated in both smoking and other forms of tobacco use, such as snuff. Smokeless tobacco, such as snuff, is also associated with higher levels of cotinine compared to smoking.[153] Many associations between smoking and some diseases are not seen for snuff use in the same diseases. When analysing cotinine as a marker for disease, it is thus important to note what type of nicotine product the individual is exposed to. In IBD, this is important since smoking, but not snuff use is associated with disease.[154]

Smoking and IBD

Smoking has long been regarded as an environmental risk factor for developing CD. For UC, smoking has until recently been considered protective against developing disease, but a recent meta-analysis (including results from Paper 1) has shown that former smoking increases the risk for both UC and CD.[21]

Cotinine is a metabolite of nicotine and an objective marker of tobacco exposure. Regardless of the advantages of using an objective marker of tobacco use, it has not been evaluated as a risk marker for IBD until now. For other diseases, such as colorectal cancer, cotinine in urine has been used as a marker of smoking and correlates better to the risk of incident neoplasia than self-reported smoking.[155]

Iron status

In this thesis, iron status includes three measured biomarkers: iron, ferritin, and transferrin, and one calculated; transferrin saturation.

Iron is a trace element essential for many biological processes, including the synthesis of DNA and ATP. It is required in many enzymes, both in humans and microorganisms, and is a constituent of many proteins. Adequate iron is essential for normal immune system function, and disruption of iron homeostasis is associated with disease. However, serum or plasma iron is not used as a biomarker for iron homeostasis or iron deficiency since it, even though it is usually low in iron deficiency, does not reflect iron storage and is highly influenced by recent intake.[156]

Ferritin is an iron storage protein where iron ions, which are toxic to body tissues in the free form, are bound to apoferritin. Most of the ferritin is found in the liver, spleen, and in the bone marrow. Ferritin is also an acute phase reactant, increasing during inflammation.[156]

Transferrin is a transport protein which binds and transports iron in the body. Transferrin is synthesised in the liver, and levels depend on hepatic function and inflammation. In the case of iron deficiency, transferrin concentrations increase.[156]

Transferrin saturation (sometimes also mentioned as iron saturation) was calculated according to the following equation.

$$\frac{\mathrm{P-Fe}\left[\frac{\mathrm{mmol}}{\mathrm{L}}\right]\mathrm{x}\,100}{\mathrm{P-Transferrin}\left[\frac{\mathrm{g}}{\mathrm{L}}\right]\mathrm{x}\,25.1}$$

It is a measure of how much iron is bound to transferrin. Iron deficiency results in a low transferrin saturation as iron concentrations decrease and transferrin concentrations increase.

Iron metabolism

Dietary iron is obtained as either heme, from animal sources, or non-heme, from plant sources. Heme iron is directly absorbed, whereas non-heme iron usually needs to be converted from Fe^{3+} to Fe^{2+} before absorption by active transportation. Depending on the iron requirement in the body, iron can either be temporarily stored in the enterocyte as ferritin (when iron availability in the body is adequate) or enter the bloodstream through ferroportin (when there is a low iron availability in the body). The uptake from the enterocyte to the bloodstream is regulated by hepcidin, a protein that can bind and inhibit the function of ferroportin. When iron enters the bloodstream through ferroportin, it binds to transferrin, the main transporter of iron in the bloodstream. If adequate iron levels in the body inhibit the uptake of iron from the enterocyte, the iron will be excreted in faeces in the enterocyte, which has a lifespan of just a few days. In the bloodstream, most of the iron is transported to the bone marrow, where blood cell synthesis occurs, which requires iron. Iron recycled from the destruction of cells, for example, in the spleen or liver, is also transferred to the bloodstream. Storage of iron in the cells is in the form of ferritin.[156]

Iron deficiency in IBD

It has long been known that anaemia is common among IBD patients. The anaemia usually results from inflammation, iron deficiency or a combination of both. Combined anaemia is the most common type in IBD patients, resulting from iron deficiency and chronic inflammation.[157] Previous studies have reported the frequency of isolated iron deficiency without anaemia in patients at diagnosis and during active disease. These studies report higher frequencies of iron deficiency among female IBD patients than in males, and generally, no comparison with a control population is presented.[158,159]

B-vitamins

There are eight different B vitamins; B1 – thiamine, B2 – riboflavin, B3 – niacin, B5 – pantothenic acid, B6 – Pyridoxine, pyridoxal and pyridoxamine, B7 – biotin, B9 – folate, and B12 – cobalamin. B vitamins are essential for normal metabolic functions and are each involved in different processes, such as DNA synthesis and the metabolism of fat and carbohydrates, see Table 5. B vitamins are not synthesised de novo in humans and are obtained from the diet either through B vitamin-containing food products or supplements or derived from gut microbiota metabolism.

Table 5. Examples of different functions for B vitamins in human metabolism.[160]

B1	Required as a coenzyme in the citric acid cycle and pentose phosphate pathway
B2	Needed for heme-protein synthesis and fat metabolism
B3	Precursor for NAD+ and NADP+
B5	Used in synthesis of cholesterol and coenzyme A
B6	Cofactor in several metabolic pathways, including the transsulfuration pathway
B7	Needed for gene regulation, cell signalling and metabolism of fatty acids, amino acids and glucose.
B9	Required in nucleic acid synthesis and erythrocyte, myelin, and neurotransmitter production
B12	Needed in erythropoiesis, myelin, neurotransmitter and lipid synthesis

Vitamin B6

Due to known changes in vitamin B6 status among IBD patients and its importance in the kynurenine pathway, it will be described in more detail.

Vitamin B6 is a collective term for three pyridine derivates, pyridoxal, pyridoxamine and pyridoxine, and their respective phosphorylated forms, pyridoxal 5'-phosphate, pyridoxamine 5-phosphate and pyridoxine 5-phosphate. Vitamin B6 acquired through diet is converted in the liver and intestine to pyridoxal 5'-phosphate or pyridoxal and catabolised to 4-pyridoxic acid, the form excreted in the urine. [161]

Pyridoxal 5'-phosphate is the vitamer used as a cofactor in vitamin B6-dependent metabolic pathways such as the kynurenine pathway.[161]

Vitamin B6 is catabolised from PL to PA, an end catabolite excreted in the urine. The catabolism from PL to PA depends on the enzymes aldehyde oxidase-1 and aldehyde dehydrogenase, which both have increased expression during oxidative stress, as is seen during inflammation.[161] The PAr index, described in statistical methods, is a marker of inflammation based on the increased vitamin B6 catabolism in inflammatory conditions.

B vitamins in IBD

Due to the risk of malabsorption and the close links to immune system functions, B vitamin concentrations among IBD patients are of interest. Previous studies have shown, for example, lower serum folate among IBD patients compared to controls.[162] Vitamin B6 has also been shown in previous studies to be low among IBD patients.[163-165]

The kynurenine pathway

The kynurenine pathway is presented in Figure 4. It is one of several ways of catabolising tryptophan. The end metabolites include kynurenic acid, xanthurenic acid, picolinic acid and NAD. Vitamin B6 is a required cofactor for several catabolic steps in the pathway, for example, the kynurenine aminotransferases that catabolise the production of kynurenic acid and xanthurenic acid. Several metabolites have immunomodulatory effects, and the pathway is upregulated in diseases with known immune activation, such as rheumatoid arthritis and cardiovascular disease. [161]

The kynurenine pathway in IBD

Tryptophan catabolism through the kynurenine pathway is known to be upregulated in several diseases with systemic immune responses, including rheumatoid arthritis and cardiovascular disease. In IBD, a study comparing serum samples from individuals with active and inactive disease found a correlation between increased tryptophan degradation through the kynurenine pathway and higher disease activity.[166] The same study found lower serum tryptophan concentrations among patients with active disease. Compared to controls, CD was associated with lower levels of the kynurenine pathway metabolites

kynurenic acid, xanthurenic acid, anthranilic acid, quinolinic acid and picolinic acid.[166]

In a DSS-colitis model in mice, intestinal inflammation was associated with lower concentrations of kynurenic acid and xanthurenic acid. Treatment with Kynurenic acid and xanthurenic acid showed anti-inflammatory effects related to AHR activation.[89]

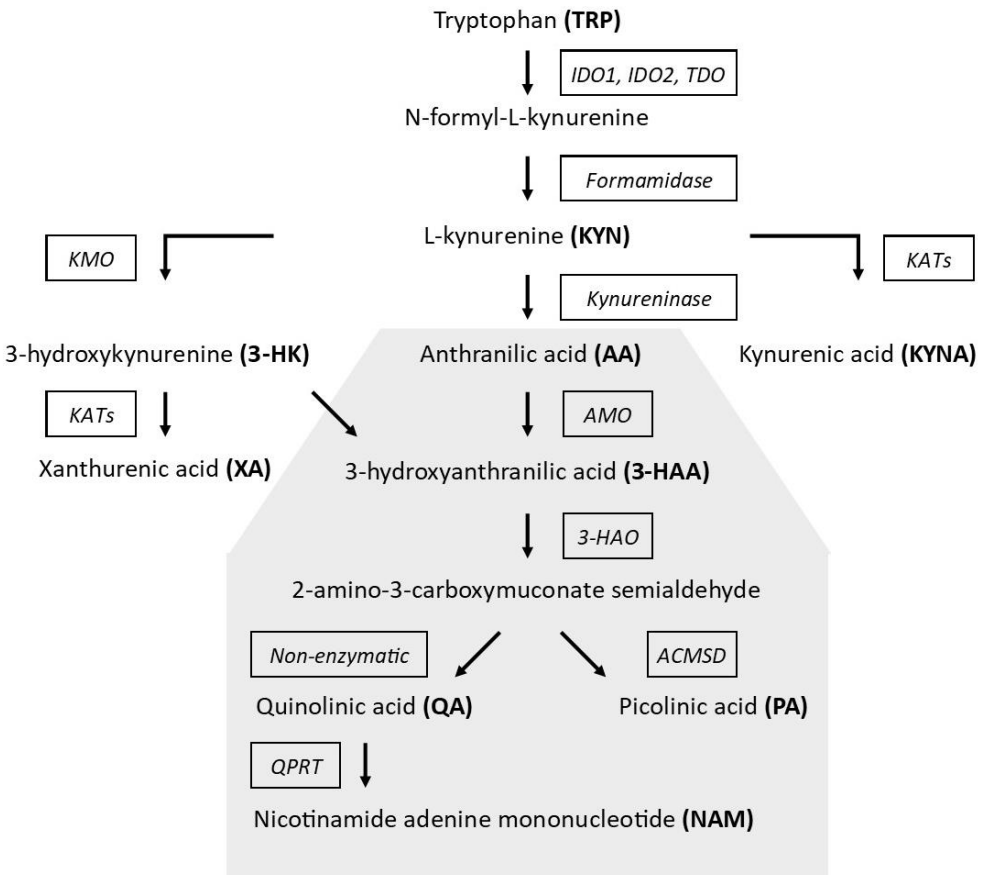

Figure 4. The kynurenine pathway of tryptophan catabolism. IDO – indoleamine (2,3)-dioxygenase. TDO – tryptophan (2,3)-dioxygenase. KATs – kynurenine aminotransferases. KMO – kynurenine 3-monooxygenase. AMO –anthranilate 3-monooxygenase. 3-HAO – 3-hydroxyanthranilate 3,4-dioxygenase. ACMSD – 2-amino-3-carboxymuconate semialdehyde dioxygenase. QPRT – quinolinate phosphoribosyltransferase.

Indexes based on the kynurenine pathway

Based on enzyme functions in the kynurenine pathway, different indexes can be calculated.

The KTR, described in statistical methods, is a marker of IDO1 activation during T_H1 immune response.[161] It was calculated as the concentration of kynurenine divided by the concentration of tryptophan, see Figure 4.

A higher KTR results from the activation of IDO, which is upregulated during inflammation and thus, a higher KTR is a marker of inflammation.

The HKr index assesses functional vitamin B6 levels and is unaffected by inflammation.[167] It was calculated as a ratio of 3-hydroxykynurenine to the different vitamin B6 dependant catabolites, see Figure 4 and statistical methods Table 6. A higher HKr indicates less catabolism and, thus, functional vitamin B6 deficiency. It might also indicate a deficiency of any of the enzymes involved in the catabolism.

Aims of Papers 1-4

This thesis aimed to expand the understanding of the pathophysiology of inflammatory bowel diseases by investigating environmental risk factors in individuals years before diagnosis. Survey data and biomarkers from biobanked plasma were used to investigate the possible effects of lifestyle on the risk of developing IBD. The aims of included Papers were:

To investigate the relationship between smoking and other lifestyle factors from survey data in relation to later developing inflammatory bowel disease

To further investigate tobacco exposure by the objective biomarker cotinine in biobanked plasma using cotinine as a potential risk marker for developing disease

The third aim was to investigate iron status before diagnosis to understand early changes in iron status, including ferritin, which is known to be affected in established disease.

And lastly, to investigate the kynurenine pathway before disease onset, a pathway known to be altered by inflammation and B vitamin status, in relation to the risk of developing inflammatory bowel disease.

Materials and Methods

Study population

The study population in all included studies was from the Northern Sweden Health and Disease Study, either from the Västerbotten Intervention Programme or the mammography screening project.

The Västerbotten intervention programme

This ongoing project invites all inhabitants in Västerbotten county aged 40, 50 and 60 years so a health screening and offers them to participate in the Västerbotten intervention programme. Detailed questionnaire data on lifestyle habits and an extensive food frequency questionnaire are obtained, and body measurements, including BMI and blood pressure and blood samples, are collected. The project started in 1985 and has since then collected over 160 000 samples. The participation rate has varied between 48-67%.[168]

The mammography screening project

This project collected blood samples and limited survey data in connection to mammography screenings for breast cancer among women, 18-82 years old, in Västerbotten between 1995-2006.

Cohorts in papers 1-4

Individuals participating between 1989 and 2008 who developed IBD at least one year after participating (or until 2010) were included. For each case, matched controls were selected from the same cohort. For Paper 1, four controls for each case, and for Papers 2-4, two controls for each case. Controls were from the same cohort as the case and matched for age, sex and time and primary care facility for health screening. This last matching reduces bias caused by preanalytical differences.

All studies included in this thesis had the same inclusion criteria for cases: participation in either the Västerbotten intervention programme or the mammography screening project at least one year before IBD diagnosis and an ICD-10 diagnosis of IBD (K50.1-9 or K51.1-9). In Paper 1, all individuals with available detailed questionnaire data were eligible,

whereas in Papers 2-4, only individuals with available plasma were included, and the detailed survey data was not required. This led to a partial overlap in the study population. Most cases were included in both cohorts except for the following exceptions: eleven cases from the cohort in paper 1 only had available survey data and no plasma data and was thus not included in the cohort for paper 2-4. Nine cases from the cohort in Papers 2-4 were from the mammography screening project and were thus not included in the Paper 1 cohort. 87 cases were included in both cohorts.

Survey data

Available survey data for the subjects from the Västerbotten intervention programme included information about smoking, snuff use, alcohol consumption, height and weight. A validated food-frequency questionnaire was available with calculated summary scores, for example, of fibre intake. Surveys were collected during the health screenings. For the mammography screening project the lifestyle survey was limited and included information about smoking and snuff use.

Sample collection and preparation

Blood samples were collected during health screenings within the participants' primary care facility. Participants rested 15 minutes before the collection of venous blood samples. After collection into Na-heparin tubes, each tube was inverted 8-10 times. Then the samples were left at room temperature for 15 minutes before being centrifuged for 15 minutes at 15000G. Plasma samples were frozen in aliquots within 1 hour and stored at -80°C until use. Of the collected samples, 80% were fasting samples with more than 8 hours of fasting time before sample collection.

Matching of cases and controls

Cases and controls were matched for age, sex, and time and area of inclusion in NSHDS. For age, there was at most one year difference between each case and control. For sex, each case was the same as its control. Area of blood sampling means that they were recruited from the same primary care facility. The time of blood sampling was within one week for cases and their respective control, reducing the effects of season. For controls with only survey data included in Paper 1, three controls to different cases had about one month difference in time.

Biochemical analyses

All biomarkers were analysed from plasma samples collected into Na-heparin tubes. Plasma is obtained from venous blood samples by using test tubes with anticoagulants to prevent coagulation. The samples were centrifuged to separate the plasma from the cellular components in the blood. The plasma contains proteins and peptides, hormones, salts and various metabolites. It differs from serum, where the blood coagulates before centrifugation, and thus, coagulation factors and fibrinogen are consumed.

Iron status

Iron status was analysed on a Cobas 8000 modular analyser (Roche Diagnostics GmbH, Mannheim, Germany). All reagents used were from Roche Diagnostics, Basel, Switzerland.

Iron

Iron was analysed with a colourimetric analysis. Acidification of samples released iron ions from the transferrin-iron complex. By the addition of ascorbic acid, Fe^{3+} ions were reduced to Fe^{2+} ions. FerroZine was added to the sample, creating a coloured complex measured by photometry. IRON Gen.2 (Cat No. 05169291 190) was used as reagent. The lowest level of detection was 1 µmol/L. Standardisation was made to primary reference material SRM 937. The total coefficients of variation were 3% at levels 12 and 40 µmol/L.

Transferrin

Transferrin was measured by an immunoturbidimetric method where an antibody against human transferrin was added to the sample, creating a complex, altering the turbidity of the sample proportionally to the transferrin concentration in the sample. The turbidity was then measured by photometry. TRSF2 (Cat No. 03015050 122) was used as reagent. The lowest level of detection was 0.20 g/L. It was standardised to the Institute for Reference Materials and Measurements, BCR470/CRM470 (Reference Preparation for Proteins in Human Serum). The total coefficients of variation were 4 and 6% at levels 1.5 and 5 g/L, respectively.

Transferrin saturation

The transferrin saturation was calculated based on the results from the iron and transferrin analyses. [Iron ×100]/[transferrin ×25.1]

Ferritin

Ferritin was analysed by adding a biotinylated monoclonal ferritin-specific antibody and a monoclonal ferritin-specific antibody labelled with a ruthenium complex to the patient sample, forming a sandwich complex. Streptavidin-covered microparticles were added and bound to the complex by streptavidin attaching to biotin. The complexes were then magnetically bound to the surface of an electrode, and unbound substances were removed. Applying electric voltage to the electrode resulted in chemiluminescent emission, measured by a photomultiplier. Ferritin (Cat No. 04491785 190) was the used reagent. The lowest level of detection was 1 µg/L. It was calibrated to NIBSC Standard # 80/602. The total coefficients of variation were 3.6% at levels 26.6 and 131.6 µg/L.

B vitamins, tryptophan metabolites and cotinine

Cotinine and the analytes in Paper 4 were analysed by Bevital AS, Bergen, Norway (www.bevial.no) either with liquid chromatography, tandem mass spectrometry (LC-MS/MS), platform D, or gas chromatography, tandem mass spectrometry (GC-MS/MS), platform B.

LC-MS/MS and GC-MS/MS

Analytes related to the kynurenine pathway (tryptophan, kynurenine, 3-hydroxykynurenin, kynurenic acid, xanthurenic acid, anthranilic acid, 3-hydroxyanthranilic acid, picolinic acid, quinolinic acid, nicotinamide and n1-methylnicotinamide), neopterin and B vitamins (pyridoxal 5' -phosphate, pyridoxal, 4-pyridoxic acid, thiamine, thiamine monophosphate, riboflavin and flavin mononucleotide) were analysed using LC-MS/MS. The method has been described previously, and details regarding the assay, including limits of detection, reported values and within-day and between-day CV%, are provided in the original publication.[169]

Analytes related to the one-carbon metabolism (homocysteine, methylmalonic acid, methionine, serine, glycine and cystathionine) were

analysed by GC-MS/MS. The method was previously described, and assay details are presented in the original publication.[170]

Microbiological assays

Cobalamin and folate were analysed with microbiological assays. The methods are based on the inability of certain microorganisms to grow normally in a media without cobalamine or folate. Thus, the growth depends on the presence of this substrate from the patient sample. The microorganisms used were *Lactobacillus leichmannii* for cobalamin and a chloramphenicol-resistant strain of *Lactobacillus casei* for folate.

The patient sample and bacteria in log phase (with exponential bacterial growth) were added to an assay buffer, and after incubation, the optical density was measured, giving a quantitative measure of cobalamin or folate in the patient sample.

A problem with using a microbiological assay is the suppression of growth expected with antibiotics in the patient sample, and since the biobanked data in this project do not include information on the use of antibiotics, this could be an issue. However, it has been shown that it is almost exclusively β-lactam antibiotics that cause interference, and thus, β-lactamase was added to all samples in the assay.[171-173] Also, patients attending the health exams were unlikely to have an active infection at the time of the visit.

Statistical analyses

Statistical analyses were performed using IBM SPSS statistical software package version 26.0 or 28.0 (IBM Corporation, New York, USA). Figures were designed with GraphPad Prism version 9.4.1 for Windows (GraphPad Software, San Diego, California, USA) with results from SPSS analyses.

For the description of demographics and analyte concentrations, medians and 25^{th}-75^{th} percentiles were presented for continuous variables and per cent for categorical variables. Differences in medians were assessed using the Mann-Whitney U-test, and differences for categorical variables were evaluated with the Chi^2-test.

Conditional logistic regression was used to assess the relationship between the different lifestyle markers, biomarkers and developing IBD. In some models, adjustments were made for known or suspected confounders. All logistic regression models were calculated separately for UC and CD patients.

For Papers 3 and 4, *z*-scored variables were used in regression models. These were created by giving the mean the value of zero, the value 1 corresponded to one standard deviation above the mean, and the value minus one corresponded to one standard deviation below the mean.

In Paper 3, sex-based ferritin quartiles were created and included in conditional logistic regression models. To assess the odds ratio over all quartiles, this categorical variable was analysed as a continuous variable, yielding a *P* value for the trend.

In Paper 4, three different indexes were calculated, see Table x. Time to diagnosis was assessed in relation to the different calculated indexes by linear regression.

Table 6. Calculated indexes in Paper 4.

PAr	4-pyridoxic acid /(pyridoxal 5ˈ-phosphate + pyridoxal)
KTR	Kynurenin/tryptophan
HKr	3-hydroxykynurenine/(kynurenic acid+ anthranilic acid + xanthurenic acid + 3-hydroxyanthranilic acid)

PAr-Pyridoxal acid ratio. KTR-Kynurenine, tryptophan ratio. HKr-Hydroxykynurenine ratio.

Ethical considerations

The included studies followed the ethical principles of the Declaration of Helsinki. Written informed consent for the data to be used in the research was obtained during the health screenings.

Since the studies were conducted on biobanked samples, and the identities of included patients were blinded to the research group, the included participants would not personally benefit from the study results. Participants were informed about this upon inclusion.

When researching biobanked material, it is crucial to acknowledge that there is only a finite amount of the biobanked sample. Thus there was a responsibility to ascertain that the maximum amount of high-quality information possible was obtained by using as little as possible of each plasma sample so that the remaining plasma could be saved for future research.

The study was approved by the Regional Ethics Committee, Umeå, Sweden (Dnr 06-024M, 2010-284-31M, 2015-78-32M).

Results

Demographics

Matching for age, sex and time and primary care facility ascertained no differences between cases and controls regarding these variables. Figure 5 shows the distribution of age and sex for the two separate cohorts used in Paper 1 and Papers 2-4, respectively. In Paper 1, most individuals were included at the age of 40, 50 and 60 years. A few individuals are also included at 30 years of age, which is because of the inclusion of 30-year-olds in the Västerbotten intervention program during the first years of the intervention. For the age distribution in Papers 2-3, the distribution was very similar, with a few extra individuals in higher age groups related to the mammography screening cohort, which included individuals to the age of 82. For sex, the distribution was relatively equal, with slightly more women among the Papers 2-4 subjects. This was related to inclusion from the mammography screening project, which only included women.

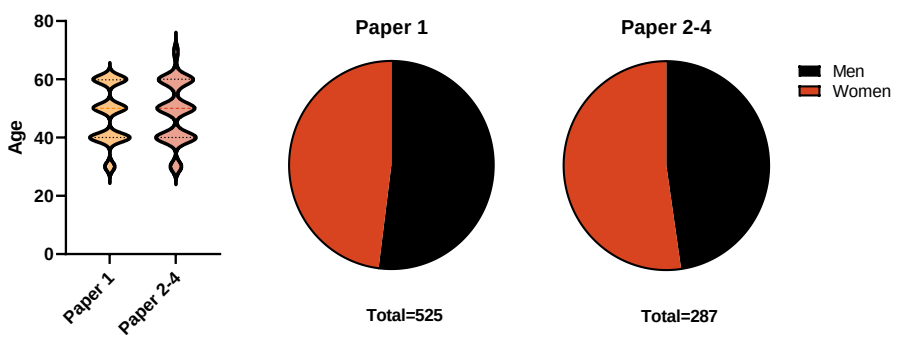

Figure 5. Distribution of age and sex for the different cohorts.

Demographics, only presented for the group with plasma samples, are seen in Table 7. Results were similar in the plasma and survey data groups since 90% of cases were included in both cohorts.

The median time from inclusion and sample collection to diagnosis was 5.26 years for UC cases and 4.76 years for CD cases. No differences were seen between cases and controls for marital status, educational level, body mass index or CRP. Most included individuals were cohabitant and had less than 12 years of education. Body mass index was of interest in IBD, especially in CD, where malnutrition is common among diagnosed

patients with the disease. In epidemiological studies, BMI has been shown to be a risk factor for CD, both in overweight and underweight individuals.[174]

Table 7. Demographical data

Ulcerative Colitis	**Case**	**Control**	***P* value***	**n**[†]
Time to diagnosis, years	5.26 (2.66-7.23)	n.a.	n.a.	70/n.a.
Marital status, cohabitant	74.3	74.1	0.49	61/129
Educational level, >12 years	18.6	20.9	0.92	63/129
Body mass index, kg/m^2	25.0 (23.2-27.5)	25.6 (23.1-27.8)	0.82	70/138
C-reactive protein, mg/L	1.1 (0.46-2.7)	0.95 (0.48-2.6)	0.70	65/138
Crohn's Disease	**Case**	**Control**	***P* value***	**n**[†]
Time to diagnosis, years	4.76 (2.50-8.08)	n.a.	n.a.	26/n.a.
Marital status, cohabitant	73.1	65.4	0.58	22/44
Educational level, >12 years	3.8	23.1	0.063	22/44
Body mass index, kg/m^2	26.1 (23.1-30.4)	25.3 (22.9-28.3)	0.43	26/52
C-reactive protein, mg/L	2.5 (0.34-8.7)	0.83 (0.31-2.1)	0.18	26/52

* Calculated with Mann-Whitney U-test for continuous and Chi^2-test for categorical variables. [†] Case/control

Table 8 shows the distribution of the Montreal classification for included cases. Most cases had a disease onset after the age of 40 years. There was a relatively even distribution of colon involvement in the UC group, with most individuals presenting with left-sided colitis or pancolitis. For CD, the distribution was also relatively even, with colonic disease being the most prevalent and with mostly non-stricturing, non-penetrating disease, which is expected in late-onset disease.

Table 8. Montreal classification of included cases divided into UC and CD.

Ulcerative Colitis (n=70)	**Number**	**%**
A2, onset 17-40 years	7	10
A3, onset >40 years	63	90
E1, proctitis	15	21
E2, left-sided colitis	29	41
E3, pancolitis	25	36
Crohn's Disease (n=26)		
A2, onset 17-40 years	4	15
A3, onset >40 years	22	85
L1, ileal disease	8	31
L2, colonic disease	10	38
L3, ileocolonic disease	8	31
B1, non-stricturing, non-penetrating disease	16	62
B2, stricturing disease	7	27
B3, penetrating disease	2	8
Bp, perianal disease	2	8

UC - ulcerative colitis, CD - Crohn's disease

Paper 1:

In paper one, the proportions of individuals in different tobacco exposure categories were evaluated, see Figure 6. A higher proportion of current smokers were seen in the total IBD group and the CD group, whereas a lower ratio of never-smokers was seen in the total IBD and the UC group compared to controls. The finding of a decreased proportion of never-smokers among UC cases was further investigated, and in univariable logistic regression, never smoking was associated with a decreased odds ratio for developing IBD, both for the UC and CD subgroups. For the CD subgroup, this was expected as smoking is a known risk factor, but for UC, this was in contrast to previous findings, which have shown a decreased risk for UC in smokers.

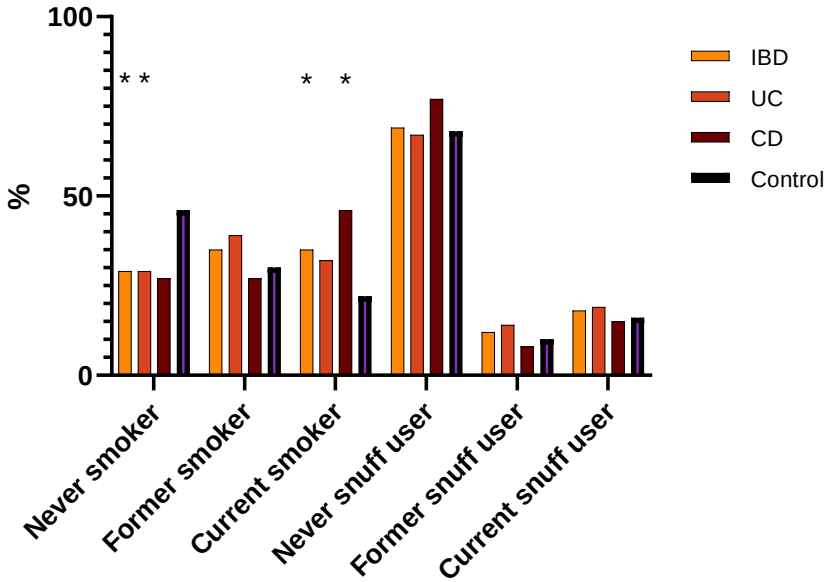

Figure 6. The proportion of cases and controls divided into categories of smoking and snuff use based on self-reported prediagnostic data.

In paper one, a few other lifestyle factors were analysed, see Figure 7. For alcohol, no significant associations with IBD were seen, even though wine consumption had a tendency towards a protective effect in the CD group only. There are several previous studies on IBD risk and alcohol, but in a meta-analysis, it was not a significant risk factor for either UC or CD.[21] A higher fibre intake was associated with a lower odds ratio for developing CD, consistent with previous findings in meta-analyses on diagnosed patients. Intake of protein and protein from animal sources was also associated with CD. However, our study did not specify what kind of animal the protein came from. Previous studies have found that fish intake is protective against CD, whereas meat intake increases the risk of CD. The significant findings are thus not unexpected.

These results show that before diagnosis, previously established risk factors can be identified in this cohort. All included data in Figure 7 were not presented in Paper 1 but were presented here further to describe the cohort and analysed environmental risk factors.

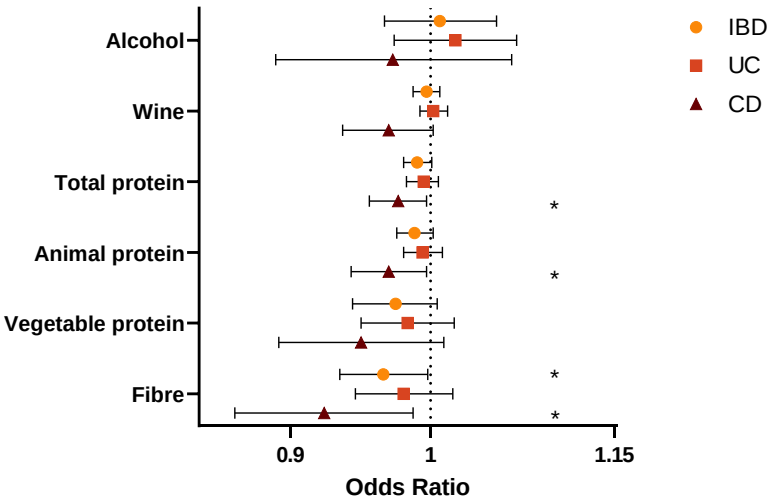

Figure 7. Univariable logistic regression showing odds ratio and 95% confidence interval for developing IBD by dietary factors. All dietary factors as g/day based on self-reported data.

In multivariable analyses, including BMI, smoking, snuff use and alcohol or wine consumption, never smoking was still associated with a decreased odds ratio for developing both UC and CD, Se Figure 8.

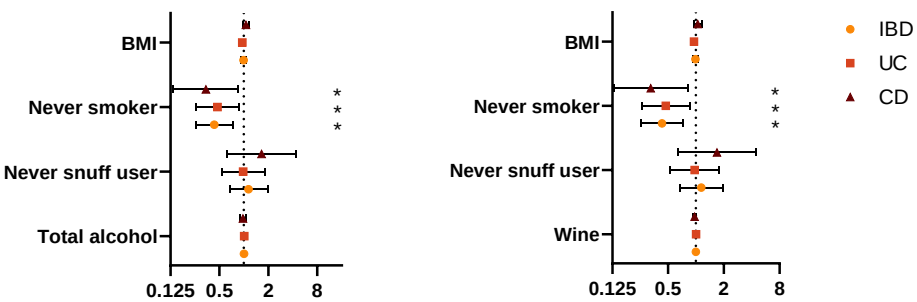

Figure 8. Multivariable logistic regression showing odds ratio and 95% confidence interval for never smoking, never using snuff, BMI and alcohol or wine consumption in prediagnostic IBD based on self-reported data.

To conclude, in Paper 1, we showed that never smokers had a decreased risk of developing both UC and CD, based on self-reported data.

Paper 2

In Paper 2, tobacco habits in relation to prediagnostic inflammatory bowel disease were further investigated. Based on plasma cotinine concentrations, individuals were categorised into four different categories, see Figure 9. A higher proportion of non-tobacco users was found in the control group, and higher proportions of tobacco users and heavy tobacco users were seen in the case group. This was consistent with the previous findings indicating an increased risk for IBD with higher tobacco exposure, specifically from smoking, based on self-reported data.

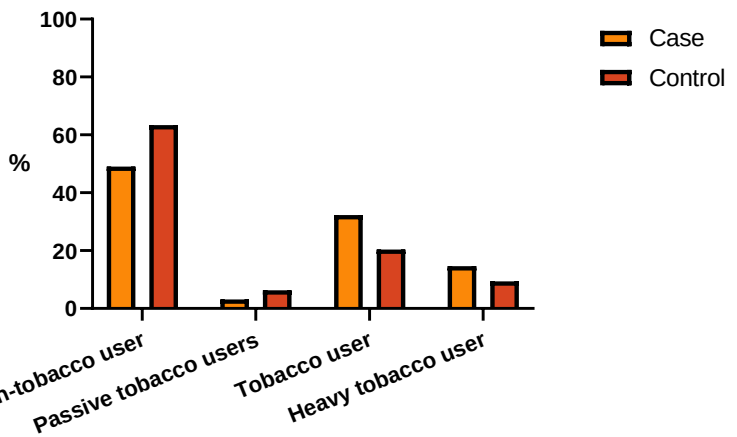

Figure 9. Distribution in categories based on cotinine concentrations. Cotinine <5 nmol/L - non- tobacco users, 5–85 nmol/L - passive tobacco users, >85–1700 nmol/L - tobacco users and >1700 nmol/L - heavy tobacco users.

Further multivariable analyses are shown in Figure 10. Higher cotinine concentrations were associated with a higher odds ratio for developing IBD, especially UC, even though the odds ratios for CD showed a non-significant tendency toward the same. Cotinine was associated with higher odds ratios for disease compared to data on self-reported smoking, which has previously been the method of assessing risk related to tobacco use. Snuff use was associated with a lower odds ratio for IBD in multivariable models with a short time from inclusion to diagnosis. Thus it is likely that it is not the tobacco exposure per se that increases the risk for IBD, but rather the smoking process. Either related to the combustion of tobacco, or the exposure to a specific tissue, i.e. the lungs.

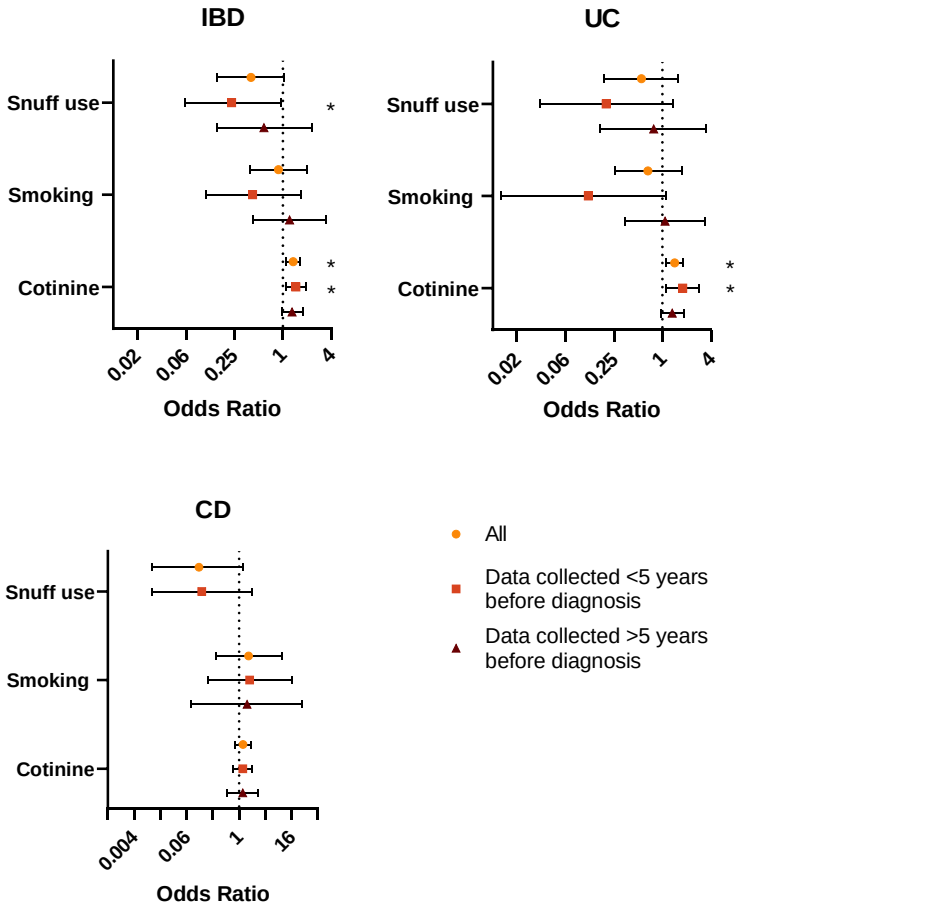

Figure 10. Univariable conditional logistic regression showing odds ratios and 95% confidence interval for developing IBD by tobacco exposure.

To summarise, Paper 2 showed an increased risk of developing IBD with higher tobacco exposure based on cotinine concentration, an objective way of assessing tobacco exposure. Associations were seen for an increased risk of developing IBD with higher cotinine and for self-reported data, but not for snuff use. This might imply that it is not nicotine or tobacco that increases the risk for IBD but rather the smoking process. The reason could be exposure to other substances from cigarette smoking, possibly related to combustion. It could also be the exposure through the lungs that leads to IBD development.

Paper 3

Paper 3 investigated the relationship between iron status and prediagnostic IBD. In Table 9, concentrations of included analytes are presented. Lower ferritin was seen in cases compared to controls, but when subdividing into two groups based on sex, this was only seen for men. No differences were seen between cases and controls for the other included analytes.

Table 9. Concentrations of iron status analytes in cases and controls.

	Case	**Control**	***P* value***
Ferritin, µg/L	73.3 (39.0-132.9)	106 (44.4-180.8)	0.032
Men	93.6 (67.2-172.7)	161.0 (117.2-269.0)	0.001
Women	49.6 (20.4-92.5)	56.9 (25.2-104.7)	0.52
Iron, µmol/L	18.1 (12.4-21.5)	17.7 (13.0-21.6)	0.62
Transferrin, g/L	2.4 (2.2-2.8)	2.4 (2.2-2.7)	0.61
Transferrin saturation, %	28.2 (19.6-35.9)	29.5 (22.5-35.4)	0.36

* Calculated with Mann-Whitney U-test

Ferritin was lower among cases, reflecting lower iron storage. Based on ferritin, iron deficiency among cases and controls was investigated. Since the difference in ferritin between cases and controls was only seen for men, further analyses of iron deficiency were performed separately for men and women, see Figure 11. Ferritin is an acute phase reactant, and when using ferritin to assess iron deficiency, it is necessary to consider possible inflammation. Based on guidelines from the European Crohn's and Colitis Organisation, inflammation was defined as CRP over 3 mg/L, and in the presence of inflammation, the ferritin cutoff, where iron deficiency could not be excluded, was elevated. The cutoff for iron deficiency was under 30 µg/L in the absence of inflammation, and with evidence of possible inflammation, iron deficiency could not be excluded with ferritin under 100 µg/L. In Figure 11, the proportions of iron deficiency and where iron deficiency could not be excluded are presented.

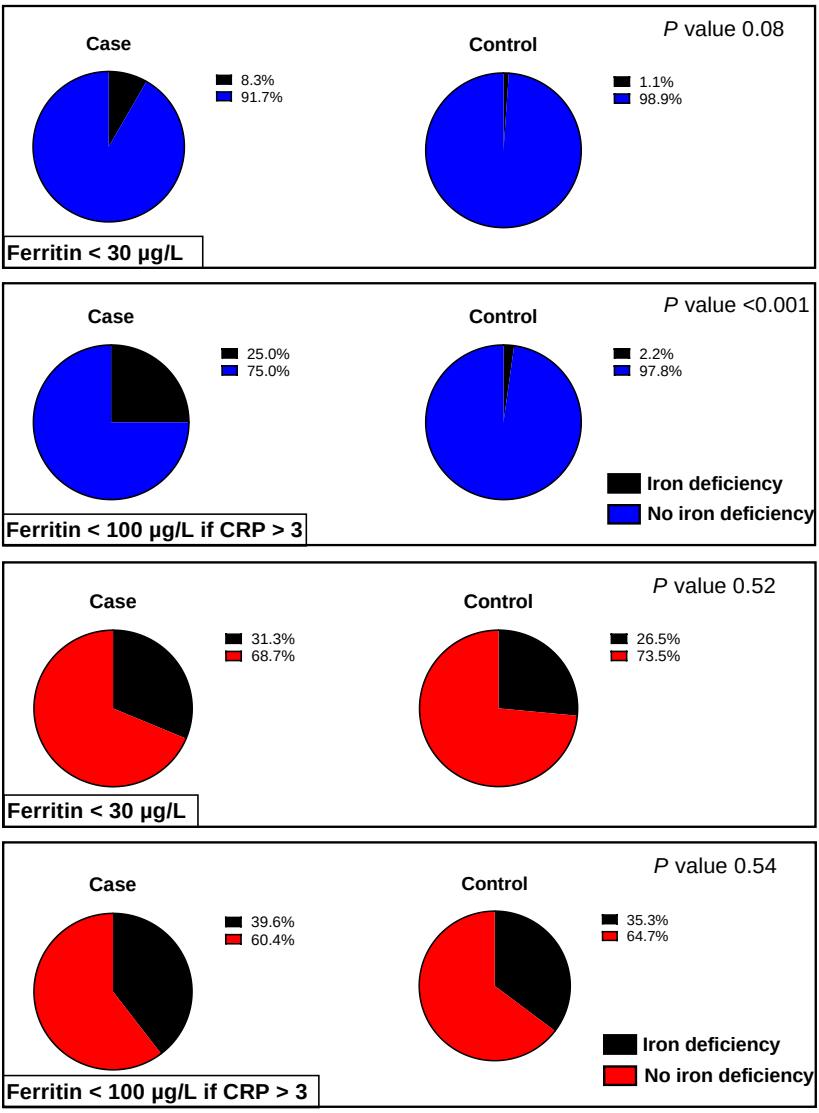

Figure 11. Proportions of iron deficiency and proportions where iron deficiency could not be excluded. Blue for men and red for women.

Iron deficiency was more common among women in all groups compared to men. Comparison between cases and controls showed that the proportion of iron deficiency and proportion where iron deficiency could not be excluded was higher among cases, although the difference was only significant for men. It is likely that among women, non-IBD-related reasons account for most of the observed iron deficiency, whereas among men, this was not the case. This indicates that among

men later developing IBD, iron deficiency is more common compared to healthy individuals.

Previous studies have reported a high prevalence of iron deficiency in women with IBD, consistent with our findings. However, in those studies, control populations are usually not included. Thus, it has not been observed that it is among men in particular that IBD can be associated with a higher prevalence of iron deficiency.

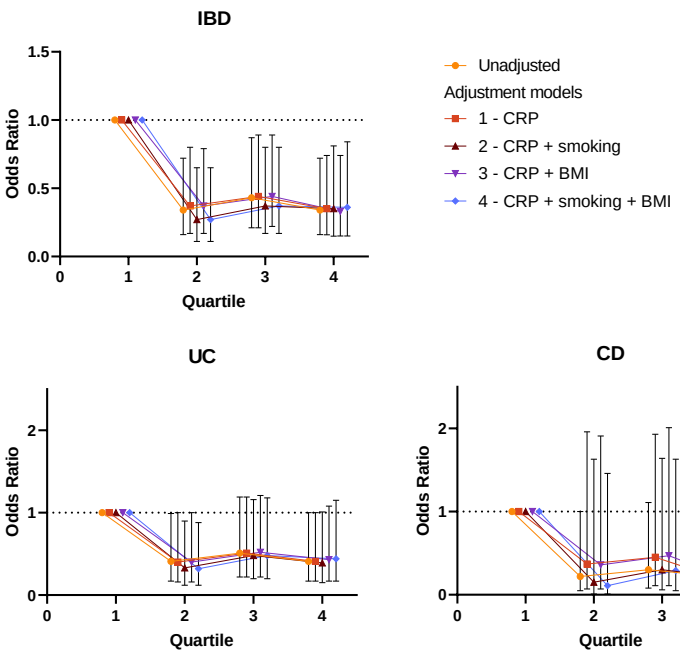

Figure 12. Multivariable logistic regression showing odds ratios and 95% confidence interval for developing IBD by quartiles of ferritin concentrations.

Further investigation into ferritin before IBD diagnosis was made by dividing individuals into sex-based quartiles of ferritin concentrations. Results are shown in Figure 12. The results indicate a threshold level where the odds ratio for IBD is affected. Individuals with ferritin in quartiles 2-4 had a lower odds ratio for developing IBD, subdivision into UC and CD resulted in similar odds ratios, although not significant for all models.

In summary, Paper 3 showed that individuals prior to IBD diagnosis have iron deficiency and lower ferritin compared to healthy controls.

Paper 4

The fourth paper focused on the kynurenine pathway and B vitamins in relation to future development of IBD. Metabolites in the kynurenine pathway were analysed using univariable and multivariable logistic regression models shown in Figure 13. For UC, higher kynurenic acid and xanthurenic acid were associated with a lower odds ratio for developing disease, with the same tendency in CD, but only significant for kynurenic acid in one adjustment model. Kynurenic acid and xanthurenic acid have both been shown to be lower among IBD patients in previous studies.[89] CD was associated with a lower odds ratio for developing disease with higher picolinic acid. Picolinic acid has previously been shown to be lower among diagnosed CD patients.[166]

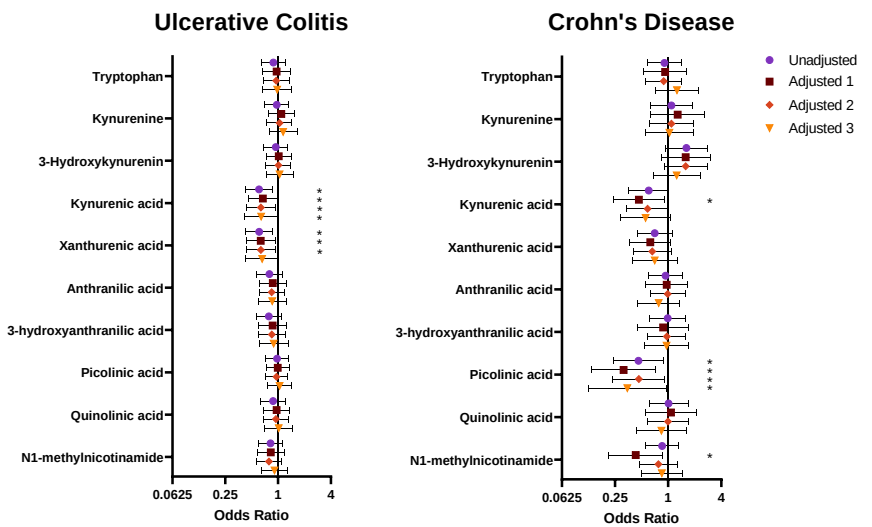

Figure 13. Kynurenine pathway metabolites, conditional logistic regression showing odds ratios and 95% confidence interval for developing UC and CD. Adjustments; Adjusted 1: BMI+Smoking, Adjusted 2: BMI+cotinine Adjusted 3: BMI+Cotinine+Albumin+CRP.

Figure 14 shows multivariable conditional logistic regression for developing IBD in relation to B vitamins. Previous studies have found alterations in B vitamins in already diagnosed patients, for example, low folate,[175] and low vitamin B6.[176] Paper 4 shows a lower odds ratio for developing IBD with higher concentrations of pyridoxal 5'-phosphate and pyridoxal, both B6 vitamers. Pyridoxal 5'-phosphate is an essential coenzyme in several steps in the kynurenine pathway.[161]

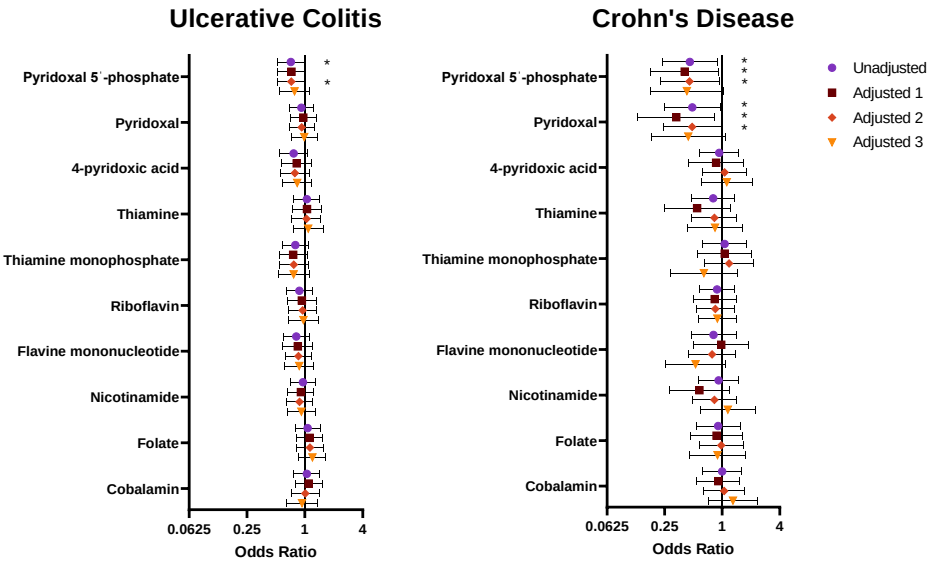

Figure 14. B vitamins, conditional logistic regression showing odds ratios and 95% confidence interval for developing IBD. Adjustments; Adjusted 1: BMI+Smoking, Adjusted 2: BMI+Cotinine, Adjusted 3: BMI+Cotinine+Albumin+CRP.

To further assess vitamin B6 and pyridoxal 5'-phosphate in particular, the HKr index, a marker for functional pyridoxal 5'-phosphate deficiency, was calculated.

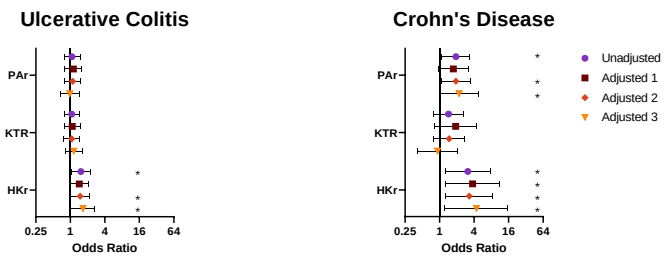

Figure 15. Calculated indexes, conditional logistic regression showing odds ratios and 95% confidence interval for developing IBD. Adjustments; Adjusted 1: BMI+Smoking, Adjusted 2: BMI+Cotinine, Adjusted 3: BMI+Cotinine+Albumin+CRP.

In multivariable conditional logistic regression models, see Figure 15, the HKr was associated with a higher odds ratio for developing both UC and

CD, indicating more prevalent functional pyridoxal 5'-phosphate deficiency among cases than controls.

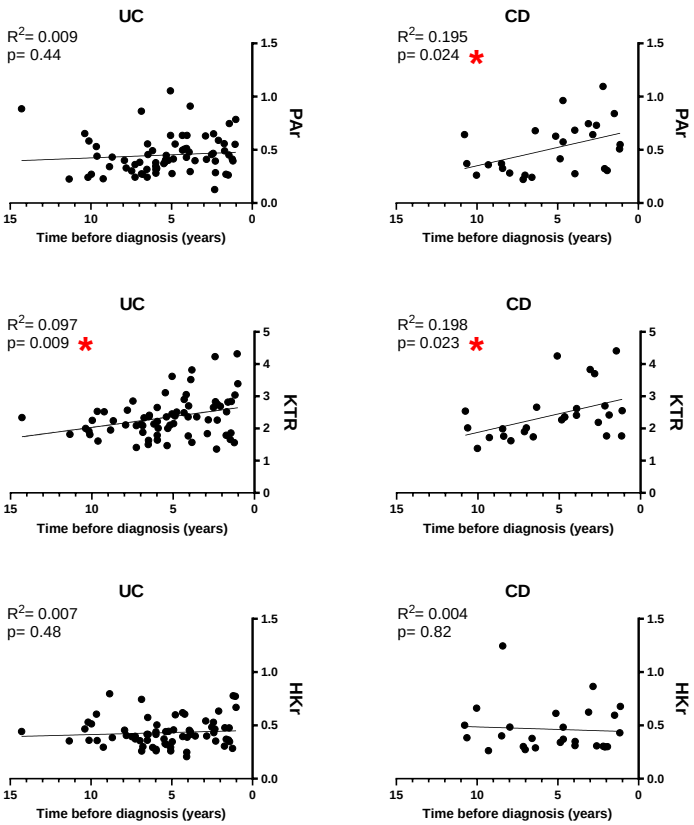

Figure 17. Linear regression, calculated indexes and time to diagnosis.

Both Kynurenine aminotransferases and kynureninase (see Figure 4) require pyridoxal 5'-phosphate, and thus the production of kynurenic acid, xanthurenic acid, anthranilic acid and 3-hydroxyanthranilic acid is vitamin B6 dependent. The HKr is sensitive to functional deficiency in vitamin B6 as it compares 3-hydroxykynurenin levels to the pyridoxal 5'-phosphate dependent metabolites. The higher ratio of HKr in our study indicates a functional vitamin B6 deficiency among individuals later developing IBD compared to controls, supported by a decreased level of pyridoxal 5'-phosphate among incipient cases. The HKr might also be affected by changes in enzyme levels of kynurenine aminotransferases and kynureninase. Serum kynurenine aminotransferase-2 protein levels are reported to be decreased in IBD patients. Still, mRNA expression of kynurenine aminotransferases 1 and 3 is increased in lymphocytes in

IBD patients and kynurenine aminotransferases 2 in UC patients.[89,177] Only kynurenic acid and xanthurenic acid are affected in our study, and this seems to support the notion that kynurenine aminotransferase deficiency may be, at least in part, a cause of the increased HKr.

Two other indexes were calculated, the PAr and the KTR. These are markers of inflammation based on vitamin B6 metabolism and tryptophan metabolism.[161] Neither of these were associated with later development of UC. For CD, the PAr index was associated with a higher odds ratio for developing IBD, see Figure 15. This is consistent with previous publications on these cases showing increased inflammatory markers in the CD but not the UC subgroup.[178] When correlating the indexes to time to diagnosis, both the KTR and PAr were associated with time to diagnosis for CD and the KTR for UC, see Figure 16.

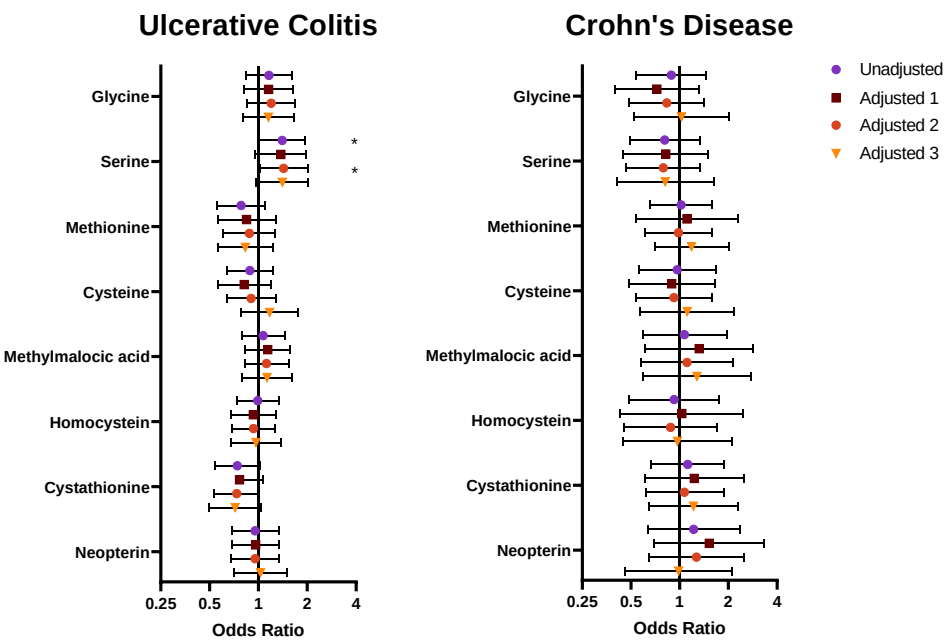

Figure 17. Neopterin and compounds related to the one-carbon metabolism, conditional logistic regression showing odds ratios and 95% confidence interval for developing IBD. Adjustments; Adjusted 1: BMI+Smoking, Adjusted 2: BMI+Cotinine, Adjusted 3: BMI+Cotinine+Albumin+CRP.

Several compounds related to the one-carbon metabolism are shown in Figure 17 in multivariable conditional logistic regression models

assessing the odds ratio for developing IBD. It has been shown that inhibition of hydrogen sulfide synthesis in the transsulfuration pathway (see Figure 18) in rats increased the severity of gastrointestinal mucosal inflammation in TNBS colitis models, whereas hydrogen sulfide donors reduced the severity.[92] Serine was higher among UC cases. De novo biosynthesis of serine and consumption through both the transsulfuration pathway and the folic acid cycle are all pyridoxal 5'-phosphate dependent,[179] and vitamin B6 deficiency has been shown to cause elevation of serine levels in humans.[180,181] Thus, a higher serine among UC cases is consistent with vitamin B6 deficiency.

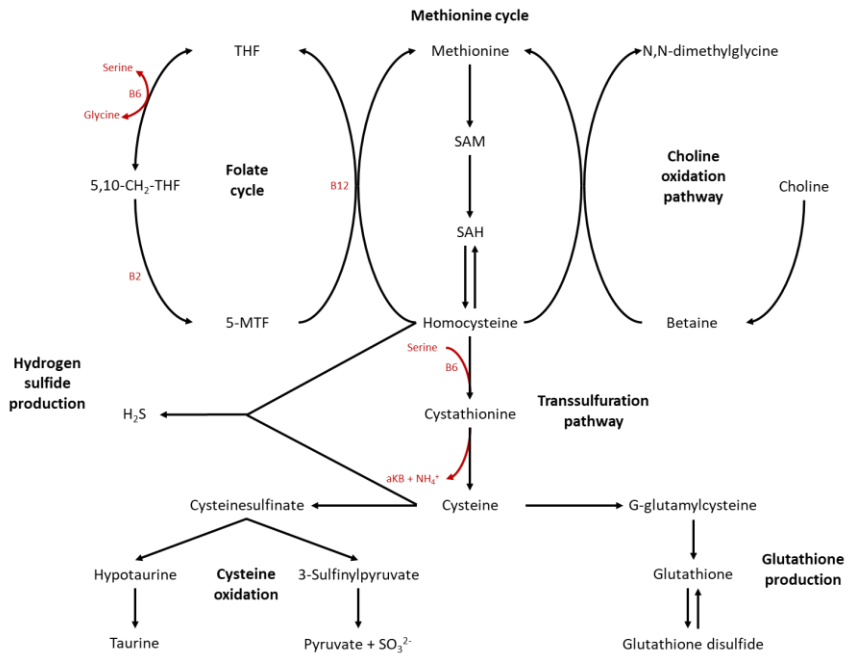

Figure 18. One carbon metabolism, including transsulfuration pathway. THF – tetrahydrofolate. 5-MTF – 5-methyltetrahydrofolate. SAM – S-adenosylmethionine. SAH – S-adenosylhomocysteine. aKB – α-ketobutyrate.

In summary, Paper 4 showed evidence of prediagnostic functional vitamin B6 deficiency among individuals later developing IBD. Lower concentrations of kynurenic acid and xanthurenic acid were also seen, which could result from vitamin B6 deficiency. In UC cases, a higher serine was associated with an increased risk of developing disease, another finding that might result from vitamin B6 deficiency. For CD cases, lower picolinic acid was associated with an increased risk of developing Disease.

Discussion

Main findings

There was an increased risk of developing IBD among individuals with smoking exposure, low fibre intake, high cotinine, low ferritin, iron deficiency among men, vitamin B6 deficiency, low kynurenic acid and low xanthurenic acid. Low picolinic acid was associated with an increased risk for CD only, and high serine was associated with an increased risk for UC. Neither was significant in the entire IBD group.

Most studies on risk factors for IBD are performed on already diagnosed patients. No previous studies have analysed cotinine, iron status, vitamin B6, kynurenic acid, xanthurenic acid, picolinic acid or serine in individuals before IBD diagnosis.

Results contradicting prior findings

Smoking is a previously known risk factor for developing CD. For UC, results are harder to interpret. At diagnosis, current smoking is generally considered protective against UC. However, former smoking is a known risk factor.[27,182] In Papers 1 and 2, never having smoked was associated with a decreased risk of developing UC, and higher cotinine (a marker of current smoking) was associated with increased risk. Previous prediagnostic data have reported an increased risk for UC with ever smoking.[154] The fact that the data is collected before diagnosis gives rise to the possibility that the smoking status of the individual changes from current to former smokers, which is a known risk factor in studies measuring risk at diagnosis. It is impossible to become a former smoker without having been a smoker. The prediagnostic data in Papers 1 and 2 indicate that ever smoking does increase the risk of developing IBD, and the reason that previous studies have not been able to show a correlation between smoking and UC may be related to that collection of data generally occurs at diagnosis.

Role in IBD pathogenesis

The potential role of the identified IBD risk factors in pathogenesis will be discussed below in relation to the mechanisms presented in the introduction. There is a possibility that environmental factors influence

disease development, and disease development may affect the measured lifestyle factors.

Smoking and cotinine

Genetic associations between IBD and smoking

Interactions between single nucleotide polymorphisms (SNP), smoking exposure, and IBD have been shown. In IBD patients, 64 SNPs differentially enriched depending on smoking status were identified. Of these, approximately 45% were close to previously identified IBD-risk SNPs. Examples of genes include NOD2, ATG16L1 and IL23R, genes with prominent functions in barrier function and autophagy.[183]

Interactions with the gut microbiota

Smoking is associated with changes in the gut microbiota. Cigarette smoke contains polycyclic aromatic hydrocarbons, nitrosamines, heavy metals and other compounds that can reach the intestinal lumen, where they may affect the gut microbiota.[184] The compounds have direct antimicrobial effects but may also act indirectly through effects on the innate and adaptive immune system. Studies on gut microbiota alterations related to smoking show conflicting results. Most studies are based on microbiota from faecal samples, and there is a lack of adjustments for confounding factors. General consensus includes a decrease in the diversity of microbial species while smoking and a relatively fast transition of the microbiota composition to normal upon smoking cessation.[185,186] A recent study on mice has shown altered bile acid metabolites related to cigarette smoke effects on the gut microbiota, which could contribute to IBD development by interactions with the farnesoid X receptor.[187] A fast transition of the gut microbiota upon smoking cessation may be a contributory factor in the development of UC, where disease onset commonly follows the cessation of smoking. Our results that ever or current smoking is a risk factor for developing UC do not exclude the possibility that subjects quit smoking prior to disease onset.

Epithelial barrier and innate immunity

The intestinal epithelial barrier is affected by cigarette smoke. Mice exposed to cigarette smoke develop more severe colitis in a TNBS colitis model. In a mouse model with cigarette smoking-induced chronic obstructive pulmonary disease, intestinal barrier dysfunction was

observed with increased permeability and increased cell death.[188] Mucin mRNA levels in mice are altered by cigarette smoke exposure,[189] and effects on paneth cells and antimicrobial peptide production have also been observed in mouse models.[184]

Mice exposed to cigarette smoke do not show alterations in tight junction protein mRNA levels.[189] However, in Caco-2 cell monolayers, tight junction protein expression was increased, and epithelial permeability was decreased following nicotine exposure.[190] This indicates that smoking, but not nicotine exposure, increases the risk for IBD, which is consistent with our findings that smoking, but not snuff use, increases the risk for IBD, even though snuff contains higher nicotine levels. Interestingly, nicotine seems to have a beneficial effect on epithelial tight junctions, which might be relevant in the development of UC, where smoking cessation increases the risk for disease onset.

Adaptive immunity

Exposure to cigarette smoke increased T_H17 cell expression in the lungs in mouse models but not in mesenteric lymph nodes or the colon. After DSS stimulation in cigarette smoke-exposed mice, T_H17 expression was, however, increased in both mesenteric lymph nodes and the colon. An increase in colonic neutrophils, weight loss and colon damage was also seen compared to mice without cigarette smoke exposure. The effects of cigarette smoke exposure were ameliorated in IL17 knockout mice, indicating a T_H17-mediated response. IL17 knockout mice, however, are not resistant to DSS-induced colitis, showing equivalent levels of pathology when exposed to DSS as wild-type mice.[191] This is consistent with anti-IL17 treatment being ineffective as a treatment for IBD.[128]

It cannot be determined whether the observed effects are due to T_H17 cell priming by smoke exposure in the lung or local effects mediated through microbiota changes, epithelial barrier defects or other changes.

Iron deficiency

Interactions with the gut microbiota

Most ingested iron (about 9 mg daily) passes through the small intestine unabsorbed, with only 1-3 mg absorbed in the duodenum. The rest of ingested iron enters the colon in relatively high concentrations (~25 mmol/L), but only a tiny portion (~0.4 mmol/L) is bioavailable due to limited solubility. Most bacteria require iron and have developed

mechanisms of obtaining iron, with some species having more effective methods, giving them a fitness advantage in iron-deficient conditions.

Oral iron supplementation in rodents exacerbates symptoms in colitis models. In infants receiving an iron-fortified diet, higher levels of calprotectin were observed. Other studies in infants and animal models have shown increases in *Enterobacteriaceae,* and some studies specifically in the pathobiont *E.coli* following oral iron supplementation.

On the other hand, low iron intake promotes microbiota changes, with a lower abundance of SCFA-producing species in rats. In vitro studies from human colonic microbiota fermenting chyme media at different iron concentrations have shown that SCFA production is hampered at low iron concentrations. Low iron concentrations also seem to favour *Enterobacteriaceae* overgrowth in mice and rats.

In germ-free mice, iron uptake was significantly decreased, related to reduced expression of iron-uptake proteins. After recolonisation, the ability of iron uptake was improved, indicating that the gut microbiota is important for iron uptake. Iron deficiency causes an increase in iron-uptake-related proteins in the colon of mice, but the significance of this on luminal iron concentrations is not known.

Both extremes of oral iron supplementation and iron restriction can induce IBD-related changes. It is, however, unlikely that such an extreme dietary iron restriction causes the iron deficiency observed in Paper 3. It is also unlikely that the slight iron deficiency observed would cause such an increased iron absorption in the colon that the microbiota would be affected in its composition. As such, the changes in iron status observed were probably not caused by microbiota changes and did probably not cause IBD through gut microbiota alterations.

Epithelial barrier and innate immunity

There is no convincing evidence of a significant impact on the intestinal epithelial barrier in iron deficiency. Regarding innate immunity, several mechanisms have been considered. HIF1α is a transcription factor important for the expression of cytokines and antimicrobial peptide production in macrophages. HIF1α is not degraded in iron deficiency conditions, leading to prolonged proinflammatory functions. Hepcidin is another iron-related protein with effects on the immune system. Hepcidin synthesis is induced in conditions with high iron availability. In hepcidin-deficient mice, inflammatory provocation resulted in

increased serum levels of cytokines, and hepcidin can cause changes in gene expression resulting in suppression of TNFα and IL6.[192]

Innate immune reactions are also described that may cause iron deficiency. Since iron is essential for many pathogenic bacteria, a natural defence mechanism against bacterial infection is to sequester iron, making it inaccessible to the bacteria. Innate immune functions focusing on this include, for example, iron chelation by calprotectin. Lactoferrin is a protein in neutrophils with iron storage and transportation functions. It is present in neutrophils, secreted in the mucosa, and it has antimicrobial properties by binding to the cell wall of the pathogen, causing membrane damage, and it binds iron to limit the iron availability of the pathogens. Another mechanism is cytokine-induced increased iron-uptake from the intestinal lumen and increased egress of iron stored in enterocytes by reduction of their life-span.[193]

Adaptive immunity

The adaptive immune system is affected during iron deficiency. Antibody response after vaccination has been shown to be decreased in iron-deficient patients. Iron deficiency in mice has been shown to skew TH-cell differentiation towards a T_H2 response that has been described as a result of a negative regulatory effect of iron on the T_H1 cytokine INFγ.[193] None of these described changes is directly implicated in known IBD pathogenesis.

Disease-related reasons for iron deficiency

There are also other possible explanations for the observed iron deficiency. These include dietary changes related to IBD symptoms or blood loss related to gastrointestinal inflammation. Since the study was conducted among healthy individuals at least one year before disease onset this is unlikely the explanation, but it cannot be ruled out.

Vitamin B6 and the kynurenine pathway

Interactions with the gut microbiota

All eight B vitamins can be synthesised by the gut microbiota.[90] Vitamin B6 can generally be synthesised by Bacteriodetes, Actinobacteria and Proteobacteria, whereas Firmicutes and Fusobacteria usually do not possess the necessary enzymes.[90] Estimations say that over 85% of the required daily vitamin B6 can be synthesised by the human gut

microbiota.[90] In IBD patients, the proportion of Actinobacteria and Proteobacteria is increased, and the proportion of Bacteroidetes and Firmicutes is decreased compared to healthy individuals.[69] The decreased proportion of Bacteroidetes may thus pose a possible explanation for decreased vitamin B6 concentrations.

Conversely, a low vitamin B6 diet in rats caused a reduction of the Bacteroides genus, with a corresponding reduction in the synthesis of SCFA,[194] and low levels of Bacteroides are associated with IBD,[195] as are low levels of SCFA[196]. Changes in the microbiota can thus cause vitamin B6 deficiency; on the contrary, B6 deficiency can cause changes in the gut microbiota. No differences were observed in dietary vitamin B6, based on survey data. Thus, it is possible that the observed differences in functional vitamin B6 concentrations were, partly at least, caused by changes in the gut microbiota.

Tryptophan metabolites from the kynurenine pathway are, to a great extent, produced by the gut microbiota. These metabolites include kynurenic acid and xanthurenic acid, both lower among cases in Paper 4, and picolinic acid, lower among CD cases in Paper 4.

Production of picolinic acid is dependent on 2-Amino 3-carboxymuconate 6-semialdehyde decarboxylase (ACMSD), which converts 2-Amino 3-carboxymuconate 6-semialdehyde (ACMS) to 2-Aminomuconic acid 6-semialdehyde (AMS). AMS is then nonenzymatically converted to picolinic acid or can enter the citric acid cycle (see Figure 4). ACMSD can be expressed by the gut microbiota.[197] The activity and levels of ACMSD determine the levels of picolinic acid. If ACMS is not converted to AMS, non-enzymatic conversion to quinolinic acid increases. Thus, the decreased levels of picolinic acid in CD cases could be caused by reduced diversion of AMS to the citric acid cycle, ACMSD activity, or decreased bacterial synthesis of picolinic acid.

Innate and adaptive immunity

Lower vitamin B6 has previously been shown to cause changes in the immune system, including a reduction in lymphocyte proliferation and alterations in T-lymphocyte and cytokine profiles.[161] Some of these changes can be attributed to alterations in the transsulfuration pathway or the kynurenine pathway.[161]

Kynurenic acid and xanthurenic acid interact with the AHR,[198] which has been implicated in IBD pathogenesis. AHR ligands in faeces are decreased in IBD patients, and mice treated with AHR ligands have

reduced the severity of DSS-induced colitis.[84,199] Activation of the AHR in the gastrointestinal tract affects survival and maintenance of ILC3s, drives the expression of IL22 in Th-17 cells, and activates intestinal homing in T-regulatory cells. IL22 from ILC3 and Th-17 cells are essential in the maintenance of intestinal epithelial cells and antimicrobial peptide secretion, which are well-described aspects of the pathogenesis of IBD.[200] Previous findings indicate that the kynurenic acid concentrations observed in Paper 4 are sufficient to activate the AHR.[201] There are also many dietary ligands, including flavonoids, glucobrassicins and indoles, as well as indole derivates from the gut microbiota, that can activate the AHR.[202] The relative importance of endogenous compared to exogenous ligands (dietary and microbiota-derived) in the pathogenesis of IBD is, however, unclear.

Picolinic acid, which was lower among CD cases in Paper 4, could also affect immune system functions. It has, for example, been shown to affect macrophages, inducing CCL3 and CCL4 (involved in JAK/STAT and MAPK signalling), inducing NO2-production, and down-regulate CCR5.[203-206]

Genetic factors

Kynurenic acid and xanthurenic acid are ligands to GPR35, which is associated with IBD in GWAS studies. However, recent studies in mouse models have shown kynurenic acid and xanthurenic acid to be protective in DSS colitis and the effect to be dependent on the AHR but independent of GPR35.[89]

In mice, ACMSD, the enzyme required for picolinic acid production, is regulated by the transcription factor HNF4α, which increases ACMSD transcription. HNF4α, in turn, is post-transcriptionally down-regulated by PPRα.[207] HNF4α is a known risk gene for UC but not for CD.[40,45] In intestinal biopsy samples, HNF4α mRNA was reduced in both UC and CD patients compared to controls. In DSS colitis models in mice, HNF4α mRNA levels are decreased.[208,209] PPRα protein levels have been shown to be increased in mouse models of DSS colitis.[210,211] Thus, low picolinic acid may result from low ACMSD activity, which in turn may be related to decreased HNF4α activity (strengthened by increased PPRα), which has been previously related to colitis. This may explain the low levels of picolinic among CD cases, although the lack of association for UC remains unexplained.

Strengths and limitations

There were several limitations in this thesis. One limitation was the absence of specific biomarkers used in general to determine the degree of disease, such as haemoglobin or a complete blood count, or creatinine. Not having available data on haemoglobin when studying iron deficiency leads to difficulty in assessing the potential clinical significance of the deficiency. It also limits the possibility of predicting whether or not the deficiency would have been noticed in a clinical setting. The lack of data on creatinine or cystatin C means no way of adjusting for glomerular filtration rate of the kidneys, which could be relevant since, for some of the measured molecules, concentrations were dependent on this.

Another limitation is that we had no available survey data or biobanked plasma at diagnosis. Therefore it was not possible to register changes in, for example, smoking status between inclusion and diagnosis. Thus the individuals marked as current smokers in our material might have become former smokers at the time of diagnosis, or those that had never smoked might have become smokers.

A further important limitation was the low number of included cases, especially CD cases. A low number of cases leads to lower statistical power, meaning a greater possibility that existing differences in the CD group could not be found. The low number of individuals has made subgroup analysis for UC and CD more difficult to interpret. Therefore, in most analyses, UC and CD were analysed together as IBD. Since there are so many differences between UC and CD, not only in clinical presentation but also regarding environmental risk factors and genetic changes, there is always a risk of losing that important information when analysing them as one group.

The thesis focused on late-onset disease. Since individuals were included at 40, 50 and 60 years of age, the results are not generalisable to the entire population of IBD. This is important since the disease phenotype and risk factors differ significantly depending on the age of onset.

A great strength of included papers is the prediagnostic data allowing a prospective approach. There is a much greater possibility of understanding the causation when analysing prediagnostic data in relation to data at diagnosis. Even though it is possible to collect retrospective data at diagnosis, that approach is more prone to recall bias.

Combining biobanked blood samples and survey data on the same individual is another great strength. It allows for comparison between self-reported data and objectively measured biomarkers in relation to risk for disease development. It is also a great asset to be able to adjust for lifestyle factors such as intake of supplements, tobacco habits, and socioeconomic status. The survey data also included a previously validated detailed food frequency questionnaire with already calculated summary scores of, for example, vitamin B6 intake and fibre intake.

Another strength was the use of biobanked plasma, making it possible to analyse prediagnostic blood samples collected as early as 1985. The blood sampling procedure and handling and storage of samples were highly standardised, generating high-quality plasma samples.

It was also a great strength to be able to match cases to controls based on possible confounding factors such as age and sex, limiting the need for excessive adjustments in statistical models.

Conclusions and future perspectives

In conclusion, prediagnostic changes in late-onset IBD related to the IBD pathophysiology have been identified.

Smoking was a risk factor for IBD, and previous data has shown that it can cause changes in the gut microbiota, interact with the epithelial barrier and affect the adaptive immune system in IBD-promoting ways. Snuff use was not associated with IBD, indicating that nicotine is not the causative agent, supported by previous findings of pathophysiological protective effects of nicotine in the mucosa. Another explanation for the increased risk for IBD for smokers, but not for snuff users, is exposure to the lungs, which can cause changes in the immune system similar to findings in IBD. We also propose cotinine as an objective marker of tobacco exposure which we have identified as a risk factor for developing IBD.

Iron deficiency and lower ferritin increased the risk of developing IBD among men. Pathophysiological processes in IBD are not completely clear regarding the role of iron, with varying effects in different studies and potential immunological consequences and microbiota changes proposed in both low and high iron conditions. The observed iron deficiency may instead be a result of an early disease process, for example, gastrointestinal bleeding or symptom-associated dietary

changes. Iron deficiency was more common in women than in men but was only significantly lower in IBD cases among men. This indicates that other reasons for iron deficiency than IBD are more common among women of this age.

Our data showed functional vitamin B6 deficiency among individuals later developing IBD, supported by lower plasma pyridoxal 5ˈ-phosphate, higher HKr, higher kynurenic and higher xanthurenic acid in IBD cases and higher serine in UC cases. The reason for the functional vitamin B6 deficiency may be related to genetic variations or changes in the gut microbiota but does not seem to be related to dietary changes (based on survey data) or defect absorption (based on previous studies). Effects of lower functional vitamin B6 include direct effects on cytokines and immune system cells and indirect effects related to changes in metabolic pathways. One such pathway is the kynurenine pathway, where our data show lower concentrations of end metabolites closely linked to IBD pathogenesis.

The data on smoking and IBD, especially UC, is clinically important since it changes the fundamental knowledge of smoking in IBD. That smoking is not beneficial is a clear statement that is easily grasped by the public, which may lead to less morbidity with fewer smoking individuals in society. The novel findings of cotinine associated with risk for IBD may lead to a future introduction of cotinine as a marker for tobacco exposure in a clinical setting.

The results from this study confirm some previously proposed pathophysiological processes in IBD by adding information about individuals before the established disease. Consequences related to this increased information can be directly linked to the future development of medications, as it is more likely that the actual pathophysiology is targeted in processes known to be affected before diagnosis.

Funding

This work was supported by: Mag-tarmfonden, The Västerbotten county council, [VLL-678111, VLL-751191, VLL-582981], and Biobank Sweden, supported by the Swedish Research Council, [VR 2017-00650].

Acknowledgements

A lot of people have contributed to making this possible. Thank you to all, and a special thanks to the following people:

Johan Hultdin, my main supervisor, thank you for guiding me through all my first researching years and for always being there to discuss everything that is important in life.

Kim Ekblom, co-supervisor, for all the rewarding conversations and for always taking the time to answer all my questions.

Pontus Karling, co-supervisor, for your engaging energy and enthusiasm and for all your good ideas and new approaches.

Kristina Stefansson, co-supervisor, thank you for always having time for my questions and for making me feel welcome in the clinical chemistry department.

Torbjörn Nilsson, thank you for sharing your experiences and for interesting discussions about anything.

Kjell Granström, for the nice lunch company and for knowing what is important and to not overthink things.

Malin Mickelsson, for making long days interesting, thanks for having time to discuss all the stupid questions and for making sure we get coffee when necessary.

Elisabet Söderström, thank you for your invaluable advice regarding the writing process.

Martin Biström, for stopping by the office just to talk,for supplying me with useful documents and for giving great advice on writing.

David Lundgren, for interesting discussions on statistics and nice company.

A warm thank you to Medicinskt Centrum Södra Lappland for an amazing work environment and support, and thank you to Peter Thorén for support and for flexibility regarding the clinical schedule, allowing me to spend all desired time on this research.

Co-authors Abdulkadir Salih, Jörn Schneede, Adrian McCann, Øivind Midttun, Arve Ulvik and Per Magne Ueland, for valuable discussions and good collaboration.

Terry, Carina, Jennica and Clas, for always being available with answers to all the questions I didn´t even know to ask.

To the clinical laboratory at NUS for help with analyses and for welcoming me to the lab and showing me how everything works. A special thanks to Eva Samuelsson, who coordinated all analyses.

To all patients participating in VIP and MA for making this work possible.

To the staff at the biobank, and a special thanks to Robert Johansson for your help with the data files.

André, you know what you did. Thank you!

References

1. Magro F, Langner C, Driessen A, Ensari A, Geboes K, Mantzaris GJ, et al. European consensus on the histopathology of inflammatory bowel disease. J Crohns Colitis 2013; 7 (10):827-851.
2. Podolsky D, Camilleri M. Yamada's Textbook of Gastroenterology Vol. 1. Chichester: Wiley-Blackwell; 2016.
3. Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. Gut 2006; 55 (6):749-753.
4. Sjoberg D, Holmstrom T, Larsson M, Nielsen AL, Holmquist L, Ekbom A, et al. Incidence and clinical course of Crohn's disease during the first year - results from the IBD Cohort of the Uppsala Region (ICURE) of Sweden 2005-2009. J Crohns Colitis 2014; 8 (3):215-222.
5. Magro F, Portela F, Lago P, Ramos de Deus J, Vieira A, Peixe P, et al. Crohn's disease in a southern European country: Montreal classification and clinical activity. Inflamm Bowel Dis 2009; 15 (9):1343-1350.
6. Gower-Rousseau C, Vasseur F, Fumery M, Savoye G, Salleron J, Dauchet L, et al. Epidemiology of inflammatory bowel diseases: new insights from a French population-based registry (EPIMAD). Dig Liver Dis 2013; 45 (2):89-94.
7. da Silva BC, Lyra AC, Rocha R, Santana GO. Epidemiology, demographic characteristics and prognostic predictors of ulcerative colitis. World J Gastroenterol 2014; 20 (28):9458-9467.
8. Rao SS, Holdsworth CD, Read NW. Symptoms and stool patterns in patients with ulcerative colitis. Gut 1988; 29 (3):342-345.
9. Rogler G, Singh A, Kavanaugh A, Rubin DT. Extraintestinal Manifestations of Inflammatory Bowel Disease: Current Concepts, Treatment, and Implications for Disease Management. Gastroenterology 2021; 161 (4):1118-1132.
10. Rothfuss KS, Stange EF, Herrlinger KR. Extraintestinal manifestations and complications in inflammatory bowel diseases. World J Gastroenterol 2006; 12 (30):4819-4831.
11. Danese S, Semeraro S, Papa A, Roberto I, Scaldaferri F, Fedeli G, et al. Extraintestinal manifestations in inflammatory bowel disease. World J Gastroenterol 2005; 11 (46):7227-7236.
12. Orchard TR, Wordsworth BP, Jewell DP. Peripheral arthropathies in inflammatory bowel disease: their articular distribution and natural history. Gut 1998; 42 (3):387-391.
13. Pardi DS, Kelly CP. Microscopic colitis. Gastroenterology 2011; 140 (4):1155-1165.
14. Kaplan GG. The global burden of IBD: from 2015 to 2025. Nat Rev Gastroenterol Hepatol 2015; 12 (12):720-727.

15. Ng SC, Shi HY, Hamidi N, Underwood FE, Tang W, Benchimol EI, et al. Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. Lancet 2017; 390 (10114):2769-2778.
16. Burisch J, Pedersen N, Čuković-Čavka S, Brinar M, Kaimakliotis I, Duricova D, et al. East-West gradient in the incidence of inflammatory bowel disease in Europe: the ECCO-EpiCom inception cohort. Gut 2014; 63 (4):588-597.
17. Büsch K, Ludvigsson JF, Ekström-Smedby K, Ekbom A, Askling J, Neovius M. Nationwide prevalence of inflammatory bowel disease in Sweden: a population-based register study. Aliment Pharmacol Ther 2014; 39 (1):57-68.
18. Sjoberg D, Holmstrom T, Larsson M, Nielsen AL, Holmquist L, Ekbom A, et al. Incidence and natural history of ulcerative colitis in the Uppsala Region of Sweden 2005-2009 - results from the IBD cohort of the Uppsala Region (ICURE). J Crohns Colitis 2013; 7 (9):e351-357.
19. Afzali A, Katz S. Inflammatory Bowel Disease in the Baby to Baby Boomer: Pediatric and Elderly Onset of IBD. Curr Treat Options Gastroenterol 2018; 16 (3):289-305.
20. Ruel J, Ruane D, Mehandru S, Gower-Rousseau C, Colombel JF. IBD across the age spectrum: is it the same disease? Nat Rev Gastroenterol Hepatol 2014; 11 (2):88-98.
21. Zhao M, Feng R, Ben-Horin S, Zhuang X, Tian Z, Li X, et al. Systematic review with meta-analysis: environmental and dietary differences of inflammatory bowel disease in Eastern and Western populations. Aliment Pharmacol Ther 2022; 55 (3):266-276.
22. Lo CH, Lochhead P, Khalili H, Song M, Tabung FK, Burke KE, et al. Dietary Inflammatory Potential and Risk of Crohn's Disease and Ulcerative Colitis. Gastroenterology 2020; 159 (3):873-883.e871.
23. Ortizo R, Lee SY, Nguyen ET, Jamal MM, Bechtold MM, Nguyen DL. Exposure to oral contraceptives increases the risk for development of inflammatory bowel disease: a meta-analysis of case-controlled and cohort studies. Eur J Gastroenterol Hepatol 2017; 29 (9):1064-1070.
24. Sun W, Han X, Wu S, Yang C. Tonsillectomy and the risk of inflammatory bowel disease: A systematic review and meta-analysis. J Gastroenterol Hepatol 2016; 31 (6):1085-1094.
25. Kaplan GG, Jackson T, Sands BE, Frisch M, Andersson RE, Korzenik J. The risk of developing Crohn's disease after an appendectomy: a meta-analysis. Am J Gastroenterol 2008; 103 (11):2925-2931.
26. Deng P, Wu J. Meta-analysis of the association between appendiceal orifice inflammation and appendectomy and ulcerative colitis. Rev Esp Enferm Dig 2016; 108 (7):401-410.

27. Mahid SS, Minor KS, Soto RE, Hornung CA, Galandiuk S. Smoking and inflammatory bowel disease: A meta-analysis. Mayo Clinic Proceedings 2006; 81 (11):1462-1471.
28. Ludvigsson JF, Neovius M, Hammarström L. Association between IgA deficiency & other autoimmune conditions: a population-based matched cohort study. J Clin Immunol 2014; 34 (4):444-451.
29. Binder V, Orholm M. Familial occurrence and inheritance studies in inflammatory bowel disease. Neth J Med 1996; 48 (2):53-56.
30. Gearry RB, Richardson AK, Frampton CM, Dodgshun AJ, Barclay ML. Population-based cases control study of inflammatory bowel disease risk factors. J Gastroenterol Hepatol 2010; 25 (2):325-333.
31. Gordon H, Blad W, Trier Møller F, Orchard T, Steel A, Trevelyan G, et al. UK IBD Twin Registry: Concordance and Environmental Risk Factors of Twins with IBD. Dig Dis Sci 2022; 67 (6):2444-2450.
32. Chen L, Li J, Yang G. A comparative review of intelectins. Scand J Immunol 2020; 92 (1):e12882.
33. Smillie CS, Biton M, Ordovas-Montanes J, Sullivan KM, Burgin G, Graham DB, et al. Intra- and Inter-cellular Rewiring of the Human Colon during Ulcerative Colitis. Cell 2019; 178 (3):714-730.e722.
34. Ntunzwenimana JC, Boucher G, Paquette J, Gosselin H, Alikashani A, Morin N, et al. Functional screen of inflammatory bowel disease genes reveals key epithelial functions. Genome medicine 2021; 13 (1):181.
35. Mohanan V, Nakata T, Desch AN, Lévesque C, Boroughs A, Guzman G, et al. C1orf106 is a colitis risk gene that regulates stability of epithelial adherens junctions. Science (New York, NY) 2018; 359 (6380):1161-1166.
36. Rana N, Privitera G, Kondolf HC, Bulek K, Lechuga S, De Salvo C, et al. GSDMB is increased in IBD and regulates epithelial restitution/repair independent of pyroptosis. Cell 2022; 185 (2):283-298.e217.
37. Zhang H, Cui Z, Cheng D, Du Y, Guo X, Gao R, et al. RNF186 regulates EFNB1 (ephrin B1)-EPHB2-induced autophagy in the colonic epithelial cells for the maintenance of intestinal homeostasis. Autophagy 2021; 17 (10):3030-3047.
38. Kaya B, Melhem H, Niess JH. GPR35 in Intestinal Diseases: From Risk Gene to Function. Frontiers in immunology 2021; 12:717392.
39. Neves SR, Ram PT, Iyengar R. G protein pathways. Science (New York, NY) 2002; 296 (5573):1636-1639.
40. Barrett JC, Lee JC, Lees CW, Prescott NJ, Anderson CA, Phillips A, et al. Genome-wide association study of ulcerative colitis identifies three new susceptibility loci, including the HNF4A region. Nat Genet 2009; 41 (12):1330-1334.
41. Castro-Dopico T, Dennison TW, Ferdinand JR, Mathews RJ, Fleming A, Clift D, et al. Anti-commensal IgG Drives Intestinal Inflammation and Type 17 Immunity in Ulcerative Colitis. Immunity 2019; 50 (4):1099-1114.e1010.

42. Goyette P, Boucher G, Mallon D, Ellinghaus E, Jostins L, Huang H, et al. High-density mapping of the MHC identifies a shared role for HLA-DRB1*01:03 in inflammatory bowel diseases and heterozygous advantage in ulcerative colitis. Nat Genet 2015; 47 (2):172-179.
43. Bernshtein B, Curato C, Ioannou M, Thaiss CA, Gross-Vered M, Kolesnikov M, et al. IL-23-producing IL-10Rα-deficient gut macrophages elicit an IL-22-driven proinflammatory epithelial cell response. Sci Immunol 2019; 4 (36).
44. Shouval DS, Ouahed J, Biswas A, Goettel JA, Horwitz BH, Klein C, et al. Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. Adv Immunol 2014; 122:177-210.
45. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. Nature 2012; 491 (7422):119-124.
46. Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science (New York, NY) 2006; 314 (5804):1461-1463.
47. Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007; 8 (9):967-974.
48. Jones SA, Jenkins BJ. Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. Nat Rev Immunol 2018; 18 (12):773-789.
49. Nielsen OH, Boye TL, Chakravarti D, Gubatan J. Selective tyrosine kinase 2 inhibitors in inflammatory bowel disease. Trends Pharmacol Sci 2022; 43 (5):424-436.
50. Harrison OJ, Srinivasan N, Pott J, Schiering C, Krausgruber T, Ilott NE, et al. Epithelial-derived IL-18 regulates Th17 cell differentiation and Foxp3$^+$ Treg cell function in the intestine. Mucosal Immunol 2015; 8 (6):1226-1236.
51. Liew FY, Girard JP, Turnquist HR. Interleukin-33 in health and disease. Nat Rev Immunol 2016; 16 (11):676-689.
52. Negroni A, Pierdomenico M, Cucchiara S, Stronati L. NOD2 and inflammation: current insights. J Inflamm Res 2018; 11:49-60.
53. Liu X, Jiang B, Hao H, Liu Z. CARD9 Signaling, Inflammation, and Diseases. Frontiers in immunology 2022; 13:880879.
54. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, et al. Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. Science (New York, NY) 2012; 336 (6086):1314-1317.

55. Hamaoui D, Subtil A. ATG16L1 functions in cell homeostasis beyond autophagy. Febs j 2022; 289 (7):1779-1800.
56. Lassen KG, McKenzie CI, Mari M, Murano T, Begun J, Baxt LA, et al. Genetic Coding Variant in GPR65 Alters Lysosomal pH and Links Lysosomal Dysfunction with Colitis Risk. Immunity 2016; 44 (6):1392-1405.
57. Liu JZ, van Sommeren S, Huang H, Ng SC, Alberts R, Takahashi A, et al. Association analyses identify 38 susceptibility loci for inflammatory bowel disease and highlight shared genetic risk across populations. Nat Genet 2015; 47 (9):979-986.
58. de Lange KM, Moutsianas L, Lee JC, Lamb CA, Luo Y, Kennedy NA, et al. Genome-wide association study implicates immune activation of multiple integrin genes in inflammatory bowel disease. Nat Genet 2017; 49 (2):256-261.
59. Sazonovs A, Stevens CR, Venkataraman GR, Yuan K, Avila B, Abreu MT, et al. Large-scale sequencing identifies multiple genes and rare variants associated with Crohn's disease susceptibility. Nat Genet 2022; 54 (9):1275-1283.
60. Steiner A, Reygaerts T, Pontillo A, Ceccherini I, Moecking J, Moghaddas F, et al. Recessive NLRC4-Autoinflammatory Disease Reveals an Ulcerative Colitis Locus. J Clin Immunol 2022; 42 (2):325-335.
61. Lange K, Buerger M, Stallmach A, Bruns T. Effects of Antibiotics on Gut Microbiota. Digestive diseases (Basel, Switzerland) 2016; 34 (3):260-268.
62. Bibbò S, Ianiro G, Giorgio V, Scaldaferri F, Masucci L, Gasbarrini A, et al. The role of diet on gut microbiota composition. Eur Rev Med Pharmacol Sci 2016; 20 (22):4742-4749.
63. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010; 464 (7285):59-65.
64. Structure, function and diversity of the healthy human microbiome. Nature 2012; 486 (7402):207-214.
65. Walter J, Ley R. The human gut microbiome: ecology and recent evolutionary changes. Annu Rev Microbiol 2011; 65:411-429.
66. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, et al. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 2011; 108 Suppl 1 (Suppl 1):4578-4585.
67. Faith JJ, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, et al. The long-term stability of the human gut microbiota. Science (New York, NY) 2013; 341 (6141):1237439.
68. Ling Z, Liu X, Cheng Y, Yan X, Wu S. Gut microbiota and aging. Critical reviews in food science and nutrition 2022; 62 (13):3509-3534.

69. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007; 104 (34):13780-13785.
70. Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol 2017; 2:17004.
71. Ni J, Wu GD, Albenberg L, Tomov VT. Gut microbiota and IBD: causation or correlation? Nat Rev Gastroenterol Hepatol 2017; 14 (10):573-584.
72. Mirsepasi-Lauridsen HC, Vallance BA, Krogfelt KA, Petersen AM. Escherichia coli Pathobionts Associated with Inflammatory Bowel Disease. Clin Microbiol Rev 2019; 32 (2).
73. Zmora N, Suez J, Elinav E. You are what you eat: diet, health and the gut microbiota. Nat Rev Gastroenterol Hepatol 2019; 16 (1):35-56.
74. Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 2016; 165 (6):1332-1345.
75. Kelley N, Jeltema D, Duan Y, He Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. Int J Mol Sci 2019; 20 (13).
76. Ianiro G, Tilg H, Gasbarrini A. Antibiotics as deep modulators of gut microbiota: between good and evil. Gut 2016; 65 (11):1906-1915.
77. Strati F, Pujolassos M, Burrello C, Giuffrè MR, Lattanzi G, Caprioli F, et al. Antibiotic-associated dysbiosis affects the ability of the gut microbiota to control intestinal inflammation upon fecal microbiota transplantation in experimental colitis models. Microbiome 2021; 9 (1):39.
78. Galazzo G, van Best N, Bervoets L, Dapaah IO, Savelkoul PH, Hornef MW, et al. Development of the Microbiota and Associations With Birth Mode, Diet, and Atopic Disorders in a Longitudinal Analysis of Stool Samples, Collected From Infancy Through Early Childhood. Gastroenterology 2020; 158 (6):1584-1596.
79. Cai S, Fan Y, Zhang B, Lin J, Yang X, Liu Y, et al. Appendectomy Is Associated With Alteration of Human Gut Bacterial and Fungal Communities. Front Microbiol 2021; 12:724980.
80. Angelakis E, Bachar D, Yasir M, Musso D, Djossou F, Gaborit B, et al. Treponema species enrich the gut microbiota of traditional rural populations but are absent from urban individuals. New Microbes New Infect 2019; 27:14-21.
81. Petnicki-Ocwieja T, Hrncir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, et al. Nod2 is required for the regulation of commensal microbiota in the intestine. Proc Natl Acad Sci U S A 2009; 106 (37):15813-15818.

82. Cohen LJ, Cho JH, Gevers D, Chu H. Genetic Factors and the Intestinal Microbiome Guide Development of Microbe-Based Therapies for Inflammatory Bowel Diseases. Gastroenterology 2019; 156 (8):2174-2189.
83. Liu H, Gao P, Jia B, Lu N, Zhu B, Zhang F. IBD-Associated Atg16L1T300A Polymorphism Regulates Commensal Microbiota of the Intestine. Frontiers in immunology 2021; 12:772189.
84. Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, et al. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. Nature medicine 2016; 22 (6):598-605.
85. Gadaleta RM, van Erpecum KJ, Oldenburg B, Willemsen EC, Renooij W, Murzilli S, et al. Farnesoid X receptor activation inhibits inflammation and preserves the intestinal barrier in inflammatory bowel disease. Gut 2011; 60 (4):463-472.
86. Wang YD, Chen WD, Wang M, Yu D, Forman BM, Huang W. Farnesoid X receptor antagonizes nuclear factor kappaB in hepatic inflammatory response. Hepatology (Baltimore, Md) 2008; 48 (5):1632-1643.
87. Larigot L, Benoit L, Koual M, Tomkiewicz C, Barouki R, Coumoul X. Aryl Hydrocarbon Receptor and Its Diverse Ligands and Functions: An Exposome Receptor. Annu Rev Pharmacol Toxicol 2022; 62:383-404.
88. Sun M, Ma N, He T, Johnston LJ, Ma X. Tryptophan (Trp) modulates gut homeostasis via aryl hydrocarbon receptor (AhR). Critical reviews in food science and nutrition 2020; 60 (10):1760-1768.
89. Michaudel C, Danne C, Agus A, Magniez A, Aucouturier A, Spatz M, et al. Rewiring the altered tryptophan metabolism as a novel therapeutic strategy in inflammatory bowel diseases. Gut 2022.
90. Magnúsdóttir S, Ravcheev D, de Crécy-Lagard V, Thiele I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front Genet 2015; 6:148.
91. Bryan PF, Karla C, Edgar Alejandro MT, Sara Elva EP, Gemma F, Luz C. Sphingolipids as Mediators in the Crosstalk between Microbiota and Intestinal Cells: Implications for Inflammatory Bowel Disease. Mediators of inflammation 2016; 2016:9890141.
92. Wallace JL, Vong L, McKnight W, Dicay M, Martin GR. Endogenous and exogenous hydrogen sulfide promotes resolution of colitis in rats. Gastroenterology 2009; 137 (2):569-578, 578.e561.
93. Liverani E, Scaioli E, Cardamone C, Dal Monte P, Belluzzi A. Mycobacterium avium subspecies paratuberculosis in the etiology of Crohn's disease, cause or epiphenomenon? World J Gastroenterol 2014; 20 (36):13060-13070.
94. Nadalian B, Yadegar A, Houri H, Olfatifar M, Shahrokh S, Asadzadeh Aghdaei H, et al. Prevalence of the pathobiont adherent-invasive

Escherichia coli and inflammatory bowel disease: a systematic review and meta-analysis. J Gastroenterol Hepatol 2021; 36 (4):852-863.
95. Castaño-Rodríguez N, Kaakoush NO, Lee WS, Mitchell HM. Dual role of Helicobacter and Campylobacter species in IBD: a systematic review and meta-analysis. Gut 2017; 66 (2):235-249.
96. Palmela C, Chevarin C, Xu Z, Torres J, Sevrin G, Hirten R, et al. Adherent-invasive Escherichia coli in inflammatory bowel disease. Gut 2018; 67 (3):574-587.
97. Boleij A, Fathi P, Dalton W, Park B, Wu X, Huso D, et al. G-protein coupled receptor 35 (GPR35) regulates the colonic epithelial cell response to enterotoxigenic Bacteroides fragilis. Commun Biol 2021; 4 (1):585.
98. Kaakoush NO, Mitchell HM, Man SM. Role of emerging Campylobacter species in inflammatory bowel diseases. Inflamm Bowel Dis 2014; 20 (11):2189-2197.
99. Underhill DM, Braun J. Fungal microbiome in inflammatory bowel disease: a critical assessment. J Clin Invest 2022; 132 (5).
100. Doron I, Mesko M, Li XV, Kusakabe T, Leonardi I, Shaw DG, et al. Mycobiota-induced IgA antibodies regulate fungal commensalism in the gut and are dysregulated in Crohn's disease. Nat Microbiol 2021; 6 (12):1493-1504.
101. Norman JM, Handley SA, Baldridge MT, Droit L, Liu CY, Keller BC, et al. Disease-specific alterations in the enteric virome in inflammatory bowel disease. Cell 2015; 160 (3):447-460.
102. Lee JY, Wasinger VC, Yau YY, Chuang E, Yajnik V, Leong RW. Molecular Pathophysiology of Epithelial Barrier Dysfunction in Inflammatory Bowel Diseases. Proteomes 2018; 6 (2).
103. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 2009; 9 (11):799-809.
104. Blair SA, Kane SV, Clayburgh DR, Turner JR. Epithelial myosin light chain kinase expression and activity are upregulated in inflammatory bowel disease. Lab Invest 2006; 86 (2):191-201.
105. Chang J, Leong RW, Wasinger VC, Ip M, Yang M, Phan TG. Impaired Intestinal Permeability Contributes to Ongoing Bowel Symptoms in Patients With Inflammatory Bowel Disease and Mucosal Healing. Gastroenterology 2017; 153 (3):723-731.e721.
106. Neurath MF, Travis SP. Mucosal healing in inflammatory bowel diseases: a systematic review. Gut 2012; 61 (11):1619-1635.
107. Turner D, Ricciuto A, Lewis A, D'Amico F, Dhaliwal J, Griffiths AM, et al. STRIDE-II: An Update on the Selecting Therapeutic Targets in Inflammatory Bowel Disease (STRIDE) Initiative of the International Organization for the Study of IBD (IOIBD): Determining Therapeutic Goals for Treat-to-Target strategies in IBD. Gastroenterology 2021; 160 (5):1570-1583.

108. Pickert G, Neufert C, Leppkes M, Zheng Y, Wittkopf N, Warntjen M, et al. STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med 2009; 206 (7):1465-1472.
109. Sommer K, Wiendl M, Müller TM, Heidbreder K, Voskens C, Neurath MF, et al. Intestinal Mucosal Wound Healing and Barrier Integrity in IBD-Crosstalk and Trafficking of Cellular Players. Front Med (Lausanne) 2021; 8:643973.
110. Melhem H, Regan-Komito D, Niess JH. Mucins Dynamics in Physiological and Pathological Conditions. Int J Mol Sci 2021; 22 (24).
111. Gubatan J, Holman DR, Puntasecca CJ, Polevoi D, Rubin SJ, Rogalla S. Antimicrobial peptides and the gut microbiome in inflammatory bowel disease. World J Gastroenterol 2021; 27 (43):7402-7422.
112. Ho S, Pothoulakis C, Koon HW. Antimicrobial peptides and colitis. Curr Pharm Des 2013; 19 (1):40-47.
113. Rubio-Tomás T, Sotiriou A, Tavernarakis N. The interplay between selective types of (macro)autophagy: Mitophagy and xenophagy. Int Rev Cell Mol Biol 2023; 374:129-157.
114. Larabi A, Barnich N, Nguyen HTT. New insights into the interplay between autophagy, gut microbiota and inflammatory responses in IBD. Autophagy 2020; 16 (1):38-51.
115. Lapaquette P, Glasser AL, Huett A, Xavier RJ, Darfeuille-Michaud A. Crohn's disease-associated adherent-invasive E. coli are selectively favoured by impaired autophagy to replicate intracellularly. Cell Microbiol 2010; 12 (1):99-113.
116. Nighot PK, Hu CA, Ma TY. Autophagy enhances intestinal epithelial tight junction barrier function by targeting claudin-2 protein degradation. The Journal of biological chemistry 2015; 290 (11):7234-7246.
117. Spalinger MR, Lang S, Gottier C, Dai X, Rawlings DJ, Chan AC, et al. PTPN22 regulates NLRP3-mediated IL1B secretion in an autophagy-dependent manner. Autophagy 2017; 13 (9):1590-1601.
118. Kabat AM, Harrison OJ, Riffelmacher T, Moghaddam AE, Pearson CF, Laing A, et al. The autophagy gene Atg16l1 differentially regulates Treg and TH2 cells to control intestinal inflammation. Elife 2016; 5:e12444.
119. Cario E. Toll-like receptors in inflammatory bowel diseases: a decade later. Inflamm Bowel Dis 2010; 16 (9):1583-1597.
120. Cario E. Bacterial interactions with cells of the intestinal mucosa: Toll-like receptors and NOD2. Gut 2005; 54 (8):1182-1193.
121. Cario E, Podolsky DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infect Immun 2000; 68 (12):7010-7017.

122. Goethel A, Croitoru K, Philpott DJ. The interplay between microbes and the immune response in inflammatory bowel disease. J Physiol 2018; 596 (17):3869-3882.
123. Saez A, Gomez-Bris R, Herrero-Fernandez B, Mingorance C, Rius C, Gonzalez-Granado JM. Innate Lymphoid Cells in Intestinal Homeostasis and Inflammatory Bowel Disease. Int J Mol Sci 2021; 22 (14).
124. Gomez-Bris R, Saez A, Herrero-Fernandez B, Rius C, Sanchez-Martinez H, Gonzalez-Granado JM. CD4 T-Cell Subsets and the Pathophysiology of Inflammatory Bowel Disease. Int J Mol Sci 2023; 24 (3).
125. Al-Sadi R, Guo S, Ye D, Rawat M, Ma TY. TNF-α Modulation of Intestinal Tight Junction Permeability Is Mediated by NIK/IKK-α Axis Activation of the Canonical NF-κB Pathway. Am J Pathol 2016; 186 (5):1151-1165.
126. Mozaffari S, Nikfar S, Abdolghaffari AH, Abdollahi M. New biologic therapeutics for ulcerative colitis and Crohn's disease. Expert Opin Biol Ther 2014; 14 (5):583-600.
127. Schmitt H, Neurath MF, Atreya R. Role of the IL23/IL17 Pathway in Crohn's Disease. Frontiers in immunology 2021; 12:622934.
128. McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 Family of Cytokines in Health and Disease. Immunity 2019; 50 (4):892-906.
129. Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature 2008; 453 (7191):106-109.
130. Keir M, Yi Y, Lu T, Ghilardi N. The role of IL-22 in intestinal health and disease. J Exp Med 2020; 217 (3):e20192195.
131. Akagbosu B, Tayyebi Z, Shibu G, Paucar Iza YA, Deep D, Parisotto YF, et al. Novel antigen-presenting cell imparts T(reg)-dependent tolerance to gut microbiota. Nature 2022; 610 (7933):752-760.
132. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 2008; 28 (4):546-558.
133. Powrie F, Leach MW, Mauze S, Menon S, Caddle LB, Coffman RL. Inhibition of Th1 responses prevents inflammatory bowel disease in scid mice reconstituted with CD45RBhi CD4+ T cells. Immunity 1994; 1 (7):553-562.
134. Clough JN, Omer OS, Tasker S, Lord GM, Irving PM. Regulatory T-cell therapy in Crohn's disease: challenges and advances. Gut 2020; 69 (5):942-952.
135. Hepworth MR, Fung TC, Masur SH, Kelsen JR, McConnell FM, Dubrot J, et al. Immune tolerance. Group 3 innate lymphoid cells mediate intestinal selection of commensal bacteria-specific $CD4^+$ T cells. Science (New York, NY) 2015; 348 (6238):1031-1035.

136. Castro-Dopico T, Colombel JF, Mehandru S. Targeting B cells for inflammatory bowel disease treatment: back to the future. Current opinion in pharmacology 2020; 55:90-98.
137. Casalegno Garduño R, Däbritz J. New Insights on CD8(+) T Cells in Inflammatory Bowel Disease and Therapeutic Approaches. Frontiers in immunology 2021; 12:738762.
138. Gerlach K, McKenzie AN, Neurath MF, Weigmann B. IL-9 regulates intestinal barrier function in experimental T cell-mediated colitis. Tissue Barriers 2015; 3 (1-2):e983777.
139. Nalleweg N, Chiriac MT, Podstawa E, Lehmann C, Rau TT, Atreya R, et al. IL-9 and its receptor are predominantly involved in the pathogenesis of UC. Gut 2015; 64 (5):743-755.
140. Baba N, Rubio M, Kenins L, Regairaz C, Woisetschlager M, Carballido JM, et al. The aryl hydrocarbon receptor (AhR) ligand VAF347 selectively acts on monocytes and naïve CD4(+) Th cells to promote the development of IL-22-secreting Th cells. Hum Immunol 2012; 73 (8):795-800.
141. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine 2015; 74 (1):5-17.
142. Spiceland CM, Lodhia N. Endoscopy in inflammatory bowel disease: Role in diagnosis, management, and treatment. World J Gastroenterol 2018; 24 (35):4014-4020.
143. Lee JM, Lee KM. Endoscopic Diagnosis and Differentiation of Inflammatory Bowel Disease. Clin Endosc 2016; 49 (4):370-375.
144. Neri B, Mossa M, Scucchi L, Sena G, Palmieri G, Biancone L. Histological scores in inflammatory bowel disease. J Dig Dis 2021; 22 (1):9-22.
145. Guglielmo FF, Anupindi SA, Fletcher JG, Al-Hawary MM, Dillman JR, Grand DJ, et al. Small Bowel Crohn Disease at CT and MR Enterography: Imaging Atlas and Glossary of Terms. Radiographics : a review publication of the Radiological Society of North America, Inc 2020; 40 (2):354-375.
146. Liu D, Saikam V, Skrada KA, Merlin D, Iyer SS. Inflammatory bowel disease biomarkers. Med Res Rev 2022; 42 (5):1856-1887.
147. Zygiel EM, Nolan EM. Transition Metal Sequestration by the Host-Defense Protein Calprotectin. Annu Rev Biochem 2018; 87:621-643.
148. Hatsukami DK, Hecht SS, Hennrikus DJ, Joseph AM, Pentel PR. Biomarkers of tobacco exposure or harm: application to clinical and epidemiological studies. 25-26 October 2001, Minneapolis, Minnesota. Nicotine Tob Res 2003; 5 (3):387-396.
149. Wall MA, Johnson J, Jacob P, Benowitz NL. Cotinine in the serum, saliva, and urine of nonsmokers, passive smokers, and active smokers. Am J Public Health 1988; 78 (6):699-701.

150. Yuki D, Kikuchi A, Miura N, Kakehi A, Onozawa M. Good relationship between saliva cotinine kinetics and plasma cotinine kinetics after smoking one cigarette. Regulatory toxicology and pharmacology : RTP 2013; 67 (2):240-245.
151. Perez-Stable EJ, Benowitz NL, Marin G. Is serum cotinine a better measure of cigarette smoking than self-report? Prev Med 1995; 24 (2):171-179.
152. Kim S. Overview of Cotinine Cutoff Values for Smoking Status Classification. Int J Environ Res Public Health 2016; 13 (12).
153. Ebbert JO, Dale LC, Nirelli LM, Schroeder DR, Moyer TP, Hurt RD. Cotinine as a biomarker of systemic nicotine exposure in spit tobacco users. Addictive behaviors 2004; 29 (2):349-355.
154. Carlens C, Hergens MP, Grunewald J, Ekbom A, Eklund A, Höglund CO, et al. Smoking, use of moist snuff, and risk of chronic inflammatory diseases. Am J Respir Crit Care Med 2010; 181 (11):1217-1222.
155. Kim NH, Jung YS, Yang HJ, Park SK, Park JH, Park DI, et al. Association Between Cotinine-verified Smoking Status and Risk of Colorectal Neoplasia. J Clin Gastroenterol 2019; 53 (3):e107-e112.
156. Yilmaz B, Li H. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. Pharmaceuticals (Basel) 2018; 11 (4).
157. Bager P, Befrits R, Wikman O, Lindgren S, Moum B, Hjortswang H, et al. High burden of iron deficiency and different types of anemia in inflammatory bowel disease outpatients in Scandinavia: a longitudinal 2-year follow-up study. Scand J Gastroenterol 2013; 48 (11):1286-1293.
158. Herrera-deGuise C, Casellas F, Robles V, Navarro E, Borruel N. Iron Deficiency in the Absence of Anemia Impairs the Perception of Health-Related Quality of Life of Patients with Inflammatory Bowel Disease. Inflamm Bowel Dis 2016; 22 (6):1450-1455.
159. Lucendo AJ, Arias Á, Roncero Ó, Hervías D, Verdejo C, Naveas-Polo C, et al. Anemia at the time of diagnosis of inflammatory bowel disease: Prevalence and associated factors in adolescent and adult patients. Dig Liver Dis 2017; 49 (4):405-411.
160. Hanna M, Jaqua E, Nguyen V, Clay J. B Vitamins: Functions and Uses in Medicine. Perm J 2022; 26 (2):89-97.
161. Ueland PM, McCann A, Midttun O, Ulvik A. Inflammation, vitamin B6 and related pathways. Molecular aspects of medicine 2017; 53:10-27.
162. Yakut M, Ustün Y, Kabaçam G, Soykan I. Serum vitamin B12 and folate status in patients with inflammatory bowel diseases. European journal of internal medicine 2010; 21 (4):320-323.
163. Hwang C, Ross V, Mahadevan U. Micronutrient deficiencies in inflammatory bowel disease: from A to zinc. Inflamm Bowel Dis 2012; 18 (10):1961-1981.
164. MacMaster MJ, Damianopoulou S, Thomson C, Talwar D, Stefanowicz F, Catchpole A, et al. A prospective analysis of micronutrient

status in quiescent inflammatory bowel disease. Clin Nutr 2021; 40 (1):327-331.
165. Saibeni S, Cattaneo M, Vecchi M, Zighetti ML, Lecchi A, Lombardi R, et al. Low vitamin B(6) plasma levels, a risk factor for thrombosis, in inflammatory bowel disease: role of inflammation and correlation with acute phase reactants. Am J Gastroenterol 2003; 98 (1):112-117.
166. Nikolaus S, Schulte B, Al-Massad N, Thieme F, Schulte DM, Bethge J, et al. Increased Tryptophan Metabolism Is Associated With Activity of Inflammatory Bowel Diseases. Gastroenterology 2017; 153 (6):1504-1516.e1502.
167. Ulvik A, Midttun Ø, McCann A, Meyer K, Tell G, Nygård O, et al. Tryptophan catabolites as metabolic markers of vitamin B-6 status evaluated in cohorts of healthy adults and cardiovascular patients. The American journal of clinical nutrition 2020; 111 (1):178-186.
168. Norberg M, Wall S, Boman K, Weinehall L. The Västerbotten Intervention Programme: background, design and implications. Glob Health Action 2010; 3.
169. Midttun Ø, Hustad S, Ueland PM. Quantitative profiling of biomarkers related to B-vitamin status, tryptophan metabolism and inflammation in human plasma by liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom 2009; 23 (9):1371-1379.
170. Midttun Ø, McCann A, Aarseth O, Krokeide M, Kvalheim G, Meyer K, et al. Combined Measurement of 6 Fat-Soluble Vitamins and 26 Water-Soluble Functional Vitamin Markers and Amino Acids in 50 μL of Serum or Plasma by High-Throughput Mass Spectrometry. Analytical chemistry 2016; 88 (21):10427-10436.
171. Kelleher BP, Broin SD. Microbiological assay for vitamin B12 performed in 96-well microtitre plates. J Clin Pathol 1991; 44 (7):592-595.
172. Molloy AM, Scott JM. Microbiological assay for serum, plasma, and red cell folate using cryopreserved, microtiter plate method. Methods Enzymol 1997; 281:43-53.
173. Kelleher BP, Scott JM, O'Broin SD. Use of beta-lactamase to hydrolyse interfering antibiotics in vitamin B12 microbiological assay using Lactobacillus leichmannii. Clin Lab Haematol 1990; 12 (1):87-95.
174. Bhagavathula AS, Clark CCT, Rahmani J, Chattu VK. Impact of Body Mass Index on the Development of Inflammatory Bowel Disease: A Systematic Review and Dose-Response Analysis of 15.6 Million Participants. Healthcare (Basel) 2021; 9 (1).
175. Pan Y, Liu Y, Guo H, Jabir MS, Liu X, Cui W, et al. Associations between Folate and Vitamin B12 Levels and Inflammatory Bowel Disease: A Meta-Analysis. Nutrients 2017; 9 (4).

176. Vagianos K, Bector S, McConnell J, Bernstein CN. Nutrition assessment of patients with inflammatory bowel disease. JPEN J Parenter Enteral Nutr 2007; 31 (4):311-319.
177. Dudzińska E, Szymona K, Kloc R, Gil-Kulik P, Kocki T, Świstowska M, et al. Increased expression of kynurenine aminotransferases mRNA in lymphocytes of patients with inflammatory bowel disease. Therap Adv Gastroenterol 2019; 12:1756284819881304.
178. Lundgren D, Widbom L, Hultdin J, Karling P. Preclinical Markers in Inflammatory Bowel Disease. A Nested Case-Control Study. Crohns & Colitis 360 2021; 3 (4).
179. Ramos RJ, Pras-Raves ML, Gerrits J, van der Ham M, Willemsen M, Prinsen H, et al. Vitamin B6 is essential for serine de novo biosynthesis. J Inherit Metab Dis 2017; 40 (6):883-891.
180. da Silva VR, Rios-Avila L, Lamers Y, Ralat MA, Midttun Ø, Quinlivan EP, et al. Metabolite profile analysis reveals functional effects of 28-day vitamin B-6 restriction on one-carbon metabolism and tryptophan catabolic pathways in healthy men and women. J Nutr 2013; 143 (11):1719-1727.
181. Park YK, Linkswiler H. Effect of vitamin B6 depletion in adult man on the plasma concentration and the urinary excretion of free amino acids. J Nutr 1971; 101 (2):185-191.
182. Calkins BM. A meta-analysis of the role of smoking in inflammatory bowel disease. Dig Dis Sci 1989; 34 (12):1841-1854.
183. Yadav P, Ellinghaus D, Remy G, Freitag-Wolf S, Cesaro A, Degenhardt F, et al. Genetic Factors Interact With Tobacco Smoke to Modify Risk for Inflammatory Bowel Disease in Humans and Mice. Gastroenterology 2017; 153 (2):550-565.
184. Berkowitz L, Pardo-Roa C, Salazar GA, Salazar-Echegarai F, Miranda JP, Ramírez G, et al. Mucosal Exposure to Cigarette Components Induces Intestinal Inflammation and Alters Antimicrobial Response in Mice. Frontiers in immunology 2019; 10:2289.
185. Antinozzi M, Giffi M, Sini N, Gallè F, Valeriani F, De Vito C, et al. Cigarette Smoking and Human Gut Microbiota in Healthy Adults: A Systematic Review. Biomedicines 2022; 10 (2).
186. Biedermann L, Zeitz J, Mwinyi J, Sutter-Minder E, Rehman A, Ott SJ, et al. Smoking cessation induces profound changes in the composition of the intestinal microbiota in humans. PloS one 2013; 8 (3):e59260.
187. Bai X, Wei H, Liu W, Coker OO, Gou H, Liu C, et al. Cigarette smoke promotes colorectal cancer through modulation of gut microbiota and related metabolites. Gut 2022; 71 (12):2439-2450.
188. Fricker M, Goggins BJ, Mateer S, Jones B, Kim RY, Gellatly SL, et al. Chronic cigarette smoke exposure induces systemic hypoxia that drives intestinal dysfunction. JCI insight 2018; 3 (3).

189. Allais L, Kerckhof FM, Verschuere S, Bracke KR, De Smet R, Laukens D, et al. Chronic cigarette smoke exposure induces microbial and inflammatory shifts and mucin changes in the murine gut. Environ Microbiol 2016; 18 (5):1352-1363.
190. McGilligan VE, Wallace JM, Heavey PM, Ridley DL, Rowland IR. The effect of nicotine in vitro on the integrity of tight junctions in Caco-2 cell monolayers. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association 2007; 45 (9):1593-1598.
191. Kim M, Gu B, Madison MC, Song HW, Norwood K, Hill AA, et al. Cigarette Smoke Induces Intestinal Inflammation via a Th17 Cell-Neutrophil Axis. Frontiers in immunology 2019; 10:75.
192. Cherayil BJ. Iron and immunity: immunological consequences of iron deficiency and overload. Arch Immunol Ther Exp (Warsz) 2010; 58 (6):407-415.
193. Haschka D, Hoffmann A, Weiss G. Iron in immune cell function and host defense. Semin Cell Dev Biol 2021; 115:27-36.
194. Mayengbam S, Chleilat F, Reimer RA. Dietary Vitamin B6 Deficiency Impairs Gut Microbiota and Host and Microbial Metabolites in Rats. Biomedicines 2020; 8 (11).
195. Zhou Y, Zhi F. Lower Level of Bacteroides in the Gut Microbiota Is Associated with Inflammatory Bowel Disease: A Meta-Analysis. Biomed Res Int 2016; 2016:5828959.
196. Zhang Z, Zhang H, Chen T, Shi L, Wang D, Tang D. Regulatory role of short-chain fatty acids in inflammatory bowel disease. Cell Commun Signal 2022; 20 (1):64.
197. Pucci L, Perozzi S, Cimadamore F, Orsomando G, Raffaelli N. Tissue expression and biochemical characterization of human 2-amino 3-carboxymuconate 6-semialdehyde decarboxylase, a key enzyme in tryptophan catabolism. Febs j 2007; 274 (3):827-840.
198. Wirthgen E, Hoeflich A, Rebl A, Günther J. Kynurenic Acid: The Janus-Faced Role of an Immunomodulatory Tryptophan Metabolite and Its Link to Pathological Conditions. Frontiers in immunology 2017; 8:1957.
199. Abron JD, Singh NP, Mishra MK, Price RL, Nagarkatti M, Nagarkatti PS, et al. An endogenous aryl hydrocarbon receptor ligand, ITE, induces regulatory T cells and ameliorates experimental colitis. Am J Physiol Gastrointest Liver Physiol 2018; 315 (2):G220-g230.
200. Stockinger B, Shah K, Wincent E. AHR in the intestinal microenvironment: safeguarding barrier function. Nat Rev Gastroenterol Hepatol 2021; 18 (8):559-570.
201. DiNatale BC, Murray IA, Schroeder JC, Flaveny CA, Lahoti TS, Laurenzana EM, et al. Kynurenic acid is a potent endogenous aryl

hydrocarbon receptor ligand that synergistically induces interleukin-6 in the presence of inflammatory signaling. Toxicol Sci 2010; 115 (1):89-97.
202. Murray IA, Perdew GH. How Ah Receptor Ligand Specificity Became Important in Understanding Its Physiological Function. Int J Mol Sci 2020; 21 (24).
203. Bosco MC, Rapisarda A, Massazza S, Melillo G, Young H, Varesio L. The tryptophan catabolite picolinic acid selectively induces the chemokines macrophage inflammatory protein-1 alpha and -1 beta in macrophages. J Immunol 2000; 164 (6):3283-3291.
204. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol 2004; 36 (10):1882-1886.
205. Bosco MC, Rapisarda A, Reffo G, Massazza S, Pastorino S, Varesio L. Macrophage activating properties of the tryptophan catabolite picolinic acid. Advances in experimental medicine and biology 2003; 527:55-65.
206. Melillo G, Cox GW, Radzioch D, Varesio L. Picolinic acid, a catabolite of L-tryptophan, is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages. J Immunol 1993; 150 (9):4031-4040.
207. Shin M, Kim I, Inoue Y, Kimura S, Gonzalez FJ. Regulation of mouse hepatic alpha-amino-beta-carboxymuconate-epsilon-semialdehyde decarboxylase, a key enzyme in the tryptophan-nicotinamide adenine dinucleotide pathway, by hepatocyte nuclear factor 4alpha and peroxisome proliferator-activated receptor alpha. Mol Pharmacol 2006; 70 (4):1281-1290.
208. Ahn SH, Shah YM, Inoue J, Morimura K, Kim I, Yim S, et al. Hepatocyte nuclear factor 4alpha in the intestinal epithelial cells protects against inflammatory bowel disease. Inflamm Bowel Dis 2008; 14 (7):908-920.
209. Chahar S, Gandhi V, Yu S, Desai K, Cowper-Sal-lari R, Kim Y, et al. Chromatin profiling reveals regulatory network shifts and a protective role for hepatocyte nuclear factor 4α during colitis. Mol Cell Biol 2014; 34 (17):3291-3304.
210. Wang L, Xie H, Xu L, Liao Q, Wan S, Yu Z, et al. rSj16 Protects against DSS-Induced Colitis by Inhibiting the PPAR-α Signaling Pathway. Theranostics 2017; 7 (14):3446-3460.
211. Zhou X, Cao L, Jiang C, Xie Y, Cheng X, Krausz KW, et al. PPARα-UGT axis activation represses intestinal FXR-FGF15 feedback signalling and exacerbates experimental colitis. Nat Commun 2014; 5:4573.