Metabolomic features and viral infections in paediatric inflammatory bowel disease

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Abstract

Background: Up to 25% of patients with inflammatory bowel disease have a paediatric onset (PIBD). The pathophysiological processes underlying PIBD are complex and largely unknown.

Aims: To investigate a hypothesized role for human enterovirus B (HEV-B) in Crohn’s disease (CD) (I). To map and compare the mucosal and plasma metabolomes in new-onset PIBD patients and controls (II). To search for a new blood-based diagnostic biomarker for PIBD (III). To investigate the effect of exclusive enteral nutrition (EEN) treatment on the mucosal and plasma metabolomes in CD patients (IV).

Methods: Immunohistochemistry and chromogen in situ hybridisation were used to search for HEV-B in surgical specimens from patients who had undergone surgery for stricturing ileocecal CD, and from volvulus patients as controls. Ultra-high-performance liquid chromatography mass spectrometry were used on biopsies and plasma from patients in the Uppsala PIBD inception cohort for metabolomic (II and IV) and lipidomic analyses (III). Patients were stratified by phenotypic subtypes and treatment responses. Symptomatic patients without PIBD were used as non-IBD controls. In Study III, two other independent PIBD inception cohorts were used for validation and confirmation.

Results: I: HEV-B was detected in epithelial cells and neuronal ganglia of the enteric nervous system, and the specific cellular Coxsackie and adenovirus receptor (CAR) was expressed in both the intestinal epithelium and the enteric nervous system. II: Alterations in two metabolic compound classes were seen: decreased levels of lysophospholipids in inflamed ileum of CD patients and altered levels of sphingolipids in inflamed ileum and colon in both CD and ulcerative colitis, as compared with non-IBD controls. III: Discovery, validation and confirmation in three independent PIBD inception cohorts of a blood-based diagnostic two-lipid signature of PIBD. IV: A generalised downregulation of the non-inflamed ileal lipid metabolism after successful remission induction with EEN, as compared with baseline, and also as compared with non-IBD controls. Reduction of several lysophospholipids was a characteristic feature of the post-EEN ileal metabolome.

Conclusions: The demonstrated presence of HEV-B supports, but does not confirm, its hypothesised role in CD. The CD-associated downregulation of mucosal metabolism both at disease onset and after successful EEN-induced inflammation resolution indicates a central role for the ileal mucosal lipid metabolism in CD, including lysophospholipids. The blood-based two-lipid signature has the potential of becoming a diagnostic tool in the clinical work-up of suspected PIBD.

Keywords: Inflammatory bowel disease, IBD, Crohn's disease, ulcerative colitis, pediatric, metabolomics, lipidomics, enterovirus, coxsackievirus

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To Hedda, Disa, Adam and Siri
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ATG16L1 autophagy related 16 like 1
AUC area under the receiver operating curve
BMI body mass index
CAR Coxsackie and adenovirus receptor
CD Crohn's disease
CBV Coxsackie B virus
CER ceramide
CI confidence interval
CISH chromogenic in situ hybridisation
CRP C-reactive protein
Da Dalton
DIABLO data integration analysis for biomarker discovery using latent components
EEN exclusive enteral nutrition treatment
ESPGHAN European Society for Paediatric Gastroenterology, Hepatology and Nutrition
F-calprotectin faecal calprotectin
FDR false discovery rate
GI gastrointestinal
HEV-B human enterovirus species B
hsCRP high sensitive C-reactive protein
IBD inflammatory bowel disease
IBD-U unclassified inflammatory bowel disease
IBSEN inflammatory bowel disease in southern Norway
IHC immunohistochemistry
IQR interquartile range
LacCer lactosylceramide
LP lysophospholipid
LPC lysophosphatidylcholine
LPE lysophosphatidylethanolamine
LPI lysophosphatidylinositol
LPSe lysophosphatidylserine
MAP2 microtubule-associated protein 2
NOD2 nucleotide-binding oligomerisation domain-containing protein2
NPV  negative predictive value
PC    phosphatidylcoline
PCA   principal component analysis
pEEN_INF  presence of macroscopic inflammation at follow up
          after EEN
pEEN_nINF absence of macroscopic inflammation at follow up
          after EEN
PLA 1 and 2 phospholipase 1 and 2
PLS-DA partial least squares discriminant analysis
PIBD  paediatric inflammatory bowel disease
PUCAI  the paediatric ulcerative colitis activity index
Q_2  prediction of the models
R_2  goodness of fit
RNA  ribonucleic acid
ROC   receiver operating characteristic curve
SCAD  smoothly clipped absolute deviation
SES-CD the simple endoscopic score for Crohn’s disease
S1P  sphingosine-1-phosphate
sPCDAI the short paediatric Crohn’s disease activity index
ssRNA single stranded ribonucleic acid
TJ    tight junctions
UC    ulcerative colitis
UK    the United Kingdom
UHPLC-Q-TOF-MS ultra-high-performance liquid chromatography
       quadrupole time-of-flight mass spectrometry
UPLC MS/MS ultra-performance liquid chromatography-tandem
mass spectrometry
VIP   variable importance in projection
Introduction

The ecosystem of the human gastrointestinal tract has developed throughout human evolution, with close interaction between the gastrointestinal motor and mucosal functions, the immune system, intestinal microflora and dietary content. This ecosystem originally developed when humans and their ancestors were hunters and gatherers. Long-term dietary changes have occurred over time, at a slow rate. A late evolutionary change for the intestinal ecosystem came with the cultural transition to agriculture about ten thousand years ago, with the introduction of a grain-based diet combined with a small but steady supply of dairy products and meat from domesticated animals\(^1\).

A contemporary – and, from an evolutionary perspective, very rapid – change for the human gastrointestinal ecosystem has followed industrialisation. Industrial mass production of food has introduced processing techniques such as pasteurisation, refining, and use of antibiotics, as well as use of both natural and synthetic chemical food additives like preservatives, dyes, texturisers and emulsifiers. With industrialisation has followed a transition to the so-called ‘Western diet’, characterised by excess energy, industrially processed foods, high levels of refined sugar and grains, saturated fats and food additives. This contemporary cultural development and thorough change of diet have radical consequences for the human gastrointestinal ecosystem, currently transforming into a dysbiotic state of reduced microbial diversity and reduced species richness, with pro-inflammatory functional properties. This profound restructuring of the human gastrointestinal ecosystem has likely contributed to the ongoing rise of immunological diseases in general and inflammatory bowel disease (IBD) in particular\(^3-9\).

IBD is a chronic condition characterised by inflammation in the gastrointestinal tract. The aetiology of IBD remains elusive, and although there is a strong epidemiological link between IBD prevalence and the Western diet, it is important to emphasise that most people in the industrialised world will never develop the disease. The pathogenesis is largely unknown; the pathophysiological process is complex and occurs in stages. According to the current paradigm, IBD develops in a genetically predisposed individual exposed to environmental risk factors, such as the Western diet, as a result of an abnormal immune response to the intestinal microflora. Complex interactions between bacteria and the immune system are central in pathophysiological process involved in IBD. However, this does not exclude the influence of other
components of the intestinal ecosystem in disease development, such as common ribonucleic acid (RNA) viruses.\textsuperscript{9-15}

Advancements in biomedical technology have led to the rapid development of several laboratory technologies that can be used to advance our understanding of IBD. Examples are metabolomics and its subdiscipline lipidomics. These are powerful tools for mapping metabolites in tissue samples and hold great promise for contributing to a better understanding of the molecular processes involved in the pathophysiology of IBD.\textsuperscript{9,14,15}

Furthermore, metabolomics and lipidomics can aid in the identification of potential biomarkers for IBD. In the clinical practice of paediatric IBD (PIBD), with the rapidly expanding arsenal of pharmacological treatment options, there is an increasing need for better biomarkers for monitoring disease activity and assessment of treatment outcomes, which can ultimately guide treatment strategy decisions.\textsuperscript{15,16}

Despite advances in clinical management over the past few decades, IBD remains an incurable disease. Pharmacological treatment options still aim only at immunological control. Nutrition therapy including exclusive enteral nutrition (EEN) has long had an established role in PIBD. Although the mechanism of action of EEN remains unclear, the application of metabolomics provides hope for a researchable path towards a better understanding of how dietary factors and the intestinal ecosystem interact both in the development of IBD and in the treatment of the disease.\textsuperscript{9,17}

Definitive cure of IBD and primary prevention of the disease are the ultimate goals of IBD research. To achieve these goals in the future, deeper insights into IBD disease mechanisms are needed. This requires mechanistic research based on new epidemiological and ecological insights, and using advanced laboratory technologies, preferably on mucosal samples, from clinically well-defined inception cohorts.

This thesis addresses four questions related to IBD:

- Is there a role for RNA viruses, or more specifically, a role for human enterovirus B, in the pathogenesis of paediatric Crohn’s disease?
- How do the mucosal and plasma metabolomes differ between children with new-onset IBD and children without IBD?
- Is there a clinically applicable blood lipidomic biomarker for PIBD?
- How does treatment with EEN influence the mucosal and plasma metabolomes in children with Crohn’s disease?
Background

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder of the gastrointestinal (GI) tract, with the predominant subtypes Crohn’s disease (CD) and ulcerative colitis (UC). A third subtype is unclassified IBD (IBD-U). The clinical presentation at diagnosis is often a combination of gastrointestinal and systemic symptoms of acute and chronic inflammation. Common symptoms are abdominal pain and diarrhoea with blood and mucus, abdominal pain, fever, fatigue, anorexia and weight loss\textsuperscript{10,12,13}.

IBD in childhood

Up to 25\% of IBD patients have a paediatric onset, i.e., before 18 years of age. Very early onset of IBD, before 6 years of age, is rare. Most PIBD patients are diagnosed from the pre-puberty period onward, with increasing incidence during puberty and adolescence\textsuperscript{18-20}.

PIBD is essentially the same disease as that in adults, although some clinical features are specific or more common in children. In general, compared with adults with IBD, children with IBD tend to have a more severe disease course. CD is relatively more common in children, as are upper GI disease involvement in CD and extensive colonic disease in UC\textsuperscript{18,21,22}.

Beyond these phenotypic differences, a child with IBD is at risk of specific paediatric complicating aspects, including growth retardation, delayed puberty, and impaired bone mineralisation. IBD during childhood or adolescence also carried a considerable psychologic burden and impaired quality of life is commonly reported\textsuperscript{18,21,22}.

Epidemiology of IBD

The prevalence rates of IBD in both children and adults have been increasing for several decades, and these increases have been associated with industrialisation, and the ‘Western diet’ in particular\textsuperscript{2-8}. The highest IBD prevalence has long been seen in Western Europe and North America. In Sweden, the overall incidence rate for IBD in 2002–2014 was 32.1/100000, with the largest
incidence peak in the age group 15–24 years\textsuperscript{23}. It is estimated that approximately one out of 40 Swedes will develop IBD during their lifetime.

However, in Sweden and other traditionally high-prevalence regions, there is a trend over the last two decades that incidence rates may be stabilising at a high level\textsuperscript{23}. Meanwhile, the incidence is still increasing in more recently industrialised parts of the world. These short-term changes in incidence cannot be explained based on genetic factors – rather, they are likely to reflect differences in exposure to environmental factors\textsuperscript{2–8}.

The intestinal ecosystem

The intestinal microbiota is the community of all commensal and pathogen microbes residing in the gastrointestinal tract, including bacteria, viruses, archaea, parasites, and fungi\textsuperscript{1,24}.

The ecosystem of the human gastrointestinal tract is defined by the complex interplay between the intestinal immune system, the microbiota and intraluminal conditions strongly influenced by dietary factors. The Western diet, with a high proportion of industrially processed food, rich in saturated fat, additives, and refined sugar, is a consequence of industrialisation and urbanisation. The current dietary driven evolution of the intraluminal environment brings about changes in the human intestinal ecosystem characterised by microbial dysbiosis: a general decrease in bacterial diversity and a change in bacterial composition. The microbial dysbiosis is shifting the function of the intestinal ecosystem towards a pro-inflammatory state. This evolution of the intestinal ecosystem is ongoing, being in different phases in different parts of the world\textsuperscript{1–8,24,25}.

Aetiology and pathogenesis

The aetiology and the pathophysiological mechanisms leading to IBD are largely unknown. Genome-wide association studies and meta-analyses of such studies have outlined IBD as a complex polygenic disease with a large number of genetic susceptibility loci. Many of these identified risk loci are linked to innate and adaptive immunity, autophagy, and intestinal barrier function\textsuperscript{11,26–28}.

According to the prevailing paradigm, IBD develops in genetically predisposed individuals from aberrant and persistent immune responses triggered by a pro-inflammatory dysbiotic intraluminal environment. The pathogenesis involves a complex interplay between the dysbiotic microbiota, the host immune system, and intraluminal triggering factors; together in a vicious cycle of dysregulated immune responses, increased dysbiosis, disruption of the mucosal barrier, and mucosal inflammation. This negative cycle eventually leads to chronic and gradually increasing inflammation and intestinal damage\textsuperscript{10–15}.
A consequence of the chronic intestinal inflammation and dysbiosis in IBD is an enhanced metabolic dysfunction of the intestinal ecosystem. Thus, the study of metabolites in mucosal tissue has become an attractive approach to increase the understanding of pathophysiological processes in IBD\textsuperscript{9,14,15}.

**Metabolomics in IBD**

Metabolites are small molecules (< 2,000 Da) that are intermediates and products of the endogenous metabolism of the host. They include, for example, carbohydrates, fatty acids, nucleotides and amino acids. The metabolome refers to the complete set of metabolites in a sample\textsuperscript{29}.

Metabolomics is the high-throughput, simultaneous systematic determination of metabolite levels in the metabolome of a biological sample. Metabolomics is a newly emerging field of ‘omics’ research and is increasingly applied in IBD research to characterise the function of the intestinal ecosystem and interactions between nutrients, intestinal mucosal metabolism, gut microbiota and host immune system\textsuperscript{9,14,15,29}.

The study of metabolites can be divided into two different approaches: targeted and untargeted (global) metabolomics. In targeted metabolomics, there is a focus on certain specific metabolites/groups of metabolites or pathways. By contrast, the global or untargeted approach aims to describe all the metabolites in a sample, or as many as possible\textsuperscript{29}.

An untargeted metabolomics workflow includes feature detection, pathway analysis, principal component analysis (PCA), biomarker discovery and validation. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is a powerful analytical technique that combines the separating power of liquid chromatography with the highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry. The identification of metabolites requires further processing and statistical analyses of the data generated from sample analyses. Online bioinformatics software tools integrated with metabolite databases are often used for this purpose. The typical workflow when using such tools begins with data visualisation with an unsupervised PCA, to identify outliers, study technical variability and detect trends and clusters. This is followed by a univariate analysis to identify statistically significant peaks, and a supervised approach, such as partial least square discriminant analysis (PLS-DA), to identify statistically significant patterns of metabolite peaks\textsuperscript{29}.

Targeted and untargeted metabolomic IBD research have reported a number of specific metabolites and molecular compound classes of interest, in relation to pathophysiology, biomarkers and/or therapeutics. Children with treatment-naïve, incident IBD, without any comorbidities, make up an ideal population for metabolomic profiling. Nevertheless, most metabolomic research has been performed on adult IBD patients with ongoing medical treatment. Various biological samples have been analysed, most commonly blood
and faeces, and only very few studies have utilised intestinal mucosal biopsies\textsuperscript{14,30-39}.

**Lipidomics in IBD**

Lipidomics has emerged as a specialised subdiscipline of metabolomics. It encompasses large-scale mapping of a wide range of lipid classes in biological samples. Like general metabolomics, lipidomics can be performed with either targeted or untargeted approaches\textsuperscript{40}.

Lipids have three main roles in the human body: energy storage, cell membrane components and bioactive functions. Lipids with this last role are called bioactive lipids, a collective term for lipid mediators with signalling and regulatory functions in inflammation and other physiological and pathophysiological processes. Altered lipid metabolism and bioactive lipids are receiving increasing attention in relation to IBD and other chronic inflammatory diseases, from the perspectives of pathophysiology, identification of biomarkers and therapeutics. Two lipid classes that have recently raised interest in relation to IBD are lysophospholipids and sphingolipids\textsuperscript{31,41-51}.

Lysophospholipids (LPs) make up a complex class including many species, of which several have been shown to exert immune regulatory and signalling functions. LPs are categorised based on their polar head group (choline: lysophosphatidylcholine (LPC), ethanolamine: lysophosphatidylethanolamine (LPE), inositol: lysophosphatidylinositol (LPI), and serine: lysophosphatidylserine (LPSer)). Alterations in various LPC, LPE, and other LP families have been linked to IBD, and to inflammation in mouse models of IBD\textsuperscript{33,41,42,49,51,52}. LPs are derived from membrane-bound phospholipids (PCs) in the cellular walls of enterocytes by phospholipases A1 and A2 (PLA1 and PLA2). These enzymes deacylates the PC into free LPs and free polyunsaturated fatty acids, PLA2 deacylation thus delivers sn-1 LPs with a potential for bioactive functions, and vice versa for PLA1\textsuperscript{41}.

Sphingolipids are increasingly recognised as important regulators of inflammation, and mucosal alterations in various sphingolipids, including ceramides and sphingomyelins, have been associated with IBD\textsuperscript{33,41,42,46,48,53}. The recent approval of a sphingosine-1-phosphate receptor modulator (ozanimod) for the treatment of moderately to severely active UC in adults underscores a role of sphingolipids in IBD, with associated therapeutic potential\textsuperscript{54}.

Lactosylceramides (LacCer) support plasma membrane stability, activate specific receptor molecules, and bind to specific bacteria. Increased plasma levels of lactosyl-N-palmitoyl-sphingosine (LacCer(d18:1/16:0)) have recently been reported in both children and adults with IBD, as well as in faecal samples of IBD patients\textsuperscript{46,48,55-57}. The mechanisms behind the increase and its role in the context of IBD are not known. However, it is known that lactosylceramide synthase is activated by pro-inflammatory cytokines to produce
lactocerylceramides, which in turn activate mucosal cell differentiation and maturation\cite{55}.

Decreased levels of phytosphingosine, another bioactive sphingolipid, were recently reported in mice with 2,4,6-trinitrobenzene sulfonic acid-induced colitis, and its anti-inflammatory effect upon oral administration was also demonstrated\cite{53}.

A potential role for RNA viruses in IBD

Eucaryotic ribonucleic acid (RNA) viruses with tropism for the human gastrointestinal tract have evolved various specific strategies to infect the mucosal epithelium. Some of these viruses alter or disrupt tight junctions (TJ) as part of their virulence. TJ are complex intra- and extracellular protein structures that link epithelial cells and have a central role in regulating paracellular permeability\cite{58,59}.

Coxsackie B viruses (CBV) are common single-stranded RNA (ssRNA) viruses belonging to the human enterovirus species B (HEV-B) family. CBV uses the TJ-regulating protein Coxsackie and adenovirus receptor (CAR) as a cellular receptor. When CBV infects an enterocyte in vitro, the CAR is translocated from the intercellular junction to the cytosol of the enterocyte along with the virus. This structural loss of CAR from the TJ leads to impaired TJ regulation and the paracellular permeability thereby increases. Whether CBV has the ability to infect the enteric nervous system, as well as to persist in neuronal tissue, is not known. However, it is well known that many HEV-B viruses, including CBV, have dual tropism for both the gastrointestinal tract and the central nervous system\cite{59-63}.

The presence of enterotropic viruses in gastrointestinal mucosal biopsies can be studied through immunohistochemistry (IHC) using specific virus antibodies. An advantage of this method is that it can be applied retrospectively to tissue sections from clinically collected paraffin-embedded tissue. Chromogenic in situ hybridisation (CISH) can be used as an independent validation method to check IHC specificity and potentially verify positive IHC findings.

Polymorphisms of the CD susceptibility genes \textit{NOD2} and \textit{ATG16L1} cause loss of the normal physiological functions of their proteins. Such genetic loss of function has implications for both innate immunity and autophagy, linking CD to HEV-B. These genes are involved in the host's immune response to ssRNA viruses, as is the ATG16L1 pathway, which is a prerequisite for CBV replication. NOD2 has been shown to function as an intracellular pattern recognition receptor for ssRNA viruses. Loss of function of these genes can lead to defective autophagy and less efficient elimination of ssRNA viruses\cite{59,64-69}.
Diagnosis of IBD

A clinical suspicion of IBD in a child requires prompt further medical investigation. The first step in primary care is a clinical examination and basic laboratory testing of biomarkers of systemic inflammation such as C-reactive protein (CRP), albumin, platelet count, and faecal (f)-calprotectin, as a marker of gastrointestinal neutrophilic inflammation.

If IBD is still a possible differential diagnosis after the primary clinical workup, the child should be referred to a paediatric gastroenterologist for a decision on endoscopic examination of the gastrointestinal tract, which is the diagnostic test for IBD. In addition, small bowel investigation with ultrasound and/or magnetic resonance tomography are often included.

In paediatric practice, the endoscopic procedure for IBD involves both the upper and lower gastrointestinal tract and is performed under general anaesthesia or deep sedation. Serial mucosal biopsies are taken during examination. The histopathological assessment of the biopsy material and the endoscopist's macroscopic assessment of the gastrointestinal tract, together with the patient's overall clinical picture, all contribute to the paediatric gastroenterologist’s balanced diagnosis of IBD\textsuperscript{70-73}.

Biomarkers in IBD

The clinical course of IBD is progressive. To prevent adverse consequences of long-term disease, a prompt diagnosis of IBD is a general goal of physicians, in both adult and paediatric healthcare. Furthermore, because IBD is a chronic disease, continuous treatment is required to maintain remission. Even with successful maintenance therapy, there is, in principle, always a risk of reactivated disease and clinical relapse. Consequently, biomarkers with sufficient sensitivity and specificity are crucial, both in the diagnostic workup of suspected IBD, to aid in the selection of patients who should qualify for endoscopy, and in monitoring disease activity in IBD patients, to guide and evaluate treatment interventions in IBD.

Over the past two decades, f-calprotectin has been established as a reliable biomarker in PIBD in combination with less sensitive and less specific blood markers of general inflammation, such as CRP and albumin. However, stool samples are not very popular among children and adolescents, although they could be a tool for continuous monitoring of disease activity. Furthermore, the specificity of f-calprotectin in monitoring IBD disease activity is lower than its diagnostic capacity, currently leaving paediatricians with an unmet need for better diagnostic and monitoring tools in IBD\textsuperscript{73,74}. 
Treatment of PIBD

The current medical treatment of IBD aims to induce and maintain clinical remission and mucosal healing. The European Crohn's and Colitis Organization/European Society for Paediatric Gastroenterology, Hepatology and Nutrition guidelines for the treatment of PIBD recommend short-term corticosteroids. In the case of CD, nutritional therapy (see next paragraph) is also recommended for remission induction. As remission-preserving maintenance therapy, a range of immuno-suppressive therapies are recommended, including thiopurines, methotrexate and monoclonal antibodies against specific pro-inflammatory mediators. The introduction of the so-called biological pharmacological treatments of IBD over the past two decades has significantly changed the lives of children with IBD.

Despite these medical advances, and the increased general understanding of disease mechanisms in IBD, the fact remains that there is still no curative treatment for IBD and that all current pharmacological IBD treatment options aim to limit and control the aberrant immunological activity. However, both corticosteroids and long-term use of immunosuppressive drugs carry the risk of short- and long-term side effects in children, including severe disease such as lymphatic and skin malignancies. It is therefore urgent to develop treatment strategies that do not aim only at immunosuppression, but instead control disease mechanisms at a more ‘upstream’ stage of the pathogenic processes of IBD\textsuperscript{73,75-79}.

Exclusive enteral nutrition treatment

Exclusive enteral nutrition (EEN) treatment is an evidence-based non-pharmacological treatment option for the induction of clinical and endoscopic remission in paediatric CD. Evidence for the treatment of UC is currently lacking. In children with new-onset CD, EEN is non-inferior to systemic corticosteroids for remission induction, and the mucosal healing rate is higher with EEN than with corticosteroids. Unlike steroids, EEN is not associated with adverse endocrinological, metabolic or other systemic side effects. In addition, the nutritional status of the patient is improved with EEN. For these reasons, the European Crohn's and Colitis Organization / European Society for Paediatric Gastroenterology, Hepatology and Nutrition recommends EEN as first-line treatment for children with newly diagnosed CD\textsuperscript{75,79,80}.

In paediatric practice, EEN involves using either tube feeding or common, oral, flavoured nutritional supplement drinks as the sole source of nutrition during the treatment period, hence the term ‘exclusive’. Support from both parents and the paediatric treatment team during the treatment period is a prerequisite for compliance and a successful treatment outcome. Although EEN is effective, most patients find the treatment very strenuous and it is only used
for a limited and predefined period, usually 6–8 weeks. Thereafter, the patient’s ordinary food is reintroduced, often in a matter of days.\textsuperscript{75,79-82}

**Action mechanisms of EEN**

EEN thus involves a radical dietary change with the capacity to effectively reverse the course of newly diagnosed paediatric CD and induce remission. However, the action mechanisms by which EEN induces remission in CD remain elusive.

A mechanistic understanding of the treatment effect of EEN at the molecular level would be highly desirable for several reasons. First, it would provide a more solid basis for developing equivalent but less demanding dietary therapies for CD than the ongoing trials of various nutritional therapies. Second, it could also enable the development of pharmacological treatment targeting the relevant biological pathways involved in the EEN treatment effect. This could possibly be an important step towards the ultimate goals of curing IBD, as well as primary prevention of the disease.

It is well known that the gastrointestinal ecosystem is highly sensitive to dietary changes and that it responds with alterations in both microbial composition and metabolic function. Consequently, a common hypothesis is that the mechanism of action behind EEN is that it induces microbial change and thereby restores the gastrointestinal ecosystem. Indeed, EEN causes changes in the faecal microbial composition. However, contrary to what was expected, several studies have shown a paradoxical enhancement of the dysbiotic profile in faecal samples, both microbially and metabolically, rather than normalisation of this profile.\textsuperscript{17}

At present, the mechanism behind the effect of EEN remains an open question. As the intestinal mucosa is the site where both the pathophysiological processes and the treatment effects occur, metabolomic profiling of mucosal biopsies should be a more attractive approach than faecal samples in order to better understand the mechanism behind the effectiveness of EEN in CD.\textsuperscript{9,17}
Aims of the thesis

The aim of this thesis was to gain deeper knowledge of molecular profiles and mechanisms involved in pathogenesis of PIBD, as well as of potential diagnostic biomarkers and nutritional therapy. The overarching idea was that the primary site of pathophysiologica l action in IBD is the intestinal mucosa. Thus, the common approach in Studies I, II and IV was to apply different biomolecular laboratory techniques to tissue samples from IBD patients. In Study III, the aim was to search for clinically applicable biomarkers, and blood samples from IBD patients were therefore utilised.

- Study I was based on a hypothesis that common primitive RNA viruses may play a role in the pathogenesis of IBD. The aim was to investigate the presence of Coxsackie B virus in surgical specimens from patients with Crohn's disease with stricturing ileocecal phenotype and paediatric onset.

In Studies II–IV, we aimed for metabolomic and lipidomic profiling of PIBD. The specific objectives were as follows:

- In Study II, the aim was to map the plasma and mucosal metabolomes of children with newly developed IBD compared with symptomatic non-IBD controls, and correlate these metabolomes with clinical and inflammatory markers.

- In Study III, the aim was to perform lipidomic profiling of blood samples from children with incident IBD and symptomatic non-IBD controls in search of new diagnostic biomarkers for PIBD.

- In Study IV, the aim was to investigate the effect of EEN on the plasma and mucosal metabolomes in children with new-onset Crohn's disease.
Study designs and patients

Study I
This was a retrospective case-control study on surgical specimens. Medical records were searched to identify patients with childhood-onset CD who had undergone surgery with ileocelecal resection due to stricturing ileocelecal disease at Uppsala University Hospital between 1997 and 2010.

Nine CD patients meeting the inclusion criteria were identified; all were invited and included after giving written informed consent. Five boys/men and four girls/women; average age at diagnosis was 11.8 (range 8.5–15.5) years, average age at surgery was 17.0 (range 9.0–24.8) years, and average time from diagnosis to surgery was 3.9 (range 0–9.3) years. Eight of out the nine CD patients were treated with immunosuppressants at the time of surgery. Mucosal biopsies from the previous diagnostic endoscopy were available from six of the patients; five of them had not received any anti-inflammatory treatment prior to the endoscopy, whereas one patient had been treated with mesalazine.

As non-IBD controls, six adult patients who had undergone surgery for small intestinal volvulus were included: two men and four women, average age at surgery 66.3 (range 43–74) years. Resection margins from their surgical specimens were used for comparison. Additionally, nine children who had undergone endoscopy without findings of inflammation were included as non-IBD controls: five boys and four girls, average age at endoscopy 14.0 (range 9–16) years. None of the non-IBD controls had received any anti-inflammatory or immunosuppressive treatment before endoscopy.

Studies II–IV
All three studies were based on the Uppsala PIBD inception cohort. This is a prospective single-centre PIBD cohort in which all children aged < 18 years with clinically suspected IBD who were referred to the Uppsala University Children’s Hospital, between 2009 and 2018, were consecutively invited to participate. The inclusion criterion was presence of gastrointestinal symptoms indicative of IBD. Exclusion criteria were a previous diagnosis of IBD, other chronic gastrointestinal diseases, and treatment with antibiotics within three months prior to inclusion.
After providing written informed consent, all children underwent a routine diagnostic workup for IBD in accordance with the European Society for Paediatric Gastroenterology, Hepatology and Nutrition/Porto criteria\textsuperscript{70}, including gastroscopy and ileocolonoscopy with serial mucosal biopsies for histology. We used the Paris classification for disease phenotyping\textsuperscript{83}, the short paediatric CD activity index (sPCDAI)\textsuperscript{84,85}, and the paediatric UC activity index (PUCAI)\textsuperscript{86,87}, to assess clinical disease activity, and the simple endoscopic score for CD (SES-CD) and the Mayo endoscopic score for UC to assess endoscopic disease activity\textsuperscript{88,89}. All recruited patients were followed prospectively in accordance with local clinical routines.

Children without endoscopic or histologic signs of acute or chronic inflammation who did not fulfil the diagnostic criteria for IBD were included as symptomatic non-IBD controls. At long-term follow-up, most of these controls were diagnosed with irritable bowel syndrome in accordance with the Rome IV criteria\textsuperscript{90}.

**Study II**

This was a prospective case-control study including 56 children with newly-diagnosed IBD (47 children with CD and 9 with UC), and 11 symptomatic non-IBD controls, all retrieved from the Uppsala PIBD cohort. Of the symptomatic non-IBD controls, one had a focal vascular malformation in the colonic mucosa, one had coeliac disease, and the remaining nine had normal endoscopy and histology on mucosal biopsies. At long-term follow-up, all these nine patients were diagnosed with irritable bowel syndrome in accordance with the Rome IV criteria.

**Study III**

This was a cross-sectional study using two independent prospective PIBD inception cohorts, the Uppsala PIBD as the discovery cohort and the IBD in South-Eastern Norway (IBSEN) III as the validation cohort, see below. Plasma samples (Uppsala PIBD) and serum samples (IBSEN III) were analysed and compared with routine clinical biomarkers. A third, independent British-Danish PIBD inception cohort was used for confirmation of the results, see below.

The discovery cohort included 58 children with IBD (CD, n = 44; UC, n = 12; IBD-U, n = 2) and 36 age-comparable symptomatic controls from the Uppsala PIBD inception cohort. The validation cohort included 79 patients with IBD (CD, n = 53; UC, n = 20; IBD-U, n = 6) and 37 non-IBD symptomatic controls from the population-based paediatric IBSEN III cohort. The confirmation cohort included a total of 164 paediatric patients with IBD (CD, n =
110; UC, n = 54) and 99 non-IBD symptomatic controls, 30 of whom were diagnosed with coeliac disease.

The IBSEN III paediatric cohort is a sub-cohort of the IBSEN III study, a population-based, prospective, inception cohort study in which all patients with suspected IBD in the South-Eastern health region of Norway in 2017–2019 were invited to participate. The diagnostic criteria for IBD, categorisation of phenotypes, and assessment of clinical and endoscopic disease activity in the IBSEN III cohort were consistent with those in the Uppsala PIBD cohort. Children and adolescents who did not meet the diagnostic criteria for IBD were included as symptomatic non-IBD controls. Patients with any other cause of acute or chronic bowel inflammation were excluded. As in the Uppsala cohort, recruited patients were prospectively followed up in accordance with routine clinical procedures.

In the confirmation cohort, paediatric patients below 18 years, from Denmark, Norway and the UK were included. The clinical assessment and diagnostic criteria for CD and UC were consistent with those used in the discovery and validation cohort. Patients were recruited at diagnosis and subsequently followed prospectively as per routine clinical care. As in the discovery and validation cohorts, symptomatic non-IBD controls were defined as patients with symptoms of IBD but lacking any endoscopic or histologic evidence of IBD.

Study IV

This was a prospective, single-centre cohort study of treatment-naïve paediatric patients with new-onset CD who were followed up after EEN as single treatment for remission induction. Eighteen CD patients were included, all of whom had undergone an eight-week period of EEN in monotherapy for remission induction and a clinical endoscopic re-assessment during the final week of EEN, i.e., before introduction of ordinary food. Both biopsies and plasma were thus collected twice from each patient: at baseline and at the end of the EEN treatment. For comparisons of EEN-related findings, we analysed baseline and follow-up samples from 11 symptomatic non-IBD controls, also recruited from the Uppsala PIBD cohort.

Ethical considerations

Studies I–IV were all approved by the Regional Ethical Review Board in Uppsala, Sweden, (2008/395). Study III was also approved by the South Eastern Regional Ethical Board, Norway (2015/946), the Ethics Committee of the Capital Region of Denmark (H-20065831), the Danish Data Protection Agency (P-2020-1065), and the Oxford Research Ethics Committee
(reference: 11/YH/0020 and 16/YH/0247). All studies were conducted in accordance with the Declaration of Helsinki. All patients received verbal and written study information before inclusion. Written informed consent was gathered from patients > 18 years and from children 12–18 years and their parents. For children < 12 years, written informed consent was gathered from parents only.
Methods

Study I
The surgical specimens and mucosal biopsies were stained IHC or immuno-fluorescence techniques with antibodies against enterovirus, CBV, echovirus or the virus receptor CAR. Pancreatic islets infected in vitro with an enterovirus were used as positive controls and uninfected pancreatic islets as negative controls. The antibody staining in the IHC sections was assessed in a semi-quantitative manner.

CISH was performed with incubation of the tissue sections with a cocktail of six different enterovirus-specific digoxigenin-labelled probes for virus detection, and with human b-actin as positive control. Tissue sections were also used for genotyping of three polymorphisms in NOD2 (rs2066844, rs2066845, and rs5743292) and one in ATG16L1 (rs2241880).

Studies II and IV
Snap-frozen mucosal biopsies were endoscopically collected from the ileum and transverse or descending colon. Blood samples were taken in immediate connection to the endoscopy and processed into plasma. All samples were stored at -80 °C.

Results of routine clinical laboratory tests and measurements of CRP, haemoglobin and f-calprotectin were retrieved from medical records.

Plasma and biopsy samples were extracted with methanol and examined through non-targeted mass spectrometry analyses using ultra-performance liquid chromatography (UPLC-MS/MS). Aliquots of the sample extracts were reconstituted in solvents compatible with one of four methods. The first two aliquots were analysed under acidic, positive ion conditions, optimised for either hydrophilic or hydrophobic compounds. The third aliquot was analysed under optimised, basic, negative ion conditions, whereas the fourth aliquot was analysed via negative ionisation. Identification of known chemical entities was based on comparison with metabolomic library entries of purified standards. Commercially available purified standard compounds were obtained for determination of detectable properties. Additional mass spectral entries were created for structurally unnamed biochemcials, which were identified based on their recurring nature (both chromatographically and mass...
spectrally). Lastly, quality-controlled data were organised into metabolic classes and pathways.

Plasma inflammatory proteins were measured by Olink Proteomics, Uppsala, Sweden, using the Proximity Extension Assay technology, with simultaneous analysis of 92 inflammation-related protein biomarkers across 96 samples. Briefly, this method involves use of pairs of oligonucleotide-labelled antibodies against each target antigen. When both antibodies bind to the same antigen in close proximity, the attached oligonucleotides hybridise. The oligonucleotide templates are extended and amplified using polymerase chain reaction.

Study III
Plasma samples from the Uppsala PIBD cohort were prepared as described in the section on Study II. In the IBSEN III study, serum samples were obtained in a similar way.

After extraction of the samples, instrumental analyses were carried out with an ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry method (UHPLC-Q-TOF-MS from Agilent Technologies (Santa Clara, CA, USA)). The analysis was done on an ACQUITY UPLC® BEH C18 column from Waters (Milford, USA). Non-targeted lipidomics raw data were pre-processed with peak detection, chromatogram deconvolution and isotopic peak grouping. Filtering with a Feature list rows filter was done in three steps, and subsequent gap filling was done with Peak finder. Comparisons with a custom database were made to match the peak list with compound names.

Statistical analyses
Continuous variables are presented as medians and interquartile ranges, and categorical data as frequencies. Univariate analyses were performed using Welch's two-sample t-test (Studies II and IV) and Wilcoxon rank-sum test (Study III). To adjust for multiple comparisons, a false discovery rate (FDR) approach was used, and FDR-adjusted p-values are reported.

Multivariate analyses included PCA, partial least squares discriminant analysis (PLS-DA) and hierarchical cluster analysis. The validity of the PLS-DA models was assessed using permutation analyses and R²/Q² analyses. Variable significance in projection plots was developed to identify important metabolites that could be used to distinguish between different groups of patients. For the multivariate analyses, patients with CD were stratified based on the presence of inflammation in the ileum and transverse colon, respectively.
Univariate and multivariate analyses were performed using MetaboAnalyst v. 5.0.31\textsuperscript{92}.

In Study III, a regularised logistic regression model was implemented in each comparison group (IBD vs symptomatic controls, CD vs symptomatic controls, UC vs symptomatic controls) using a supervised machine learning method: smoothly clipped absolute deviation (SCAD) regularised logistic regression. The diagnostic lipidomic signature models were built using data from the discovery cohort and validated using data from the validation cohort. The top validated differential lipidomic signatures were used for prediction, and the area under the receiver operating curve (AUC) with 95\% confidence interval (95\% CI) was reported\textsuperscript{93}.

For the integration analyses in Study II, we used DIABLO (Data Integration Analysis for Biomarker discovery using Latent Components)\textsuperscript{94}. Correlation coefficients between important variables in each dataset were calculated, and positive and negative correlations were visualised in Circos plots.
Results

Study I

Genetic analysis showed that 3/9 ICD patients were heterozygous for disease-associated missense (rs2066844/rs2066845) or frameshift (rs5743293) mutations in NOD2 (Table 1), whereas one was homozygous. Four out of nine patients were homozygous and four patients were heterozygous for a disease-associated missense mutation (rs2241880) in ATG16L1. One patient (patient 6) had no disease-associated mutations in either NOD2 or ATG16L1.

Table 1. Data from genetic analyses of CD-associated polymorphisms in NOD2 and ATG16L1 genes in nine patients with stricturing ileocecal Crohn's disease.

<table>
<thead>
<tr>
<th>CD patients</th>
<th>NOD2</th>
<th>ATG16L1 A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rs2066844</td>
<td>G/G</td>
</tr>
<tr>
<td>2</td>
<td>rs2066845</td>
<td>A/G</td>
</tr>
<tr>
<td>3</td>
<td>rs5743293</td>
<td>G/G</td>
</tr>
<tr>
<td>4</td>
<td>rs2066844</td>
<td>A/G</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>A/A</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>A/G</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>G/G</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>A/G</td>
</tr>
</tbody>
</table>

Analysis of the surgical specimens revealed the presence of mucosal inflammation and myenteric plexitis (Figures 1a and b). No signs of mucosal inflammation or myenteric plexitis were observed in resection margins from the ileocecal region of patients who had undergone surgery due to intestinal volvulus (Figure 1c).
Figure 1 Myenteric plexitis in patients with ileocecal Crohn’s disease (ICD). (a) Representative image of a haematoxylin- and eosin-stained transmural section from the ileocecal region of a patient with ICD, showing inflammation in both the mucosa (Muc) and the enteric nervous system (myenteric plexitis (P)) within the muscularis externa (ME) layer of the submucosa (SubM). (b) Representative high-magnification image showing plexitis in a myenteric ganglia (G) in the ileocecal region of a patient with ICD. (c) Representative high-magnification image showing no signs of P in a volvulus patient. Bars 1/4 (a) 500 mm; (b, c) 100 um.

To study the presence of HEV-B, we stained surgical specimens through IHC using antibodies specific for CBV and echovirus. Positive cytoplasmic immunostaining for CBV was detected in crypt epithelial cells of the mucosa, and in both neurons and glial cells of myenteric ganglia in patients with ICD (Figures 2a and b). No positive staining for CBV was detected in surgical specimens from volvulus patients (Figures 2c and f). High-magnification images revealed a granular, perinuclear staining pattern of CBV in the nerve cell ganglia in ileocecal resections from patients with ICD (Figure 2d). A similar type of staining was detected for echovirus in crypt epithelial cells of the mucosa (data not shown) and in myenteric ganglia (Figure 2e) in patients with ICD, but not in volvulus patients (Figure 2g).
**Figure 2.** Detection of human enterovirus species B (HEV-B) in patients with ileocelecal Crohn’s disease (ICD) using immunohistochemistry (IHC). (a) Representative image of a transmural section of the ileocecal region of a patient with ICD, stained through IHC with an antibody specific for Coxsackie B virus (CBV). Positive staining for CBV was detected both in the crypt epithelium of the mucosa (Muc) and in myenteric ganglia (G) within the muscularis externa (ME) layer of the submucosa (SubM). Representative images showing (b) positive, perinuclear staining of CBV in G of a patient with ICD and (c) myenteric plexitis on a haematoxylin- and eosin-stained section. Representative high-magnification images showing positive, perinuclear staining of (d) CBV and (e) echovirus in G of a patient with (d, e) ICD and (f, g) negative staining in a patient with volvulus. Bars 1/4 (a) 500 mm; (b, c) 100 mm; (d–g) 20 mm. Cox, Coxsackie B virus; Echo, echovirus.
We also found positive staining for HEV-B in intestinal epithelial cells of colon biopsies from all six ICD patients from whom baseline endoscopy biopsies were available, i.e., samples taken before any immunosuppressive treatment. In addition, positive staining for HEV-B was detected in intestinal epithelial cells of colon biopsies from another nine CD patients at the time of diagnosis. Positive staining for CVB and echovirus was detected in 4 of 15 CD patients at the time of diagnosis.

CISH using probes recognising a broad range of HEV-B revealed positive staining for HEV-B (positive-stranded RNA) in myenteric ganglia (Figure 3a), which further supported the presence of HEV-B in the enteric nervous system in patients with ICD. The replication template (negative-stranded RNA) was also positive in ICD patients but was weaker than the virus template (Figure 3b), suggesting that virus replication occurred at a slow rate. These findings were further supported by positive immunostaining for double-stranded RNA, which forms during viral replication, and for the interferon-alfa-induced enzymes oligoadenylate synthetase and protein kinase R, both induced during virus infection. CISH detected no positive signal for HEV-B in ileocecal resections from patients with volvulus (Figure 3c).

**Figure 3.** Detection of human enterovirus species B (HEV-B) in patients with ileocecal Crohn’s disease (ICD) through chromogen in situ hybridisation (CISH). (a–c) Representative high-magnification images showing (a, CISH PLUS) positive, perinuclear CISH staining for HEV-B virus template, (b, CISH MINUS) weaker staining for the replication template, and (c) negative CISH staining in a patient with volvulus. Bar 1/4 20 mm.
Table 2. Summarized data from IHC and CISH analysis revealed the presence of myenteric plexitis in all patients with ICD, but in none of the volvulus patients (Table 2). Positive stainings for CBV and Echovirus in both crypt epithelial cells and myenteric ganglia were detected in seven out of nine ICD patients by IHC and in all ICD patients by CISH. Staining for CBV was negative in all volvulus patients. Staining for Echovirus was positive in one out of six volvulus patients by IHC, but not by CISH.

<table>
<thead>
<tr>
<th>Patients</th>
<th>IHC</th>
<th>CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plexitis</td>
<td>CBV, c</td>
<td>CBV, g</td>
</tr>
<tr>
<td>ICD patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Present</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>2 Present</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3 Present</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4 Present</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>5 Present</td>
<td>3</td>
<td>2.3</td>
</tr>
<tr>
<td>6 Present</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7 Present</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>8 Present</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>9 Present</td>
<td>0</td>
<td>0_1</td>
</tr>
<tr>
<td>Volvulus patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Absent</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2 Absent</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 Absent</td>
<td>0</td>
<td>0_1</td>
</tr>
<tr>
<td>4 Absent</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 Absent</td>
<td>0</td>
<td>0_1</td>
</tr>
<tr>
<td>6 Absent</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

_c, crypt epithelium; CBV, Coxsackie B virus; CD, Crohn’s disease; CISH, chromogen in situ hybridization; g, ganglia; ICD, ileocecal Crohn’s disease; IHC, immunohistochemistry._

Immunofluorescence staining of ileocecal resections and subsequent confocal microscopy revealed positive staining for CAR in both crypt epithelial cells and myenteric ganglia (Figure 4). The staining pattern of CAR in ganglia was membranous and was observed in both glial cells and neurons; the latter was verified by co-staining for the microtubule-associated protein 2 found in neurons. These results reveal a mechanism by which CBV could enter the enteric nervous system.
Figure 4. Immunofluorescence analysis of the expression of the Coxsackie and adenovirus receptor (CAR) in the mucosa and submucosa of the ileocecal region in patients with ileocecal Crohn’s disease (ICD). Representative high-magnification images showing positive staining for CAR in (a) tight junctions and (c) negative staining for microtubule-associated protein 2 (MAP2), a marker for nerve cell ganglia, in crypt epithelial cells (C) of the mucosa (e is a merged image of a and c). Representative high-magnification images showing positive membrane staining for (b) CAR and (d) positive cytoplasmic staining for MAP2, in myenteric ganglia (G) of the submucosa (f is a merged image of b and d).

Study II

We used untargeted mass spectrometry to map the mucosal and plasma metabolomes in the study group. Unsupervised multivariate analyses compared treatment-naïve paediatric patients with incident CD and UC with symptomatic non-IBD controls, stratified based on biological specimen, i.e., plasma, ileal and colonic biopsies. PCA plots showed a discrete separation between CD patients with ileal involvement (L1/L3) and non-IBD controls (Figure 5A). When colonic biopsies were examined, the CD patients with inflamed colonic mucosa (L2/L3) and non-IBD controls were partly separated (Figure 5B), whereas CD patients with ileal involvement (L1) were highly similar to
non-IBD controls. Plasma analyses did not show any clear patterns or separations between the investigated groups (Figure 5C).

**Figure 5.** Principle component analysis (PCA) for the metabolomics data showing the degree of discrimination between children with Crohn’s disease (phenotypes L1–L3) and symptomatic non-inflammatory bowel disease (nIBD) controls in A) ileal biopsies, B) colonic biopsies, and C) plasma samples. The lower row shows the corresponding PCA for children with ulcerative colitis (UC) versus symptomatic nIBD controls in D) ileal biopsies, E) colonic biopsies and F) plasma samples.

A comparison of inflamed colonic biopsies from UC patients and biopsies from non-IBD controls revealed clear separation (Figure 5E), whereas no separation was observed in plasma or ileal samples for UC patients (Figure 5D, 5F). Collectively, these unsupervised analyses showed some disease-associated metabolomic profiles in inflamed mucosa of CD and UC patients, but not in non-inflamed tissue or plasma.

Next, we examined levels of individual metabolites stratified by type of biological material.
CD, Crohn’s disease; UC, ulcerative colitis.

Red indicates decreased levels and blue increased levels compared with non-IBD controls. In the univariate analyses, there were no significantly altered metabolites in ileal biopsies in UC patients or in plasma samples in either CD or UC patients. The data are false discovery rate-adjusted p-values for each metabolite.

Univariable results of stratified analyses by biological material are shown in Table 3. Several dysregulated metabolites were identified in inflamed ileal and colonic biopsies from CD and UC patients as compared with biopsies from symptomatic non-IBD controls, with a fold change of at least 2 and FDR-adjusted p-values < 0.05. In contrast, no metabolites were found to be
significantly altered in the plasma samples. The metabolite class with the greatest alterations was lipids. The subclasses of upregulated lipids were mainly sphingolipids, plasmalogens and glycerophospholipids, whereas most of the downregulated lipids were LPs and monoacylglycerols. In the inflamed colonic samples, some amino acids or their modifications were also upregulated.

Figure 6 Volcano plots of metabolites from A) ileal biopsies, B) colonic biopsies and C) plasma samples in ulcerative colitis patients compared with symptomatic non-inflammatory bowel disease controls. Altered metabolites are annotated and were selected based on log₂-fold change (≥2) and significance (false discovery rate-adjusted p < 0.05). The last three panels show mean (+/-3 standard deviations) levels of the top four altered metabolites for each comparison for D) ileum, E) colon and F) plasma.

Lastly, we aimed at identifying disease-associated metabolites at the site of inflammation, i.e., colonic or ileal mucosa, which correlated with plasma metabolites. In CD patients versus non-IBD controls, our DIABLO model with
integration of metabolites from inflamed ileum and plasma had an error rate of 15%, with 16 and 8 ileal metabolites and 8 and 5 plasma metabolites in components 1 and 2, respectively. The corresponding model of inflamed colonic and plasma metabolites comprised two components with 10 and 5 colonic metabolites and 5 and 5 plasma metabolites, respectively. The error rate of this model was 4%. For UC, the final model, integrating colon and plasma metabolites, included two components with 12 colon metabolites and 18 plasma metabolites and had an error rate of 13%. The error rate for the model with ileum and plasma metabolites in UC was 16% and included 30 ileal and 14 plasma metabolites.

Study III

The lipidomics measurements yielded 663 plasma lipid species in the discovery cohort and 687 serum lipid species in the validation cohort, of which 169 were matched to known annotations, passed quality control, and were found in both cohorts. These 169 annotated individual lipids represented a broad range of lipid compound classes, including diacylglycerols, triacylglycerols, phosphatidylcholines (PCs), phosphatidylethanolamines, phosphatidylserines, phosphatidylinositols, ceramides, lactosyl- or hexosylceramides and sphingomyelins.

We identified 45 altered molecular lipids in our univariable comparison of IBD patients with symptomatic controls in the discovery cohort. With the use of machine learning, a diagnostic lipidomic signature that distinguished patients with IBD from symptomatic controls was identified. The SCAD model for distinguishing patients with IBD from symptomatic controls comprised 30 lipids (Figure 7).
Figure 7. Molecular lipid signatures of IBD. Variable selection of diagnostic lipidomic signatures using the SCAD model in the discovery cohort (N = 94). The bars represent the effect estimates (beta [95% CI]) of the corresponding molecular lipids selected by the model during 5-fold cross-validation. The left and right lines of the boxes indicate the first and third quartiles, the lines in the middle represent the median, and the whiskers extend to the most extreme points within 1.5 times of the interquartile range. Information about the variable importance projection (VIP, %) for each molecular lipid is provided on the right-hand side of each forest plot. (a) Through the comparison of IBD patients vs SC, a diagnostic lipidomics signature consisting of 30 molecular lipids was selected. Abbreviations: IBD, inflammatory bowel disease; SC, symptomatic controls.

The diagnostic 30-lipid signature was validated in the IBSEN III cohort, achieving an AUC of 0.85 (95% CI 0.77–0.92) in distinguishing patients with IBD from symptomatic controls (Table 4). This model had a diagnostic accuracy that was significantly higher than high-sensitivity CRP (hsCRP) (AUC = 0.73, 95% CI 0.63-0.82, P < 0.001).
Table 4 Diagnostic accuracy (area under the curve, AUC) of hsCRP and lipidomic signatures in predicting paediatric inflammatory bowel disease in the validation cohort.

<table>
<thead>
<tr>
<th>Evaluated models</th>
<th>AUC (95% CI)</th>
<th>P-value vs hsCRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsCRP</td>
<td>0.73 (0.63–0.82)</td>
<td>reference</td>
</tr>
<tr>
<td>Full model (30 molecular lipid species)</td>
<td>0.85 (0.77–0.92)</td>
<td>0.001</td>
</tr>
<tr>
<td>LacCer(d18:1/16:0)</td>
<td>0.76 (0.67–0.84)</td>
<td>0.53</td>
</tr>
<tr>
<td>hsCRP + LacCer(d18:1/16:0)</td>
<td>0.79 (0.70–0.87)</td>
<td>0.09</td>
</tr>
<tr>
<td>PC(18:0p/22:6)</td>
<td>0.71 (0.61–0.81)</td>
<td>0.80</td>
</tr>
<tr>
<td>hsCRP + PC(18:0p/22:6)</td>
<td>0.76 (0.66–0.85)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PC(18:0p/22:6) + LacCer(d18:1/16:0)</td>
<td>0.86 (0.78–0.92)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>hsCRP + LacCer(d18:1/16:0) + PC(18:0p/22:6)</td>
<td>0.86 (0.77–0.92)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Abbreviations: AUC, area under the curve; CI, confidence interval; hsCRP, high-sensitivity C-reactive protein.

In clinical practice, a diagnostic 30-lipid signature would not be useful. Therefore, we went back to the discovery cohort and applied forward stepwise logistic regression to evaluate the potential for establishing a shorter lipidomic signature for distinguishing patients with IBD from symptomatic controls.

We found the highest diagnostic accuracy for a short lipidomic signature of only two lipids, LacCer(d18:1/16:0) and PC(18:0p/22:6) (AUC = 0.93, 95% CI 0.87–0.98). Applying this two-lipid signature to the validation cohort yielded an AUC of 0.86 (95% CI 0.78–0.92) (Table 4, with a sensitivity of 84% and a specificity of 78%. Compared with this two-lipid signature, the diagnostic accuracy of hsCRP was significantly lower (AUC 0.73, P < 0.001, Table 4), with a sensitivity of 68% and a specificity of 70%. The comparison with f-calprotectin was limited by the fact that one-third (39/117) of the patients in the IBSEN III cohort did not provide a faecal sample. Among those who did (n = 77), there was no statistically significant difference between the diagnostic performance of the two-lipid signature (AUC = 0.88 95% CI 0.80–0.95) and that of f-calprotectin (AUC = 0.93, 95% CI 0.87–0.99, P = 0.22).
Figure 8. Receiver operating characteristic (ROC) curve illustrating the diagnostic prediction of paediatric inflammatory bowel disease (IBD) in the validation cohort using logistic regression. Abbreviations: BMI, body mass index; hsCRP, high-sensitivity C-reactive protein.

The last step was to confirm the two-lipid signature in a third, independent paediatric inception cohort, using targeted quantification employing calibration curves and surrogate internal standard liquid chromatography coupled to multiple reaction monitoring tandem mass spectrometry. Both molecular lipids were confirmed to be significantly different in the comparison of patients with IBD vs symptomatic controls in the third cohort (\(\beta\)LacCer(d18:1/16:0) = 1.08, 95% CI 0.73–1.42, \(P < 0.001\); \(\beta\)PC(18:1p/22:6) = -0.55, 95% CI -0.83 to -0.27, \(P < 0.001\)) (Figures 6a-b).

**Study IV**

Clinical remission (sPCDAI 0–10) was achieved in 15/22 patients (68%). Fecal protectin was available at follow-up endoscopy post-EEN in 12/20 patients, and was reduced by >50% in 8/12 patients (67%). Endoscopic response (SES-CD reduction >50%) was achieved in 10/22 patients (45%). Endoscopic deep remission (SES-CD ≤ 2) was achieved in 8/22 patients (36%). Of children with ileal inflammation at baseline, 14/20 (70%) had segmental SES-CD ≤ 2 at follow-up endoscopy, whereas 3/9 patients (33%) with transverse colonic
inflammation at baseline had segmental SES-CD ≤ 2 at follow-up endoscopy post-EEN.

To assess the effect of EEN on the overall paediatric CD metabolome, baseline and follow-up samples from all EEN-treated CD patients were compared. The analyses were stratified by specimen type, examining plasma, ileal and colonic biopsies separately. PCA plots, shown in Figures 9A-C, did not reveal any clear separations between pre- and post-EEN samples.

![Figure 9. Principle component analysis (PCA) for the metabolomics data showing the degree of discrimination between samples obtained at baseline and after 7–8 weeks of exclusive enteral nutrition (EEN) treatment from A) ileum and, B) colon and C) plasma in children with incident Crohn's disease.](image)

To evaluate the metabolomic effect of EEN on mucosal inflammation based on treatment response, we compared the metabolomic profiles of inflamed baseline biopsies with their corresponding follow-up biopsies, stratified by treatment response, i.e., endoscopic remission or not. In this analysis, we added samples from symptomatic non-IBD controls as a reference group.

The PCA for ileal biopsies revealed a slight tendency for separation between inflamed and non-inflamed baseline samples, whereas no clear separations were observed in the PCA for colonic biopsies and plasma samples, respectively (Figures 10A-C).
A hierarchical cluster analysis of the top 50 differentially regulated baseline metabolites from the analysis of ileal tissue revealed distinct clustering between inflamed baseline biopsies and matched non-inflamed follow-up biopsies. A similar clustering was observed for the corresponding plasma samples, whereas no clear separations of groups were seen in the corresponding cluster analysis of colonic biopsies. (Figures 10A-C).
Figure 10. Principle component analysis (PCA) and heat maps of the top 50 metabolites for the global metabolomics data showing the degree of discrimination between inflamed mucosal biopsies obtained at baseline and follow-up biopsies after 7–8 weeks of exclusive enteral nutrition (EEN) treatment. Data stratified by presence of macroscopic inflammation (pEEN_INF) or not (pEEN_nINF) at follow-up from A) ileum, B) colon and C) the corresponding plasma samples in children with incident Crohn's disease. Of note, inflamed ileal biopsies after EEN treatment (pEEN_INF) were available from only one patient, therefore not included in the analysis shown in A).

The observation that the heat maps of the top 50 altered metabolites showed a distinct separation in both ileum and plasma before and after EEN treatment when stratified for presence of mucosal inflammation prompted us to examine individual metabolites.

In these univariable analyses of biopsies and plasma samples, a general pattern of downregulation of multiple metabolic compounds was observed in non-inflamed ileal biopsies after 7–8 weeks of EEN therapy compared with at baseline (Figures 11A-C). The post-EEN mucosal metabolome in these non-inflamed ileal biopsies was dominated by a general decrease in lipids, including several sub-classes of bioactive lipids, such as LPs and sphingolipids. In contrast, both upregulation and downregulation of compounds were observed in post-EEN non-inflamed colonic biopsies and plasma samples.

Nine metabolic compounds maintained statistical significance after adjustment for multiple comparisons with a fold change of at least 2 and FDR-adjusted p-values below 0.05 (Table 5). Except for one decreased ileal metabolite, N1,N8-acetylsperrmidine, all significantly altered metabolites post-EEN were detected in plasma; among these were increased levels of gamma-CEHC (vitamin E), pantothenate (vitamin B5), and pyridoxate (vitamin B6), as well as phosphatidylcholine 1-oleoyl-2-linoleoyl-GPC (18:1/18:2). The plasmalogens 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4) was decreased.

In order to contextualise the findings, the post-EEN non-inflamed metabolomes of CD patients were compared with the corresponding metabolomes of symptomatic non-IBD controls. Volcano plots for ileum, colon and plasma are presented in Figures 11D-F.

Also in this comparison, a generalised downregulation of metabolites, dominated by lipid reduction, was observed in non-inflamed ileal biopsies post-EEN (Figure 11D). Both upregulation and downregulation of metabolites were observed in plasma samples and non-inflamed colonic biopsies post-EEN compared with symptomatic non-IBD controls. FDR-adjusted results from these analyses are listed in Table 5.
Figure 11. Volcano plots of metabolites obtained from patients with incident Crohn's disease and symptomatic non-IBD controls.  
A) Non-inflamed ileal biopsies after 7–8 weeks of EEN vs inflamed ileal biopsies at baseline [raw p-values < 0.05].  
B) Non-inflamed colonic biopsies after 7–8 weeks of EEN vs inflamed colonic biopsies at baseline [raw p-values < 0.05].  
C) Plasma samples from CD children without endoscopic inflammation post-EEN vs plasma samples from CD children at baseline, [raw p-values < 0.05].  
D) Non-inflamed ileal biopsies after 7–8 weeks of EEN vs non-inflamed ileal biopsies from symptomatic non-IBD controls [raw p-values < 0.05].  
E) Non-inflamed colonic biopsies after 7–8 weeks of EEN vs non-inflamed colonic biopsies from symptomatic non-IBD controls [raw p-values < 0.05].  
F) Plasma samples from CD children without endoscopic inflammation post-EEN vs plasma samples from symptomatic non-IBD controls [raw p-values < 0.05].
Table 5. Univariable analyses of single metabolites in non-inflamed biopsies from ileum and plasma samples in Crohn’s disease patients after EEN showing statistically significantly altered metabolites and their compound classes when compared with a) corresponding samples collected at the diagnostic endoscopy before EEN, and b) symptomatic non-inflammatory bowel disease (non-IBD) controls.

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<th>PLASMA</th>
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Discussion

A common thought in Studies I, II and IV was that mechanistic IBD research at some point must ‘dig’ where the pathogenic processes take place, i.e., in the mucosa of the human gastrointestinal tract. Using clinically well-characterised prospective PIBD inception cohorts in Studies II–IV increased the chances of obtaining relevant, unconfounded research results.

Each study gained some new information, discussed in detail in the specific sections below. However, none of the four studies had the resources, design or power to deliver any major gap-filling knowledge. Still, the results might provide information that in addition to future research will ultimately contribute to an improved understanding and management of PIBD.

Study I

This study was based on the hypothesis that RNA viruses with a tropism for the gastrointestinal tract, such as viruses of the human enterovirus B family (HEV-B), may have pathogenic roles in the development of IBD. This limited retrospective study was not designed for hypothesis testing, but overall the results were supportive rather than contradictory. With IHC, we were able to demonstrate the presence of Coxsackie virus B (CVB) and echovirus, two HEV-B species, in ileocaecal resections from all patients with childhood-onset CD and advanced ileocaecal disease. No viral presence could be detected in any of the patients in the control group of adult volvulus patients. For the IHC, we used different HEV-B antibodies, both those broadly directed against several different subspecies of HEV-B and those specific for CBV and echovirus. Virus findings were validated by the findings of positive viral footprints. In addition, the presence of HEV-B at the RNA level was validated through CISH.

HEV-B was detected in both epithelial cells and neuronal ganglia of the enteric nervous system, consistent with the known tropism of these viruses for both the human central nervous system and the gastrointestinal tract\textsuperscript{63}. We also showed that the specific cellular receptor for CBV, CAR, is expressed both in TJs between intestinal epithelial cells and in enteric nervous system neurons in myenteric ganglia. CAR expression in the intestinal epithelium is known from previous studies\textsuperscript{60,61}. That CAR is also expressed in myenteric ganglia in
the nervous system has not previously been described in humans. This finding thus suggests a possible mechanism for CBV entry into the enteric nervous system. The perinuclear distribution of HEV-B in neurons and positive IHC for negative-stranded RNA virus indicate a possibility that HEV-B can also replicate in the enteric nervous system. HEV-B has previously been shown to replicate, albeit at a slow rate, in non-dividing cells, such as neurons\textsuperscript{63}. In addition, HEV-B can establish latent infections in human myocytes and persist as double-stranded RNA\textsuperscript{69}. Together with our current findings, there is thus some support suggesting that HEV-B infections could persist in the enteric nervous system and possibly also be reactivated under certain conditions\textsuperscript{59,63,69}.

IHC was also performed on endoscopic biopsies collected at the time of CD diagnosis. All CD patients showed clear epithelial positivity for HEV-B, whereas specific positivity for CVB and echovirus could only be seen in four of these 15 patients. This indicated presence of mixed subtypes of HEV-B in the intestinal mucosa of the CD patients already at the time of diagnosis, and could be interpreted as suggesting that the virus presence at the time of surgery was not necessarily caused by the then-ongoing immunosuppressive treatment.

All but one CD patient had disease-associated polymorphisms in NOD2 or \textit{ATG16L1} or both. Heterozygous mutations in these specific single nucleotide polymorphisms of NOD2 are associated with an increased risk of developing CD, which is even higher in patients with homozygous or compound heterozygous mutations. Ideström et al. reported that NOD2 mutations are rare in Swedish children with CD, and the high frequency of NOD2 mutations in our study may reflect that NOD2 mutations are more common in ileocecal CD. Four of the CD patients were homozygous (GG) for the risk allele in \textit{ATG16L1}, which is associated with a more than threefold increased risk of developing CD compared with heterozygous mutations\textsuperscript{11,26,27,95}.

It is possible that persistent or recurrent infections with HEV-B in the gastrointestinal tract may contribute to a dysfunctional intestinal barrier\textsuperscript{68,69}. In CD, increased paracellular permeability is well described. Barrier dysfunction leads to increased paracellular permeability, which in turn is associated with loss of function or altered expression of TJ proteins\textsuperscript{58,59}.

Taken together, the results of this study provide some support for the hypothesis that ssRNA viruses of the HEV-B family may have a pathogenetic role in paediatric CD.

Studies II and IV

In these studies, we explored the plasma and mucosal metabolomes in newly diagnosed children with IBD; the first was a baseline study with samples from the diagnostic endoscopy, whereas the latter was a follow-up study of a
subcohort from the first study with CD patients who had received EEN in monotherapy. To our knowledge, these two studies are the first untargeted explorations of the mucosal metabolomes in biopsies collected at the diagnostic endoscopy for paediatric IBD, as well as at a follow-up endoscopy after EEN in patients with newly diagnosed paediatric CD.

In Study II, we demonstrated specific patterns of metabolomic alterations in the inflamed mucosa of treatment-naive children with new-onset IBD compared with symptomatic non-IBD controls. The main findings were alterations in two compound classes comprising many bioactive lipids, decreased levels of LPs in inflamed ileal and colonic biopsies from children with CD, and altered levels of sphingolipids in inflamed ileal and colonic biopsies from children with both UC and CD. Correlations were shown between the mucosal metabolomes, plasma metabolites and inflammatory proteins.

LPs are a complex class of lipids with several subclasses and a large number of species, of which many have bioactive functions – see the background section for details. In both Study II and Study IV, we observed reduced mucosal levels of several lipid species of the LPC, LPE, LPI and LPSe subclasses in children with CD, both in inflamed ileum at baseline and in non-inflamed ileum after EEN. In Study II, the integrated correlation analyses showed that in CD patients at baseline, ileal LPC, LPE and LPI levels correlated positively with plasma levels of N-acetyltryptophan, N-acetylleucine, cysteinylglycine and glycerate. Furthermore, in CD patients, colonic LPC and LPI levels correlated positively with plasma levels of amino acid analogues, trimethylamine N-oxide and hexadecadienoate (16:2n6). These findings in Study II together indicate that LP dysregulation in inflamed mucosa is associated with systemic metabolic alterations in children with new-onset CD. In contrast, no statistically significant LP alterations were identified in children with UC.

In Study II, the decreased LP species were solely of sn-1 types, indicating that disease onset of CD was associated with reduced PLA2 enzymatic activity, as PLA2 deacetylates membrane-bound phospholipids into free sn-1 LPs and free polyunsaturated fatty acids.41 The observed decreased colonic levels of polyunsaturated fatty acid linoleate (18:2n6), and the increase of some phospholipid precursors (phosphatidylcholine (PC) 16:0/16:0, PC 16:0/18:0, PC 16:0/20:4n6, phosphatidylethanolamine 16:0/20:4) further supported this hypothesis.

Altered sphingolipids was the other dominant IBD-related metabolomic pattern in Study II, where we observed an increase in several mucosal sphingolipids in inflamed mucosa in both CD and UC. In line with recent reports from both paediatric and adult IBD populations,48,55,46 we observed an increase in LacCer(d18:1/16:0) in inflamed mucosa of children with CD and UC. In UC, the correlation analysis also identified positive correlations of mucosal LacCer(d18:1/16:0) with its plasma levels and with three plasma bile acids. Further, we observed a decrease of N-palmitoyl-phytosphingosine (t18:0/16:0) in inflamed mucosa of children with UC and CD compared with
in non-IBD controls. These associations were supported by the PLS-DA, where levels of LacCer(d18:1/16:0) and N-palmitoyl-phytosphingosine (t18:0/16:0) in colonic biopsies were identified as the most important variables for distinguishing children with UC from non-IBD controls. In UC, mucosal levels of N-palmitoyl-phytosphingosine (t18:0/16:0) negatively correlated with three plasma bile acids, and this finding was in line with a recent report. A pathophysiological role of sphingolipids in IBD was further supported by the results of the multivariable analyses, showing correlations between CD mucosal sphingolipid levels and plasma levels of various metabolites, including sphingolipids, bile acids and N-acetylglucosamine/N-acetylgalactosamine. Impaired N-acetylation of glucosamine and metabolic perturbations of other N-acetylated metabolites have previously been linked to IBD. In line with this observation, we identified correlations between plasma levels of several N-acetylated metabolites, including N-acetylleucine, and metabolomic alterations of both lysophospholipids and sphingolipids in ileal mucosa of patients with CD.

In Study IV, we found that EEN in monotherapy was more effective in ileal CD with a segmental endoscopic remission rate of 70% compared with 40% in the transverse colon. Based on the main finding in Study II of a mucosal metabolome characterised by a reduction of LPs in inflamed ileal biopsies, we had expected a normalisation of the mucosal lipidome in EEN-induced endoscopic remission in the ileum. We were therefore first surprised to find a generalised downregulation of the non-inflamed ileal mucosal metabolism compared with at baseline after successful remission induction with EEN. In colonic biopsies, the findings were less clear. Obviously, the metabolomic pattern associated with mucosal inflammation in the terminal ileum at baseline was not directly resolved through EEN. On the contrary, a consistent and even more pronounced reduction of LP and other lipid classes such as sphingolipids, was the dominating pattern of the post-EEN mucosal metabolome in non-inflamed ileum. When we compared the metabolomes in biopsies and plasma in patients with endoscopic remission after EEN with the corresponding samples from the symptomatic non-IBD controls, a similar pattern appeared. In this comparison too, the post-EEN ileal metabolome was downregulated, and most of the reduced ileal compounds were lipids, including 14 different species of significantly reduced LPs.

This generalised metabolic downregulation in ileal mucosa after EEN-induced endoscopic remission may seem paradoxical. However, the findings are in line with a number of studies of the faecal gut microbiota that have shown that EEN is associated with a reduction in the number, richness and diversity of faecal microbial species. In other words, in these studies, EEN induced or enhanced a dysbiotic intestinal ecosystem that has been associated with active CD. Many of these studies were conducted based on the hypothesis that EEN exerts its effect by normalising the intestinal microbiota. However, a reduced bacterial load and diversity after EEN should not be surprising, instead
it is a logical consequence of the altered nutritional situation with EEN, including an expected reduction of luminal energy supply in the distal ileum and colon. An alternative but less explored hypothesis for the action mechanism of EEN in CD focus on cellular starvation, i.e. that not only the microflora is stressed by energy depletion, but also the enterocytes in the distal ileum and colon, as the epithelial cells in both these intestinal segments depend on luminal energy uptake. The cellular stress caused by starvation induces autophagy, which may be beneficial for the anti-inflammatory response in CD. In this perspective, our findings of a generalised downregulation of ileal mucosal metabolism during EEN appear as a consequential adaptation to a general energy depletion of the intestinal ecosystem.

Collectively, these observations associate EEN-induced remission in paediatric CD patients with a general downregulation of the lipid metabolism in the mucosa of the terminal ileum, and the mucosa-associated microbiota. This suggests that the anti-inflammatory effects of EEN are at least partly mediated by manipulation of the intestinal ecosystem including a downregulation of the mucosal metabolism at the ileal level.

Furthermore, the findings in this study suggest that analyses of mucosal biopsies from multiple segments are needed in order to advance the understanding of the action mechanisms of EEN and other dietary interventions in CD and other diseases. A biopsy from a single mucosal segment cannot reflect the segmental variation in interventional impact on the intestinal ecosystem and its metabolic response – and a faecal sample even less.

The few metabolomic EEN studies reported thus far have been performed on faecal and blood samples, and their findings are scarce and often inconsistent. In an untargeted study of the plasma metabolome in 14 paediatric CD patients, an EEN-associated increase in several diet-related plasma metabolites was reported. We also noted significant changes in some diet-related compounds in plasma at endoscopic remission after EEN, such as increased levels of gamma-CEHC (vitamin E), pantothenate (vitamin B5) and pyridoxate (vitamin B6) in children who achieved EEN-induced endoscopic remission. Low levels of circulating vitamin E have been associated with active CD in children. Reduced pyridoxate levels are common in CD patients, and also associated with a more severe disease course. Both pantothenate and pyridoxate are ingredients of the nutrition drinks used for EEN therapy, and the observed increase of these plasma metabolites in our study thus indicated adherence to EEN. Phosphatidylcholine 1-oleoyl-2-linoleoyl-GPC (18:1/18:2) was increased, whereas the plasmalogen 1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4) was decreased. In Study II, phosphatidylcholine 1-oleoyl-2-linoleoyl-GPC (18:1/18:2) was decreased in colonic biopsies of children with new-onset IBD, which was coherent with our finding of increased systemic levels in EEN-induced CD remission. The plasmalogen has been described as a systemic biomarker for red meat consumption, and obviously, this reduction was an expected effect of an eight-week course of meat-free EEN.
In ileal mucosa, a significant decrease in N1,N8-acetylsppermidine was observed after EEN treatment. This polyamine metabolite is involved in intracellular ageing processes by inducing autophagy, and increased levels are also a biomarker of several cancers and Parkinson’s disease\textsuperscript{104,105}. Although autophagy is a key pathophysiological process in IBD, an association of N1,N8-acetylsppermidine with IBD is novel.

**Study III**

In this study, we used untargeted mass spectrometry-based lipidomics to identify a diagnostic signature of PIBD with only two molecular lipid species, i.e., LacCer(d18:1/16:0) and PC(18:0p/22:6). This was superior to hsCRP in distinguishing paediatric patients with IBD from symptomatic controls. The two-lipid signature demonstrated an improved negative predictive value (NPV) to rule out PIBD when compared with hsCRP at its clinically established cut-off (NPV 76% vs 40%). The diagnostic performance of the two-lipid signature was similar to that of f-calprotectin among patients who had provided a faecal sample. However, a blood-based test may be preferred to a faecal test by children and teenagers. By using three independent paediatric inception cohorts for discovery, validation and confirmation, the diagnostic signature was shown to be robust and consistent across different populations.

Thus, the results of this study implied that this diagnostic two-lipid signature should have the potential to complement the current biomarkers in everyday clinical work-ups of children and adolescents presenting with gastrointestinal symptoms suggestive of IBD.

The pathophysiological role for LacCer(18:1/16:0) remains to be elucidated. In Study II, we demonstrated significantly increased levels of LacCer(18:1/16:0) in inflamed mucosal biopsies in both colon and ileum in children with newly diagnosed IBD compared with symptomatic non-IBD controls. However, plasma levels were not significantly altered, which may be explained by lack of statistical power. Other studies have reported increased levels of LacCer(18:1/16:0) in both serum and faecal samples of IBD patients. Taken together, these findings suggest that LacCer(18:1/16:0) is both a relevant biomarker for mucosal inflammation in IBD and a target for further mechanistic IBD research.

We have not found any reports linking IBD to decreased levels of PC(18:0p/22:6), and the physiological role of this specific lipid compound is unclear. PCs are not only the main structural components of biological membranes but are also involved in cellular signalling\textsuperscript{41,42}. More specifically, this long-chain unsaturated plasmalogens consists of one chain of plasmalogen and one chain of omega-3 docosahexaenoic acid. Plasmalogen PCs have structural roles in cell membranes and function as endogenous antioxidants. Some species have known functions as bioactive lipids involved in cell differentiation.
and signalling pathways\textsuperscript{41,42,106}. Reduced levels of other PCs and LPs, including plasmalogens and other alkyl ether PCs, have been reported in Study II and other studies of IBD patients, in both plasma and mucosal biopsies\textsuperscript{46,47,102}. 
Strengths and limitations

The two main limitations of this thesis are a) the lack of healthy controls in all four studies, and b) the limited statistical power due to small cohort and sample sizes, particularly in Studies I, II, IV, less so for Study III. For obvious ethical reasons, intestinal tissue from healthy children is not obtainable; cohort and sample sizes were restricted by the single-centre approach. Regardless of the reasons, these limitations contributed to a limited possibility of drawing well-founded conclusions.

Study I

The retrospective design, treatment with different anti-inflammatory drugs and the small study group were major limitations. IHC has methodological limitations in terms of specificity, and investigator-dependent visual assessment was a source of potential bias. The lack of an age-matched healthy control group was a limitation of the study, although the complete lack of positive staining for HEV-B in the control group strengthened the specificity of the positive results in the study group.

The use of different laboratory methods was a strength, as was the detection of HEV-B with the two independent methods IHC and CISH.

Studies II and IV

The small cohorts created one major limitation. In Study II, this was especially the case for the UC sub-group and the group of symptomatic non-IBD controls. In Study IV, the entire study cohort was small, and subgrouping for EEN treatment response resulted in minimal subgroups. The lack of healthy control groups was another major limitation, and the absence of validation cohorts called the generalisability of the results into question. A lack of gut microbiome data was a limitation in both studies.

The prospective design and use of a PIBD inception cohort, with sample collection before treatment, were main strengths of both studies. The unique design of Study IV was a major strength, with baseline endoscopy sampling, use of EEN in strict monotherapy, a repeat sampling at the follow-up
endoscopy performed before re-introduction of normal food. This meant that biopsies and plasma samples were obtained at both baseline and follow-up. Using the Metabolon platform in both studies allowed assessment of a large set of metabolites, providing comprehensive coverage of metabolic pathways.

Study III

This was the statistically most well-powered study in the thesis. Despite this, the number of patients within each Paris phenotype subgroup was insufficient for stratified analyses of CD or UC phenotypes. Because of prospective patient recruitment, matching IBD patients with symptomatic non-IBD controls by sex, age and date of sampling was not possible. The use of both serum and plasma in different cohorts was methodologically questionable.

The use of three independent inception cohorts for discovery, validation and confirmation was a major strength. Consistent use of strict diagnostic criteria and the prospective collection of samples before treatment were other strengths. In contrast to the other studies in the thesis, the control group of symptomatic non-IBD patients was a relative advantage in this study, as it showed the clinical relevance of the diagnostic two-lipid signature.
Conclusions

Study I
This study demonstrated the presence of HEV-B both in the intestinal mucosa and in the enteric nervous system of patients with severe ileocecal CD. Furthermore, we showed that CAR, the receptor for CBV, is expressed both in the intestinal mucosa and in the ganglia of the enteric nervous system. Further studies are warranted to confirm the presence of HEV-B in other CD cohorts, and to determine whether HEV-B has an etiological significance for CD.

Study II
Newly diagnosed PIBD was associated with specific metabolomic alterations in inflamed mucosa compared with in symptomatic non-IBD controls. Perturbations of mucosal levels of LPs and sphingolipids that correlated with plasma metabolites were the most prominent IBD-associated metabolomic patterns. The findings indicate that mucosal lipid metabolism is involved in the pathophysiology at an early stage of PIBD. Studies of mucosal metabolomic patterns provide a basis for improved understanding of IBD pathogenesis.

Study III
In this study, examinations of blood samples from three independent PIBD inception cohorts identified and validated a two-lipid signature, Lac-Cer(d18:1/16:0) and PC(18:0p/22:6), that could distinguish children with IBD from children with symptoms that indicated IBD but who did not have the disease. This blood-based two-lipid signature has a potential of becoming a diagnostic tool to be used in combination with existing established biomarkers in the clinical work-up of suspected PIBD.

Study IV
This study demonstrated that successful remission induction with EEN in newly diagnosed paediatric CD patients, also has an impact on the mucosal
metabolome. EEN was more effective in inducing endoscopic remission in ileum than in colon. In accordance, EEN-induced endoscopic remission was associated with a specific metabolomic pattern in the ileum, i.e. a generalised downregulation of the mucosal metabolism, a pattern that was not seen in the colonic mucosa. The metabolomic pattern in the non-inflamed ileum was dominated by a generalised reduction of lipids, particularly LPs, and the results suggest that downregulation of lipid metabolism could constitute a key mechanism in EEN-induced remission of CD with ileal involvement.
Future perspectives

As previously mentioned, this thesis confirms the necessity of using mucosal tissue from the gastrointestinal tract for mechanistic studies of pathogenic mechanisms in IBD in future research, ideally from large prospective inception cohorts of both paediatric and adult IBD patients. An unsolved problem in that perspective is the lack of healthy controls.

Study I

The future goal of elucidating whether CBV or other HEV-B have any significance for CD aetiology is not easy to achieve. A necessary first step involves studies to confirm the presence of HEV-B in other cohorts of CD patients. The next step involves well-powered prospective studies, utilising up-to-date sequencing techniques in combination with IHC and CISH, to produce more specific data on the virome in relation to IBD, virus receptors, involvement of the enteric nervous system, et cetera. Furthermore, there is a need for studies to determine whether the current practice of using immunomodulating pharmacological treatment is specifically linked to impaired clearance and persistence of HEV-B or other relevant viruses in patients with IBD. The same applies to CD-related polymorphisms.

Studies II and IV

These studies showed the importance of mucosal metabolomic research to advance the understanding of pathophysiological pathways of IBD in the complex relation to the intestinal ecosystem. The central findings in these studies – that the lipid metabolism at the mucosal level in the ileum is perturbed at diagnosis of paediatric CD, and that the lipid alteration is even more pronounced after successful remission induction with EEN – have indicated researchable paths that promise mechanistic insights regarding both pathogenesis and action mechanisms for nutrition therapies in paediatric CD.
Study III
For clinical translation of the molecular two-lipid signature, method validation as well as stability, repeatability, reproducibility and interlaboratory studies are required for clinical implementation and regulatory approval. Furthermore, clinical cut-offs and corresponding likelihood ratios for various clinical scenarios need to be established. Thus, further work is required to ultimately translate the findings of Study III into an assay for clinical use.
Sammanfattning på svenska

Inflammatorisk tarmsjukdom (inflammatory bowel disease, IBD) är ett samlingsnamn för sjukdomar som orsakar kronisk inflammation i mag-tarmkanalen; de två vanligaste diagnoserna är Crohns sjukdom (Crohn’s disease, CD) och Ulcerös colit (UC). Dessa sjukdomar är olika vanliga i olika delar av världen, och det finns ett tydligt samband mellan förekomst av IBD och industrialiseringensgrad, västerländsk livsstil och kosthållning. Sverige utgör tillsammans med övriga västeuropa och nordamerika områden där dessa sjukdomar är särskilt vanliga, och det uppskattas att ungefär en av 40 svenskar utvecklar sjukdomen under sin livstid, varav ungefär en fjärde del innan 18 års ålder.

Sjukdomarna orsakar både generella kroppssymtom och symtom från mag-tarmkanalen, ofta i form av buksmärta och blodig diarré. IBD är naturligt progressiv och leder till successiv försämring vid utebliven behandling. Jämfört med vuxna tenderar barn med IBD att ha ett mer aggressivt sjukdomsförlopp, mer utbredd sjukdom, samt att CD är förhållandevis mer vanlig än UC. Därtill medför IBD i barnomen även risk för specifika barnrelaterade problem, såsom tillväxtlämning, förserad pubertet, utebliven skelettminerlising och nedsatt livskvalitet. Vid misstanke om IBD hos ett barn krävs omfattande medicinsk utredning för att bekräfta eller utesluta diagnos.

IBD kan i dagsläget inte botas. Behandlingen inriktas istället mot att uppnå och bevara remission, d v s att sjukdomen är stabilt inaktiv och patienten lever ett normalt liv utan komplikationer. För att uppnå detta mål krävs i regel kontinuerlig antiinflammatorisk läkemedelsbehandling i kombination som anpassas individuellt för patienten. Ett undantag är så kallad exklusiv enteral nutritionbehandling (EEN), en iclek-farmakologisk näringsbehandling som är effektiv vid CD, men en sådan behandling är krävande för patienten och används endast under en begränsad tid, vanligtvis cirka åtta veckor.

Trots decennier av omfattande IBD-forskning som medför stor kunskap är det långsiktiga målet avläget, d v s att kunna bota IBD liksom att kunna förebygga sjukdomen. Det är fortfarande mycket som är oklart om hur och var för IBD utecklas, även om komplexiteten i sjukdomsorsakande processer är uppenbar. De fyra delstudierna i den här avhandlingen berörde olika kunskapsluckor för IBD hos barn.

Delstudie I: Studien utgick från frågeställningen om en viss form av vanligt förekommande tarmvirus kan ha en roll i sjukdomsutvecklingen av CD.
Studien kunde påvisa förekomst av de aktuella virustyperna i både tarmslmhinan och i nervvävand i tarmväven hos patienter som insjuknat i CD som barn och som senare opererats för tarmförträngning orsakad av sjukdomen. Ytterligare en upptäckt som gjordes var att den proteinstruktur som viruset använder som receptor för att infektera cellen finns i både tarmslmhinnan och i nervvävand i tarmväven.

Delstudie II: Studien syftade till att kartlägga vilka småmolekyler (metaboliter) som finns i tarmslmhinnan och i blodet hos barn som nyinsjuknat i IBD. Vi använde prover från tarmslmhinna och blod från barn som utretts för missänkt IBD, den ena gruppen fick IBD-diagnos medan kontrollgruppen var de som inte hade IBD. Grupperna jämfördes och då hittades en rad sjukdomsrelaterade avvikelser i den inflammerade slmhinnan hos barn med IBD, främst när det gällde olika fettmolekyler (lipider).

Delstudie III: Studiens målsättning var att undersöka om det går att hitta enkel mätbara avvikelser i blodprov hos barn som nyss insjuknat i IBD, som skulle kunna användas för att förbättra och förenkla diagnostiken vid misstanke om IBD hos barn som söker hjälp i primärvård. Vi använde blodprover från tre olika provsamlingsar från barn som nyinsjuknat i IBD, från Uppsala, Oslo, respektive en norsk-dansk-brittisk provsamlning. Sammantaget hittades en enkel så kallad signatur bestående av endast två lipider som med hög säkerhet visades kunna skilja ut barn med IBD från barn med symtom utan IBD.

Delstudie IV: Här byggdes vidare på delstudie II och syftet var att öka kunskapen om verkningsmekanismen bakom EEN vid CD genom att undersöka effekten på tarmslmhinna och blod på barn som nyinsjuknat i CD och som genomgått behandling med EEN. Metoden var densamma som i delstudie II, d v s vi använde avancerade mätmetoder för att kartlägga metaboliter i proverna som tagits för och efter behandling med EEN. Trots att materialet var begränsat fann vi ett mönster av generellt reducerad metabolism samt en minskning av lipidmolekyler i framförallt tunntarmsslmhinna där inflammationsläkt ut med EEN.
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