Transcriptomics and Metabonomics in Precision Medicine of Inflammatory Bowel Disease: Diagnostics, Phenotypes, and Novel Treatment Strategies

By

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The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in Medicine.

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Content

Preface

This dissertation is submitted for evaluation to the Faculty of Health and Medical Sciences at the University of Copenhagen, Denmark, with the purpose of obtaining the degree of Doctor of Medical Science (DMSc).

The thesis is based on research carried out at the Department of Gastroenterology, Herlev Hospital, Denmark, from 2014 to 2022. This research was made possible primarily by patients willing to participate in scientific studies - not for the benefit of themselves but for the benefit of future patient management. Furthermore, research grants were kindly provided by the Danish Agency for Higher Education and Science, Aase og Ejnar Danielsens Fond, Civilingeniør Frode V. Johanne Nygaard og Hustrus Fond, Aage og Johanne Louis-Hansens Fond, Colitis-Crohn Foreningen, Memorial Foundation of Solveig Høymann Jacobsen, and Frimodt-Heineke Fonden.

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Finally, I am especially grateful to my wife, Camilla, and children, Hannah, Ole, and Eva, for always being there for me and letting me satisfy my scientific curiosity and accepting the countless hours I have spent doing research outside regular clinical work.

Summary in English

Inflammatory bowel diseases (IBDs) consist of primarily ulcerative colitis (UC) and Crohn's disease (CD), which are lifelong chronic or recurrent states of intestinal inflammation without a clear etiology or pathophysiology. IBD precision medicine seeks to improve the disease stratification and timing of healthcare through biomarker identification and molecular characterization of disease-specific pathways using omics strategies. The aim of this dissertation is to apply IBD precision medicine using transcriptomics and metabonomics analyses to improve IBD diagnostics and molecularly characterize the inflammatory process, UC-associated mucosal dysplasia, and colonic mucosal wound healing with the ultimate goal of identifying potential novel therapeutic targets.

This dissertation demonstrates that omics-based diagnostic IBD tests are not currently applicable because of insufficient sensitivity and specificity. However, the dissertation also demonstrates that this lack of sensitivity and specificity might be the consequence of a too simple phenotyping of IBD. Thus, the inflammatory process in pancolitis and UC-associated dysplasia are found to be molecularly distinct from those in left-sided UC and consequently should be considered unique subphenotypes needing tailored treatment strategies. To this end, the dissertation also provides potential treatment targets, some of which are currently being tested.

Omics analyses also reveal the existence of quiescent UC as a distinct molecular phenotype at the metabonomics level. This challenges our current clinical treatment goal of mucosal healing, potentially raising the bar and forcing us to reach beyond to achieve molecular healing - that is, if it can be correlated with an improved clinical course of UC in future clinical studies.

To achieve molecular healing in the ulcerated intestines, mucosal wound healing is required. To be able to describe the kinetics and molecularly characterize the mucosal wound-healing process, this dissertation applies a novel human in vivo colonic mucosal wound-healing assay. The model demonstrates the presence of a hyperresponsive innate immune system in the colonic mucosa of patients with UC in remission and a subsequent delayed wound healing that correlates with a distinct lipidomic trajectory. Dampening this hyperresponsiveness and promoting the healing process using lipid-based treatments represent innovative therapeutic avenues that need to be tested and validated.

Serum metabonomics analyses identify a proatherogenic lipid profile in patients with active IBD, and as a consequence, the dissertation recommends that it is time for future IBD treatment algorithms to include advise on lifestyle interventions and statin treatment in young patients with frequent flares or chronically active disease.

With this dissertation, I propose the existence of more detailed molecular phenotypes in IBD, the need for more ambitious treatment goals in terms of molecular healing, and novel wound-healing treatment strategies, which will all support the notion of precision medicine in IBD.

Summary in Danish

Inflammatoriske tarmsygdomme (IBD) udgøres primært af colitis ulcerosa (UC) og Crohns sygdom (CD), som begge er livslange kroniske eller tilbagevendende tilstande af tarmbetændelse uden en klar ætiologi eller patofysiologi. Ved individualiseret IBD-behandling søges sygdomsstratificeringen og timingen af behandlingen forbedret via biomarkøridentifikation og molekylærbiologisk karakterisering af sygdomsspecifikke mekanismer ved hjælp af forskellige omics-strategier. Formålet med denne afhandling er at anvende individualiseret IBD-behandling via transkriptom- og metabonom-analyser for derved at forbedre IBD diagnostikken og molekylærbiologisk karakterisere den inflammatoriske proces, den UC-associeret slimhindedysplasi samt tyktarmsslimhindens sårhelingsproces med det ultimative mål at identificere potentielle nye terapeutiske behandlingsprincipper.

Afhandlingen viser, at omics-baserede diagnostiske IBD-tests ikke synes anvendelige på grund af utilstrækkelig sensitivitet og specificitet. Den viser imidlertid også, at dette formentlig er konsekvensen af en utilstrækkelig fænotyping af IBD. Således findes den inflammatoriske proces ved pancolitis og UC-associeret dysplasi molekylærbiologisk signifikant forskellig fra venstresidig UC, og dermed bør disse betragtes som unikke underfænotyper, som kræver skræddersyede behandlingsstrategier. Til dette formål leverer afhandlingen også potentielle behandlingsmuligheder, hvor af en enkelt er under klinisk afprøvning.

Omics-analyser identificerer også patienter med UC i remission som en særskilt molekylærbiologisk fænotype på metabonomics-niveau. Dette udfordrer vores nuværende kliniske behandlingsmål, som blot er slimhindeheling, idet det potentielt kan hæve barren for behandling såfremt molekylærbiologisk slimhindeheling kan korreleres til forbedring af det kliniske forløb af UC i fremtidige kliniske studier.

For at opnå molekylærbiologisk slimhindeheling i den inflammerede tarm er sårheling påkrævet. Med henblik på at kunne beskrive kinetikken og molekylærbiologisk karakterisere slimhindens sårhelingsprocessen anvender afhandlingen derfor en nyligt udviklet human in vivo sårhelingsmodel. Denne model demonstrerer tilstedeværelsen af et hyperresponsivt innat immunsystem i tyktarmsslimhinden blandt patienter med UC i remission og en efterfølgende forsinket sårheling, som korrelerer med specifikke lipidændringer. Hæmning af denne hyperresponsivitet og understøtning af helingsprocessen ved hjælp af lipidbaseret behandling repræsenterer innovative terapeutiske muligheder, som skal testes og valideres.

Serum-baseret metabonomanalyser identificerede en proatherogen lipidprofil hos patienter med aktiv IBD, og som følge heraf anbefaler denne afhandling, at fremtidige IBD-behandlingsalgoritmer, som noget nyt, bør omfatte rådgivning om livsstilsinterventioner og statinbehandling hos unge patienter med hyppig opblussen eller kronisk aktiv sygdom.

Med denne afhandling foreslår jeg eksistensen af mere detaljerede molekylærbiologiske fænotyper ved IBD, behov for mere ambitiøse behandlingsmål med molekylærbiologisk heling og nye behandlingsstrategier for sårheling, hvilket samlet set vil understøtte individualiseret IBD-behandling.

Abbreviations

ANOVA	analysis of variance
AOM/DSS	azoxymethane/dextran sulfate sodium
AUC	area under the curve
BCAA	branched chain amino acid
CAGE	cap analysis of gene expression
CD	Crohn's disease
CI	confidence interval
CPMG	Carr-Purcell-Meiboom-Gill
CRC	colorectal cancer
eIF4E	eukaryotic initiation factor 4E
FFPE	formalin-fixed paraffin-embedded
FMT	fecal microbiome transplantation
GWAS	genome-wide association study
HB	Harvey-Bradshaw
HR	histologic remission
IBD	inflammatory bowel disease
IBDu	inflammatory bowel disease unclassified
IECs	intestinal epithelial cells
IFX	infliximab
IL	interleukin
INSRA	insulin receptor alpha
IEC	intestinal epithelial cells

IOIBD	International Organization for the Study of IBD
LPC/PC	lyso/phosphatidylcholine
LPA/PA	lyso/phosphatidic acid
MAPK	mitogen-activated protein kinase
MES	Mayo endoscopic score
MS/LC	mass spectrometry/liquid chromatography
MKNK	MAP kinase interacting serine/threonine kinase
NMR	nuclear magnetic resonance
OPLS-DA	orthogonal partial-least-squares regression-discriminant analyses
OR	odds ratio
PBMCs	peripheral blood mononuclear cells
PCA	principal component analysis
PD-L1	programmed death ligand 1
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PGD_2	prostaglandin D_2
PGE_1	prostaglandin E_1
PI	phosphatidylinositol
PI3K	phosphatidylinositide 3-kinase
PS	phosphatidylserine
PSC	primary sclerosing cholangitis
qPCR	quantitative polymerase chain reaction
RIN	RNA integrity number

RT-PCR	real-time polymerase chain reaction
RNA	ribonucleic acid
SCCAI	simple clinical colitis activity index
SCFA	short-chain fatty acid
SES	simplified endoscopic score
SIBDCS	Swiss IBD Cohort Study
STRIDE	Selecting Therapeutic Targets in IBD
SNP	single-nucleotide polymorphism
TNF	tumor necrosis factor
TH	transmural healing
UC	ulcerative colitis
UCEIS	ulcerative colitis endoscopic index score
VOCs	volatile organic compounds

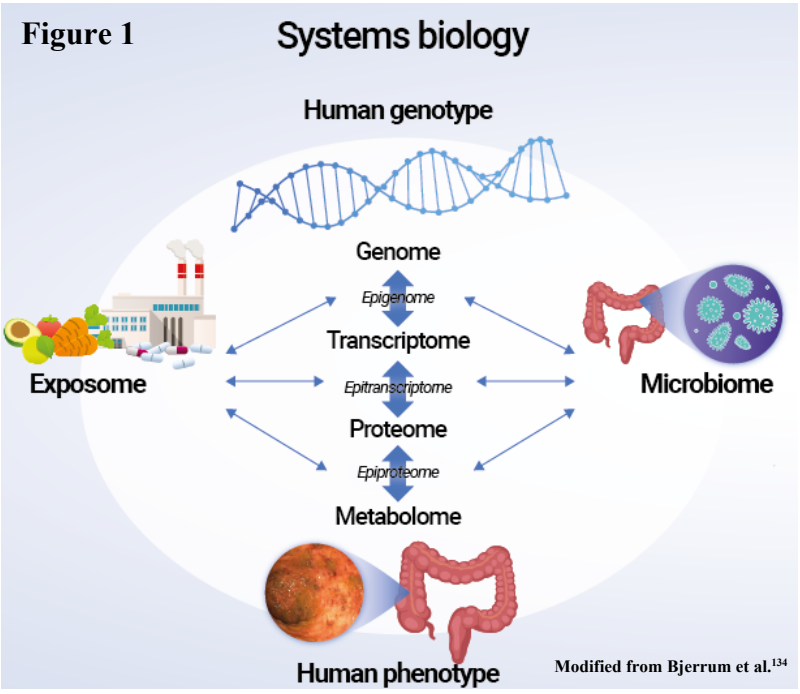
1. Introduction

Ulcerative colitis (UC)[1] and Crohn's disease (CD)[2] are the two main entities of inflammatory bowel disease (IBD), which is a lifelong chronic or recurrent state of intestinal inflammation with symptoms of bloody diarrhea, abdominal distress, and anorexia. The incidence of IBD is increasing worldwide, even though high-income countries seem to have reached a plateau in recent years.[3] IBD is associated with significant healthcare costs, especially within the first year of the diagnosis, because of numerous diagnostic procedures, frequent hospitalizations, and treatment initiation with biologics.[4] Furthermore, long-standing and extensive UC and CD localized to the colon have an approximately two- to threefold increased risk of colorectal cancer (CRC), with the highest incidence among patients with concomitant primary sclerosing cholangitis (PSC).[5] Even IBD patients with a diverted rectum have a 10-fold increased risk of rectal cancer 10 years after colectomy.[6] The etiology of IBD is still unknown but is believed to be the consequence of an imbalanced immunologic response to environmental and microbial components in genetically susceptible individuals.[7]

UC is localized to the colonic mucosa and extends continuously from the rectum to include either the rectosigmoid, the left side of the colon, or more extensively past the left colonic flexure, whereas CD is a transmural segmental inflammation that can be localized anywhere in the gastrointestinal tract and may be complicated by strictures, fistulas, and/or abscesses. Although UC and CD share a range of characteristics, they are evidently two distinct diseases requiring exact diagnostics, differentiation, and phenotyping for tailored successful treatment.[8] However, the diagnostic procedure is hampered by a multidisciplinary approach using biomarkers, radiology, endoscopy, and histology, which are all cumbersome, time consuming, and to some extent inefficient, because the approach leaves ~10% of patients with

the diagnosis of IBD unclassified (IBDu).[9] After five years, this is only reduced to 7%, which potentially has serious clinical consequences because successful clinical management and the patient's quality of life depend on early and correct diagnosis and, accordingly, subsequent treatment.[10]

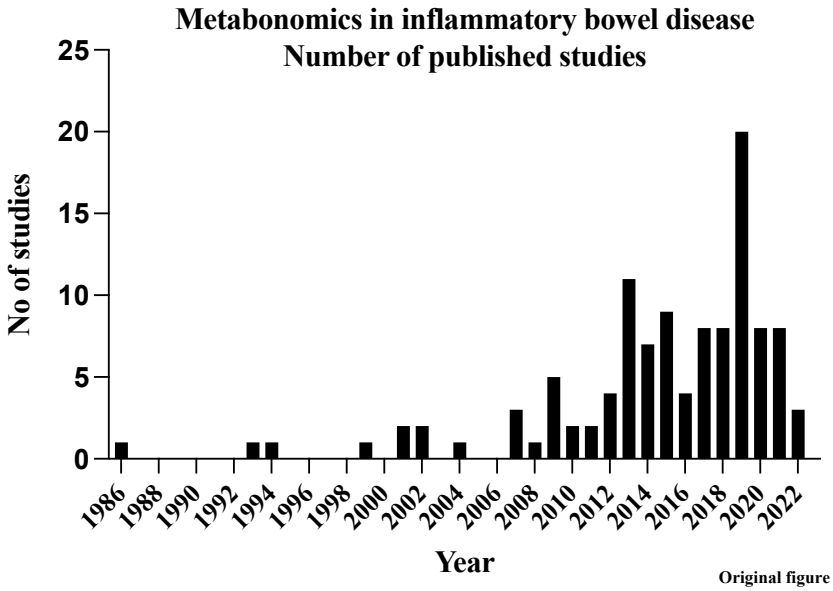
Regardless of the medical therapies applied, clinical response and remission rates are comparable between drugs and only reach approximately 60%, indicating that a therapeutic maximum has been reached with our current treatment regimens, and novel insight into the pathology of IBD and new treatment strategies are needed to overcome this therapeutic maximum. Among these strategies is precision medicine, an important concept that aims to improve stratification and timing of healthcare through biomarker identification and molecular characterization of disease-specific pathways by use of genomics, transcriptomics, proteomics, and metabonomics.[11] This approach, however, is an extremely challenging task because the dissection of genotype-to-phenotype relations is not straightforward (Fig. 1). Genome-wide association studies (GWAS) have identified >240 loci containing IBD-associated variants, but IBD-associated single-nucleotide polymorphisms (SNPs) explain a mere 15%–20% of the disease phenotypes, and ~70% of IBD-associated SNPs are noncoding.[12] Furthermore, integrative metabolome genome-wide analyses



estimate that solely ~50% of total phenotypic differences at the metabolite level are due to genetic variance, however, with a significantly different heritability across metabolite classes.[13] In a recent IBD serum metabolome GWAS, 1292 metabolites were identified, of which 173 were acknowledged as genetically controlled metabolites, but only 17 were notated as being controlled by genetic variants in IBD risk loci.[14] The actual phenotype is consequently the result of multiple downstream regulatory processes that take place from the genome to the metabolome and the concomitant interactions with the exposome and microbiome (Fig. 1), where the latter is increasingly recognized as a significant component of the actual pathogenesis[15] as well as the risk profile of IBD.[16]

Thus, the complexity of the network increases toward the metabolome, which is the entire set of both intermediates and end products of metabolism, that is, the life-sustaining chemical processes of the biological system. Accordingly, the metabolome is highly reflective of the actual (patho-) physiologic phenotype. With this acknowledgment alongside ongoing technological and bioinformatic advances, adult IBD omics research has focused more and more on the downstream "omes," making IBD transcriptomics and especially IBD metabonomics studies (Fig. 2) increasingly relevant in precision medicine.

Figure 2



IBD Transcriptomics

Transcriptomics aims to identify and quantify the complete set of RNA transcripts that are transcribed from the genome in a specific cell or biological system at a given time and under specific circumstances such as disease states or different treatments. As with any other omics, transcriptomics is descriptive by nature and consequently a hypothesis-generating tool.

The initial IBD transcriptomics studies were published around the millennium and applied simple but at the time state-of-the-art microarray technology containing relative few transcripts.[17–23] The studies were often characterized by inclusion of few study subjects, use of tissue from intestinal resections, and the need for pooled tissue samples. The first studies using unpooled pinch biopsies from well-characterized IBD cohorts and control subjects were performed by Castello et al.[24] and Okahara et al.,[25] whereas Burczynski et al.[26] used peripheral blood mononuclear cells (PBMCs). These authors identified comprehensive sets of novel candidate transcripts not previously associated with IBD and provided new molecular insights into the pathophysiology of IBD but also underlined the polygenic and complex landscape of IBD. I[27–34] and others[35–59] have subsequently confirmed and elaborated significantly on the multifaceted polygenic nature and have correlated unique transcript profiles to UC or CD and subphenotypes, for example, active and quiescent disease, extend of disease, and IBD-associated CRC. These studies clearly demonstrate the diagnostic potential of IBD transcriptomics and the differential power to discriminate between UC and CD. They also demonstrate its limitations, because clinical use is compromised by the need for quite a substantial number of transcripts to achieve sufficient discriminative power, as illustrated by our most recent transcriptomics study,[29] which was performed as a multidisciplinary collaborative work between specialized research groups. Here a panel of 35 biomarkers was needed to reach an overall accuracy of 85% in an

external validation cohort of IBD patients with active disease. However, von Stein et al.[60] identified a panel of only seven genes with a unique expression pattern in UC and CD that was able to correctly classify UC and CD in >92% of cases. This is the closest any biomarker panel has come to the accuracy of conventional clinical diagnosis of IBD, and the panel is currently commercially available as a patented DiBiCol test. Nevertheless, the reliability and clinical usefulness of this panel must be tested and prospectively validated in an independent cohort.[61]

The molecular characterization achieved with IBD transcriptomics has also revealed the existence of a potential continuous inflammatory state in the colonic mucosa of patients with quiescent UC but not in CD. Wu et al.[35] were the first to show that endoscopically unaffected intestinal pinch biopsies from patients with UC might be transcriptionally different from inflamed intestinal tissue, but also from that of healthy subjects. These unaffected or uninvolved biopsies originated from patients with active IBD (i.e., from a more proximal uninvolved colonic segment), which questions the actual status of "unaffected." I[27,28] and others[41,56,62] have, however, subsequently verified the presence of a distinct colonic transcriptional profile in patients with clinical and endoscopically quiescent UC that offers a molecular stratification of patients with UC and, consequently, an opportunity to improve IBD therapy and maintain remission to prevent long-term disease progression.

The Selecting Therapeutic Targets in IBD (STRIDE) initiative of the International Organization for the Study of IBD (IOIBD) has recently updated its proposed treatment targets for adult patients with IBD.[63] It is recommended that endoscopic healing in both UC and CD should be a long-term therapeutic target, with a Mayo endoscopic score (MES) = 0 (previous recommendation 1) and a simple endoscopic score (SES) < 3, respectively. Despite ample

evidence, histologic remission (HR)[64] and transmural healing (TH), which we in our intestinal ultrasound research group[65,66] and others[67,68] have defined, are surprisingly not recommended as treatment targets in UC. However, if deeper layers of remission beyond HR and TH actually exist in terms of molecular remission at the omics level, for example, transcriptomics or maybe even metabonomics, then the opportunity exists to change the natural history of especially UC by setting the treatment targets in accordance with the molecular stratification.

Importantly, this also might reduce the risk of UC-associated CRC, because a recent meta-analysis demonstrates a pooled odds ratio (OR) of 2.6 (95% confidence interval [CI], 1.5–4.5; $p = 0.01$) in UC patients with histologic inflammation compared with no inflammation at all.[69] It has been assumed that the neoplastic processes are inflammation driven and that the chronic inflammatory load is the central element in the increased risk of UC-associated CRC.[70] Based on multiple epidemiologic studies, extensive UC or pancolitis is also repeatedly recognized as a prognostic risk factor for UC-associated CRC, but why the inflammation in pancolitis results in a higher risk of CRC compared with less extensive UC, that is, left-sided UC, is yet unknown.[71] One hypothesis is linked to the enhanced number of epithelial cells in the pancolitic mucosa exposed to carcinogenic insults. Alternatively, the inflammation per se may be more carcinogenic in the pancolitis mucosa, and if this is the case, yet another opportunity exists for the development of more rational and individualized treatments options for UC patients with pancolitis, thus supporting precision medicine.[32]

IBD Metabonomics

Metabonomics or metabolomics (terms often used interchangeably) is the study of metabolic changes in response to internal and external stimuli in an integrated biological system.[72]

Metabonomics aims to quantify the global dynamic metabolic response of living organisms to biological stimuli or disease perturbations, thus seeking to describe the systemic changes taking place through time in complex multicellular systems.

An overview of currently available adult IBD metabonomics studies is presented by sample type in Tables I–V. Most of these studies, especially the early metabonomics studies, focus on IBD diagnosis, differentiation between UC and CD, and biomarker identification. Bezabeh at al.[73] were the first to use metabonomics on colonic biopsies, and this research group differentiated between patients with active UC, active CD, and control individuals with a sensitivity and specificity ranging from 96% to 100%. When samples from patients with inactive IBD were validated using their classifier, only 82% could be categorized unambiguously, and 42% of these, with the majority being patients with UC, were described as abnormal or inflamed despite endoscopically and histologically definite quiescent disease. I conducted a methodologically analogous study and differentiated active UC and healthy subjects based on their metabolic profiles, but 36% of endoscopically assessed inactive UC patients were categorized as inflamed.[74] Sharma et al.[75] used paired colonic biopsies from unaffected and affected tissue from the same patients with UC and demonstrated that the metabolic profile of apparently macroscopically normal tissue was indistinguishable from that of inflamed colonic mucosa. These observations suggest that the involvement of the colonic mucosa is far more extensive at the molecular level, and they indicate that the continuous inflammatory state described at the transcriptomics level of quiescent UC also may be present at the metabonomics level.

Traditionally, the different omics approaches have been applied independently because of time-consuming and costly technologies. The development of relative low-cost and high-

throughput analyses has advanced the use of multiomics, providing a systems biology approach or holistic view, the advent of which has been elegantly demonstrated, especially in recent metabologenomics studies.[76] Here correlation of metagenomics and metabonomics enables large-scale identification of gene clusters responsible for the biosynthesis of expressed metabolites delivering putative mechanistic associations,[77–83] which can be further integrated using host omics to reveal parts of the integrate web of molecular interaction that characterize IBD phenotypes and subphenotypes.[14,15,84] A similar integrative approach using both transcriptomics and metabonomics on colonic mucosal biopsies is expected to be highly synergistic and thus improve IBD diagnostics and the molecular characterization of clinically relevant phenotypes, including the proposed continuous inflammatory state in quiescent UC.[33,34]

The major drawback of using colonic biopsies is the invasive endoscopic procedure needed to acquire the samples. Noninvasive testing is generally always preferred for diagnostics and surveillance of disease activity because of the higher degree of compliance and ultimately better disease control. Through minimally invasive sampling, breath volatomics,[85–96] that is, detection of volatile organic compounds (VOCs), and urine metabonomics[74,79,97–109] have to some extent shown that people with active IBD can be separated from healthy control subjects. However, in my previous metabonomics study, I was unable to differentiate between active UC, quiescent UC, and control subjects using urine metabonomics,[74] and inconsistent results generally characterize the breath- and urine-based metabonomics studies attempting to differentiate between UC and CD. Stephens et al.[106] found a clear difference in the urine metabolome of patients with UC and CD, but once corrected for confounding elements, such as by surgery and medication, the differential power was lost. Other studies have also tracked the metabolic changes taking place in urine during treatment to try and predict treatment response[79] or

relapse[97,109] but so far with limited success. Ding et al.[79] found a bile acid profile and cysteine levels to be predictive of a response to anti–tumor necrosis factor (TNF) treatment in patients with CD, but with an area under the curve (AUC) of only 0.70 in both cases. Although breath and urine seem to be the ideal noninvasive biological sample types for diagnostics and prognostics, the results so far have been disappointing. By contrast, it is now well-established that the gut microbiome and host-derived metabolites are central elements in IBD,[110] and as a consequence, the majority of the early metabonomics studies using metabolic profiles of fecal samples have consistently been able to differentiate between IBD and healthy control subjects, as well as between IBD subtypes.[111–117] Substantiating these findings and further characterization of the fecal metabolome might make fecal metabonomics an extremely useful clinical diagnostic tool and may provide an opportunity to elaborate on the extreme complexity of the gut microbiome and fecal metabolites.[118]

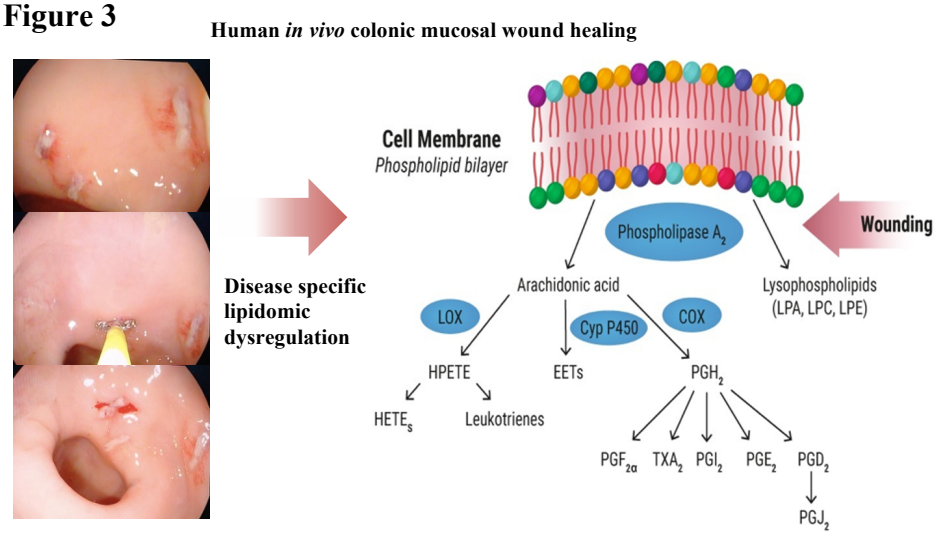
Although the microbiome-derived metabolome and fecal samples are getting increased attention, blood samples are thus far the most studied biofluid in IBD metabonomics (Table III). Serum and plasma samples are easy and convenient to collect and probably represent the obvious choice for biomarker identification in a clinical setting. However, the intestinal inflammation is not necessarily reflected in detectable changes in the blood; for example, patients with mild to moderate active UC often have a C-reactive protein (CRP) level within the normal range.[119] Some of the early studies, including my own,[74,120] investigated the potential differences in isolated PBMCs as well as stimulated monocyte-derived macrophages in patients with quiescent or moderately active IBD versus healthy control subjects. As could be expected, these studies did not identify any differences in the metabolic profiles. Williams et al.,[121] in contrast, demonstrated significant differences in the metabolic profile of serum in quiescent UC and CD as well as in

control subjects, with an AUC > 0.90 for all three models (e.g., UC vs. controls, CD vs. controls, and UC vs. CD). However, all subsequent nuclear magnetic resonance (NMR)-based studies have been unable to differentiate between UC and CD,[105,107,122–125] although many of the metabolic perturbations have been affirmed in patients with active disease. No endoscopic evaluation or indirect measures of active IBD were provided (e.g., CRP or calprotectin) in the study by Williams et al.,[121] which means that a potential presence of subclinical inflammation might explain the observed differences during clinically quiescent disease. This raises interesting questions regarding the metabolic trajectories during resolution of the inflammation: Are these identical or different in UC and CD; can they be used to define a serologic molecular state of quiescent disease, and/or can they potentially be used to predict treatment response?[126] The latter is of great clinical and socioeconomic importance when treatment is prescribed with biologics such as infliximab (IFX). One-third of patients with IBD do not respond to IFX induction therapy at all,[127] which might be explained by a completely different immunological drive or pharmacodynamics in these patients or that other processes than the inflammation are at play (e.g., compromised wound healing). Thus, predicting treatment response and characterizing the underlying mechanisms are the very essence of precision medicine.[126]

In this respect, it has become evident that one of the main risk factors for ongoing disease activity in spite of anti-inflammatory treatment is sustained wound formation and that several anti-inflammatory strategies actually may impair vital pathways involved in mucosal tissue regeneration.[128] Other mechanisms than inflammation consequently seem to be in play, but our understanding of the normal and pathologic responses to intestinal mucosal injury and subsequent healing in vivo in humans is still poorly understood.[129] Research on wound healing in the healthy colon and in patients with UC is consequently critical in order to identify potential

therapeutic targets that could modify the disease course through promotion of tissue regeneration and bring us closer to even higher overall response rates than the ones seen with the current anti-inflammatory treatment strategy. I have recently demonstrated that patients with quiescent UC respond to mucosal breaks by an innate hyperresponse engaging resident regulatory cells and a subsequent adaptive activation when compared with healthy control subjects.[130] Inspired by the Segal team,[131] I participated in the development of a human in vivo model for the study of flare initiation, but the model is also applicable as a subsequent wound-healing assay,[132] which we consider to be of significant importance because all other studies on colonic mucosal healing have been performed in vitro or in animal colitis models.[130] The most recent animal models on wound-healing have focused on lipidomics[133] - a subset of the metabolome that is generally receiving increased attention in IBD metabonomics research.[134] These studies have shown that eicosanoids and phospholipids are vital elements in the various stages of wound-healing and that they display an omnipresent and surprisingly complex bioactivity providing promising treatment targets in animal colitis[135,136] and human studies[137] (Fig. 3).

Figure 3



Abstract figure from Bjerrum et al.[132]

Aims

The overarching aim of the dissertation and included studies is to support the notion of precision medicine in IBD. This is achieved by transcriptomics and metabonomics analyses of disease-relevant sample types, that is, colonic and wound biopsies, feces, and blood. With such techniques I try to improve IBD diagnostics, differentiate between UC and CD during both active and quiescent disease, and to molecularly characterize the phenotypes in relation to the inflammatory process, dysplasia, and wound-healing - with the ultimate goal of identifying potential novel therapeutic avenues.

2. Materials and Methods

Patient Population and Healthy Volunteers

All patients with IBD were recruited from the Department of Gastroenterology, Herlev Hospital, University of Copenhagen, Denmark, except for one study[32] where samples partly originated from the Swiss IBD Cohort Study (SIBDCS). Only patients with an affirmed diagnosis of UC or CD established according to well-established criteria[1,2] were included. All patients with IBDu,[9] a subsequent diagnosis of microscopic colitis or coexistence thereof, or infectious colitis were excluded. Furthermore, in the case of a discrepancy between the colonoscopy and histology (e.g., normal macroscopic colonoscopy but inflamed by histology in patients with an otherwise affirmed IBD diagnosis), the patient was excluded to avoid any misclassification of disease activity status.

Clinically, the disease activity was assessed using the Mayo score[138] (Mayo scores of 0–1, inactive UC; 2–4, mild UC; 5–8, moderate UC, and 9–12, severe UC) or the Harvey–Bradshaw (HB) score[139] (HB scores of 0–4, inactive CD; 5–8, mild CD; 9–16, moderate CD; and >16,

severe CD). In studies using tissue samples,[32–34,61,132] the clinical scores were correlated with endoscopic activity scores (Mayo endoscopic score for UC[138] and the simplified endoscopic score for CD [SES-CD][140]) and histologic scores.[141–143] Thus, patients classified as having quiescent disease were in complete endoscopic and histologic remission (e.g., Mayo endoscopic score = 0 and Geboes score < 0.1[142] for patients with UC in remission). Similarly, none of the included control subjects had any signs of endoscopic or histologic inflammation. These individuals were undergoing colonoscopy because of gastrointestinal symptoms, but all clinical investigations turned out to be normal. Patients and control subjects were not matched for age or gender.

Exclusion criteria included age <18 years or >70–80 years (depending on the study); clinical evidence of any infections; recent (within 14 days) use of antibiotics or probiotics; pregnancy or lactation; severe mental illness that would affect decision making; special food regimens such as a diet with low content of fermentable oligosaccharides, disaccharides, monosaccharides, and polyols; and a diabetic or gluten-free diet. Ongoing maintenance treatment was typically allowed with 5-aminosalicylic acid, immunomodulators (e.g., azathioprine, mercaptopurine, or methotrexate), and biologics on a stable dosing minimum two months prior to inclusion.

Sampling and the Human In Vivo Wound-Healing Model

All fecal samples,[118] blood samples,[126] and colonic biopsies[32–34,61,132] were collected prospectively, as described previously. In brief, fecal samples were placed in a sealed insulated container, immediately put on ice, and stored at –80°C. Within 3 hours of sampling, the serum was collected after centrifugation (2500×*g* for 5 minutes at ambient temperature) and stored at

–80°C.

Colonic mucosal pinch biopsies were obtained during endoscopy from the left side of the colon in each patient using routine endoscopic forceps. The left side was explicitly preferred to avoid any intersegmental variation in the transcriptome or metabolome and because this is the most frequent area of inflammation in patients with UC. The biopsies were immediately stabilized in RNAlater (Ambion, Austin, TX) or snap frozen in liquid nitrogen and stored at –80°C for subsequent transcriptomics or metabonomics analyses, respectively. Adjacent biopsies were used for histopathologic evaluation. In one study colonic biopsies also originating from a biobank in the SIBDCS collected and stored in a similar fashion to the above-described procedure.[144]

To study the kinetics and molecular dynamics of acute colonic mucosal injury and the subsequent healing process, the previously mentioned human wound-healing assay and concomitant wound scores were utilized.[130,132] In short, index wounds were generated in the rectosigmoid colon of healthy subjects and patients with UC using a biopsy forceps (Radial Jaw 4, 2.8 mm, Boston Scientific, Marlborough, MA). The wound-healing processes were documented and scored by successive macroscopic high-definition imaging via a standard endoscope at given predefined

Figure 4

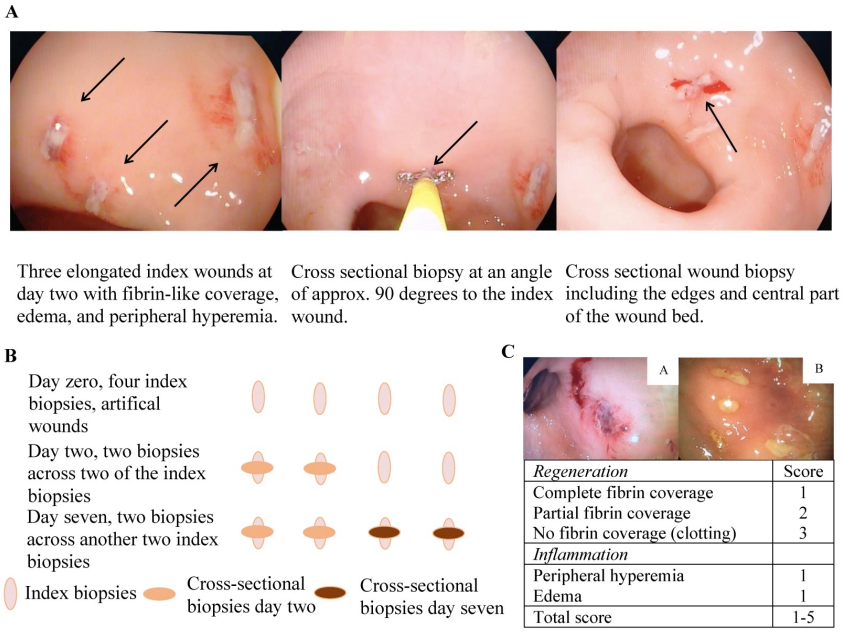


Figure 1 from Bjerrum et al.[132]

postwounding time points. Subsequent molecular characterization was made possible by obtaining biopsies across the index wounds with the use of a biopsy forceps angled 90 degrees to the initial index wounds (Fig. 4). Based on previous experience from skin-excision wound models,[145] a scoring system was developed evaluating barrier breach exudate appearance and signs of inflammation (i.e., peripheral edema and erythema) and wound-healing (i.e., closure of the defect). The model consequently allowed a description of the kinetics of the initial injury and subsequent wound-healing in normal and diseased human colonic mucosa on both the macroscopic and molecular levels.

Transcriptomics Analyses

RNA extraction from colonic mucosal biopsies was performed identically in all studies using the NucleoSpin RNA/Protein Mini Kit (Macherey-Nagel, Düren, Germany) and in the case of formalin-fixed paraffin-embedded (FFPE) biopsies with the RNeasy FFPE Kit (Qiagen, Hilden, Germany) applied in accordance with the manufacturers' protocols. Integrity and purity were verified with an Agilent Bioanalyzer (Agilent, Palo Alto, CA), and only RNA integrity numbers (RIN) > 7 were accepted, except for the FFPE biopsies, where RIN values are inherently low.

At the time of the initiation of these studies, state-of-the-art transcriptome-wide analysis was performed using microarray technology simultaneously measuring the expression levels of thousands of RNA transcripts. Thus, in these experiments performed at the Centre for Genomic Medicine, Rigshospitalet, Copenhagen, Denmark, the Affymetrix GeneChip Human Genome U133 Plus 2.0 was applied, containing more than 47,000 transcripts. This method was fast and affordable, but the obvious disadvantage compared with current technologies, such as RNA sequencing or cap analysis of gene expression (CAGE), used in my more recent IBD

transcriptomics studies[29,130] is the limited number of transcripts because only known transcripts are applied to the array. Array technology also has a smaller dynamic range because hybridization is limited by background noise at the low end and signal saturation at the high end, and finally, it has a relative low sensitivity and specificity due to small detection rates of differentially low expressed genes and of rare and low-abundance transcripts.

Subsequent validation of the array analyses was performed with quantitative real-time polymerase chain reaction (RT-PCR), and protein expression was investigated by immunohistochemistry.

Metabonomics Analyses

Metabolic profiles were generated from colonic mucosal biopsies,[33,34] fecal samples,[118] and blood samples,[126] using ^{1}H NMR spectroscopy as the preferred approach. High-resolution NMR spectroscopy quantifies the metabolites in an untargeted approach and simultaneously carries molecular structural information that allows identification of the metabolites. Furthermore, NMR-based metabonomics is a robust and reliable technique with high reproducibility, minimal sample preparation, low running costs, and high throughput; is nondestructive; and, importantly, the only technique capable of analyzing intact tissue. However, NMR spectroscopy has a low intrinsic sensitivity and heavy signal overlap, limiting the number of metabolites that can be detected. This has to be acknowledged when interpreting NMR-based metabonomics.

In my most recent metabonomics study based on intestinal wound biopsies,[132] I used another commonly applied technology for metabolic profiling, namely mass spectrometry (MS) coupled with the separation technique liquid chromatography (LC), as a targeted approach to characterize the metabolic trajectory during wound healing in the colonic mucosa of healthy

subjects and patients with UC. The targeted approach selectively quantifies specific metabolites, in this case lipids, which requires the addition of internal standards for identification and quantification purposes. The advantage of this approach is that the MS conditions can be optimized for ideal separation and detection, resulting in identification of metabolites in the picomole range with a superior sensitivity to NMR-based metabonomics. However, the technique requires a priori knowledge and valid hypotheses regarding the samples of interest, and more care should be taken when conducting MS experiments because the reproducibility of MS is intrinsically low and quality control strategies are necessary to obtain reproducible results. It is consequently essential to acknowledge the limitations of metabonomics because none of the existing technologies can identify the entire metabolome. Moreover, the metabolome is an ever-changing and dynamic structure due to the continuous influence of upstream omics and the environment. Thus, the results from metabonomics studies consequently need to be interpreted under these conditions and with caution.[134]

3. Results and Discussion

IBD Diagnostics and Differentiation of UC and CD

Intestinal Transcriptomics

The need for novel, reliable, and clinically suitable methods for diagnosing IBD is evident, and a diagnostic model based on the expression pattern of a limited number of genes would be an ideal tool, as suggested by von Stein et al.[60] Their diagnostic DiBiCol test was independently investigated in a subsequent Swedish study including 38 probable cases of UC, 18 probable cases of CD, and 22 cases of IBDu.[146] Using DiBiCol as the "gold standard" test, sensitivity and specificity for the clinical diagnosis were found to be 77% and 66% and 89% and 92% for UC

and CD, respectively. However, because the final diagnosis was reached by including the results from the DiBiCol test, this raises the question of confounding by diagnosis. Furthermore, the study was performed in an unblinded fashion. In a more recent Swedish retrospective analysis of the clinical application of the DiBiCol test on a real-world data set, the sensitivity and specificity of the test were found to be 97% and 92% for UC and 78% and 88% for CD.[147] However, in a subset of these retrospectively collected patients, duplicate colonic biopsies were taken at the same time in 300 patients, and in 1 of every 5 patients the test result was different between the two biopsies.

To validate this seven-gene model in a prospective Danish cohort, I included 119 patients with IBD and divided them into a training cohort (n = 58) and a test cohort (n = 61) to assess the

Tabel 1

Sensitivity, specificity and AUC

	Present study	Von Stein *et al.* Retrospective study	Von Stein *et al.* Prospective study
	UC (n = 21)	UC (n = 33)	UC (n = 38)
	Non-UC (n = 40)	Non-UC (n = 110)	Non-UC (n = 48)
	CD (n = 20)	CD (n = 22)	CD (n = 28)
	Non-CD (n = 41)	Non-CD (n = 121)	Non-CD (n = 58)
	IBD (n = 41)	IBD (n = 55)	IBD (n = 75)
	Non-IBD (n = 20)	Non-IBD (n = 88)	Non-IBD (n = 20)
Sensitivity (UC vs. non-UC)	43% (22–66%)	88%	90%
Specificity (UC vs. non-UC)	83% (67–93%)	99%	86%
AUC (UC vs. non-UC)	0.82 (0.71–0.93)	0.98	0.94
Sensitivity (CD vs. non-CD)	60% (36–81%)	70%	68%
Specificity (CD vs. non-CD)	68% (52–82%)	98%	92%
AUC (CD vs. non-CD)	0.71 (0.55–0.87)	0.96	0.92
Sensitivity (IBD vs. non-IBD)	95% (83–99%)	85%	95%
Specificity (IBD vs. non-IBD)	90% (68–99%)	94%	95%
AUC (IBD vs. non-IBD)	0.92 (0.84–1.00)	0.95	1.00

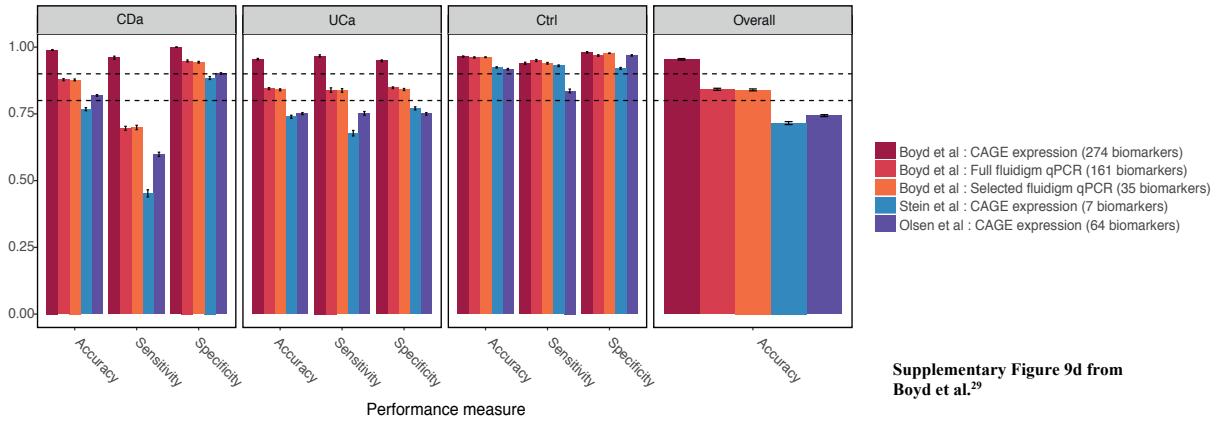
Sensitivity, specificity and area under the curve (AUC) from the two studies. The von Stein study is composed of three sub-studies: a pilot study used to evaluate the potential of the seven genes as a diagnostic tool, a retrospective study used as validation, and finally a prospective study used for confirmation. The results of the retrospective and prospective studies are presented here. Values for this study are given with 95% confidence intervals in parentheses. CD, Crohn's disease; UC, ulcerative colitis. Note that the non-UC group contains both CD and non-IBD controls, and the non-CD group contains both UC and non-IBD controls.

Table 1 from Bjerrum et al.[61]

reliability and clinical usefulness of this tool.[61] The resulting gene expression pattern of the seven genes was identical to the one found by von Stein et al.[60] except for *RegIV*, but the subsequent classification of the test cohort with or without *RegIV* resulted in high misclassification rates and consequently a low sensitivity and specificity (Table 1). The seven-gene panel does distinguish patients with IBD from healthy control subjects, but the differential power with respect to CD versus UC was found to be inadequate for diagnostic purposes, and the results indicate that the genes merely reflect nonspecific inflammation rather than specific markers of IBD.

For comparison, the classifying power of a previously identified gene expression panel by Olsen et al.[27] and the gene expression markers from von Stein et al.[60] were tested in our most recent transcriptomics CAGE data set.[29] The expression of these genes were measured by summing CAGE tags across corresponding gene models in the CAGE cohort and trained and evaluated by a random forest model for each gene set using a fivefold cross-validation system iteratively 1000 times to ensure stable results. For comparison, the same analysis was made on (1) the initial 274 CAGE-defined markers, (2) the 161 Fluidigm targets, measured by quantitative (q)PCR, and (3) the final 35 Fluidigm targets measured by qPCR. The average accuracy, sensitivity, and specificity are shown in Figure 5 for each group (CDa: active CD;

Figure 5



Supplementary Figure 9d from Boyd et al.[29]

UCa: active UC; Ctrl: healthy control individuals) as bar plots along with the overall accuracy. The seven-gene panel performed consistently worse than the CAGE data.

Recent large meta-analyses of previously published gene expression studies on colonic mucosa from patients with IBD demonstrate an overall lack of significant differences between CD and UC,[48,52] which to some extent is also confirmed by more up-to-date transcriptomics studies.[44,46] These results, altogether, show the difficulties encountered in efforts to reduce the complexity of IBD into a comprehensive and clinically applicable seven-gene panel.[61]

Fecal Metabonomics

Therefore, I took a completely different approach and collected stool samples from 113 individuals - 48 patients with UC (19 active, 29 inactive), 44 patients with CD (13 active, 31 inactive), and 21 healthy control subjects - and analyzed the fecal water extracts with NMR spectroscopy.[118] The initial results indicated that IBD patients and healthy control subjects were easily separated based on fecal metabonomics, but no differences between UC and CD were demonstrated. Furthermore, excluding patients who have had intestinal surgery, which coincided with anti-TNF- treated patients, resulted in an extremely poor differential potential because only active UC was found to be distinct from inactive UC and healthy control subjects and with corresponding predictive capabilities (Table 2, Fig 6).[118]

Table 2

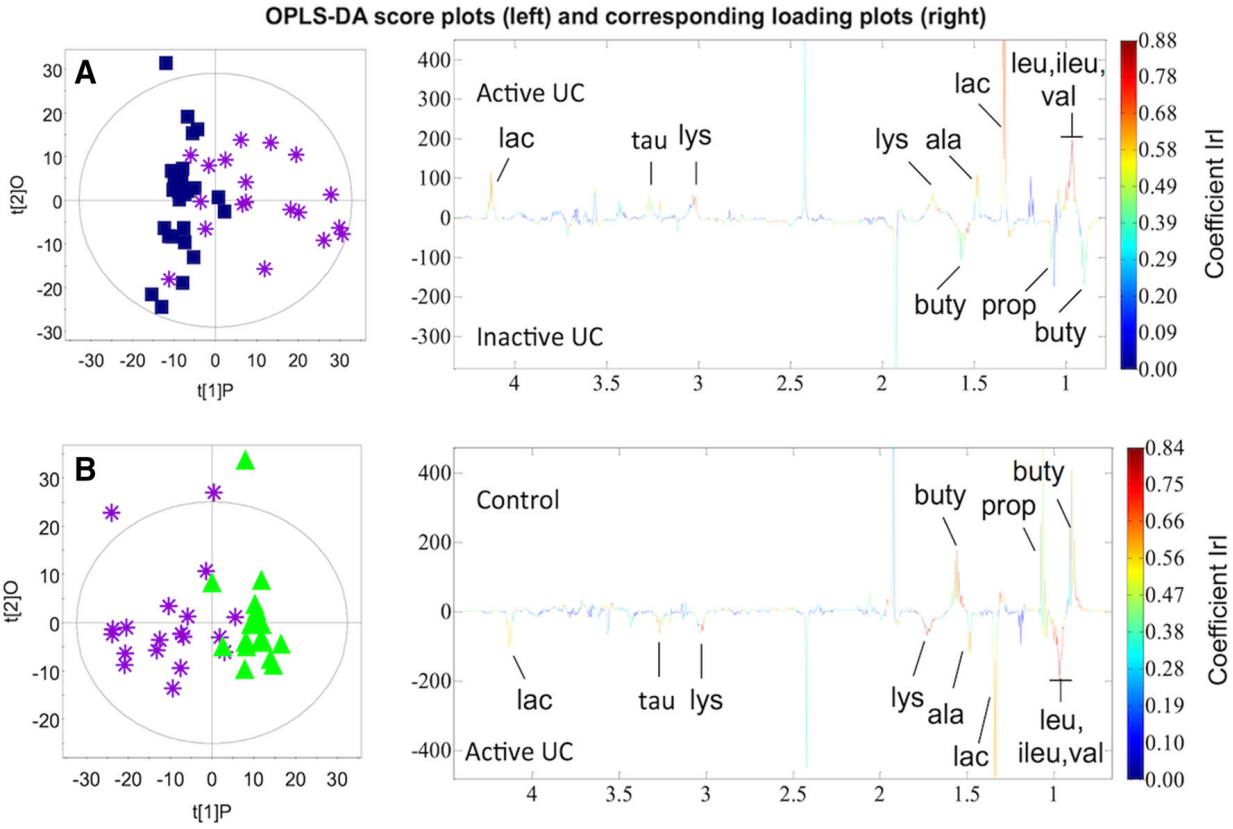
Predictive capability of the models

Models corrected for surgery	Area under the curve	
Active UC vs. inactive UC	0.813	Good
Active UC vs. controls	0.876	Good
Inactive UC vs. controls	0.654	Poor
Active UC vs. active CD	0.693	Poor
Inactive UC vs. inactive CD	0.659	Poor
Active CD vs. inactive CD	0.529	Poor
Active CD vs. controls	0.667	Poor
Inactive CD vs. controls	0.726	Fair

Prediction performance estimates presented as area under the curve (AUC) for each model

Table 4 from Bjerrum et al.[118]

Figure 6



OPLS-DA score plots of patients with UC and without intestinal surgery; blue squares inactive UC; purple stars active UC; green triangles controls. The corresponding back-scaled loading plots reflect the class differences in the NMR spectra; upright peaks indicate a relatively increased intensity of metabolites, and downright peaks a decreased intensity. The colors shown on the plot are associated with the significance (above a 0.44) of metabolites in separating the samples. ala alanine; buty butyrate; ileu isoleucine; lac lactate; leu leucine; lys lysine; prop proprionate; tau taurine; val valine.

Figure 2 from Bjerrum et al.[118]

Today we know that the diversity of the microbiome and metabolome is reduced in patients with IBD who have been subjected to intestinal surgery.[81] However, at the time, these results were surprising because the first-ever metabonomics study based on fecal samples and NMR spectroscopy by Marchesi et al.[112] differentiated between patients with active UC, active CD, and healthy control subjects. This initial study was methodologically similar to study V except that all patients with IBD received treatment with prednisolone (40 mg/day) and 5-aminosalicylic acid (0.8–1.6 g/day) in their study. The metabolites holding differential power were primarily increased levels of branched-chain amino acids (BCAAs) in CD (i.e., isoleucine,

leucine, and valine), reduced short-chain fatty acids (SCFAs) in CD (i.e., butyrate and acetate) and UC (i.e., butyrate only), and reduced tri/methylamine in both CD and UC. These findings were initially confirmed in my study, but after correction for surgery and medication, only a higher abundance of BCAAs (lysine, alanine, taurine, and lactate) and low levels of SCFAs (propionate and butyrate) remained in patients with flaring UC. The large number of amino acids observed in active disease in the two studies can be explained by malabsorption caused by the inflammation, whereas the low levels of SCFAs seem to be the consequence of an inflammation-driven intestinal dysbiosis.

Elegantly designed and executed metabologenomics studies[15,77,80,82,116,148–151] have demonstrated reduced biodiversity and dysbiosis in IBD and identified, among others, *Faecalibacterium prausnitzii* and *Clostridium coccoides* in a lower abundance in IBD. These species are important participants in the bacterial fermentation of polysaccharides and, consequently, in the production of SCFAs. Usually SCFAs are produced in large amounts in the colon and absorbed because especially butyrate is a preferred energy source for colonocytes, and along with propionate, it exhibits anti-inflammatory characteristics.[152] Thus the dysbiosis resulting in low levels of SCFAs becomes a self-promoting vicious spiral leading to chronic inflammation.

A double-blinded randomized, controlled trial used the probiotic inulin to treat patients with active and quiescent CD and healthy control subjects.[153] Patients receiving inulin had a subsequent significant increase in butyrate and acetaldehyde, but whether these changes were of clinical significance is not disclosed. Other studies[117,154] have also employed gas chromatography-mass spectrometry (GC/MS) but reached completely diametral results, with increased levels of SCFAs, including butyrate, in active CD. During standard medication in

active CD, these levels, however, decreased and normalized in comparison with control individuals, but no differences in the volatile metabolites could be identified between patients with active UC and irritable bowel syndrome (IBS) and control subjects.[117] Similarly, enteral feeding leads to a significant reduction in SCFAs and the disease activity, as scored by the HB index.[154] A few other GC/MS-based metabonomics studies[113,155] have also found changes in SCFAs and their derivatives, but whereas one study[113] could use this to differentiate between patients with UC and infectious colitis and control subjects, another study[71] observed a less distinct separation between patients with active UC and quiescent UC and control subjects. The reason for these opposing findings, especially in GC- and GC/MS-based studies, is unclear, but they highlight the importance of an exact description and validation of the technological approach[156] and characterization of the cohorts under investigation with respect to demographics and clinical features (e.g. surgery, medications, diet, smoking, etc.) when performing metabonomics studies. Nevertheless, the results have animated medical trials with supportive local treatment of colitis using SCFA enemas, which were investigated for the first time 30 years ago.[157] Perhaps not so surprising, the outcomes were somewhat disappointing, although SCFAs actually seem to ameliorate the inflammation, but they might at the same time impede the wound-healing process.[158]

SCFAs, together with a well-described bile acid dysmetabolism,[77,79,111,114,116,148,159] in IBD also seem to predict response to both fecal microbiome transplantation (FMT) and anti-TNF treatment.[134]

Thus, IBD metabonomics based on fecal samples has been unable to provide the desired diagnostic accuracy in differentiating UC and CD, especially when confounding factors, such as the systemic effect of medication and even minor intestinal surgery, are considered.

Serum Metabonomics

In my longitudinal cohort study of NMR spectroscopy–based metabolic profiling of serum from patients with IBD treated with IFX, I included successive serum samples collected during IFX induction treatment (i.e., weeks 0, 2, 6, and 14) from 87 patients (UC, *n* = 38; CD, *n* = 49) and 37 healthy control subjects to identify potential diagnostic biomarkers that could hold differential power.[126] The study confirms previously published studies[105,107,123,160] and generally displays significant differential diagnostic power in models comparing patients with active IBD with healthy control subjects, but as with tissue and fecal metabonomics differentiation between UC

Table 3

Validation of PLS-DA and O-PLS-DA models

Model	PLS-DA permutation test n = 200	O-PLS-DA CV-ANOVA	Area under the ROC curve
CD(0) vs. UC(0)	$Q^2 = 0.095$	$Q^2 = -0.182$	0.44
	×	× $P = 1$	
CD(0) vs. Control	$Q^2 = 0.637$, r = 0.32	$Q^2 = 0.7$	0.96
	✓	✓$P < 0.001$	
UC(0) vs. Control	$Q^2 = 0.583$, r = 0.32	$Q^2 = 0.383$	0.94
	✓	✓$P < 0.001$	
CD Rem(0) vs. Control	$Q^2 = 0.63$, r = 0.37	$Q^2 = 0.66$	0.96
	✓	✓$P < 0.001$	
CD Rem(2) vs. Control	$Q^2 = 0.69$, r = 0.37	$Q^2 = 0.66$	0.95
	✓	✓$P < 0.001$	
CD Rem(6) vs. Control	$Q^2 = 0.52$, r = 0.37	$Q^2 = 0.63$	0.91
	✓	✓$P < 0.001$	
CD Rem(14) vs. Control	$Q^2 = 0.48$, r = 0.40	$Q^2 = 0.62$	0.90
	✓	✓$P < 0.001$	
UC Rem(0) vs. Control	$Q^2 = 0.56$, r = 0.47	$Q^2 = 0.60$	0.91
	✓	✓$P < 0.001$	
UC Rem(2) vs. Control	$Q^2 = 0.60$, r = 0.48	$Q^2 = 0.60$	0.94
	✓	✓$P < 0.001$	
UC Rem(6) vs. Control	$Q^2 = 0.25$	$Q^2 = 0.52$	0.71
	×	✓$P < 0.001$	
UC Rem(14) vs. Control	$Q^2 = 0.17$	$Q^2 = 0.37$	0.64
	×	✓$P < 0.001$	

The models were only considered valid if the permutation test and the CV-ANOVA test ($p < 0.05$) were satisfied at the same time
(0), before 1st infusion of infliximab; (2), before 2nd infusion; (6), before 3rd infusion; (14), before 4th infusion
Q2, predictability of the model; r correlation coefficient
✓, valid model
X invalid model
CD Crohn's disease, *CV-ANOVA* analysis of variance of the cross-validated residuals, *O-PLS-DA* orthogonal-projection to latent structure discriminant analysis, *PLS-DA* projection to latent structure-discriminant analysis, *Rem* remission, *ROC* receiver operating characteristics, *UC* ulcerative colitis

Table 3 from Bjerrum et al.[126]

and CD is still challenging whether active or quiescent disease is considered (Table 3). The few studies where inactive IBD has been identified with distinct metabolic profiles compared with healthy control subjects, the disease activity has been based only on clinical scores (e.g., Williams et al.[121]) or indirect measures such as a mean fecal calprotectin of 234.5 μg/g in the study by Notarargio et al.,[125] and the risk of subclinical inflammation is consequently present. One study, however, has demonstrated distinct metabolite profiles in patients with UC and CD and healthy control subjects and through variable selection created a diagnostic model containing only four metabolites (i.e., oxalate, 3-hydroxy-butyrate, ribulose, and 1,6-anhydroglucose) holding enough differential power to distinguish between UC and CD with a sensitivity and specificity of 85% and 97%. respectively.[161] This study also produced a UC activity assessment model with only two metabolites (*p*-hydroxybenzoic acid and histidine; AUC = 0.97) that correlated with the clinical activity score (active UC vs. quiescent UC). In the subsequent prospective monitoring study, three patients with later flares had a simultaneous increase in the UC assessment index and four patients who went into remission had a reduction, suggesting that this model could be a clinically applicable monitoring tool.[161] It is important to note that treatment with sulfasalazine was found to be positively correlated with high levels of *p*-hydroxybenzoic acid, which was one of the metabolites holding strong differential power, and that no cohort with non-IBD intestinal inflammation was included in the study. Histidine, in contrast, is consistently reported at low levels in patients with IBD. In a prospective study of patients with quiescent UC, and among the investigated metabolites, low levels of histidine have been exclusively identified to be predictive of relapse within a 1-year period.[162] Nevertheless, it should be noted that remission was based on a clinical score and that no information on endoscopy or fecal calprotectin was available. Furthermore, in a recent study, the ulcerative

colitis endoscopic index score (UCEIS) and histologic severity (Nancy index) were correlated with high and low UCEIS (accuracy, 77% ± 5%) and Nancy index (65% ± 6%) metabolic profiles. These profiles were characterized by decreased lipoproteins and increased BCAAs, glucose, and myo-inositol in high UCEIS and Nancy index, demonstrating for the first time that serum-based metabonomics can distinguish between mild and severe active UC.[163] Interestingly, the patients were followed prospectively, and a distinct baseline metabolic profile was identified that predicted deterioration of symptoms within a period of 6 months, and again low levels of histidine were found to be predictive.

Lipidomic studies have similarly found profound changes in the lipid profile of patients with both UC and CD compared to control subjects.[164–168] One study used these profound differences in the lipid profile of especially fatty acids and combined those with serum proteomic data to create highly predictive models for UC and CD compared with control subject with 100% accuracy, but for unknown reasons, no direct models were created for UC versus CD.[169] A similar study could differentiate between patients with IBD and control subjects, but no valid predictive models were created for UC versus CD based on the untargeted bioinformatics approach.[170] The fact that differentiation between UC and CD is difficult despite apparent differences in the lipid profiles highlights the importance of continued optimization of the analytical strategies for serum metabonomics,[171] rigorous phenotyping, and integration of omics. Recent studies by Borren et al.[83,172] elegantly illustrated the advent of such an approach, because patients with quiescent IBD were characterized with metagenomics and serum proteomics and metabonomics. Based on stringent phenotyping, the authors identified four metabolic markers (i.e., propionyl-L-carnitine, carnitine, sarcosine, and sorbitol) predictive of relapse within two

years. The AUC of this models was only 0.70, but combined with a similar proteomics model, the AUC increased to an acceptable 0.83, which was superior to both models alone.

Intestinal Omics

The merge of the most vital discriminative information from different omics levels seems to significantly improve the diagnostic power. I consequently integrated transcriptomics and metabonomics data generated from colonic mucosal pinch biopsies from patients with active UC (n = 22) and inactive UC (n = 21) and healthy control subjects (n = 15) to improve diagnostics and through variable selection to evaluate the prediction performance of relatively small (~20 variables) multivariate biomarker panels.[33] By means of orthogonal partial-least-squares regression-discriminant analyses (OPLS-DA) in combination with class-balanced Monte Carlo cross-validation, I assessed the predictive performance as the AUC for each comparison and each individual and combined data set. As seen in Table 4, the best predictive performances were observed with active UC versus inactive UC (AUC > 0.95 across all data sets) and active UC versus control subjects (AUC > 92 across all data sets), whereas with inactive UC versus control subjects, the classification performance varied between the data sets, with the metabonomic (nuclear overhauser effect, NOE) performing the best. I subsequently assessed predictive

Table 4

Prediction performance

Analysis name	Metabonomics (NOE)	Metabonomics (CPMG)	Transcriptomics	Omics
ActiveControl	0.95	0.92	0.97	0.97
InactiveControl	0.79	0.65	0.57	0.58
InactiveActive	0.98	0.95	0.96	0.96
SiSd	0.73	0.76	0.80	0.78
DurationLess10	0.43	0.35	0.63	0.63
DebutLess25	0.43	0.34	0.38	0.36

Prediction performance estimates presented as area under the curve (AUC) for each sub-population and for each full data set, including combined data sets: Omics. AUC estimates are based on classification using OPLS-DA and Monte Carlo cross-validation

NOE nuclear Overhauser effect, *CPMG* Carr–Purcell–Meiboom–Gill, *Si* steroid independence, *Sd* steroid dependence, *DurationLess10* disease duration less than 10 years, *DebutLess25* age at diagnosis less than 25 years

Table 3 from Bjerrum et al.[33]

performance under variable selection for each subgroup, and the results indicate that it is actually possible to differentiate between patients with active UC, those with inactive UC, and control subjects; between steroid-independent (Si) and steroid-dependent (Sd) disease; and between early and late disease onset based on small subsets of transcriptomics and metabonomics variables (Table 5). However, the expected diagnostic benefits of combining omics in this case

Table 5

Prediction performance of small candidate biomarker panels

Analysis name	Metabonomics (NOE)	Metabonomics (CPMG)	Transcriptomics	Omics
ActiveControl	0.93	0.90	0.97	0.96
InactiveControl	0.76	0.68	0.68	0.76
InactiveActive	0.94	0.89	0.93	0.93
SiSd	0.62	0.71	0.63	0.70
DurationLess10	0.54	0.50	0.38	0.38
DebutLess25	0.49	0.67	0.71	0.69

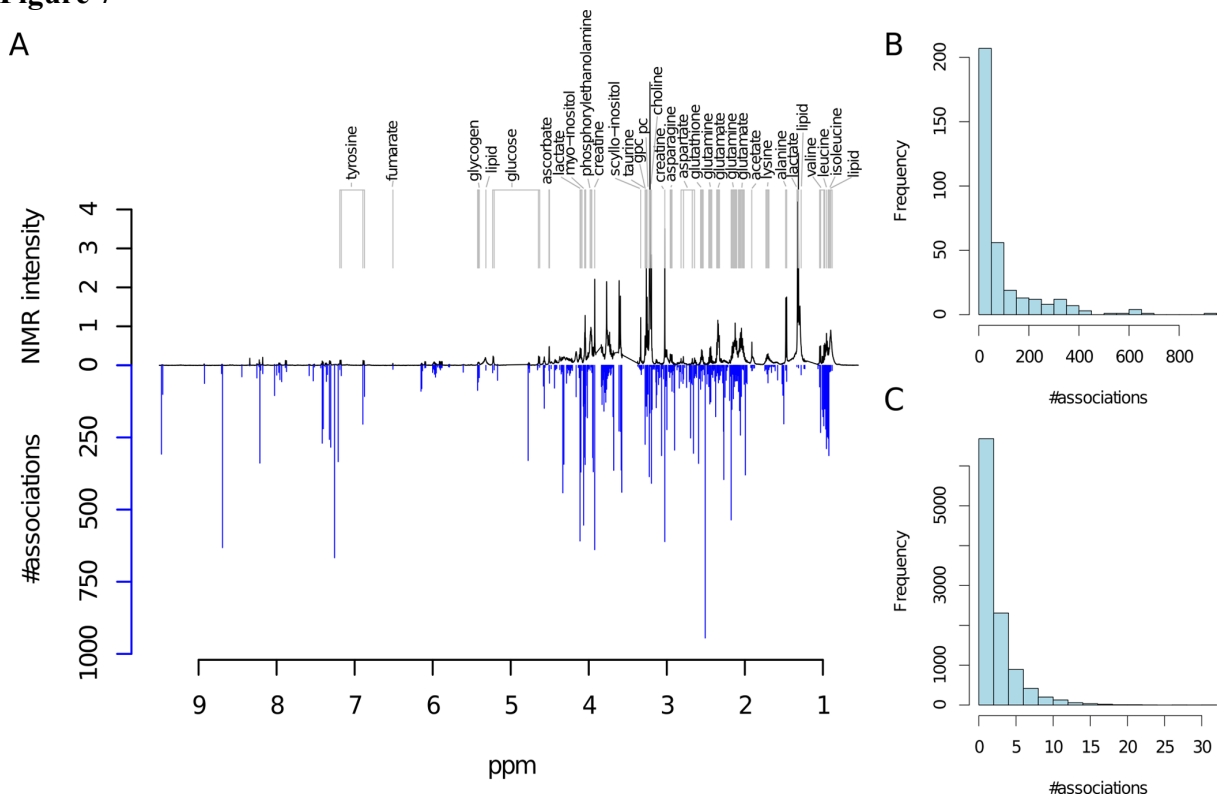
Prediction performance estimates presented as area under the curve (AUC) for each sub-population under selection of small candidate biomarker panels for each full data set and the combined data sets: Omics. AUC estimates are based on classification using logistic regression fitted by lasso and a nested Monte Carlo cross-validation procedure

NOE nuclear Overhauser effect, *CPMG* Carr–Purcell–Meiboom–Gill, *Si* steroid independence, *Sd* steroid dependence, *DurationLess10* disease duration less than 10 years, *DebutLess25* age at diagnosis less than 25 years

Table 4 from Bjerrum et al.[33]

remained elusive, which might be explained by a potentially low correlation between the two omics. To test this further, I conducted a metabolome-transcriptome-wide association analysis to investigate linkage between the metabolite abundances and gene expression levels and to furthermore pay special attention to assessing differential expression in candidate genes reported in previous IBD GWASs.[34] A substantial proportion of associations between metabolite abundances and gene expression levels was found with metabolic spectral features associated with hundreds of transcripts and others with only very few transcripts (Fig. 7). These results suggest that colonic tissue from patients with UC share an interconnected global molecular phenotype across both the transcriptome and metabolome that reflects the state of the biological system. It also indicates that both omics data sets actually can be combined in the development of diagnostic biomarker panels or in the molecular characterization of the phenotypes in UC. In

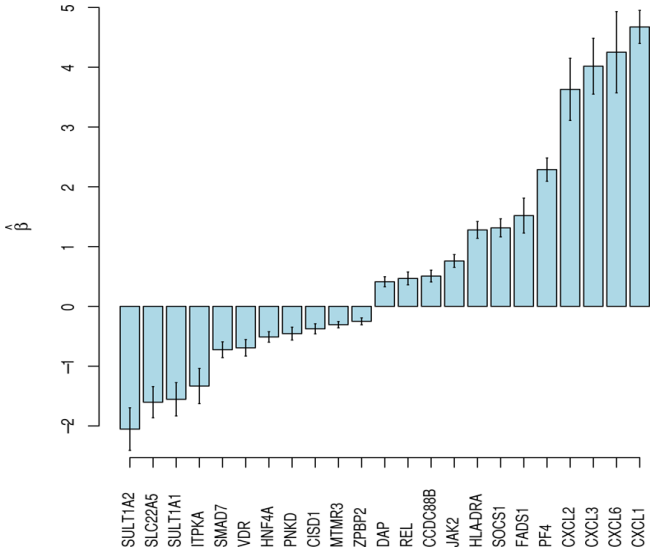
Figure 7



Metabolomic−transcriptomic-wide association analysis. (A) ^{1}H NMR CPMG spectral profile plotted together with annotations. Blue bars indicate the number of significantly (FDR adjusted p-value < 0.05) associated mRNA transcripts for each spectral feature. (B) Empirical distribution of the number of associations for each mRNA variable. (C) Empirical distribution of the number of associations for each ^{1}H NMR spectral feature.

Figure 4 from Rantalainen et al.[34]

Figure 8



Association analysis of mRNA expression levels for GWAS candidate genes with UC active disease and control status. Effects size estimates (coefficient) and associated standard errors from the robust linear model are displayed for significant associations. A positive effect size (beta) indicates a higher expression level in the active UC group. For abbreviations, please see paper IV

Figure 3 from Rantalainen et al.[34]

contrast, although a number of IBD GWAS candidate genes were differentially expressed in the UC phenotypes (Fig. 8), I found no evidence of a significant enrichment of differentially

expressed genes in the subset of GWAS candidate genes. This is in line with subsequent transcriptome-wide expression analyses in more recent studies by one of our own research groups (i.e., CAGE data)[29] and others,[173] where transcriptome data from intestinal biopsies in 300 healthy European individuals were correlated to IBD GWAS signals and provided evidence that 63 of the known IBD loci actually reflect the activity of common regulatory variants that preferentially drive multigenic modules. This just confirms that the estimated genetic effect sizes are relatively small and that other molecular risk factors (e.g., epigenetic or environmental effects) may play a larger role. The need for integration of IBD multiomics is consequently becoming more and more evident, which is also reflected in the increase in multiomics data availability.[174,175]

IBD Phenotypes

Response to Treatment

My longitudinal cohort study of serum metabonomics from patients with IBD treated with IFX demonstrated that the metabolic profiles in active IBD were clearly discriminated from the healthy control subjects before anti-TNF therapy.[126] After induction therapy (i.e., 14 weeks of treatment), the metabolic profile of patients going into clinical remission became almost indistinguishable from that of healthy control subjects (Table 3), whereas responders stayed significantly different, as expected (Table 6). Surprisingly, primary nonresponders, especially nonresponding patients with UC, did not seem to have a significantly different metabolic profile from healthy control subjects, neither before nor after treatment (Table 6). Subsequent analyses comparing the metabolic profiles of the different response groups did not reveal any differences, presumably because of the small numbers in each group, nor did I identify an explanation in the

demographics or clinical scores because these were comparable between the response groups. Thus, the primary nonresponders to IFX seem to constitute a unique molecular phenotype, although not identified with the current technological approach. Another recent study, however, has used liquid chromatography/mass spectrometry (LC/MS)-based metabonomics on serum and feces and identified lipid profiles with increased levels of phosphocholine, ceramide, sphingomyelin, and triglycerides in nonresponders to anti-TNF treatment.[79] Besides confirming a destinct molecular signature for primary nonresponders, this study also pinpoints a possible pharmacodynamic explanation for the primary anti-TNF nonresponse because the dominant inflammatory process might correlate with a specific metabolic phenotype, in which some are driven by TNF and others by lipid. This kind of a priori knowledge would bring us one step closer to real-world precision medicine and let us choose between TNF inhibitors and other regimens from the therapeutic armamentarium (further discussed in Bjerrum et al.[134]).

Tabel 6 **Validation of PLS-DA and O-PLS-DA models**

Model	PLS-DA Permutation test n=200 Q^2	O-PLS-DA CV-ANOVA Q^2
CD Res(0) vs. Control	0.692 ✓	0.637 ✓, p<0.001
CD Res(2) vs. Control	0.632 ✓	0.586 ✓, p<0.001
CD Res(6) vs. Control	0.598 ✓	0.574 ✓, p<0.001
CD Res(14) vs. Control	0.568 ✓	0.557 ✓, p<0.001
CD NRes(0) vs. Control	0.706 ✓	0.731 ✓, p<0.001
CD NRes (2) vs. Control	0.339 ×	0.498 ✓, p<0.001
CD NRes (6) vs. Control	0.390 ✓	0.502 ✓, p<0.001
CD NRes (14) vs. Control	——	——
CD Rem(0) vs. CD Res(0)	0.097 ×	0.035 ×, p=0.89
CD NRes (0) vs. CD Res(0)	0.628 ✓	0.558 ×, p=0.05
CD NRes (0) vs. CD Rem(0)	0.195 ×	-0.143, × p=1.00
UC Res(0) vs. Control	0.477 ✓	0.544 ✓, p<0.001
UC Res(2) vs. Control	0.388 ✓	0.552 ✓, p<0.001
UC Res(6) vs. Control	0.453 ✓	0.419 ✓, p<0.001
UC Res(14) vs. Control	0.471 ✓	0.424 ✓, p<0.001
UC NRes (0) vs. Control	0.272 ×	0.313 ✓, p=0.003
UC NRes (2) vs. Control	0.597 ✓	0.584 ✓, p<0.001
UC NRes (6) vs. Control	0.155 ×	0.052 ×, p=0.734
UC NRes (14) vs. Control	0.380 ×	0.289 ✓, p=0.013
UC Rem(0) vs. UC Res(0)	-0.21 ×	-0.296 ×, p=1
UC NRes (0) vs. UC Res(0)	-0.175 ×	0.022 ×, p=1
UC NRes (0) vs. UC Rem(0)	-0.102 ×	-0.573 ×, p=1

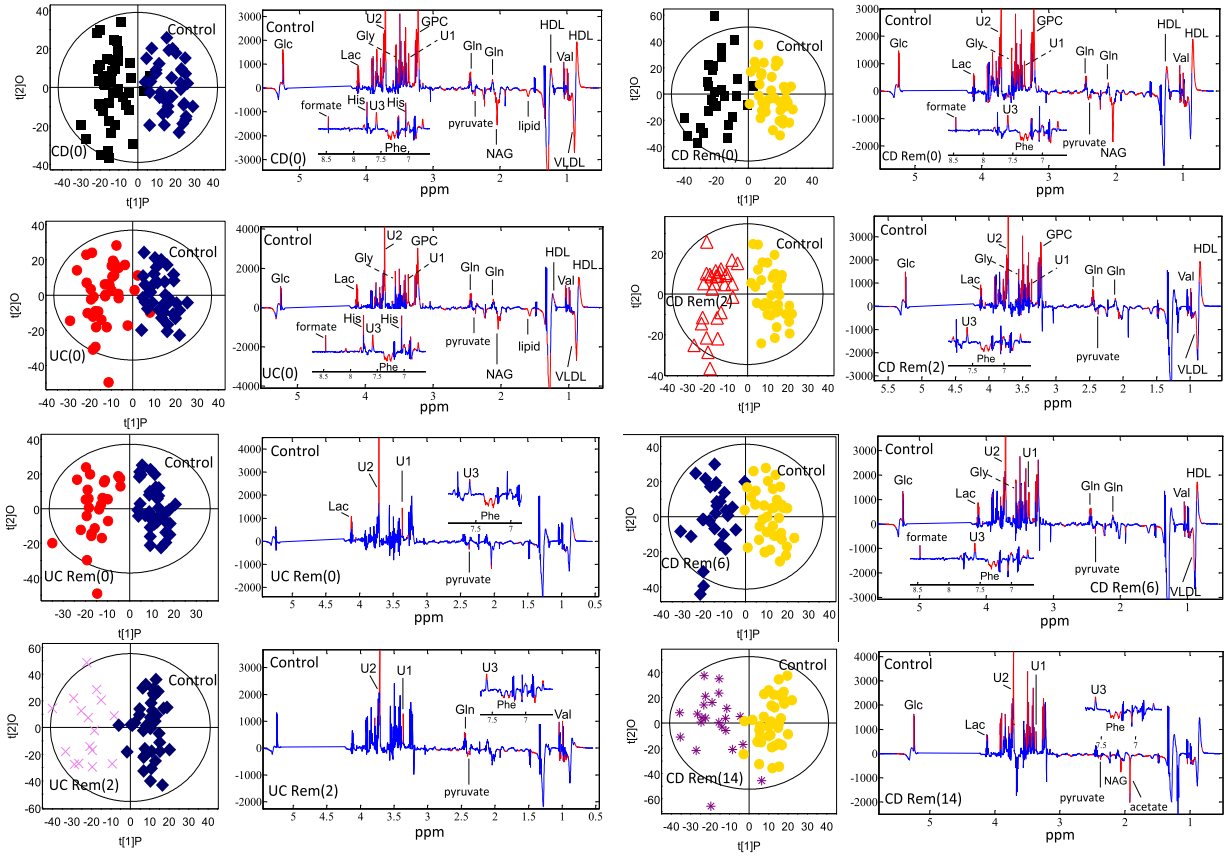
The models were only considered valid if the permutation test and the CV-ANOVA test (p<0.05) were satisfied at the same time
CD, Crohn's disease; CV-ANOVA, analysis of variance of the cross-validated residuals; NRes, non-responder, O-PLS-DA, orthogonal-projection to latent structure-discriminant analysis; PLS-DA, projection to latent structure-discriminant analysis; Rem, remission; Res, responder; UC, ulcerative colitis
(0), before 1st infusion of infliximab; (2), before 2nd infusion; (6), before 3rd infusion; (14), before 4th infusion
Q^2, predictability of the model; r correlation coefficient
✓ valid model
X invalid model
-- Not enough samples to perform statistics

Table S2 (Additional file 4) from Bjerrum et al.[126]

A Proatherogenic Lipid Profile and Increased Risk of Cardiovascular Disease

Chronic inflammation is a dominant driver of thrombosis and atherosclerotic cardiovascular disease, and several chronic conditions, such as rheumatoid arthritis and psoriasis, are associated with progressive atherosclerosis.[176,177] The nationwide Danish landmark cohort study of 4.3 million people, including 28,833 patients with IBD followed for up to 13 years, also associated IBD with cardiovascular heart disease as an independent risk factor.[178] Another Danish study of 20,795 patients demonstrated that the risk for myocardial infarction, stroke, and cardiovascular death was significantly higher during IBD flares, whereas it was similar to that in control subjects during remission.[179] Furthermore, the risk for atherosclerotic cardiovascular disease seems to be particularly increased during the first year after IBD diagnosis, presumably due to disease activity. The exact genesis still remains elusive, although a proatherogenic serum lipid profile seems to play a central part.[180] Thus increased very low-density lipoproteins (VLDLs) and decreased high-density lipoproteins (HDLs) are found repeatedly in patients with active IBD and appear to be the consequence of increased transit times, malabsorption, and the inflammatory setting itself.[107,121,126] Moreover, in my longitudinal study monitoring the metabolic trajectory during induction treatment of IBD patients with IFX, this proatherogenic lipid profile was ameliorated in the context of patients with IBD brought into remission, which was, however, not the case for nonresponders (Fig. 9).[126] These results underline the importance of rigorous and ambitious treat-to-target strategies to avoid not only intestinal complications but also cardiovascular morbidity, especially among young adults. It also merits consideration regarding concomitant statin treatment and early lifestyle interventions during frequent flares or chronically active disease. No international IBD treatment algorithms currently recommend such preventive strategies, but with the current evidence this might soon change.

Figure 9



O-PLS-DA score plots and loading plots. O-PLS-DA score plots and corresponding coefficient-coded loading plots obtained from metabolic profiles of ^{1}H NMR spectra of the serum samples. The score plots display the first PLS component and one orthogonal component for each model. A two-way separation of the samples is demonstrated in all plots. The corresponding back-scaled loading plots reflect the class differences in the NMR spectra. Upright peaks indicate a relatively increased intensity of metabolites, and downright peaks a decreased intensity of metabolites. The colors shown on the plot are associated with the significance of metabolites in separating the samples; red indicating significance at a level of $P < 0.05$. (0), before first infusion of infliximab; (2), before second infusion; (6), before third infusion; (14), before fourth infusion. Glc glucose, Gln glutamine, GPC glycerophosphocholine, Gly glycine, HDL high-density lipoproteins, His histidine, Lac lactate, NAG N-acetyl glycoprotein, Phe phenylalanine, U1, U2, U3 unknown metabolite, Val valine, VLDL very-low density lipoproteins, CD Crohn's disease, UC ulcerative colitis, Rem remission

Figure 3 from Bjerrum et al.[126]

Quiescent UC and Omics Remission

The distinct colonic transcriptional profile of patients with quiescent UC previously described by us (i.e., Olsen et al.[27] and Bjerrum et al.[28]) and others[41,56,62] has been difficult to replicate in other

cohorts.[29,33,34] In my initial integrative omics study,[33] the predictive transcriptomic model for differentiating patients with quiescent UC and healthy control subjects was poor, with an AUC of only 0.57 (Table 4), and subsequent analyses identified only two differentially expressed transcripts among quiescent UC patients and healthy control subjects.[34] The reason for this discrepancy between studies often lies within the definition of quiescent disease; quiescent UC was defined as a Mayo score of <2 and no evidence of microscopic inflammation (i.e., a Geboes score of 0), whereas a Mayo score <4 and a Matts score[181] <3 were considered as quiescent disease in the Planell study.[56] In a more recent study, the mean Geboes score for patients in remission was 1.07, and the significant fold change was only set at 0.5.[41] Thus an actual unique transcriptionally defined state of remission in UC does not seem to exist with stringent histologic remission criteria. It is, however, intriguing that the few differences generated in study IV produce significant KEGG pathways such as DNA replication, mismatch repair, cell cycle, and oxidative phosphorylation that indicate compromised colonic mucosal cell turnover even in quiescent UC and, consequently, wound-healing issues, as described previously in UC.[182]

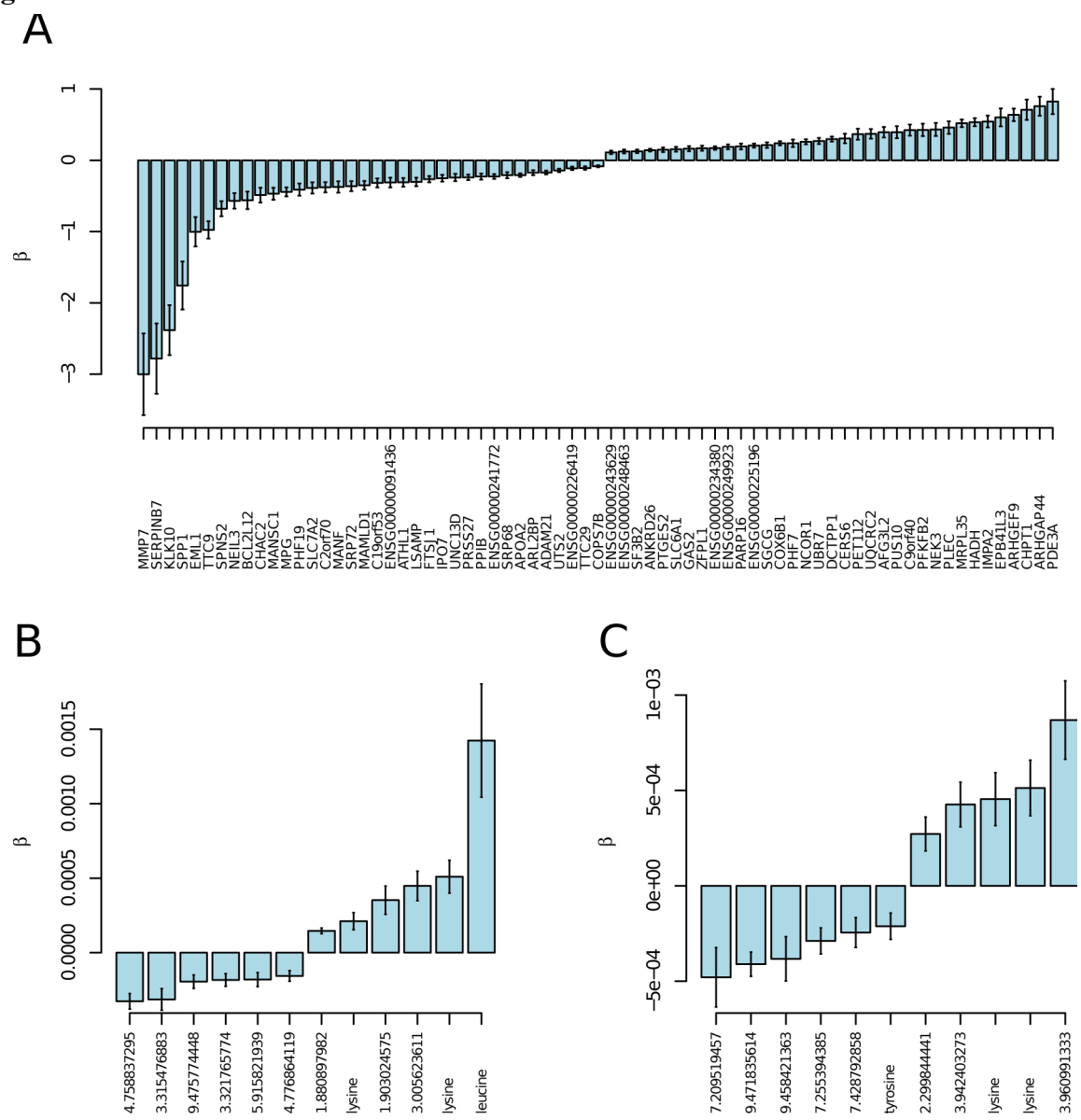
At the metabonomics level, differentiating patients with quiescent UC and healthy control subjects was possible, with an AUC of 0.79 (Table 4, NOE data[33]), even though no significant associations were found between single metabolites and the model comparing patients with quiescent UC and healthy control subjects in the subsequent integrative study.[34] Nevertheless, these results complement earlier findings from me[74] and others[73,75] indicating the existence of an even deeper molecular state of quiescent disease in terms of metabonomics remission (see detailed discussion in Bjerrum et al.[132]). An unfavorable course of IBD with frequent flares and an increased risk of colectomy and CRC is conditioned by sustained wound formation and inflammation. Although not currently recommended by the STRIDE initiative, histologic

remission is becoming the new treatment target in IBD, and large ongoing trials (e.g., ClinicalTrials.gov identifier NCT05157750) are comparing endoscopic remission, histologic remission, and barrier healing for the prediction of long-term disease behavior.[183] However, with the existence of a potential metabonomics remission in IBD, a future treatment target might be set at the molecular level to achieve even better long-term outcomes, but large-scale prospective studies based on metabonomics and intestinal biopsies are warranted to validate this hypothesis.

Glucocorticoid-Dependent UC

Oral or intravenous glucocorticoids are used for the treatment of moderate to severe UC.[184] After initial exposure to glucocorticoids, approximately two-thirds of patients will require reintroduction, and one-third will become glucocorticoid dependent over time. A glucocorticoid-dependent phenotype is characterized by flares recurring during glucocorticoid tapering or within 3 months after glucocorticoid discontinuation and is associated with a higher risk of colectomy.[185,186] A priori knowledge of glucocorticoid dependency would allow for early intervention with timely concomitant treatment with immunomodulators or initiation of treatment with biologics. My integrative studies demonstrate that it is actually possible to molecularly characterize and consequently identify a glucocorticoid-dependent phenotype (Tables 4 and 5).[33,34] Interestingly, the transcripts and metabolites that molecularly dominate this phenotype are all involved in the homeostasis of the extracellular matrix (Fig. 10) and, as a consequence, are part of wound-healing and development of CRC.[187–192] Thus, glucocorticoid-dependent patients with UC seem to have an imbalance in the extracellular matrix potentially resulting in insufficient mucosal wound-healing, which ultimately leads to the increased risk of CRC. This hypothesis obviously needs to be elucidated in future studies.

Figure 10



Glucocorticoid dependency status association analysis. Effect size estimates (coefficient) and associated standard errors (robust linear model) for (A) mRNA expression data, (B) ^{1}H NMR CPMG spectral data, and (C) ^{1}H NMR NOESY spectral data. Positive coefficients (beta) indicate a higher expression level in the steroid independent group relative to that in the steroid dependent group. (Labels: HGNC gene identifiers are used for mRNA transcript probes when available, otherwise Ensembl IDs are provided. For NMR spectral features, annotated metabolites are provided where available, otherwise the chemical shift (ppm) is used.) For abbreviations, please see Rantalainen et al.[34].

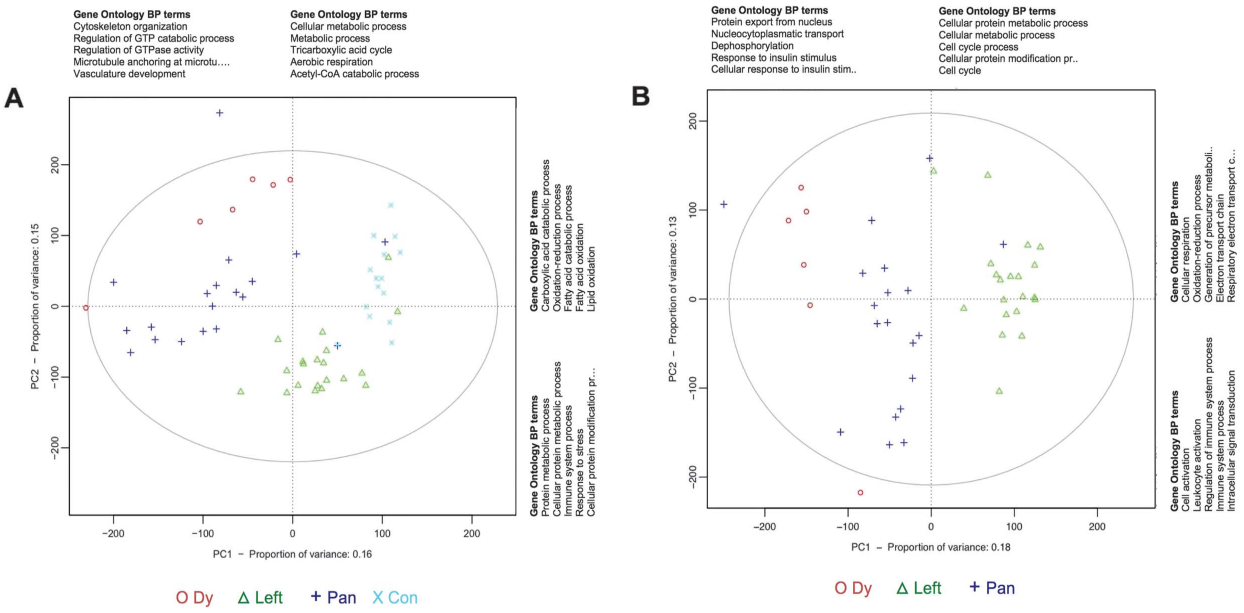
Figure 2 from Rantalainen et al.[34]

Novel Therapeutic Targets and Regimens

Transcriptomics

To characterize the inflammatory processes and to identify transcripts of importance for the carcinogenic behavior of left-sided UC, pancolitis, and UC-associated dysplasia, I enrolled three cohorts - one exploratory microarray cohort and two validation cohorts, including 40 patients with active pancolitis, 47 patients with active left-sided colitis, 12 patients with UC-associated dysplasia, and 15 healthy control subjects in all.[32] The clustering of each group of patients in the principal components analysis (PCA) score plot suggests that each of them has a unique transcriptional profile and that the inflammatory processes of pancolitis are more similar to those of dysplasia than of left-sided colitis (Fig. 11).[32] Focusing on transcripts based on their known involvement in inflammatory and neoplastic processes, I subsequently identified and validated

Figure 11

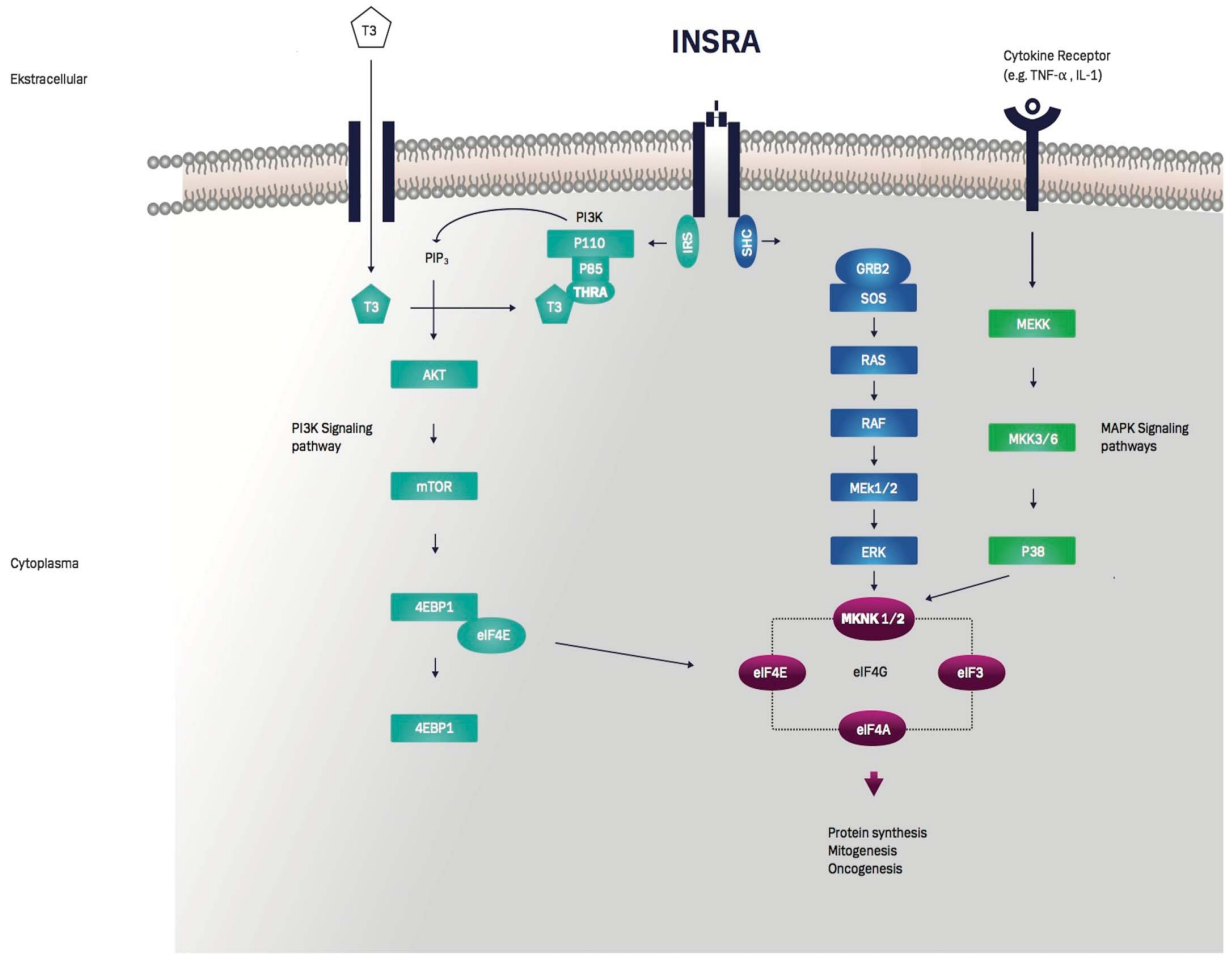


PCA score plots of the microarray data. A, The PCA score plot illustrates separate clustering of the 4 sample types. B, The PCA score plot illustrates a 3-way separation of the samples in accordance with class belonging. Each axis in both PCA score plots is functionally annotated with the 5 most significant (P, 0.005) Gene ontology terms. BP, biological processes

Modified from Figure 1 from Bjerrum et al.[32]

insulin receptor alpha (INSRA) and MAP kinase interacting serine/threonine kinase 2 (MKNK2) as highly overexpressed in dysplastic tissue and pancolitis versus left-sided UC. The two transcripts (i.e., INSRA and MKNK2) are part of the same intracellular signaling pathways: the phosphatidylinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways (Fig. 12) are important regulators of cellular growth, metabolism, and survival in normal functional cells. The signaling pathways congregate on MKNK2 like several other pathways of inflammation-associated cytokine receptors (e.g., interleukin 1 [IL-1] and TNF-α). Once activated, MKNK2 and MKNK1 (MKNKs) phosphorylate eukaryotic initiation factor 4E (eIF4E), which is the rate-limiting step in the activation of the eIF4G complex that subsequently guides the synthesis of proteins and cytokines. Previous studies have identified overexpressed and phosphorylated eIF4E as an oncogenic accelerator and phosphorylation by MKNKs as vital for the oncogenic features of eIF4E,[193] whereas it seems unessential for development and growth in normal tissue.[194] Based on these observations, I singled out MKNKs to be ideal and potential targets in both cancer and IBD treatments.[32] Ultimately, MKNK inhibitors could serve as a novel therapeutic chemopreventive avenue in personalized tailored treatment of pancolitis with potentially very few side effects. Indeed, subsequent and recent work emphasize the strong potential for agents that modulate regulators of mRNA translation integrating signals from oncogenic and immune signaling pathways through phosphorylation of eIF4E and other mRNA binding proteins.[195–198] Thus eFT508, tomivosertib, is such a potent agent that is a highly

Figure 12



Schematic representation of the intracellular signaling pathways in which INSR and MKNK2 are pivotal elements. Activated INSRA stimulates the tyrosine kinase activity of the receptor, which phosphorylates several substrates including IRS1/2 (insulin receptor substrate 1/2) and Shc/Grb2/Sos (adaptor protein Src homology 2 domain-containing/shc/growth factor receptor bound protein-2/Son of Sevenless protein) complexes. IRS proteins and the ligand bound THRA are able to interact with the regulatory subunit (p85) of PI3K (phosphatidylinositide 3-kinase) resulting in an activation of the catalytic subunit (p110) and subsequently activation of AKT and mammalian target of Rapamycin (mTOR). Activated mTOR liberates eIF4E (eukaryotic initiation factor 4E) from the inhibitory binding protein 4EBP1 (eIF4E-binding protein 1), and eIF4E is free to engage the scaffolding protein eIF4G and be phosphorylated by MKNKs, which is the rate-limiting step in the activation of the eIF4F translation initiating complex (eIF4G, eIF4E, and eIF4A). The activated Shc/Grb2/Sos complex along with ligand bound cytokine receptors trigger the MAPK (mitogen-activated protein kinases) pathways of ERK (extracellular signal–regulated kinase) and p38, which activate MKNKs promoting protein synthesis, mitogenesis, and oncogenesis

Figure 6 from Bjerrum et al.[32]

selective and orally bioavailable MKNK1 and MKNK2 inhibitor that significantly reduces multiple proinflammatory and potentially tumorigenic cytokines (i.e., TNF-α, IL-6, and IL-8).[198] Furthermore, eFT508 reverses the aggressive and metastatic characteristics of liver tumors in a mouse model by efficiently eliminating eIF4E phosphorylation without affecting global protein

synthesis. This ultimately leads to a significant and selective downregulation of programmed death ligand 1 (PD-L1) protein abundance.[199] PD-L1 is a transmembrane protein expressed on the cell surfaces of antigen-presenting cells such as macrophages and monocytes and is a ligand of the immune-checkpoint receptor programmed death 1 (PD-1) on T cells. PD-L1/PD-1 interaction leads to negative regulation of T-cell proliferation and is a part of the normal immunologic homeostasis.[200] Cancer cells hide from the immune system by cloaking themselves with expressed PD-L1 on the surface, thus inactivating and inhibiting T-cell proliferation, effectively evading the immune system. Immune checkpoint inhibitor treatment has, however, revolutionized cancer treatment within the past decade, and by blocking the PD-L1/PD-1 interaction, and thus unmasking the cancer cells, a significant increase in survival rates has been documented in an array of microsatellite-instability-high or mismatch-repair-deficient tumors, including CRC[201] and non–small cell lung cancer.[202] By adding selective inhibitors of mRNA translation, such as eFT508, and thus selectively further downregulating PD-L1, the therapeutic efficacy of existing checkpoint inhibitors might increase considerably. A range of clinical trials has been or is currently being undertaken with eFT508 in both lymphoma (ClinicalTrials.gov identifier NCT02937675) and solid cancers (identifiers NCT03616834, NCT03690141, NCT04261218, NCT03318562, NCT02605083, and NCT03258398), including non–small cell lung cancer (NCT04622007), the latter in combination with anti-PD-L1 therapy. While the MKNK1/2 inhibitors are being thoroughly investigated for their potential in cancer therapy, their anti-inflammatory prospects so far remain unexploited.

The INSRA found highly expressed in dysplasia and pancolitis[32] is also commonly expressed in human cancers and binds insulin to promote tumorigenesis[203] via upregulation of the PI3K signaling network, providing tumor cells with enhanced capacities for growth,

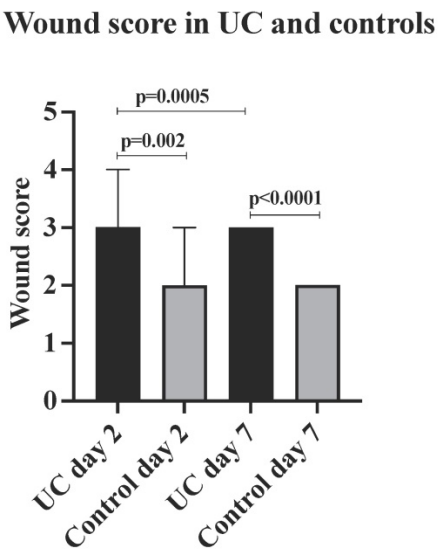
proliferation, survival, and migration.[204,205] Thus abnormal insulin levels have been connected to several malignancies, including CRC, pancreatic cancer, prostate cancer, breast cancer, melanoma, osteosarcoma, and childhood malignancies.[204] Several clinical trials in cancer with small-molecule inhibitors against PI3K report promising results.[206] In spite of this, the effects of insulin and INSRA interaction are not straightforward. A recent in vitro study demonstrated that insulin enhances the effect of chemotherapeutic agents in CRC and that the underlying mechanism is downregulation of PI3K.[207] This inconsistency in the effect of insulin may be the consequence of a local temporal pattern of action with high doses of insulin and a short duration of action accounting for the observed effect. The increased expression of INSRA in the intestinal epithelial cells (IECs) of patients especially with pancolitis consequently may not be a sign of a neoplastic conversion but rather a cytoprotective mode of action during inflammation. This hypothesis was tested in a spin-off study from my initial work[32] employing mice with IEC-specific inactivation of the INSR in an azoxymethane/dextran sulfate sodium (AOM/DSS) colitis–associated CRC model.[208] The study revealed that mice with ablated INSR signaling did not display any signs of a deviant phenotype, implying that epithelial INSR signaling is not essential for the development of a normal colon. The ablation did, however, result in more severe inflammation and higher susceptibility to the DSS treatments, which might explain the increased incidence of IBD seen in patients with both type 1 and 2 diabetes mellitus.[209,210] The ablation also resulted in the development of significantly larger (i.e., tumor > 2 mm) and higher numbers of colonic tumors. This intense inflammation and concomitant neoplastic tendency seem partly to be the consequence of compromised regeneration and wound-healing in inactivated INSR IECs because transcript levels of the IEC differentiation marker cytokeratin 20 and lymphocyte antigen 6A (which is a marker of repairing intestinal epithelium) were significantly lower during

DSS-induced inflammation. As could be expected, genes expressed at high levels were annotated with gene ontology terms linked to inflammation. In contrast, wild-type mice had high expressed genes linked to lipid metabolism during inflammation, signifying the importance of lipids in the inflammatory and wound-healing processes. Subsequent rectal administration of insulin in wild-type mice ameliorated DSS-induced colitis and significantly reduced the number and size of intestinal tumors. Thus, it seems that the anti-inflammatory and wound-healing effects per se of epithelial INSR signaling are chemopreventive. Based on these results, a phase I study has been initiated in which patients with active UC are treated with rectally administered insulin as an add-on treatment to their already established anti-inflammatory therapy, and a phase 2 study is currently being designed.

Metabonomics

Accumulating evidence from my studies suggests that mucosal wound-healing is vital for achieving completely quiescent disease and that lipids are central elements in the process. My most current work consequently focuses on the lipidomic trajectory during wound-healing in the colonic mucosa of healthy control subjects and patients with UC in histologic remission.[132] I included 21 patients with UC and 9 healthy control individuals and subjected them to the wound-healing assay. With this approach, I was able to describe kinetically a more aggressive inflammatory response to intestinal wounding (Fig. 13). This is in line with previous findings by others[131]

Figure 13



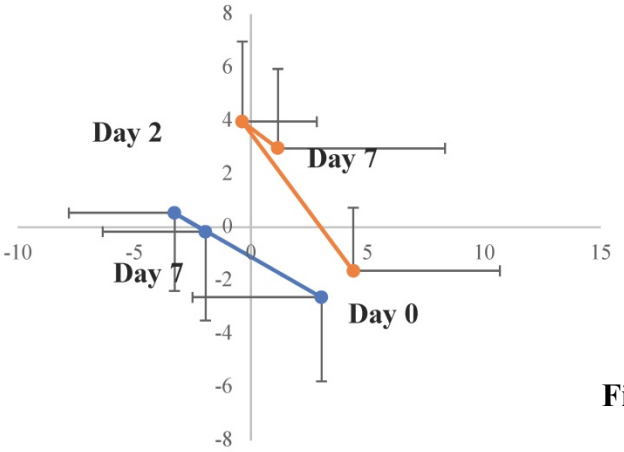
Modified from Figure 2A from Bjerrum et al.[132]

including one of my own studies,[130] in which patients with quiescent UC have a pronounced postinjury response dominated by early neutrophil engagement contrasting a more diminished innate response in the normal human intestine with no significant recruitment of neutrophils within 24 hours. Interestingly, the changes in neutrophil recruitment to innate stimuli does not seem to be restricted to the intestine in UC because similar findings have been seen using a model of acute skin injury in patients with UC.[131,211,212] This is in contrast to patients with CD in whom a delayed neutrophil response is dominant,[131] which illustrates a profound difference in the pathophysiology between UC and CD during flare initiation. Thus, dampening the hyperresponsive mode of action in quiescent UC could be a completely new approach to long-term maintenance treatment and a potential concomitant treatment strategy to conventional anti-inflammatory treatment algorithms. As a proof of concept, my colleagues and I demonstrated an enrichment of the innate lymphoid cell 3 (ILC-3) marker CD117 prior to injury in patients with quiescent UC, and after injury, a significant increase was seen in the ILC-3-secreted IL-17A and IL-22.[130] ILCs are morphologically comparable to T cells but without antigen-specific receptors,[213] and they play a vital part in the innate responses against invading pathogens through secretion of these cytokines,[214] which stimulate epithelial cells to produce antimicrobial peptides and chemokines attracting neutrophils.[215] Colitis models propose that augmented ILC-3 activation exacerbates experimental colitis through the effects of IL-17A and IL-22.[216] In contrast, deficiency of IL-22 and impaired ILC-3 function also aggravates experimental colitis and various microbial infections.[217] These opposing effects of IL-22 seem to be the consequence of spatiotemporal circumstances during inflammation in which coexpressed IL-17A and IL-22 act synergistically to promote chemokine expression, neutrophil recruitment, and inflammation, whereas, in the absence of IL-17A, IL-22 conveys tissue-protective functions by promoting the

integrity of the epithelial barrier.[218] As a consequence, UTTR1147A (IL-22Fc, a fusion protein of human IL-22 linked to a crystallizable fragment [Fc]) is being developed as a novel, nonimmunosuppressive treatment to stimulate epithelial healing and repair and has gone through phase I[219] and II trials (ClinicalTrials.gov identifier NCT03558152). The phase 2 trial recruited patients with moderate to severe active UC, and publicly available data are eagerly awaited because this might be the first-ever IBD drug supporting the wound-healing process.

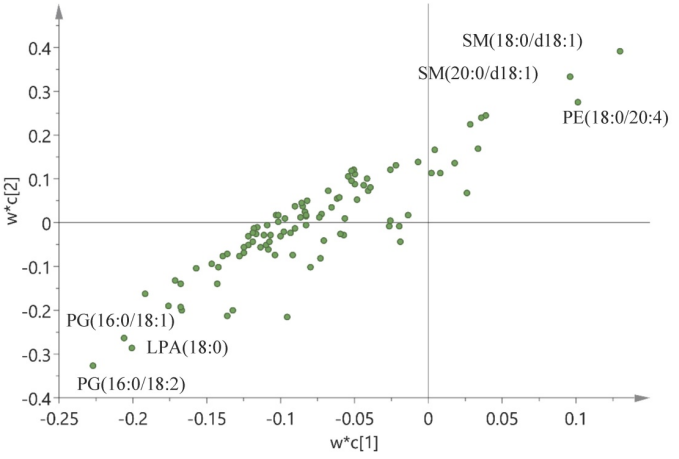
The prolonged observation and sampling period (2–7 days after wounding) enabled me to describe for the first time the normal colonic wound-healing process and thus also a delayed epithelial restitution (Fig. 13) in patients with UC that correlated with distinct changes in the lipidomic profile of especially phospholipids (Figs. 14 and 15).[132] Ultimately, these results may help to guide future medical treatment regimens because several potential therapeutic opportunities for the recovery from active disease and subsequent regeneration were identified, especially with respect to the metabolites of lyso/phosphatidylcholine (LPC/PC),[137,170,220–227] lyso/phosphatidic acid (LPA/PA),[12,136,228–231]

Figure 14



Metabolic trajectory generated from the average of PCA scores of controls (orange color) and UC (blue) during wound healing **Figure 5I from Bjerrum et al.[132]**

Figure 15



Correlation between the wound score (X-matrix, w*c[1]) and phospholipids (Y-matrix, w*c[2]) Lysophosphatidic acid (LPA), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM). **Figure 7 from Bjerrum et al.[132]**

phosphatidylinositol (PI),[232,233] phosphatidylglycerol (PG),[234,235] phosphatidylethanolamine (PE),[236] phosphatidylserine (PS),[237] prostaglandin E_1 (PGE_1),[238,239] and PGD_2. [240] It is, however, extremely difficult to predict the treatment outcome with these metabolites or combinations thereof because they are only poorly described in relation to intestinal wound-healing. Furthermore, no similar human in vivo studies have ever been performed, and our current knowledge of lipids and intestinal wound-healing is based only on in vitro and animal studies. Nevertheless, a few human studies have previously demonstrated that lipid-based treatment strategies may be an alternative or concomitant treatment regime. Thus, orally delivered PC has been shown to improve mucosal wound-healing and may subsequently help to reconstitute the structure and density of the mucus to serve as a protective mechanical shield in patients with UC.[241,242] Finally, in line with my hypothesis on an insufficient wound-healing process in the steroid-dependent UC phenotype, a phase 2 study with PC in steroid-refractory patients with UC (i.e., chronically active disease in spite of ongoing steroid treatment) demonstrated significant improvement or successful steroid withdrawal in 50% of PC-treated patients compared with 10% in the placebo group ($p = 0.002$).[243] In a subsequent phase 3 study, the primary endpoint was met with a significantly higher absolute reduction in the simple clinical colitis activity index (SCCAI) in patients treated with PC compared with placebo. More important, histologic remission was achieved in 41% of the PC-treated group compared with 20% of the placebo-treated group ($p = 0.016$).[137]

4. Conclusion and Future Perspective

With the introduction of high-throughput transcriptomics and metabonomics as well as advanced multivariate analyses, I and others expected to be able to apply and combine these technologies

and bioinformatics to develop clinically useful diagnostic tools in the field of IBD. I now know that the creation of omics-based diagnostic IBD tests with meaningful sensitivities and specificities is a challenging task in the current clinical setting - but why? Based on the data presented herein, an obvious explanation could be an almost rudimentary simplified phenotyping of IBD (i.e., UC and CD). At the molecular level, my studies clearly show that the inflammatory process in left-sided UC is distinct from pancolitis and should be considered as unique phenotypes with differences in their prognosis and need for tailored treatment strategies. IBD precision medicine consequently has the opportunity to advance significantly because a range of novel treatment targets particularly in pancolitis was identified (i.e., INSRA and MKNK), of which insulin enemas are currently being tested in a phase I trial. However, based on current knowledge, MKNK might be an even better treatment target because it is a centrally located hub in several inflammation-associated signaling pathways and may function as both an anti-inflammatory and a chemopreventive treatment strategy.

Omics analyses also revealed quiescent UC as a distinct molecular phenotype, at least at the metabonomics level. It could be argued that this does not represent an actual phenotype but rather is a consequence of unambitious treatment targets in UC. Currently, mucosal healing is the ultimate treatment target in UC, in accordance with the updated STRIDE recommendations, but I believe that with these results, future treatment goals should be set at the molecular level. This obviously requires that future studies can correlate metabonomics remission (i.e., molecularly quiescent disease) with an improved course of UC in terms of extraintestinal manifestations, flares, hospitalization, colectomy, and development of CRC.

A prerequisite for molecular healing is mucosal wound-healing. Here I have demonstrated the presence of a hyperresponsive innate immune system in the colonic mucosa of patients with

UC in remission and subsequent delayed wound-healing that correlates with a distinct lipidomic trajectory. Dampening the hyperresponsiveness (e.g., via IL-22Fc treatment) and promoting the healing process using lipids (e.g., via PC treatment) either as local enemas or in delayed-release formulas as polymeric nanoparticles represent novel therapeutic avenues that needs to be tested and validated. However, the molecular characterization of the wound-healing process has only just been initiated, and further metabologenomics and transcriptomics analyses are highly warranted.

Serum metabonomics analyses identified a proatherogenic lipid profile in patients with active IBD that was eliminated with successful biologic treatment. Based on these findings and the known increased cardiovascular morbidity among young adults, I recommend that future IBD treatment algorithms include advice on lifestyle interventions and statin treatment in young patients with frequent flares or chronically active disease.

The same serum-based metabonomics study also indicated that patients with UC not responding to biologic treatment might have a unique metabolic profile. Subsequent and more recent metabonomics studies have confirmed this in serum and feces, and fecal-based metabolic profiles of bile acids, lipids, and SCFAs are already available for clinical testing and validation. The mechanism by which biologics induce these above-mentioned changes is, however, unknown and requires further molecular characterization of the mucosa-related microbiota and its interplay with host omics during treatment.

Lastly, it should be noted that a challenging aspect of IBD phenotypes is the apparent dynamic structure: patients with IBD initially responding to anti-TNF treatment will lose their response as a result of pharmacodynamic changes in the primary inflammatory driver of their disease,[244] and a significant fraction of patients will experience either more extensive disease or

regression (i.e., from left-sided colitis to pancolitis or vice versa).[245] Not only do we need to accept the existence of far more complex IBD phenotypes, we also need to acknowledge the dynamic structure and thus the concept of adaptive precision medicine.

With this dissertation I propose the existence of more detailed molecular phenotyping, ambitious molecular treat-to-target actions, novel wound-healing treatment strategies, and the use of adaptive precision medicine.

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6. Currently Available Human Adult IBD Metabonomics Studies

Table I. Metabonomics studies based on intestinal biopsies from patients with inflammatory bowel disease

Study	Patients (no)	Technique	Primary results
Bezabeh et al.[73] 2001	UC (46), CD (39), C (25)	NMR	Differentiation of UC, CD and C
Ehehalt et al.[221] 2004	UC (11), CD (7), C (21)	ESI/MS	Decreased levels of (lyso)phosphatidylcholine in quiescent UC, not in CD
Braun et al.[220] 2009	UC (21), CD (10), C (29)	ESI/MS	Decreased levels of (lyso)phosphatidylcholine in quiescent and active UC, not in CD
Balasubramanian et al.[246] 2009	UC (31), CD (26), C (26)	NMR	Decreased levels of amino acids and membrane components in IBD
Bjerrum et al.[74] 2010	UC (68), C (25)	NMR	Differentiation of UC and C Increased levels of amino acids and decreased levels of membrane components in UC
Sharma et al.[75] 2010	UC (5), CD (5), C (26)	NMR	Decreased levels of amino acids and membrane components in IBD
Ooi et al.[247] 2011	UC (22)	GC/MS	Decreased levels of amino acids and TCA cycle molecules in UC
Sewell et al.[120] 2012	CD (5), C (5)	ESI/MS	Decreased phosphatidylinositol in CD
Masoodi et al.[248] 2013	UC (54)	LC/MS	Increase in eicosanoids in inflamed tissue compared to paired uninflamed tissue
Pearl et al.[249] 2014	UC (69), C (69)	LC/MS, GC/MS	Changes in polyunsaturated fatty acids in inflamed tissue, changes correlate with severity of inflammation.
Bjerrum et al.[III] 2014	UC (43), C (15)	^{1}H NMR	Differentiation of active UC, inactive UC and C Identification of UC sub phenotypes Integration of omics
Rantalainen et al.[IV] 2015	UC (43), C (15)	NMR	Molecular characterization of UC sub phenotypes with combined metabolomics and transcriptomics
Gobbetti et al.[250] 2017	IBD (10), C (11)	LC/MS	Identification of increased levels of a range of resolvins in the colon of patients with active IBD compared to controls
Diab et al.[251] 2019	UC (20), C (10)	LC/MS	Increase in eicosanoids and decrease in endocannabinoids in active UC
Bazarganipour et al.[166] 2019	UC (59)	LC/MS	Decreased levels of sphinganine and dihydroceramides in inflamed colonic tissue compared with noninflamed tissue from the same patients

Diab et al.[225] 2019	UC (33), C (14)	LC/MS	Differentiation of active UC, inactive UC and C Changes of phospholipids in both active and inactive UC
Diab et al.[252] 2019	UC (28), C (14)	LC/MS, GC/MS	Altered metabolic pathways in UC; phospholipids, linoleic acid, glutamate, tryptophan, butyrate, and glutathione
Santoru et al.[253] 2021	UC (82), CD (50), C (51)	GC/MS	No differentiation between UC and CD, but UC vs C and CD vs C with higher concentrations of aspartate, glutamate, glutamine, glycine, and ornithine, low levels of fumarate, glycerol, phosphate, lactate, myo-inositol, aoleic acid in IBD vs C
Adegbola et al.[254] 2021	CD (20), C (30)	LC/MS	Differentiation between CD fistulas and idiopathic fistulas with 19 and 2 differentially expressed metabolites and lipids, respectively
Frau et al.[255] 2021	UC (20), CD (23), C (20)	GC/MS	Correlation between fungi microbiome and level of BCFA
Bjerrum et al.[VIII] 2022	UC (21), C (9)	LC/MS	Lipidomic trajectories characterize delayed colonic mucosa healing

Branched chain fatty acid (BCFA), controls (C), Crohn´s disease (CD), electrospray ionization (ESI), gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), tricarboxylic acid (TCA), ulcerative colitis (UC)

Table II. Metabonomics studies based on fecal samples from patients with inflammatory bowel disease

Study	Patients (no)	Technique	Primary results
Kruis et al.[111] 1986	UC (6), CD (10), C (5)	GC	Increased bile acids in CD and decreased in UC compared with C Increase in primary bile acids and decrease in secondary bile acids in IBD compared with C
Marchesi et al.[112] 2007	UC (10), CD (10), C (13)	NMR	Differentiation of active UC, active CD, and C Increased levels of amino acids in IBD Decreased levels of SCFA, methylamine and trimethylamine in especially CD
Garner et al.[113] 2007	UC (18), C (30)	GC/MS	Differentiation of UC and C Lower range of VOCs in UC than C High number of alkenes and low number of alkanes and nitrogen- and sulfate containing compounds in UC

Jansson et al.[114] 2009	14 (CD), C (20)	ICR-FT/MS	Differentiation of quiescent ilieal CD, colonic CD, and C, young and adult Differences in the metabolism of tyrosine, phenylalanine, bile acids, fatty acids, arachidonic acid, and prostaglandins
Le Gall et al.[115] 2011	UC (13), IBS (10), C (22)	NMR	Differentiation of UC and C Increased levels of taurine, cadaverine and to some extent lactate
Vigsnaes et al.[116] 2013	UC (8), C (4)	LC/MS	Increase in bile acids, tryptophan, and phenylalanin
Walton et al.[117] 2013	UC (20), CD (22), IBS (26), C (19)	GC/MS	Differentiation of CD and C Increase in ester and alcohol derivatives of SCFA Normalization of VOCs after treatment
De Preter et al.[153] 2013	CD (67), C (49)	GC/MS	Differentiation of CD and C Decreased levels of median-chain fatty acids in CD Inulin induced increase in butyrate and acetaldehyde,
Duboc et al.[148] 2013	UC (30), CD (12), C (29)	LC/MS	Impaired metabolism of bile acids in IBD characterized by defective deconjugation, transformation and desulphation
Kumari et al.[149] 2013	UC (26), C (14)	GC-FID	Decreased levels of SCFA in severe active UC
Machiels et al.[151] 2014	UC (127), C (87)	GC/MS	Decreased levels of SCFA in UC
Bjerrum et al.[V] 2015	UC (48), CD (44), C (21)	NMR	Differentiation of active UC, quiescent UC and C, Increased levels of amino acids and lactate in UC, Decreased levels of SCFA in UC
De Preter et al.[256] 2015	UC (68), CD (83), C (40)	GC/MS	Differentiation of UC, CD, and C Decreased levels of median-chain fatty acids in IBD
Bussche et al.[156] 2015	UC (8), CD (5), C (10)	LC/MS	Differentiation UC, CD and C Validation of the metabolic fingerprinting workflow for feces and in vitro digestive fluids
Ahmed et al.[155] 2016	UC (100), CD (117), C (109)	GC/MS	Differentiation of active CD, quiescent CD and C, but not active UC, quiescent UC and C
Walton et al.[154] 2016	CD (17)	GC	Enteral feeding reduces SCFA, 1-propanol. 1-butanol, and methyl and ethyl esters of SCFSs
Lee et al.[257] 2017	UC (22), CD (31), C (19)	FT-ICR/MS	Differentiation of UC, CD and C. Oral iron therapy differentially affects the fecal metabolome compared with iv therapy
Santoru et al.[150] 2017	UC (82), CD (50), C (51)	GC/MS, LC/MS, and NMR	Differentiation of IBD and C, not between UC and CD, increased amino acids and lipids in IBD and decreased b group vitamins
Franzosa et al.[77] 2019	UC (76), CD (88), C (56)	LC/MS	Differentiation of IBD and C In IBD increased sphingolipids and bile acids, decreased triacylglycerols and tetrapyrols

Lloyd-Price et al.[84] 2019	UC (38), CD (67), C (27)	LC/MS	Reduced diversity and changes in bile acids, SCFA, and other metabolites (NB pediatric and adult cohort, mixed activity)
Weng et al.[159] 2019	UC (107), CD (173), C (42)	GS/MS, LC/MS	Decrease in especially long- and medium-chain fatty acids, bile acids, and vitamin compounds in IBD
Aden et al.[78] 2019	UC (6), CD (3)	LC/MS	Characterize the changes (butyrate and acetaldehyde) in the fecal metabolome in response to anti-TNF treatment,
Paramsothy et al.[258] 2019	UC (26)	LC/MS	Responders to FMT increased SCFA and secondary bile acids
Ding et al.[79] 2020	UC (10), CD (76), C (13)	LC/MS	Candidate biomarker identification (bile acids and lipids) predicting anti-TNF treatment response in CD
Sinha et al.[80] 2020	UC pouch (17), C pouch (7)	LC/MS	Low levels of the secondary bile acids deoxycholic acids and lithocholic acid
De Freitas Lins Neto et al.[259] 2020	UC (11), CD (10), C (15)	NMR	Unknown activity status of IBD patient, Differentiation of CD and C, but not UC and C, CD vs UC not explored
Fang et al.[81] 2021	UC (50), CD (79)	LC/MS	Surgery lowers the metabolome diversity
Yang et al.[82] 2021	UC (32), C (23)	LC/MS	Low microbiome diversity and high primary bile acids
Mills et al.[15] 2022	UC (40+ 73), CD (117), C (20)	LC/MS	Multi-omics identify B. vulgatus derived proteolysis contributing to UC severity
Di´Narzo et al.[14] 2022	UC (484), CD (464), C (465)	LC/MS	Integrative genetics and metabolomics identify 173 genetically controlled metabolites some associated with clinical and endoscopic disease status

Controls (C), Crohn´s disease (CD), flame ionization detector (FID), Fourier-Transform Ion-Cyclotron-Resonance (FI-ICR), gas chromatography (GC), irritable bowel syndrome (IBS), liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), short chain fatty acid (SCFA), tricarboxylic acid (TCA), tumor necrosis factor (TNF), ulcerative colitis (UC), volatile organic compounds (VOC)

Table III. Metabonomics studies based on blood samples from patients with inflammatory bowel disease

Study	Patients (no)	Technique	Primary results
Forrest et al.[260] 2002	UC (7), CD (5), C (12)	LC	Increased levels of kynurenine in IBD
Bene et al.[167] 2007	CD (100), C (94)	ESI/MS	Carnitine ester profiles differ significantly in CD
Gnewuch et al.[261] 2009	UC (161), CD (197), C (310)	LC/MS	Total bile acid, total bile acid conjugate, and total bile acid glycoconjugate levels decreased only

			in CD, total unconjugated bile acid levels decreased only in UC
Bjerrum et al.[74] 2010	UC (74), C (25)	NMR	No difference between active UC, quiescent UC, and C in PBMCs
Ooi et al.[247] 2011	UC (13), CD (21), C (17)	GC/MS	Differences in 27 amino acids including TCA cycle metabolites in a mixed cohort of active and quiescent UC and CD
Williams et al.[121] 2012	UC (20), CD (24), C (23)	NMR	Differentiation of quiescent UC, CD, and C with changes in primarily lipids, choline metabolites, and amino acids
Schicho et al.[105] 2012	UC (20), CD (20), C (40)	NMR	Differentiation of active IBD and C, but not between active UC and active CD
Gupta et al.[262] 2012	CD (25), C (11)	LC	Decreased levels of tryptophan in active CD
Sewel et al.[120] 2012	CD (41), C (24)	LC/MS	No difference between quiescent CD and C in unstimulated and stimulated macrophages from PBMCs
Zhang et al.[101] 2013	UC (20), C (19)	NMR	Differentiation of active UC and C with increased levels of glucose, 3-hydroxybutyrate, and phenylalanine, and reduced levels of lipids
Fathi et al.[123] 2013	CD (26), C (29)	NMR	Differentiation of active CD and C with a low level of lipid in CD
Duboc et al.[148] 2013	UC (30), CD (12), C (29)	LC/MS	No difference in bile acids between IBD and C except for low levels of secondary bile acids in active IBD
Iwamoto et al.[263] 2013	UC (10), CD (21), C (26)	LC/MS	Bile acid malabsorption in CD associates with deactivation of pregnane X receptor and its anti-inflammatory properties
Dawiskiba et al.[107] 2014	UC (24), CD (19), C (17)	NMR	Differentiation of active IBD and C with reduced lipids and choline and increased phenylalanine, acetoacetate, formate, 3-hydroxybutyrate, and lactate, no difference between UC and CD
Fathi et al.[124] 2014	CD (26), C (29)	NMR	Differentiation of CD and controls primarily based on high levels of isoleucine and low levels of valine in CD
Kohashi et al.[161] 2014	UC (120), CD (39), C (120)	GC/MS	Differentiation of active UC, quiescent UC, CD, and C with decreased levels of TCA-related metabolites, urea-related metabolites and several amino acids
Yau et al.[264] 2014	UC (19), CD (25), C (9)	LC/MS, GC/MS	Increased levels of angiotensin IV, diphthamide, and GM3 ganglioside in CD. Quinolinic acid increased in active CD and correlated with CRP and CDAI
Wilson et al.[265] 2015	UC (33), CD (73), C (373)	LC/MS	Low level of trimethylamine-*N*-oxide in IBD, especially in active UC. High

			level of choline in IBD, and no difference in carnitine levels
Fan et al.[165] 2015	UC (16), CD (24), C (84)	LC/MS	Profound differences in lipid profile of CD, but not in UC compared to C
Hisamatsu et al.[162] 2015	UC (355)	LC/MS	Low level of histidine predicts 1-year relapse
Nikolaus et al.[266] 2017	UC (211), CD (224), C (291)	LC	Targeted metabonomics with inverse correlation between tryptophan and disease activity
Bjerrum et al.[VI] 2017	UC (38), CD (49), C (37)	NMR	Distinct metabolic trajectory in UC and CD during infliximab treatment with changes in lipids, phospholipids, and pyruvate metabolites
Scoville et al.[164] 2018	UC (20), CD (20), C (20)	LC/MS	Profound differences in amino acids, lipids and TCA cycle metabolites of CD, but only few in UC compared with C
Murakami et al.[267] 2018	UC (12), CD (14), C (30)	LC/MS	Decreased Clostridium subcluster XIVa correlates with reduced primary bile acids
Sofia et al.[268] 2018	UC (99)	LC/MS, GC/MS	Increased kynurenine/tryptophan ratio correlates with degree of endoscopic inflammation in UC
Murgia et al.[170] 2018	UC (78), CD (50), C (60)	LC/MS	Differentiation of IBD and C with changes in primarily phosphatidylcholine, lysophosphatidyl-choline, fatty acids, and several amino acids
Probert et al.[163] 2018	UC (40)	NMR	Metabolic profiles predict progression and differentiate between low and high endoscopic and histological activity
Lai et al.[269] 2019	CD (20), C (10)	LC/MS	Enhanced ß-oxidation of fatty acids (docosahexaenoic acid, linolenic acid, arachidonic acid) and altered amino acid metabolism (tryptophane, histidine, phenylalanine)
Whiley et al.[270] 2019	UC (19), C (10)	LC/MS	Decreased levels of picolinic acid and xanthurenic acids and increased level of kynurenine in UC
Sun et al.[271] 2019	UC (48), C (30)	LC/MS	Low level of trimethylamine-*N*-oxide in UC, especially in active UC. High level of sphingosine-1-phosphate in UC and associated with Roseburia, Klebsiella, and Escherichia-Shigella
Bazarganipour et al.[166] 2019	UC (98), C (25)	LC/MS	Changes in sphingolipids, free fatty acids, lysophosphatidylcholines, and triglycerides correlate with disease severity in UC
Roda et al.[272] 2019	UC (40), CD (40), C (29)	LC/MS	Secondary bile acids increase after anti-TNF treatment in CD patients and restore the bile acid profile
Dudzinska et al.[273] 2019	UC (27), CD (28), C (50)	LC	Low level of tryptophane in IBD and low level of kynurenic acid during remission compared with active disease

Manfredi et al.[169] 2019	UC (13), CD (15), C (17)	LC/MS, GC/MS	Differentiation of active UC, active CD, and C using integrated proteomics and lipidomics analyses and associated with coagulation, fibrinolysis, and acute phase response processes and low levels of free fatty acids
Guan et al.[224] 2020	IBD (129), C (81)	LC/MS	Differentiation of IBD and C with profound changes in bile acids, eicosanoids, and glycerophospholipids,
Giovanni et al.[171] 2020	CD (35), C (33)	GS/MS	Optimization of the analytical workflow
Tefas et al.[168] 2020	UC (17), CD (5), C (24)	LC/MS	Differentiation between UC and CD and between IBD and C with changes in glycerophospholipids, linoleic acids, and sphingolipids
Ding et al.[79] 2020	UC (19), CD (76), C (13)	LC/MS	Candidate biomarker identification (bile acid and lipids) predicting anti-TNF treatment response in CD
Krzystek-Korpacka et al. 2020	UC (48), CD (52), IBS (18), C (40)	LC/MS	Identification of deregulated arginine/Nitric oxide pathway in both active and quiescent CD and UC
Borren et al.[172] 2020	UC (56), CD (108)	LC/MS	Biomarker identification predicting relapse over 2 years in patients with quiescent disease
Borren et al.[83] 2021	UC (60), CD (106)	LC/MS	Fatigue in quiescent IBD associates with reduced levels of methionine, tryptophan, proline, and sarcosine including a lower alpha diversity of the microbiome
Horta et al.[274] 2021	UC (20), CD (27)	LC/MS	Lipidome of UC and CD fatigue with decreased levels of phospholipids and eicosanoids compared to non-fatigue
Notararigo et al.[125] 2021	UC (9), CD (18), C (10)	NMR	Homoserine/methionine and isobutyrate discriminate inactive CD and C, creatinine, proline, tryptophane discriminate inactive UC and C
Santoru et al.[253] 2021	UC (82), CD (50), C (51)	GC/MS	No differentiation between UC and CD, but UC vs C and CD vs C with higher concentrations of hydroxybutyrate, citrate, lactate, proline, threonine, urea, and sorbitol
Di´Narzo et al.[14] 2022	UC (484), CD (464), C (465)	LC/MS	Integrative genetics and metabolomics identify 173 genetically controlled metabolites some associated with clinical and endoscopic disease status

Controls (C), Crohn´s disease (CD), Crohn´s disease activity index (CDAI), C-reactive protein (CRP), electrospray ionization (ESI), gas chromatography (GC), irritable bowel syndrome (IBS), liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), peripheral blood mononuclear cells (PBMCs), tricarboxylic acid (TCA), tumor necrosis factor (TNF), ulcerative colitis (UC)

Table IV. Metabonomics studies based on urine samples from patients with inflammatory bowel disease

Study	Patients (no)	Technique	Primary results
Cracowski et al.[102] 2002	CD (23), C (23)	GC/MS	Increased F_2-isoprostanes in CD
Stanke-labesque et al.[103] 2008	UC (28), CD (32), C (30)	LC/MS	Increased leukotriene E_4 in in active UC and CD
Williams et al.[104] 2009	UC (60), CD (86), C (60)	NMR	Differentiation of UC, CD and C Low levels of hippurate and 4-cresol, high levels of formate in CD.
Bjerrum et al.[74] 2010	UC (74), C (25)	NMR	No difference between active UC, quiescent UC, and C.
Schicho et al.[105] 2012	UC (20), CD (20), C (40)	NMR	Differentiation of IBD and C, not between UC and CD Low levels of Hippurate and low levels of lactate and tryptophan.
Stephens et al.[106] 2013	UC (30), CD (30), C (60)	NMR	Differentiation of UC, CD, and C Corrected for surgery and anti-TNF treatment the difference disappeared between UC and CD.
Dawiskibat et al.[107] 2014	UC (24), CD (19), C (17)	NMR	Differentiation of active IBD and quiescent IBD, active IBD and C, and quiescent UC and C
Alonso et al.[108] 2016	UC (402), CD (399) C (289)	NMR	Differentiation of UC, CD and C Differentiation of active and quiescent CD Low levels of hippurate, citrate, unknown 7, and 3-hydroxyisovaleric acid
Keshteli et al.[109] 2017	UC (20)	LC/MS, NMR	Urine metabonomics predicts UC relapse within 12 months.
Keshteli et al.[97] 2018	CD (38)	LC/MS, NMR	Postoperative unique urinary metabolomic fingerprint in patients with endoscopic recurrence compared to remission.
Alothaim et al.[98] 2018	UC (40), C (17)	LC/MS	Development of LC/MS for optimized detection of SCFA
Li et al.[101] 2019	CD (9)	NMR	Significant increase in indoxyl sulfate, 4-hydroxyphenylacetate, creatinine, dimethylamine, glycylproline, hippurate, and trimethylamine oxide at the first fecal microbiota transplantation
Keshteli et al.[99] 2019	UC (53), IBS (39), C(21)	GC/MS, LC/MS	Differentiation between IBS, quiescent UC and C.
Piestansky et al.[100] 2019	CD (13), C (10)	CE/MS, LC/MC	Validation of the metabolic profiling of 20 amino acids
Ding et al.[79] 2020	UC (10), CD (76), C (13)	LC/MS	Candidate biomarker identification (histidine and cysteine) predicting anti-TNF treatment response in CD.

Controls (C), Crohn´s disease (CD), gas chromatography (GC), irritable bowel syndrome (IBS), liquid chromatography (LC), mass spectrometry (MS), nuclear magnetic resonance (NMR), short chain fatty acid (SCFA), tumor necrosis factor (TNF), ulcerative colitis (UC)

Table V. Metabonomics studies based on breath samples from patients with inflammatory bowel disease

Study	Patients (no)	Technique	Primary results
Kokoszka et al.[85] 1993	UC (1), CD (14), Non-IBD (17)	GC	Increased pentane predicts intestinal inflammation
Sedghi et al. [86]1994	UC (17), C (14)	GC	Increased level of ethane, but not pentane in active UC
Pelli et al.[89] 1999	UC (10), CD (10), C (10)	GC	Increased levels of pentane, propane and ethane in active IBD
Wendland et al.[90] 2001	CD (37), C (37)	GC	Increased levels of pentane, ethane, and isoprostane in both active and inactive CD
Dryahina et al. [91]2013	UC (28), CD (20), C (140)	SIFT/MS	Increased levels of pentane in IBD patients in an on-line, real-time, single breath test
Bodelier et al.[94] 2015	CD (191), C (110)	GC/MS	Differentiation between active CD, inactive CD and, C based on a set of 10 discriminatory VOCs
Hicks et al.[95] 2015	UC (20), CD (18), C (18)	SIFT/MS	Differentiation of UC, CD and C based on six discriminatory VOCs
Rieder et al.[88] 2016	UC (11), CD (24), non-IBD (6), IPAA (30), C (53)	SIFT/MS	Differentiation of IBD, non-IBD, IPAA, and C. Not between UC and CD
Smolinska et al.[96] 2017	UC (72), non-UC (22)	GC/MS	Differentiation of active UC, quiescent UC, and non-UC based on 11 VOCs
Dryahina et al.[92] 2017	UC (51), CD (136), C (14)	SIFT/MS	Increased levels pentane, acetate, propanoate, butanoate and hydrogen sulphide in CD
Smolinska et al.[87] 2018	CD (68)	GC/MS	Differentiation of active CD and quiescent CD and correlation of VOCs and the microbiome
Tiele et al.[93] 2019	UC (16), CD (14), C (9)	GC/IMS	Differentiation of IBD and C based on butanoate and ethanoate

Controls (C), Crohn´s disease (CD), gas chromatography (GC), ion mobility spectrometer (IMS), ileal pouch-anal anastomosis (IPAA), mass spectrometry (MS), selected ion flow tube (SIFT), ulcerative colitis (UC), volatile organic compounds (VOC)