CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS
Abstract

Inflammatory bowel diseases (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), are chronic inflammatory diseases of the gastrointestinal tract. Patients with IBD experience alternating episodes of active and quiescent disease, which vary greatly and are difficult to treat. Currently, there is no cure for IBD and its exact pathogenesis remains incompletely understood. IBD is a complex, heterogeneous and unpredictable condition, which not only complicates its early detection and diagnosis, but also the prediction of disease course and response to therapy. This emphasizes the urgent need for biomarkers, which are objectively measured indicators of (ab)normal biological processes or – systems. In the context of IBD, biomarkers are crucial for a variety of purposes, including (early) diagnosis, assessment of disease activity and complications, and prediction of therapeutic response, disease course and prognosis. This thesis aims to identify and apply novel biomarkers in patients with IBD, while simultaneously evaluating their utility to predict clinically relevant outcomes (e.g. disease activity and therapeutic response) and their amenability to nutritional and drug-based interventions. For that purpose, biomarkers are explored from different biological systems and -mechanisms, including (antimicrobial) immunity (Part I), inflammation and fibrosis (Part II), and oxidative stress (Part III). Subsequently, the utility and dynamics of several of these biomarkers are assessed in relation to nutritional and drug-based interventions (Part IV). Simultaneously, this thesis also aims to deepen our understanding of the complex interactions of pathophysiological factors underlying the pathogenesis of IBD. Finally, the scientific and clinical sequelae of the recent coronavirus disease 2019 (COVID-19) pandemic are discussed in the context of biomarkers and IBD (Part V). Ultimately, this thesis aims to contribute to the characterisation and validation of novel biomarker signatures in patients with IBD by adopting a multimodal approach, consisting of multiple biological systems and -mechanisms and a combination of in vitro, in vivo and in silico strategies to interrogate them.
Inflammatory Bowel Disease: a complex and heterogeneous disease

Inflammatory bowel diseases (IBD) are chronic ulcerative inflammatory diseases of the gastrointestinal (GI) tract, characterized by a considerable degree of heterogeneity and pathophysiological complexity.1 Crohn's disease (CD) and ulcerative colitis (UC) are the two main clinicopathological subtypes of IBD. In CD, ulcerations and inflammation can occur in any part of the gastrointestinal (GI) tract, whereas in UC, inflammation is generally limited to the colon. CD is endoscopically characterized by discontinuous or ‘patchy’ involvement, ulcerative inflammation (where the terminal ileum is most frequently affected), a ‘cobblestone appearance’ of the intestines, and (peri-)anal lesions, whereas UC typically presents with rather superficial mucosal inflammation.2,3 Currently, IBD affects approximately 1 in 1,000 individuals, and its global incidence is steadily rising, especially in European and North American countries. This trend is fairly similar for men and women, but shows varying patterns depending on genetic background.4 The peak age of onset lies within the second to fourth decade of life. Although the exact disease origin is unknown, an interplay between genetic factors, the gut microbiota, the host immune system and environmental triggers (e.g. lifestyle and diet) is believed to underlie the initiation of IBD.5,6

Clinical manifestations of IBD include abdominal pain, diarrhoea (with or without blood loss), fatigue, weight loss, and a series of extraintestinal manifestations, e.g. arthralgia or arthritis, uveitis, and skin abnormalities. However, clinical symptoms of IBD vary greatly between patients and in time. This heterogeneous clinical symptomatology makes a clinical suspicion of IBD rather nonspecific, which may lead to a delay in medical diagnosis and necessitates the use of additional diagnostic modalities. According to the European Crohn’s and Colitis Organisation (ECCO) and European Society of Gastrointestinal and Abdominal Radiology (ESGAR) diagnostic consensus guidelines, the diagnosis of CD or UC should be based on a combination of clinical, biochemical, stool, endoscopic, cross-sectional imaging, and histological investigations.7 Upon initial investigations of a patient presenting with GI complaints and a raised inflammatory marker in stool, the performance of an invasive ileo-colonoscopy (i.e., endoscopy) procedure with biopsies is considered as the gold standard for diagnosing IBD.

The disease course of patients with IBD is characterised by a ‘relapse-remitting’ pattern, where episodes of mild disease, sometimes in the absence of any symptoms, alternate with periods of active and/or severe disease, which often require urgent medical or surgical intervention. Therapeutic interventions are aimed at resolving intestinal inflammation, maintaining disease remission, and preventing long-term disease complications, hospitalisation and the need for surgery. Despite the increasing availability of effective treatments, it remains challenging to control the disease course. This is evidenced by the fact that long-term surgery rates only slightly decreased over the past decade; even up to one third of patients require surgery within the first ten years post-diagnosis.8 As a consequence, patients with IBD still need life-long treatment.

The need for biomarker-based disease evaluation of IBD

The term ‘biomarker’ is defined as “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention”.9,10 In the context of IBD, biomarkers could potentially be used for a variety of disease management purposes (Figure 1), many of which will be touched upon in subsequent sections.
Figure 1 | Simplified overview of biomarker targets and utilities alongside the disease progression pathway in IBD. Targets of biomarkers may be found in the main determinants of IBD susceptibility (e.g. genetics, the gut microbiota, host immunity, and environmental triggers such as lifestyle and dietary habits), the routinely used outcome parameters of intestinal inflammation (e.g. endoscopy as ‘gold standard’, followed by fecal calprotectin, C-reactive protein, imaging modalities such as intestinal ultrasonography, and symptom-based scores), or in disease complications such as intestinal fibrosis. From early risk factors towards irreversible disease complications, the utility of biomarkers may range from the early detection and initial diagnosis of IBD to the prediction of fibrotic complications and post-surgical disease course. The usefulness or value of a given biomarker generally decreases further downstream the disease progression pathway, since the disease becomes increasingly resistant against the established therapeutic arsenal. Abbreviations: CRP, C-reactive protein; FCal, fecal calprotectin; IBD, inflammatory bowel disease.

In this thesis, biomarkers will be explored that may potentially be useful for elucidating disease pathophysiology and/or IBD susceptibility to improve the initial disease diagnosis. In addition, biomarkers will be evaluated for assessment of intestinal inflammatory disease activity, assessment of disease complications, for prediction of response to established medical treatment, monitoring of therapeutic efficacy, as potential targets for novel therapeutic interventions, and for prediction of disease course and/or disease prognosis.

Improving the initial diagnosis of IBD using immune-based biomarker signatures
As clinical symptomatology is rather non-specific for diagnosing IBD, biomarkers form an integral part in establishing the initial diagnosis of IBD. Every patient suspected for IBD undergoes a
biochemical assessment that comprises a full blood count, inflammatory biomarkers such as C-reactive protein (CRP), platelets, electrolytes, liver- and kidney function tests, albumin, and provides a stool sample for microbiological culture (e.g. for ruling out bacterial, viral or parasitic infection) and for measurement of fecal calprotectin (FCal) levels. FCal is a neutrophil protein that can be detected in stool upon intestinal inflammation. It is highly sensitive in detecting IBD-associated intestinal inflammation, since it associates well with disease activity as established by endoscopy. In addition to biochemical measures that aid in the diagnosis, there are also several serological biomarkers that may support diagnosis of IBD. Well-known examples of these markers include anti-
Saccharomyces cerevisiae antibodies (ASCA, most specific to CD) and perinuclear antineutrophil cytoplasmic antibodies (pANCA, most specific to UC). However, their diagnostic accuracy is often limited. Hence, serological testing is clinically not recommended for the routine diagnostic make-up of CD or UC. Likewise, antimicrobial antibodies such as anti-CBir1 or anti-OmpC are currently not being used as their additional diagnostic value is considered only marginal. Although their exact clinical value remains elusive, these markers are still included in serum biomarker panels that are used to detect IBD, and have previously been tested for their predictive ability with regard to the development of IBD. Based on the notion that specific antibody responses may constitute one of the earliest pathogenic events in IBD, the characterisation of immune-based biomarker signatures may facilitate the early detection of IBD development and improve the initial disease diagnosis.

**Assessment and monitoring of intestinal inflammatory disease activity**

Patients with IBD often suffer from long-lasting mucosal disease activity that is difficult to detect, to monitor and to promptly treat. Other means are required to allow accurate disease activity monitoring and early therapeutic intervention, since ongoing disease activity negatively affects the disease course by increasing the risk of hospitalization and the need for surgery. Importantly, this also negatively impacts patient-reported quality of life as well as rates of socioeconomic participation. Endoscopy is still the ‘gold standard’ diagnostic procedure to assess the presence and severity of intestinal inflammatory disease activity in patients with IBD. However, endoscopic procedures entail a high patient burden, are time-consuming and very expensive. In light of these considerations, there is an urgent need for alternatives, i.e. biomarkers that accurately reflect endoscopic disease activity in IBD. Clinical symptoms are usually non-specific for disease activity, and non-endoscopic clinical scoring systems, such as the Harvey-Bradshaw Index (HBI) for CD and the Simple Clinical Colitis Activity Index (SCCAI) for UC, usually do not correlate well with endoscopically active disease. Blood C-reactive protein (CRP) and fecal calprotectin (FCal) levels are currently the most widely used disease activity biomarkers in IBD, but they still demonstrate inconsistent associations with mucosal inflammation as evaluated by endoscopy and have limited specificity. For example, FCal lacks the specificity to distinguish between IBD and other types or causes of intestinal inflammation. Thus, additional, preferably minimally invasive biomarkers are needed that are capable of reflecting intestinal inflammation.

Inflammation is a key pathophysiological process in IBD, which may range from local mucosal inflammation to widespread systemic inflammation. Blood CRP and FCal levels are
characteristic examples of inflammatory biomarkers in IBD, but as alluded to earlier, they are clinically deemed insufficient and carry their own limitations. In recent years, new, innovative and highly-sensitive techniques such as electrochemiluminescence (ECL) detection, inductively coupled plasma mass-spectrometry (ICP-MS) and high-throughput proximity extension assay (PEA) technology have become available and further optimized, enabling us to quantify the concentrations of circulating biomarkers in a highly sensitive, validated and efficient manner. Using these techniques, we have evaluated the utility of inflammatory biomarkers (cytokines, chemokines, markers for angiogenesis and vascular injury), and intestinal permeability ($^{52}$Cr-EDTA, a non-radioactive, orally administered tracer for intestinal permeability) in relation to intestinal inflammatory disease activity in patients with IBD. Inflammatory biomarkers, or combinations thereof, may confer predictive value in relation to disease activity, in a similar way as CRP or FCal are used nowadays. Similarly, intestinal permeability may also be a potential biomarker for disease progression.33 Numerous intestinal permeability biomarkers have previously been studied, including orally administered tracers such as $^{51}$Cr-labeled ethylenediaminetetraacetic acid ($^{51}$Cr-EDTA). However, the role of the latter radioactive tracer is not yet entirely clear as relatively small and heterogeneous cohorts of patients with IBD were used for evaluation.34-36 Recently, a new alternative tracer was developed, in which the $^{52}$Cr stable isotope was incorporated to create $^{52}$Cr-EDTA.

Oxidative stress is another key pathophysiological process in IBD and is defined as an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of physiological redox signalling.37 Oxidative stress is associated with reduced levels of serum free thiols (R-SH) since these compounds are rapidly oxidized by reactive oxygen species (ROS). Although the role of R-SH compounds had already been evaluated in a number of oxidative stress-mediated diseases38-40, their role in IBD was not yet thoroughly investigated. Therefore, this biomarker became of special interest, since oxidative stress may be a key therapeutic target in IBD, and related biomarkers may serve to reflect disease activity or therapeutic efficacy. Considering intestinal oxygen handling, the hypoxia-inducible factor (HIF) pathway may also be of particular interest, since it is a key player in intestinal homeostasis by modulating intestinal barrier and immune function.41,42 Under physiological circumstances, the intestinal mucosa is characterized by an oxygen gradient along the crypt-villus axis, with higher levels of oxygen towards the mucosa and lower levels towards the predominantly anaerobic intestinal lumen.43 In inflamed circumstances, this physiological hypoxia becomes more extensive and severe, culminating in pathophysiological hypoxia.44 To restore this pathophysiological state, HIF becomes transcriptionally active, and activates a number of target genes that counteract the resultant hypoxic state or oxidative stress.41 Activation of HIF also influences systemic and cellular iron homeostasis, and iron itself has a direct impact on regulating the transcriptional activity of HIF. As patients with IBD frequently suffer from impaired iron homeostasis, leading to iron deficiency and anaemia, it would be highly interesting to examine the interplay between iron and HIF pathway activation. Moreover, this could have potential clinical implications, as the HIF pathway is a potential therapeutic target to improve patients' responsiveness to iron supplementation and decrease intestinal inflammation.
Assessment of disease behaviour and disease complications

Long-lasting disease activity of IBD may progress to the development of disease complications, such as stricturing (e.g. intestinal stenosis) or penetrating disease (e.g. intestinal fistulae, perforations or formation of abscesses). In patients with CD, these complications eventually occur in up to 70% of patients.\textsuperscript{45,46} In terms of pathophysiology, fibrosis is considered as the primary driver of these disease complications, which is defined as excessive deposition of extracellular matrix (ECM), mainly collagens, together with aberrant remodelling of ECM as a result of chronic intestinal inflammation and impaired wound healing.\textsuperscript{47} Fibrosis is a process regulated by mucosa-residing (myo)fibroblasts, which proliferate and differentiate in the development (or progression) of intestinal stricture formation.\textsuperscript{48} Abnormal ECM remodelling is the result of an impaired balance between the production of structural ECM proteins (e.g., collagen) and turnover (e.g. protease-mediated breakdown of the ECM). For instance, matrix metalloproteinases (MMPs), which are collagenases able to break down constituents of the ECM, are produced by immune cells like T-lymphocytes and granulocytes and contribute to mucosal damage, disruption of epithelial barrier integrity, and eventually the formation of intestinal fistulae.\textsuperscript{49,50} Formation of fistulae may occur when healing of chronic ulcerations is hampered by increased activity of immune cells that exhibit high protease activity, leading to chronic tracts of granulation tissue between two epithelial-lined surfaces after re-epithelialization of penetrating ulcers.\textsuperscript{51,52}

Cross-sectional imaging techniques such as intestinal ultrasound, computed tomography (CT) or magnetic resonance (MR) enterography are clinically used to detect intestinal stricture formation, internal penetrating disease and intra-abdominal abscess formation with varying accuracy, where MRI is the preferred technique for evaluation of deep internal or pelvic fistulae/abscesses.\textsuperscript{53-55} However, the diagnostic accuracy of these modalities for the detection of disease complications is limited. Since there are few studies available demonstrating their diagnostic accuracy in varying clinical contexts, their performance methods remain unstandardised and insufficiently validated. As such, these methods are considered suboptimal in determining the degree of intestinal fibrosis in clinical practice.\textsuperscript{56} In addition, none of these techniques have proven to be helpful in precisely assessing the degree of inflammatory versus fibrotic tissue in a stenosis in order to guide disease management.\textsuperscript{7} Taken together, there is yet no consistent approach for monitoring intestinal stricture and/or fistulae formation over time. Currently, no biomarkers exist that are able to assess the degree of fibrosis in a given patient, which complicates the early detection of these disease complications. The identification of adequate biomarkers could aid in prompt initiation of therapeutic intervention, selection of alternative treatments, and ultimately the prevention of more severe disease.
Improving the therapeutic landscape of IBD with assessment of biomarkers and nutritional interventions

Using biomarkers to monitor and select drug-based interventions
The main treatment goals in the management of IBD are to induce and to maintain disease remission.\(^5^7\) Both in CD and UC, induction of remission is usually achieved with (intravenous) corticosteroids, often alongside or followed by the initiation of maintenance medications, which are principally targeting immune system components. Commonly prescribed drugs include 5-aminosalicylates, immunomodulators (including thiopurines, methotrexate or calcineurin inhibitors), and biological therapies (including TNF-α-antagonists such as infliximab [IFX], anti-integrins such as vedolizumab [VEDO] or anti-IL-12/23 inhibitors).\(^2^,^3\) Recently, a new class of maintenance drugs, referred to as the small molecules or JAK-STAT inhibitors, has been added to the therapeutic arsenal of IBD, of which the drugs tofacitinib and upadacitinib are good examples.\(^5^8,^5^9\) For most of these medical treatments, particularly in the case of biologicals, it is challenging to determine which patients will benefit the most from which type of treatment. Unfortunately, due to the heterogeneity of the disease, the response rates for several types of these drugs are rather low. For example, up to 30-40% of patients demonstrate non-response or loss-of-response after induction therapy with TNF-α-antagonists, and up to 40-50% of patients show non-response or loss-of-response to VEDO induction therapy.\(^6^0,^6^1\) Clinical factors have demonstrated poor predictive value with regard to treatment response, and there is a lack of accurate biomarkers that may be used to predict disease relapse and response to treatment.\(^6^2,^6^3\) In clinical practice, it therefore remains challenging to predict which patients will respond to which specific treatment, underscoring the need for biomarkers to discriminate between future responders and non-responders against specific therapies. Furthermore, many of these treatments are intensive, unpredictable for patients, and expensive, which further emphasizes the importance of accurately predicting therapeutic responses. Another reason for improving response- and remission rates against medical treatments in IBD is the risk of surgical intervention in these patients. The majority of patients with IBD (CD: 70-90%, UC: 25-30%) eventually undergo intestinal resection during their disease course, mainly because they became refractory to the available medical treatments, or because of strictureing and/or penetrating disease complications or the development of colorectal cancer.\(^6^4,^6^5\)

Nutritional interventions
Apart from developing novel medical treatments, there is growing interest in the field of lifestyle- and nutritional interventions in IBD. As for the latter, diet plays an important role in the development of IBD, and dietary intake patterns have appeared to be strongly associated with disease activity and the risk of disease relapse.\(^6^6-^6^8\) In this context, a striking example is the effect of exclusive enteral nutrition (EEN) that has been demonstrated to induce disease remission in paediatric patients with CD.\(^6^9\) To make further progress in this field, it is of utmost importance to study both the effects of single food ingredients (e.g. vitamins or dietary fibres) and those of complete (anti-inflammatory) diets that are designed especially for IBD, i.e. diets that have the appropriate evidence-based properties in order to be potentially beneficial for patients with IBD. Single nutrients as nutritional therapeutic agents (neutraceuticals) require thorough assessment.
of their potential anti-inflammatory, antioxidant or gut microbiota-modulating properties, which should ideally be investigated in both \textit{in vitro} and \textit{in vivo} research settings. Besides, there is a need for prospective clinical trials evaluating the efficacy of complete diets or dietary patterns, since all nutrients that we consume may behave differently as part of our total habitual dietary intake, e.g. they may act synergistically or antagonistically with each other. It is important to assess the efficacy of nutritional interventions in IBD and compare this to the existing drug-based therapeutic armamentarium.

**The importance of a well-established data- and biobank infrastructure**

In the quest for novel biomarkers for a heterogeneous disease like IBD, it is crucial to make use of well-documented, deeply phenotyped patient- and control cohorts in order to allow detailed phenotypic patient stratification. Although a systems biology approach of IBD is increasingly advocated for the identification of disease biomarkers and therapeutic targets, a major challenge is posed by the fact that cohort characteristics and/or interindividual patient differences frequently explain the majority of biomarker variation\textsuperscript{70,71}. In order to address this issue, it is critical to carefully assess phenotypic characteristics of patients, including demographic, clinical, disease-specific, dietary, and environmental factors. This allows an integrative study of biomarker behaviour and may provide rationale for stratification based on specific phenotypic factors that are relevant to a particular study.

The majority of the studies presented in this thesis have been conducted using data and biomaterials that were collected within various cohorts and biobanks. In total, nine different cohorts and biobanks have been used or were newly established as part of my doctoral research (Table 1). The most frequently used cohorts in this thesis include the 1000IBD cohort and the Dutch IBD Biobank. The 1000IBD cohort, or ‘1000IBD project’, is a cohort consisting of patients with IBD treated in the University Medical Centre Groningen (UMCG), of which detailed phenotypic information and several multi-omics profiles have been collected for over 1,000 patients. It features a wealth of biological data including genetics (whole-exome sequencing [WES], genome-wide genotyping [global screening array, GSA]), gene expression (bulk RNA-sequencing of intestinal biopsies), the gut microbiome (fecal metagenomics, 16S rRNA gene sequencing of intestinal biopsies), the plasma proteome (proteomics of plasma inflammation-related proteins), humoral immunity (phage-display immunoprecipitation sequencing, PhiP-seq, generating antibody epitope repertoires), but also detailed phenotypic and lifestyle data.\textsuperscript{72}

Different subsets have been used from the Dutch IBD Biobank, which constitutes a prospective, nationwide biobank of patients with IBD who are treated in one of the eight Dutch university medical centres.\textsuperscript{73} For the establishment of this biobank, a new national institute was founded by the Dutch Federation of University Medical Centres (NFU) to facilitate its logistic infrastructure, referred to as the ‘String of Pearls initiative’ (Dutch: ‘Parelsoer Instituut’, abbreviated as PSI).\textsuperscript{74} Using this biobank infrastructure, and together with two fellow PhD-students, I established two unique, specialized cohorts consisting of patients with IBD who received induction therapy with the biologicals infliximab or vedolizumab, who were extensively phenotyped and had biomaterials (serum/plasma) available for biomarker analysis before and during therapy. Furthermore, we performed a study in which data and biomaterials were prospectively collected from patients with
CD who received riboflavin (vitamin B<sub>2</sub>) supplementation in the ‘RISE-UP’ clinical trial. Similarly, two other intervention cohorts consisting of healthy volunteers were newly established and used to study health effects of vitamin B<sub>2</sub> and vitamin C supplementation in the ‘Ribogut-study’ and vitamin C-study, respectively. Finally, four cohorts of healthy volunteers or population-based individuals have been used in this thesis, either serving as control cohorts (300BCG and LifeLines-DEEP) or as exploratory cohorts with longitudinal (follow-up) data (PREVEND and COVID-HOME).

Table 1 | Overview of cohorts/biobanks and corresponding data and/or biomaterials used for the studies as presented in this thesis.

<table>
<thead>
<tr>
<th>Cohort/Biobank</th>
<th>Participants</th>
<th>Number (n)</th>
<th>Data/biomaterials used or collected</th>
<th>Relevant to chapters:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000IBD cohort, UMCG, the Netherlands</td>
<td>IBD patients</td>
<td>1,000-1,200</td>
<td>WES, GSA, proteomics, bulk RNA-seq, fecal metagenomics, metabolomics, mucosal 16S rRNA seq, PhiP-seq, clinical data, dietary data, environmental data</td>
<td>3,4,6,11-13,18,19</td>
</tr>
<tr>
<td>Dutch IBD Biobank or ‘Parelsoer’, the Netherlands (nationwide)</td>
<td>IBD patients</td>
<td>1,000-1,500</td>
<td>Serum, plasma, clinical data</td>
<td>5,7-10,16-18,28,30</td>
</tr>
<tr>
<td>Infliximab (IFX) cohort</td>
<td>IBD patients</td>
<td>70-100</td>
<td>Serum, plasma, clinical data</td>
<td>23-26</td>
</tr>
<tr>
<td>Vedolizumab (VEDO) cohort</td>
<td>IBD patients</td>
<td>50-80</td>
<td>Serum, plasma, clinical data</td>
<td>23-26</td>
</tr>
<tr>
<td>RISE-UP study, UMCG</td>
<td>CD patients</td>
<td>79</td>
<td>Serum, plasma, urine, fecal metagenomics, FISH</td>
<td>22</td>
</tr>
<tr>
<td>Ribogut study, UMCG</td>
<td>Healthy volunteers</td>
<td>99</td>
<td>Serum, plasma, fecal metagenomics, FISH</td>
<td>21</td>
</tr>
<tr>
<td>Vitamin C study, UMCG</td>
<td>Healthy volunteers</td>
<td>20</td>
<td>Fecal 16S rRNA gene sequencing</td>
<td>20</td>
</tr>
<tr>
<td>LifeLines(-DEEP), Groningen</td>
<td>Population individuals</td>
<td>167,000 (total) 1,443 (LL-DEEP)</td>
<td>PhiP-seq, fecal metagenomics, WES/GSA, plasma, phenotypic data</td>
<td>3,4,5,19</td>
</tr>
<tr>
<td>PREVEND study, UMCG</td>
<td>Population individuals</td>
<td>8,592 (total)</td>
<td>Serum, plasma, urine, clinical data</td>
<td>15</td>
</tr>
<tr>
<td>300BCG study, Radboud UMC</td>
<td>Healthy volunteers</td>
<td>148</td>
<td>Plasma, clinical data</td>
<td>11</td>
</tr>
<tr>
<td>COVID-HOME, UMCG</td>
<td>Population individuals</td>
<td>&gt;200</td>
<td>Serum/plasma, clinical data</td>
<td>29</td>
</tr>
</tbody>
</table>

Abbreviations: GSA, global screening array; FISH, fluorescence in-situ hybridization; IBD, inflammatory bowel disease; PREVEND, PhiP-seq, phage-display immunoprecipitation sequencing; Prevention of Renal and Vascular EnD-stage Disease; UMCG, University Medical Center Groningen; WES, whole-exome sequencing.
COVID-19 and IBD
During the period in which the studies described in this thesis were performed, coronavirus disease 2019 (COVID-19), caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), spread globally and posed a major challenge to our society and public health. Simultaneously, it had a great impact on science, and triggered actions to unravel COVID-19 pathophysiology, to develop diagnostic methods, and to deliver therapeutic agents and vaccinations in order to combat the disease. Soon it became clear that COVID-19 also affected the gastrointestinal tract, and this triggered us to investigate its impact on patients with IBD. In the final part of this thesis, we present a series of studies that resulted from these efforts, highlighting consequences of COVID-19 for patients with IBD and the importance of biobanks and biomarkers in medicine.

Aim of this thesis
The overall aim of this thesis is to identify and apply novel biomarker signatures in patients with IBD, while also assessing their utility to predict clinical outcomes (e.g., disease activity or response to established medical treatment) and their potential for therapeutic modulation (e.g., through dietary interventions). By exploring potential disease markers from different biological systems (e.g., the immune system) and biological mechanisms (e.g., inflammation, oxidative stress, fibrosis), together with detailed phenotypic stratification, this thesis is aimed to contribute to the characterisation and validation of translational, evidence-based, and clinically applicable biomarker signatures in IBD by adopting both experimental and clinical approaches (Figure 2). Concomitantly, this thesis aims to unravel complex interactions between pathophysiological factors and to gain more insight into the pathogenesis of IBD.
Overall, the aim of this thesis is to discover and apply novel biomarker signatures in patients with IBD, and simultaneously assessing their utility in relation to clinically relevant outcomes for patients with IBD and their potential to be therapeutically modulated (e.g. through dietary- or drug-based interventions). Distinct biological systems and -mechanisms were selected for biomarker exploration. In Part I, (adaptive) immunity and immunological interactions with the gut microbiota gain central attention. In Part II, the search for biomarkers is focused on inflammation, gut permeability and intestinal fibrosis. In Part III, oxidative stress and redox signalling as well as biomarkers of iron metabolism are investigated. Part IV covers studies involving both nutritional and drug-based (biological) interventions, where the (predictive) potential of several of the explored biomarkers and their dynamics upon therapeutic intervention will be assessed. In the final part of this thesis (Part V), the scientific and clinical sequelae of the recent coronavirus disease 2019 (COVID-19) pandemic are discussed in the context of biomarkers and IBD. To summarize, both targeted and untargeted approaches are adopted in this thesis to explore biomarker signatures of distinct pathophysiological processes and biological systems in the context of IBD.
Outline of this thesis

PART I: IMMUNE-BASED BIOMARKERS IN IBD
In Part I of this thesis, we focus on immune-based biomarker signatures in the context of IBD, with a special emphasis on antimicrobial immune activity. In Chapter 2, we review the current knowledge about the main humoral immunological alterations in IBD and currently available serological indicators. Furthermore, we present the state-of-the-art antibody profiling technologies and their clinical potential through systematic and in-depth profiling of the human antibody epitope repertoire. In Chapter 3 and Chapter 4, we characterise the blood antibody epitope repertoire by leveraging a phage-display immunoprecipitation sequencing (PhIP-Seq) workflow, which allowed profiling of circulating antibodies against 344,000 different microbial, immune- and food antigens. In Chapter 3, we study the role of genetic, lifestyle and intrinsic factors in shaping the human antibody epitope repertoire in a large cohort of population-based individuals. Subsequently, in Chapter 4, we compare antibody profiles of these individuals with those from patients with IBD. Specifically, in patients with IBD, we study the relationships between antibodies and disease-specific phenotypes, the capacity of antibody epitope repertoires to discriminate patients from controls, as well as the associations with (disrupted) fecal microbiome composition. In Chapter 5, we use a different approach to study antimicrobial immune responses, by combining magnetic-activated cell sorting (MACS), flow cytometry (FC) and 16S rRNA sequencing techniques to quantify fractions of IgG-coated bacteria after incubating fecal samples with autologous serum. Following this, we aimed to investigate to which bacterial groups the humoral IgG immune response is directed to in patients with IBD and healthy individuals. In Chapter 6, we switch from serological markers to mucosal (transcriptional) signatures of immune regulation, by studying the interplay between mucosal (immune) gene expression signatures and the mucosa-attached microbiota composition.

PART II: INFLAMMATORY- AND FIBROSIS BIOMARKERS IN IBD
In Part II of this thesis, we focus on the utility of inflammatory, gut permeability and fibrosis biomarkers with respect to disease activity and disease behaviour in the context of IBD. In Chapter 7, we study the associations between a series of Th1- and Th17-associated pro- and anti-inflammatory cytokines, chemokines, adhesion molecules and markers for vascular injury and repair versus the levels of fecal calprotectin in stool samples of patients with CD. In Chapter 8, we take a rationally-selected panel of inflammatory biomarkers (based on findings described in Chapter 7) and study their associations with measures of clinical, biochemical and endoscopic disease activity in a larger cohort of patients with IBD. In this chapter, we present a distinct set of four circulating inflammatory biomarkers that demonstrate a reasonable ability to discriminate between patients with mild vs. patients with moderate-to-severe endoscopic disease activity. In Chapter 9, we focus on 52Cr-EDTA as a potential biomarker for gut permeability in patients with CD, and present its association to disease activity as reflected by fecal calprotectin levels. In Chapter 10, we examine the potential of extracellular matrix and collagen remodelling biomarkers for discriminating between disease behaviour subclasses in patients with CD. In addition, we also aimed to study their capability to predict future disease course, i.e. the future progression of
stricturing and penetrating disease complications. Recent technological advances have facilitated biomarker discovery by enabling high-throughput, high-resolution characterization of circulating proteins (proteomics) as well as circulating antibodies (immunosequencing). In this part, we continue our exploration of biomarkers by leveraging proximity extension assay technology, which was used to quantify 92 different inflammation-related plasma proteins in over 1,000 patients with IBD. Furthermore, we aimed to study the impact of genetics, disease phenotypes and dietary habits on protein variation in patients with IBD, since these factors may potentially affect inflammatory protein levels and thereby reveal relevant pathophysiological pathways and drug targets. In Chapter 11, we study the influence of phenotypic patient characteristics and patient genotypes on plasma inflammatory proteins in patients with IBD. In Chapter 12, we use the same technology to search for potential inflammatory biomarkers for fatigue in patients with clinically and biochemically quiescent IBD, and in Chapter 13 we study the influence of dietary habits of patients with IBD on their plasma inflammatory protein profile.

PART III: OXIDATIVE STRESS BIOMARKERS IN IBD
In Part III of this thesis, we shift our focus to the role of oxidative stress and hypoxia in IBD, with special attention for the role of systemic sulfhydryl groups (R-SH, reduced or “free” thiols) and HIF pathway activity as markers for systemic oxidative stress and hypoxia, respectively. In Chapter 14, we review the current knowledge on the role of oxidative stress in IBD, present a novel theoretical framework of redox signalling - referred to as the Reactive Species Interactome -, and discuss the potential of oxidative stress biomarkers and redox-modulating therapeutics in the context of IBD. In terms of biomarkers, systemic free thiol levels are mainly highlighted, as they represent the central components of the extracellular antioxidant machinery, form important transducers of redox-regulated events and capture the balance between total oxidant burden and antioxidant capacity in humans. Since free thiols rapidly scavenge ROS, lower levels of free thiols are generally associated with oxidative stress, whereas higher levels represent a favourable “healthy” redox status. In Chapter 15, we show that serum free thiols comprise a useful predictive tool with regard to the occurrence of future oxidative stress-mediated disease, in this case cardiovascular disease and all-cause mortality, in individuals from the general population. In Chapter 16, we test the hypothesis that serum free thiols may be reduced in patients with IBD and are associated with inflammatory disease activity. In Chapter 17, we further build on these findings by examining the discriminative capacity of serum free thiols with regard to endoscopic disease activity in patients with IBD. Finally, in Chapter 18, we zoom in on the interplay between HIF pathway activation and systemic iron status, as this may be underlying iron deficiency in patients with IBD. In this study, we investigate associations between serum iron status and activation of the HIF pathway and related target genes in mucosal biopsies of patients with IBD.
PART IV: EVALUATING THERAPEUTIC EFFECTS ON BIOMARKERS IN IBD

In Part IV of this thesis, we zoom in on the effects of therapeutic interventions, consisting of nutritional interventions and treatment with biological therapies.

First, we focus on the therapeutic effects of single food ingredients in patients with IBD and in healthy volunteers, including vitamin B₂ (riboflavin), vitamin C (ascorbic acid), and dietary fibres (inulin-type fructans). In these studies, therapeutic effects are assessed in relation to a number of relevant outcome parameters, including many parameters that were explored earlier in this thesis, e.g. markers of inflammation, oxidative stress and the gut microbiota. In Chapter 19, we start with examining prebiotic modulation of the gut commensal bacterium Faecalibacterium prausnitzii, of which a decreased abundance is associated with IBD and which is often considered as a microbial biomarker for gut health. Using an in vitro co-culture system (called the ‘Human-oxygen Bacteria-anaerobic’ system, abbreviated as ‘HoxBan’), we supplemented dietary fibres, including inulin-type fructans, to F. prausnitzii and analysed the effects of its resulting metabolites (e.g. butyrate and fructose) on gene expression and viability of intestinal epithelial cells (e.g. inflammation- and oxidative stress-marker expression). In Chapters 20 and 21, we evaluated the in vivo effects of riboflavin (vitamin B₂) and vitamin C supplementation on systemic oxidative stress and on gut microbiota composition in healthy volunteers, respectively. In Chapter 22, we subsequently tested the effect of riboflavin supplementation on the gut microbiota composition, while simultaneously assessing changes in multiple inflammatory- and oxidative stress biomarkers, in patients with CD (referred to as the ‘RISE-UP’ clinical trial). In this study, we hypothesised that riboflavin supplementation may lead to a reduction in clinical symptoms through beneficial modulation of the gut microbiota composition (specifically by increasing F. prausnitzii abundance), reducing oxidative stress and inflammation, and alleviating CD-specific symptoms (as evaluated by clinical scores and quality of life (QoL) parameters).

Second, we explore the potential of biomarkers of inflammation, oxidative stress, fibrosis and iron status to predict response to biological therapy in patients with IBD, focusing on IFX and VEDO induction therapy. In addition, we aimed to study the changes of these biomarkers upon treatment with these biologicals in order to gain insight into the dynamics of the pathophysiological processes that these biomarkers represent. In Chapter 23, we assess the early effects of IFX and VEDO induction therapy on markers of systemic iron status in patients with IBD, and evaluated their diagnostic capacity in relation to iron deficiency, that may in turn lead to anaemia in these patients, which is a common extraintestinal manifestation. Further, we study the relationships between systemic iron indices and markers of inflammation, oxidative stress and hypoxia in patients with active IBD. In Chapters 24 and 25, we continue studying the effects of IFX and VEDO induction therapy, but then focus on markers of fibrosis and collagen remodelling. In these studies, we aimed to assess the potential of biomarkers of collagen formation and degradation in monitoring and/or predicting response to these biological therapies in patients with CD, since CD is particularly marked by ECM alterations and the onset of fibrosis. Finally, in Chapter 26, we evaluate the predictive value of mucosal eosinophils and the serum biomarker eotaxin-1, a selective chemoattractant for eosinophils, in relation to response to VEDO induction therapy in patients with IBD. VEDO has the capacity to bind to several types of immune cells,
including eosinophils, and may inhibit eosinophil trafficking to the intestinal mucosa. This led us to hypothesise that VEDO induction therapy may influence gut mucosal eosinophil abundance as well as serum eotaxin-1 levels, which might therefore be used as potential biomarkers for response to therapy.

PART V: COVID-19 AND IBD
In the final part of this thesis, we focus on COVID-19 in the context of IBD and the scope of this thesis. In Chapter 27, we present a comprehensive overview of COVID-19 pathophysiology and the role of the ACE2 protein. Since patients with IBD often use immunosuppressive drugs and may have uncontrolled disease activity, they are at increased risk of viral infections. In Chapter 28, we present a case series of individuals admitted to Dutch hospitals who contracted COVID-19 but clinically presented with symptoms that typically occur in IBD, either being a first presentation of the disease or a flare of pre-existing IBD. Here, we share our clinical experience with these first patients and propose suitable management strategies with the goal to initiate the discussion on the efficacy and safety of IBD treatments in the context of COVID-19. Since oxidative stress emerged as a key pathophysiological process in COVID-19, we set out to investigate the potential of serum free thiols in individuals with COVID-19, which is presented in Chapter 29. In 2021, vaccination campaigns were globally initiated, but there was uncertainty about seroconversion rates and, thus, the adequacy of the serological immune response against SARS-CoV-2 upon vaccination in patients with IBD. In Chapter 30, we present a study where we investigated the effects of different types of IBD medication on the magnitude of the serological antibody response upon vaccination in patients with IBD.

In Chapter 31, I summarize the main findings of this thesis and discuss their mutual relations and implications for clinical practice. In addition, I will propose several suggestions for future research and outline future perspectives.
References


PART I

IMMUNE-BASED BIOMARKERS IN IBD