University of Groningen

A combination of fecal calprotectin and human beta-defensin 2 facilitates diagnosis and monitoring of inflammatory bowel disease

Gacesa, R.; Vich Vila, A.; Collij, V.; Mujagic, Z.; Kurilshikov, A.; Voskuil, M. D.; Festen, E. A. M.; Wijmenga, C.; Jonkers, D. M. A. E.; Dijkstra, G.

Published in:
Gut Microbes

DOI:
10.1080/19490976.2021.1943288

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2021

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Gacesa, R., Vich Vila, A., Collij, V., Mujagic, Z., Kurilshikov, A., Voskuil, M. D., Festen, E. A. M., Wijmenga, C., Jonkers, D. M. A. E., Dijkstra, G., Fu, J., Zhernakova, A., Imhann, F., & Weersma, R. K. (2021). A combination of fecal calprotectin and human beta-defensin 2 facilitates diagnosis and monitoring of inflammatory bowel disease. *Gut Microbes*, *13*(1), [1943288]. https://doi.org/10.1080/19490976.2021.1943288

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Gut Microbes

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/kgmi20

A combination of fecal calprotectin and human beta-defensin 2 facilitates diagnosis and monitoring of inflammatory bowel disease

R. Gacesa, A. Vich Vila, V. Collij, Z. Mujagic, A. Kurilshikov, M.D. Voskuil, E.A.M. Festen, C. Wijmenga, D.M.A.E. Jonkers, G. Dijkstra, J. Fu, A. Zhernakova, F. Imhann & R.K. Weersma

To cite this article: R. Gacesa, A. Vich Vila, V. Collij, Z. Mujagic, A. Kurilshikov, M.D. Voskuil, E.A.M. Festen, C. Wijmenga, D.M.A.E. Jonkers, G. Dijkstra, J. Fu, A. Zhernakova, F. Imhann & R.K. Weersma (2021) A combination of fecal calprotectin and human beta-defensin 2 facilitates diagnosis and monitoring of inflammatory bowel disease, Gut Microbes, 13:1, 1943288, DOI: 10.1080/19490976.2021.1943288

To link to this article: https://doi.org/10.1080/19490976.2021.1943288

© 2021 The Author(s). Published with license by Taylor & Francis Group, LLC.

View supplementary material

Published online: 27 Jul 2021.

Submit your article to this journal

Article views: 506

View related articles

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=kgmi20

GUT MICROBES
2021, VOL. 13, NO. 1, e1943288 (18 pages)
https://doi.org/10.1080/19490976.2021.1943288

RESEARCH PAPER

OPEN ACCESS

A combination of fecal calprotectin and human beta-defensin 2 facilitates diagnosis and monitoring of inflammatory bowel disease

R. Gacesa[a,b], A. Vich Vila[a,b], V. Collij[a,b], Z. Mujagic[c], A. Kurilshikov[b], M.D. Voskuil[a,b], E.A.M. Festen[a,b], C. Wijmenga[b], D.M.A.E. Jonkers[c], G. Dijkstra[a], J. Fu[a,d], A. Zhernakova[b], F. Imhann[a,b*], and R.K. Weersma[a*]

[a]University of Groningen, University Medical Center Groningen, Department of Gastroenterology and Hepatology, Groningen, The Netherlands; [b]University of Groningen and University Medical Center Groningen, Department of Genetics, Groningen, The Netherlands; [c]Maastricht University Medical Center, Division of Gastroenterology-Hepatology, NUTRIM School for Nutrition and Translational Research in Metabolism, Maastricht, The Netherlands; [d]University of Groningen and University Medical Center Groningen, Department of Pediatrics, Groningen, The Netherlands

ABSTRACT

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) show a large overlap in clinical presentation, which presents diagnostic challenges. As a consequence, invasive and burdensome endoscopies are often used to distinguish between IBD and IBS. Here, we aimed to develop a noninvasive fecal test that can distinguish between IBD and IBS and reduce the number of endoscopies.

We used shotgun metagenomic sequencing to analyze the composition and function of gut microbiota of 169 IBS patients, 447 IBD patients and 1044 population controls and measured fecal Calprotectin (FCal), human beta defensin 2 (HBD2), and chromogranin A (CgA) in these samples. These measurements were used to construct training sets (75% of data) for logistic regression and machine learning models to differentiate IBS from IBD and inactive from active IBD. The results were replicated on test sets (remaining 25% of the data) and microbiome data obtained using 16S sequencing.

Fecal HBD2 showed high sensitivity and specificity for differentiating between IBD and IBS (sensitivity = 0.89, specificity = 0.76), while the inclusion of microbiome data with biomarkers (HBD2 and FCal) showed a potential for improvement in predictive power (optimal sensitivity = 0.87, specificity = 0.93). Shotgun sequencing–based models produced comparable results using 16S-sequencing data. HBD2 and FCal were found to have predictive power for IBD disease activity (AUC ≈ 0.7).

HBD2 is a novel biomarker for IBD in patients with gastro-intestinal complaints, especially when used in combination with FCal and potentially in combination with gut microbiome data.

ARTICLE HISTORY
Received 16 November 2020
Revised 21 May 2021
Accepted 7 June 2021

KEYWORDS
Gut microbiome; inflammatory bowel disease; biomarkers; human beta defensin 2; noninvasive diagnosis; machine learning

Introduction

Inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) are two of the most common gastrointestinal (GI) disorders, affecting 0.3–0.5% and 7–21% of the population, respectively.[1–3] Patients with IBD and IBS often present with similar symptoms, such as diarrhea, constipation, bloating, abdominal pain, and abdominal discomfort, but their pathophysiology differs.

IBD is characterized by chronic intestinal inflammation in which flare-ups in disease activity, which cause gut complaints including diarrhea and bloody stools, are followed by quiescent periods in which patients have fewer gut complaints. The main clinical types of IBD are Crohn's disease (CD) which can affect any part of the GI tract and ulcerative colitis (UC), which is typically limited to the colon. Unlike IBD, IBS is defined as a combination of GI symptoms without a causative anatomical or biochemical abnormality that can be used to make a definitive diagnosis.[2,4] In the absence of known biochemical tests, IBS diagnosis is based on the presence of a combination of symptoms described by the Rome committee.[4]

Since IBD and IBS often show overlap in their symptoms, gastroenterologists face diagnostic dilemmas on a daily basis. These include how to

CONTACT F. Imhann f.imhann@umcg.nl University of Groningen, University Medical Center Groningen, Department of Gastroenterology and Hepatology, Groningen, The Netherlands

*Equal contribution

Supplemental data for this article can be accessed on thepublisher's website.

© 2021 The Author(s). Published with license by Taylor & Francis Group, LLC.
This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

diagnose IBS without missing a diagnosis of IBD and how to correctly diagnose an exacerbation in IBD patients while avoiding unnecessarily invasive, burdensome, and costly endoscopic testing.[5] Although the Rome committee emphasizes that IBS is not a diagnosis of exclusion, and should be based on symptom criteria, invasive diagnostic procedures like colonoscopy are still frequently used in daily clinical practice to exclude other GI disorders like IBD, before the IBS diagnosis is made.[2]

The relapsing-remitting nature of IBD makes it very important to quickly and accurately diagnose flare-ups as these disease episodes require a quick escalation in anti-inflammatory treatments.[1] Diagnosing IBD exacerbations can, however, be complicated because clinical disease activity scores and currently used biomarkers, such as C-reactive protein in blood and fecal Calprotectin (FCal) often do not accurately capture inflammatory activity in IBD.[6–8]

FCal is a well-known fecal biomarker that is used to help both distinguish IBD from IBS and to determine whether an IBD patient has an exacerbation.[9] During intestinal inflammation, FCal is secreted by neutrophils in the gut, leading to an increase in FCal levels in fecal samples.[9] While FCal is currently used in patient care, it is far from a perfect test, with an area under the curve (AUC) of 0.73 for distinguishing IBD from IBS and a sensitivity of 0.80 and a specificity of 0.82 for detecting exacerbations in IBD (for an FCal cutoff of 250 μg/g).[3,10] Moreover, FCal is degraded in the gut and is therefore not a good predictor of ileal inflammation, which makes it less reliable in CD in which the terminal ileum is commonly inflamed.[11]

The lesser known fecal protein chromogranin A (CgA) and human beta-defensin 2 (HBD2) have only been used as biomarkers for IBD and IBS in scientific research settings. CgA is a member of the granite peptides, which are secreted in nervous, endocrine, and immune cells under stress. In the gut, CgA is secreted by entero-endocrine cells and shows co-expression with serotonin, suggesting it has a role in the gut-brain axis, and elevated levels of fecal CgA have been associated with IBS and with ulcerative colitis (UC).[12–15] HBD2 is a human antimicrobial peptide produced by intestinal epithelial cells in response to bacteria and inflammatory cytokines. Reduced levels of HBD2 have been reported in IBD patients compared to healthy controls, whereas elevated levels of HBD2 have been reported in patients with IBS.[15,16]

In addition to the aforementioned fecal proteins, the gut microbiota composition can also be used as a biomarker because it has been shown to differ between IBD patients, IBS patients, and the general population.[3,17] We previously found that the gut microbiome composition and function significantly differs between IBD patients and IBS patients, with IBD patients displaying strong dysbiosis in the gut.[3] While we also observed significant alterations in gut microbiota between patients with CD and UC, these differences were less pronounced than in IBD-associated dysbiosis.[3] We also demonstrated that the gut microbiome can distinguish IBD from IBS, and that a predictive model combining FCal with gut microbiota composition increases the diagnostic yield of FCal.[3]

In this study, we expand on these results by increasing the size of the IBD cohort to 447 patients, newly measuring fecal biomarkers CgA and HBD2 alongside FCal in 447 IBD patients,[18] 169 IBS patients,[15] and 1044 population controls,[19] evaluating diagnostic yield of these biomarkers, and combining them with different layers of gut microbiome data to construct diagnostic models for noninvasive discrimination between IBD and IBS. We also evaluate the performance of fecal protein and microbiome biomarkers for noninvasive monitoring of IBD activity. Finally, we test the microbiome models constructed using shotgun metagenomic sequencing on the 16S sequencing data we generated for the same cohorts to estimate if this, less expensive method of profiling microbiome provides a viable approach for the implementation of gut microbiome measurements in the clinical environment. Our ultimate goal is to improve noninvasive diagnostic yields in order to reduce the number of unnecessary colonoscopies.

Methods

Cohorts

This study used data pooled from three Dutch cohorts: the 1000IBD cohort consisting of patients with IBD from the University Medical Center Groningen (UMCG),[18] the Lifelines-Deep cohort

(LLD) representative of the general population of the northern the Netherlands,[19] and the Maastrich IBS cohort (MIBS) consisting of clinically diagnosed patients with IBS and their age and sex-matched healthy controls from the Maastricht University Medical Center.[15] The results of 16S sequencing of LLD and 1000IBD Cohorts were used as replication data for testing the IBD prediction models. Detailed descriptions of these cohorts have been published previously,[15,18,19] and the cohorts are summarized in Table 1.

Disease assessment and determination of IBD disease activity

Diagnosis of IBD was made after expert evaluation by gastroenterologists based on endoscopic, histopathological, and radiological characteristics. Diagnosis of IBS was made by gastroenterologists after extensive follow-up to exclude other explanations for the GI complaints in these patients. We defined IBD activity based on the expert opinion of a gastroenterologist as established in our previous work.[20] This assessment was based on IBD-associated complaints, clinical disease activity scores (Harvey–Bradshaw index (HBI)[21] or Simple Clinical Colitis Activity Index (SCCAI)[22]), visits to the outpatient clinic, examination of the inflammatory markers C-reactive protein, FCal, and leucocytes derived from blood, medication prescriptions such as the start of corticosteroids and results of endoscopy and pathology.

Sample collection, DNA extraction, and sequencing

Our protocol for fecal sample collection and profiling of gut microbiota was previously published.[3] In short, participants produced, collected, and stored stool samples at home in a freezer or refrigerator. The samples collected and transported to the UMCG facility and stored at −80°C until DNA extraction. Fecal DNA was isolated using the

Table 1. Summary of cohorts. Summary statistics of the data used in this study. Summary is sub-divided based on diagnosis, and parameters that are significantly different from control group are indicated by * (FDR < 0.05 for Mann–Whitney U test or Chi-Squared test). Abbreviations: IBD = Inflammatory bowel disease patients, CD = Crohn's disease patients, UC = patients with Ulcerative colitis, IBD-U = patients with undetermined type of IBD, IBS = patients with irritable bowel syndrome, BMI = body mass index, NSAIDs = non-steroid anti-inflammatory drugs, PPIs = proton-pump inhibitors, SSRIs = selective serotonin reuptake inhibitors, IBS-C = constipation-dominated IBS, IBS-D = diarrhea-dominated IBS, IBS-M = mixed-presentation IBS, N/A: data not available.

Variable	IBD	*CD*	*UC*	*IBD-U*	IBS	*IBS-C*	*IBS-D*	*IBS-M*	Healthy Controls	*Total*
Sample size	447	250	168	29	169	30	61	69	1044	1660
Anthropometrics										
Age[a]	44 [33; 56]	38 [30; 49] *	46 [35; 57]	41 [27; 50]	44 [29; 58]	44 [25; 58]	46 [35; 58]	43 [28; 58]	46 [35; 56]	44 [33; 56]
Sex (Male)[b]	41% *	34% *	50%	48%	30% *	27%	36%	29%	47%	44%
BMI[a]	24.5 [22; 27.6]	23.8 [21.7; 27.4]	25.2 [22.7; 28.2]	24.3 [23.3; 27.5]	24.5 [21.7; 28]	24.1 [21.2; 26.6]	25 [22;29]	23.6 [21;28]	24.5 [22; 27.2]	24.5 [22.1; 27.4]
Medication use										
Laxatives (Y)[b]	4.7% *	6.4% *	3.0%	3.4%	18.3% *	43.3%*	8.20%	14.50%	1.1%	3.9%
NSAIDs (Y)[b]	4.8%	7.2%	1.2%	6.9%	11.8% *	6.60%	13.10%	14.50%	4.4%	5.3%
PPIs (Y)[b]	16.3%	18.9% *	12.4%	17.2%	24.9% *	23.30%	21.30%	28.90%	6.5%	11.0%
SSRIs (Y)[b]	1.0%	0.8%	1.8%	6.9%	17.8% *	6.60%	14.80%	24.60%	2.5%	3.8%
Immunosuppressive medication (Y)[b]	44.5% *	49.4% *	37.3% *	27.6% *	0	0	0	0	0	11.70%
Antidiarrheal (Y)[b]	10.2% *	14.5% *	4.1% *	3.4% *	4.7% *	0	8.20%	4.30%	0	3.10%
Antibiotics (Y)[b]	1.4%	1.2%	1.8%	0	0.6%	0.00%	3.30%	0.00%	1.1%	1.1%
IBD phenotypes										
Active disease (Y)[b]	12.10%	10.40%	14.80%	10.30%	N/A	N/A	N/A	N/A	N/A	N/A
Ileal resection (Y)[b]	6.50%	11%	0	4.20%	N/A	N/A	N/A	N/A	N/A	N/A
Colonic resection (Y)[b]	8.90%	11.70%	5.30%	4.20%	N/A	N/A	N/A	N/A	N/A	N/A
Ileocecal resection (Y)[b]	21.6% *	37.1% *	0	4.20%	N/A	N/A	N/A	N/A	N/A	N/A
Bowel movements per day	2.6 [1; 3]*	2.8 [1; 3] *	2.5 [1; 3] *	2.5 [1; 3] *	N/A	N/A	N/A	N/A	1.4 [1;2]	1.8 [1;2]
Active IBD	4.0 [1; 6]*	3.5 [1; 4] *	4.3 [2; 6] *	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Inactive IBD	2.5 [1; 3]	2.2 [1; 3]	2.7 [1; 3]	N/A	N/A	N/A	N/A	N/A	N/A	N/A

AllPrep DNA/RNA Mini Kit (Qiagen; cat. # 80204) with the addition of bead-beating as previously described.[23] Library preparation for metagenomic sequencing was performed at the Broad Institute of Harvard and MIT (Cambridge, Massachusetts, USA), and DNA was sequenced using the Illumina HiSeq platform, generating mean coverage of 30 million reads (3 Gb) per sample.

The 16S rRNA gene sequencing of the V4 variable region was performed on separate aliquots of these samples. Sequencing was done on the Illumina MiSeq platform, as previously reported,[19] and generated 175 bp paired-end reads.

Profiling of microbiome composition and function using metagenomic sequencing

The KneadData toolkit (v0.5.1) was used to trim the raw metagenomic reads to PHRED quality 30 and to remove Illumina adapters. The KneadData-integrated Bowtie2 tool (v2.3.4.1)[24] was used to remove trimmed reads that aligned to the human genome (GRCh37/hg19), and the quality of the processed metagenomes was examined using the FastQC toolkit (v0.11.7).[25] The taxonomic composition of metagenomes was profiled by the MetaPhlAn2 tool (v2.7.2)[26] using the MetaPhlanAn database of marker genes (v. mpa_v20_m200). Profiling of genes encoding microbial biochemical pathways was performed using the HUMAnN2 pipeline (v0.11.1)[27] integrated with the DIAMOND alignment tool (v0.8.22),[28] UniRef90 protein database (v0.1.1)[29] and ChocoPhlAn pangenome database (v0.1.1). Analyses were performed using locally installed tools and databases from CentOS (release 6.9) on the UMCG high-performance computing infrastructure.

Profiling of microbiome composition using 16S rRNA gene sequencing

Profiling of results of 16S sequencing was performed using the mothur pipeline[30] (v.1.40.0), following a protocol based on the mothur standard operating procedure guidelines (https://mothur.org/wiki/454_SOP): 1) duplicate input 16S sequences (in FASTA format) were discarded, 2) unique sequences were aligned to the SILVA database[31] (release 132), 3) sequences that aligned to the SILVA database with less than 90% of the total length were discarded, 4) columns in the alignment that contained missing data were removed, 5) aligned sequences were pre-clustered and chimeric sequences were removed using the chimera.uchime tool and the SILVA-aligned version of the gold database (release 132), 6) aligned sequences were assigned taxonomical lineage by the mothur Bayesian classifier utilizing the mothur trainset (v.16_022016) with a classification cutoff of 80, 7) potential contaminants were removed by discarding all sequences classified as mitochondria, chloroplasts, archaea, eukaryote, or unknown taxa, and 8) sequences were clustered into operational taxonomical units based on a mothur-calculated distance matrix with a cutoff of 0.15 and each cluster was assigned taxonomy based on the "majority-consensus" taxon.

Measurements of fecal biomarkers

FCal concentrations were measured in fecal samples at the UMCG using the commercial enzyme-linked immunosorbent assay (ELISA, Bühlmann Laboratories, Switzerland). CgA levels in fecal samples were measured using the commercial radio-immunoassay (RIA, Euro-Diagnostica, Sweden) and HBD2 levels in fecal samples were measured using a commercial enzyme-linked immunosorbent assay (ELISA, Immunodiagnostik AG, Germany) at the Medische Laboratoria Dr Stein & Collegae, the Netherlands.

Filtering, covariate selection, and data normalization

Data was prepared for modeling as follows:

(1) Samples with 50% or higher abundance of "unclassified" microbial taxa were deemed low quality and discarded. Samples from patients with a stoma or pouch of the gut were discarded.[32] Self-reported IBS and IBD cases in the general population cohort were also discarded from further analysis as these samples lacked clinical diagnosis but were considered a potential source of bias. In total, 1660 samples (1044 population-based controls, 169

IBS cases, and 447 IBD cases) were used for modeling.

(2) Microbiome features (taxa and pathways) detected in less than 10% of samples and features with a mean relative abundance lower than 0.01 were deemed unreliable and discarded from further analysis. A total of 244 taxa (131 species, 55 genera, 29 families, 14 orders, 10 classes, and 5 phyla) and 277 pathways were then used as covariates for training the prediction models.

(3) The distributions of the relative abundances of microbial taxa and microbial pathways were normalized using the arcsine square root transformation. Normalized abundances were corrected using multivariate linear regression for the potential confounding effect of population characteristics (sex, BMI, and age), use of medications known to affect the gut microbiota (protein-pump inhibitors (PPIs), antibiotics, and laxatives)[12] and sequencing depth. Fecal FCal, HBD2, and CgA values were normalized by natural logarithm transformation. All covariates were scaled and centered by subtracting the mean of the covariate and dividing values by the standard deviation of the covariate.

Model training, optimization, and testing

A training set was established that includes a randomly sampled pool of 75% of the data (both patient and healthy), which left a test set made up the remaining 25% of the data. The training set was used for construction and optimization of prediction models and assessment of predictive power by internal cross-validation while the test set was used for testing of model performance.

Two different machine-learning procedures were used: a multivariate logistic regression model and a support vector machine model with radial kernel function. Model training and optimization were performed using three repeats of 10-fold internal cross-validations on the training set, with the goal of optimizing Cohen's Kappa value, a balanced metric of positive and negative predictive values.[33] The models were optimized by recursive feature elimination, with resampling to minimize potential overfitting bias and maximize performance.[34] The optimized set of variables was selected as the set with the minimum number of variables that achieved 98% of the maximum Kappa value observed during recursive feature elimination. Learning curves (plots of the performance of the model on test and training sets versus training set size) were used to evaluate the model training and assess potential over-fitting. Optimized models were tested on the test set composed of 25% of the total cases and controls not used in model training, and the performance of classification was reported as area under receiver–operator curve (AUC), sensitivity, and specificity. All prediction performance metrics in the main manuscript are reported for the test set. All metrics for internal cross-validation and the test set are reported in Supplement S2.

Models were trained, optimized, and tested using the *Caret* package (v.6.0–80, https://topepo.github.io/caret/) for the statistical programming language R (v.3.4.3; https://www.r-project.org/). Source codes are available at https://github.com/GRONINGEN-MICROBIOME-CENTRE/Groningen-Microbiome/tree/master/Projects/IBD_Predict.

ROC analysis and determination of biomarker cutoffs

To determine optimal values of biomarkers for separation of IBD and IBS, we performed receiver operating curve (ROC) analysis using the *pROC* package for R and selected the value with the highest combination of specificity and sensitivity as the optimal biomarker cutoff. These values were validated by examining regression and classification tree (CART) models with a maximum depth 1 (for single biomarkers) or 2 (for combination of biomarkers). CART models were trained using 1000 bootstraps of the input data.

Comparison of performance of machine learning models

Areas under the ROC curves of prediction models were compared using DeLong's test, nonparametric test for comparing AUCs of ROC curves

implemented in *roc.test* function from *pROC* package for R.

Evaluation of impact of sequencing technology on IBD diagnosis models

To evaluate if models constructed on microbiome data generated by shotgun metagenomic sequencing retain predictive power when applied on 16S sequencing data, we tested models for identification of IBD using the 16S sequencing generated from our cohorts of healthy controls and IBD patients. We constructed an IBD-diagnosis model using the relative abundances of microbiome genera of 447 IBD patients and 1044 healthy controls and tested this model using 16S sequencing data of the same cohorts.

Results

Cohort description

This study included 1660 participants: 1044 population-based controls from the LLD cohort[19] and healthy controls from the MIBS cohort,[15] 169 clinically diagnosed IBS patients from the MIBS cohort[15] and 447 patients with IBD from the UMCG 1000IBD cohort.[18] The average age of CD patients was significantly lower than controls, and patients with IBD or IBS had a higher proportion of females compared to controls, but no significant differences were observed in BMI of patient groups and controls (Table 1). In addition, the use of prescription drugs was higher in disease cohorts compared to the healthy controls. Patients with IBS showed increased use of five drugs (laxatives, non-steroid anti-inflammatory drugs (NSAIDs), selective serotonin reuptake inhibitors (SSRIs), proton-pump inhibitors (PPIs), and antidiarrheal drugs) compared to controls. Patients with IBD showed increased use of laxatives, immunosuppressants, and antidiarrheal drugs (Table 1). IBD patients also had higher use of immunosuppressants and laxatives and lower use of SSRIs than IBS patients ($FDR < 0.05$ for Chi-Squared tests).

Fecal biomarker HBD2 is a strong predictor of IBD

For all 1660 participants in this study, we measured fecal concentrations of HBD2, CgA, and FCal (Figure 1) and determined the taxonomical and functional profiles of gut microbiota using shotgun metagenomic sequencing of fecal samples. Biomarker measurements are summarized in Table 2. Summary statistics of the gut microbiome profiling are shown in Supplement S1. Concentrations of FCal and HBD2 were significantly elevated in IBD patients as compared to healthy controls or IBS patients (Mann–Whitney U test, p-values <1.0e-5, Figure 1). The levels of both biomarkers were significantly higher in each subgroup of IBD (CD, UC, and IBDU) compared to the control cohort (Table 2). HBD2 was also found to have a significant decrease in IBS patients (Mann–Whitney U test p-value 0.004) as compared to healthy controls and IBD patients. The concentration of CgA was, however, only found to be elevated in IBS cases compared to controls (Mann–Whitney U test p-value 3.0e-5). No relation between IBD and CgA was identified. Within IBD cases, we identified an increase in FCal in patients with IBD with active disease. We also observed a significant negative correlation between HBD2 and the number of bowel movements per day (adjusted R-squared of linear model = 0.014, F-Test p-value = 0.006), which suggests that the lower HBD2 in patients with active IBD might be a result of lower colonic transit time and looser stools, which can dilute the fecal concentration of the biomarker. We also examined associations between medication use and biomarker levels in IBD patients (Table 2) and found that CgA levels are significantly decreased in laxative users.

Next, we evaluated the predictive power of these biomarkers for discrimination between IBD and IBS (Figure 2). We constructed multivariate logistic regression models in the randomly selected 75% of the data and used these models to classify the remaining 25% of the data. FCal (Sensitivity = 0.76, Specificity = 0.67, AUC = 0.78), and HBD2 (Sensitivity = 0.89, Specificity = 0.76, AUC = 0.91) were found to be highly accurate in separating patients with IBD from patients with IBS, while the predictive power of CgA was considerably lower (Sensitivity = 0.73, Specificity = 0.05, AUC = 0.59). A model comprised age, sex, and BMI – factors known not to be predictive of IBD[3] – was used as a negative control to identify potential bias in the data and showed no predictive power (AUC ≈ 0.5). Combining FCal

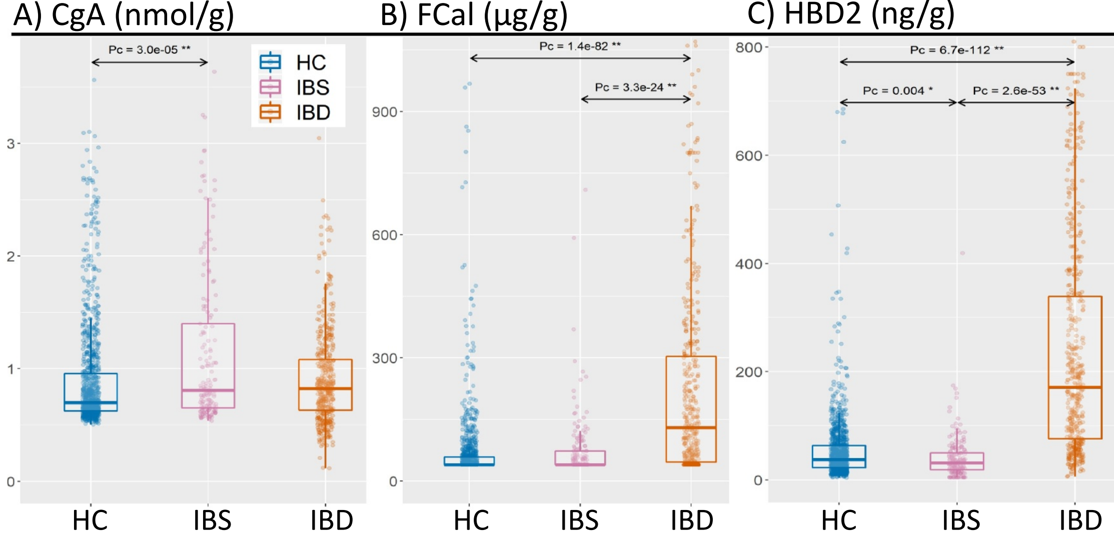

Figure 1. Fecal biomarker measurements. Biomarker measurements in fecal samples from healthy controls (blue), IBS patients (pink), and IBD patients (orange). Statistically significant differences (Mann–Whitney U test, adjusted for multiple testing by Bonferroni correction) are shown in the plots.

and HBD2 led to a considerable increase in predictive power when compared to single-biomarker models (Sensitivity = 0.86, Specificity = 0.83, AUC = 0.93) while adding CgA to the FCal and HBD2 models did not further improve the predictive power (AUC ≈ 0.93). We also constructed binary classification models using biomarker cut-offs obtained by ROC analysis and training classification and regression tree models to determine the optimal separation of IBD and IBS cases. Optimal values for IBD diagnosis using a single biomarker were found to be FCal ≥111 µg/g (Sensitivity = 0.86, Specificity = 0.58) or HBD2 ≥41 ng/g (Sensitivity = 0.9, Specificity = 0.64), while a classification tree using HBD2 and FCal achieved Sensitivity = 0.9 and Specificity = 0.72 with a simple decision tree that classifies a sample as IBD if HBD2 ≥41 ng/g or FCal ≥377 µg/g.

Inclusion of microbiome has potential to increase optimal specificity and sensitivity of fecal biomarker tests

To assess if the gut microbiome can be used to diagnose IBD in patients with gut complaints, we trained prediction models for identification of IBD using gut microbiome profiles obtained by metagenomic sequencing of these samples. We trained separate prediction models using relative abundances of microbial phyla, genera, species, biochemical pathways, and combinations of these profiles. We then compared these models to the models using our biomarker panel (FCal, HBD2, and CgA). We also assessed the additive value of the gut microbiome in the biomarker panel. Training was performed using a randomly selected training set of 336 IBD cases and 127 IBS cases. Testing was performed on the remainder of the data (111 IBD cases and 42 IBS cases).

A comparison of the models showed that the predictive power of the model increases when the microbiome is profiled at a lower taxonomic level (e.g. genus- or species-level as opposed to phylum-level). The use of bacterial biochemical pathways did not improve predictive power (Figure 3/A), and no significant difference was observed between the support vector machine model and multivariate logistic regression model (Supplement S2). Examination of these models identified that optimal performance was achieved using 20 bacterial genera (Supplement S3/A) or 23 bacterial species (Supplement S3/B). Of these, the strongest signal was observed for the species *Eubacterium rectale, Collinsella aerofaciens, Lactobacillus delbrueckii, Odoribacter sp.* and *Ruminococcus sp.* and for genera *Eubacterium, Collinsella, Sutterellaceae, Adlercreutzia*, and *Streptococcus*. Our microbiome-

Table 2. Summary of biomarker measurements. Summary statistics of biomarker measurements, grouped per diagnosis. Measurements significantly different from healthy control group (Mann–Whitney U test or Chi-Squared test) are marked as * for *p*-value <0.05 and as ** for *p*-value < 1.0E-5. Control groups are Healthy controls (for comparison with IBS patients, all IBD cases, UC cases, CD cases, and IBD-U cases), male group (for Sex), positive cases for binary variables (IBD activity and resections), and lowest class for Montreal classifications and HBI/SCCAI values. Abbreviations: M: Montreal classification, HBI: Harvey–Bradshaw index, SCCAI: Simple Clinical Colitis Activity Index.

Variable	Sample size	FCal (µg/g) median [Q1;Q3]	*p*-value	HBD2 (ng/g) median [Q1;Q3]	*p*-value	CgA (nmol/g) median [Q1;Q3]	*p*-value
IBS patients	**169**	**40 [40; 73.2] ***	**0.0076**	**31.6 [19.1; 50.0]***	**5.10E-04**	**0.82 [0.65; 1.50] ***	**< 1.0e-5**
IBS-D	61	40 [40;75.9]	0.24	25.9 [15; 46.9]	0.24	0.9 [0.67; 1.55]	0.36
IBS-C	30	40.1 [40; 73.6]	0.91	30.8 [21.9; 37.1]	0.91	0.77 [0.69; 1.23]	0.64
IBS-M	69	40 [40; 74.3]	0.21	33.9 [20.7; 52.1]	0.21	0.83 [0.633; 1.4]	0.6
all IBD cases	447	150 [50; 380] **	< 1.0e-5	172 [77; 342] **	< 1.0e-5	0.83 [0.63; 1.08]	0.1
Sex: Male	183	160 [56.5; 458]	0.31	189 [89; 390]	0.11	0.81 [0.62; 1.1]	0.49
Sex: Female	264	142 [49.5; 320]	N/A	161 [71; 314]	N/A	0.83 [0.65; 1.1]	N/A
IBD activity: Active	54	340 [104; 822] **	4.00E-04	106 [57; 214] *	0.01	0.81 [0.63; 1]	0.51
IBD activity: Inactive	393	135 [48; 335]	N/A	185 [86; 348] *	N/A	0.83 [0.64; 1.1]	N/A
Ileal resection(s): N	343	150 [50.5; 405]	N/A	176 [80.5; 334]	N/A	0.84 [0.66; 1.1]	N/A
Ileal resection(s): Y	24	167 [58.5; 275]	0.77	119 [66.6; 179]	0.11	1.02 [0.78; 1.26]	0.11
Colonic resection(s): N	337	150 [50; 390]	N/A	176 [77.6; 333]	N/A	0.84 [0.66; 1.11]	N/A
Colonic resection(s): Y	33	180 [54; 575]	0.63	156 [72.4; 203]	0.4	0.99 [0.76; 1.36]	0.09
Ileocecal resection(s): N	290	158 [54.2; 411]	N/A	185 [87; 356]	N/A	0.84 [0.66; 1.07]	N/A
Ileocecal resection(s): Y	80	125 [44.1; 366]	0.27	139 [64.3; 222] *	0.018	0.93 [0.73; 1.32] *	0.017
IBD location: colon	215	155 [52.1; 390]	N/A	188 [84; 379]	N/A	0.80 [0.63; 1.05]	N/A
IBD location: ileum	86	123 [44; 242]	0.07	169 [90.7; 285]	0.39	0.87 [0.64; 1.12]	0.27
IBD location: ileocolonic	100	192 [63.2; 475]	0.42	164 [60.2; 303]	0.08	0.88 [0.65; 1.17]	0.12
IBD: Laxative use: Y	22	148 [78; 229]	0.60	176 [76; 345]	0.3	0.67 [0.47; 0.80] *	0.0015
IBD: NSAID use: Y	22	152 [71; 285]	0.92	135 [83; 392]	0.63	0.79 [0.54; 0.91]	0.11
IBD: PPI use: Y	73	255 [125; 500] *	0.005	176 [94; 440]	1.00	0.73 [0.6; 0.94]	0.3
IBD: Immunosuppresives: Y	194	132 [40; 340]	0.14	165 [83; 332]	0.91	0.84 [0.67; 1.1]	0.051
IBD: Antidiarrheal drugs: Y	44	175 [60; 475]	0.55	141 [63; 303]	0.32	0.89 [0.7; 1.2]	0.11
Crohn's disease cases	**249**	**157 [57; 359] ****	**< 1.0e-5**	**176 [74; 344] ****	**< 1.0e-5**	**0.85 [0.66; 1.14]***	**6.60E-04**
Active CD	26	178 [98.5; 730]	0.09	104 [32.7; 213] *	0.03	0.83 [0.70; 1.05]	0.62
Inactive CD	223	150 [52.5; 330]	N/A	187 [80; 361]	N/A	0.86 [0.66; 1.15]	N/A
M/B3 (Penetrating disease)	31	225 [83.5; 390]	0.27	158 [77.2; 223]	0.36	1.01 [0.73; 1.43]	0.04
M/B2 (Stricturing disease)	85	155 [61; 310]	0.79	187 [68; 303]	0.37	0.85 [0.66; 1.27]	0.21
M/B1 (Uncomplicated CD)	133	160 [44; 340]	N/A	185 [79.7; 387]	N/A	0.83 [0.64; 1.05]	N/A
HBI 10 or higher	20	305 [165; 684] *	0.02	133 [59.5; 270]	0.22	0.88 [0.71; 1.14]	0.76
HBI 6–9	25	125 [70; 340]	0.99	127 [49.1; 297]	0.19	0.85 [0.67; 1.02]	0.75
HBI 5 or less	202	145 [51.2; 336]	N/A	187 [80.6; 378]	N/A	0.84 [0.65; 1.14]	N/A
Ulcerative colitis cases	**169**	**133 [41; 420] ****	**< 1.0e-5**	**170 [80; 331] ****	**< 1.0e-5**	**0.80 [0.60; 1.02]**	**0.87**
Active UC	25	385 [115; 750] *	0.005	150 [60.8; 209]	0.18	0.77 [0.70; 1.05]	0.62
Inactive UC	144	111 [40; 336]	N/A	178 [91.3; 337]	N/A	0.86 [0.66; 1.15]	N/A
M/S0-1 (Mild UC or remission)	63	90.2 [40; 265]	N/A	170 [86.2; 340]	0.18	0.77 [0.62; 0.93]	N/A
M/S2 (Moderate UC)	74	195 [64.7; 462] *	0.04	184 [69.2; 375]	0.99	0.81 [0.546; 1.04]	0.77
M/S3 (Severe UC)	29	170 [48; 505]	0.19	127 [76.9; 207]	0.1	0.86 [0.635; 1.07]	0.22
SCCAI 0–1	127	130 [44; 333]	N/A	180 [92.3; 380]	N/A	0.80 [0.62; 1.03]	N/A
SCCAI 2–4	47	130 [44.5; 610]	0.87	167 [85.8; 292]	0.3	0.80 [0.59; 1.02]	0.49
SCCAI 5 or higher	18	562 [374; 1060] *	3.00E-04	96.2 [58; 177] *	0.01	0.87 [0.67; 0.98]	0.51
Undetermined IBD cases	**29**	**155 [91; 440] ****	**< 1.0e-5**	**157 [90; 345] ****	**< 1.0e-5**	**0.82 [0.65; 1.14]**	**0.46**
Healthy Controls	**1044**	**40 [40; 58.3]**	**N/A**	**38 [23; 64]**	**N/A**	**0.70 [0.62; 0.97]**	**N/A**
Sex: Male	489	40 [40; 57.5]	0.5	40 [25; 72] *	1.00E-04	0.70 [0.62; 1.03]	0.75
Sex: Female	555	40 [40; 58.9]	N/A	35 [22; 57]	N/A	0.7 [0.63; 0.93]	N/A

based IBD prediction performance was comparable to that found by previous studies using 16S sequencing to predict pediatric IBD[35] or using metagenomics sequencing to predict IBD in American[36] and Chinese[37] cohorts.

Models trained on the fecal biomarkers and microbiome data were found to be more accurate than models utilizing biomarkers or microbiome alone (Figure 3/B). The increase in performance was significant in cross-validation test for biomarker models combined with bacterial species, bacterial genera, or bacterial species and pathway (DeLong's test p-value <0.05, Supplements S2 and S3/E). The difference was not statistically significant on the test sets, possibly due to the limited sample sizes of the test sets. However, the models combining biomarkers and the microbiome showed increased optimal specificity and sensitivity and required fewer features to achieve high predictive power. For example, the biomarker-only model showed optimal Sensitivity = 0.90 and Specificity = 0.74, while the model combining biomarkers with five microbiome genera (*Collinsella, Coprococcus, Ruminococcus, Methanobrevibacter*,

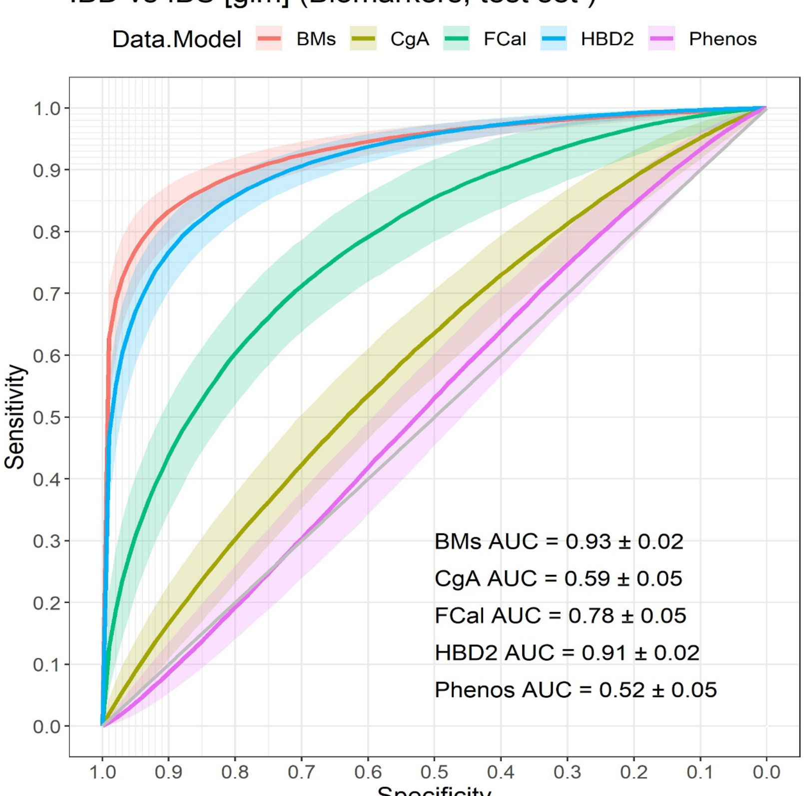

Figure 2. Model for differentiation of IBD from IBS using fecal biomarkers. Receiver operating characteristics (ROC) and area under the curve (AUC) of logistic regression models are trained to separate IBD and IBS cases based on age, sex, and BMI (Phenos), individual biomarkers (CgA, FCal, and HBD2) and a combination of HBD2 and FCal (BM). The predictive power of these models was calculated on a test set of 25% of the samples (n = 153) not used for model training and optimization. The shaded area represents one standard deviation of the ROC obtained using 1000 bootstraps.

and *Alistipes*) had optimal Sensitivity = 0.93 and Specificity = 0.87 (Supplement S3/C). The biomarker and species model showed optimal performance (Sensitivity = 0.93, Specificity = 0.81) when FCal, HBD2, and eight bacterial species (*Eubacterium rectale*, *Veillonella atypica*, *Lachnospiraceae sp.*, *Collinsella aerofaciens*, *Streptococcus sanguinis*, *Dorea longicatena*, *Clostridium nexile*, and *Bacteroides fragilis*; Supplement S3/D) were included into the model.

Models based on metagenomics sequencing–generated data are compatible with 16S rRNA sequencing

To evaluate if simpler and less expensive gut microbiome profiling using 16S rRNA sequencing of fecal samples is a viable strategy for diagnosis of IBD, we performed a method replication experiment where the IBD-prediction model was trained on whole-genome shotgun metagenomic sequencing-generated microbiome profiles of 447 IBD patients and 1044 population controls and then tested using 16S rRNA tag sequencing-generated microbiome profiles of the same cohort. The replication experiment identified that the model trained on 21 bacterial Genera (Supplement S4) successfully separated the IBD from controls using 16S microbiome profiles (Figure 4), with an only minor loss of accuracy compared to metagenomic data (Sensitivity = 0.9, Specificity = 0.63, AUC = 0.86 for metagenomic test set; Sensitivity = 0.82, Specificity = 0.65, AUC = 0.83 for 16S test set).

Microbiome and HBD2 are limited predictors of IBD activity

To determine if our biomarker panel and microbiome data can be used to monitor the IBD disease course, we evaluated disease activity in our IBD

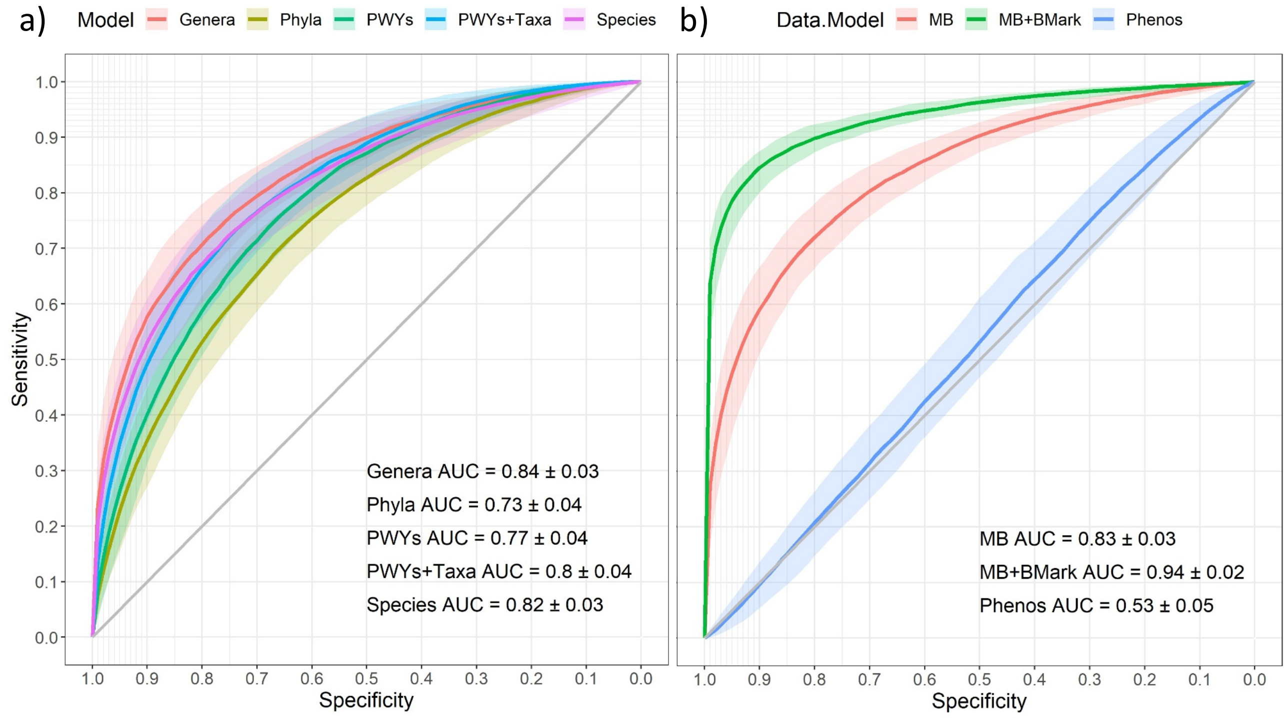

Figure 3. IBD vs IBS prediction models based on microbiome and fecal biomarkers. A) Comparison of ROC curves and areas under the curves (AUC) of microbiome-based models for prediction of IBD built on different levels of taxonomical and functional profiling of gut microbiome: microbial Phyla, Genera, Species, Metacyc pathways (PWYs), and combination of Pathways and taxonomy (PWYs+Taxa). B) Comparison of models trained on the gut microbiome profiled at the genus level (MB), a combination of microbial Genera and biomarkers (MB+BMark) and only age, sex and BMI (Phenos model). The ROC curves were calculated on a test set of 415 samples not used for model training and optimization. Shaded area represents one standard deviation of ROC, obtained using 1000 bootstraps.

cohort using clinical records, colonoscopy, FCal, and the expert opinion of the gastroenterologist using the methodology we previously employed in the analysis of flare-ups in CD.[20] Comparison of biomarkers in patients with active IBD at the moment of fecal sampling (N = 54) and patients with inactive disease (N = 393) identified that the concentration of FCal was significantly elevated (Mann–Whitney U test FDR = 0.001) in patients with active disease, that the concentration of HBD2 was significantly decreased in patients with active IBD (FDR = 0.03), and that CgA was not significantly different between groups (Figure 5).

The predictive power of FCal and HBD2 was found to be significant but limited (AUC = 0.7 for logistic regression model with both biomarkers; AUC □ 0.6 for individual biomarkers), whereas CgA and the model using age, sex, and BMI were not predictive for IBD activity (AUC ≅ 0.5). Additionally, combining the biomarkers with the microbiome did not significantly increase the predictive power of these models (Supplement S2).

Discussion and conclusions

We analyzed the predictive potential of the fecal biomarkers FCal, HBD2, and CgA and the gut microbiome in order to: 1. distinguish patients with IBD from healthy controls, 2. distinguish patients with IBD from individuals with GI complaints, e.g., patients with IBS, and 3. distinguish active from inactive disease within patients with IBD. We identified that fecal HBD2 is highly elevated in patients with IBD and better distinguishes IBD from controls and IBS patients than the currently used FCal. We also discovered that gut microbiome composition and function are predictive of IBD, and the predictive power is increased when the microbiome profile is combined with HBD2 and FCal.

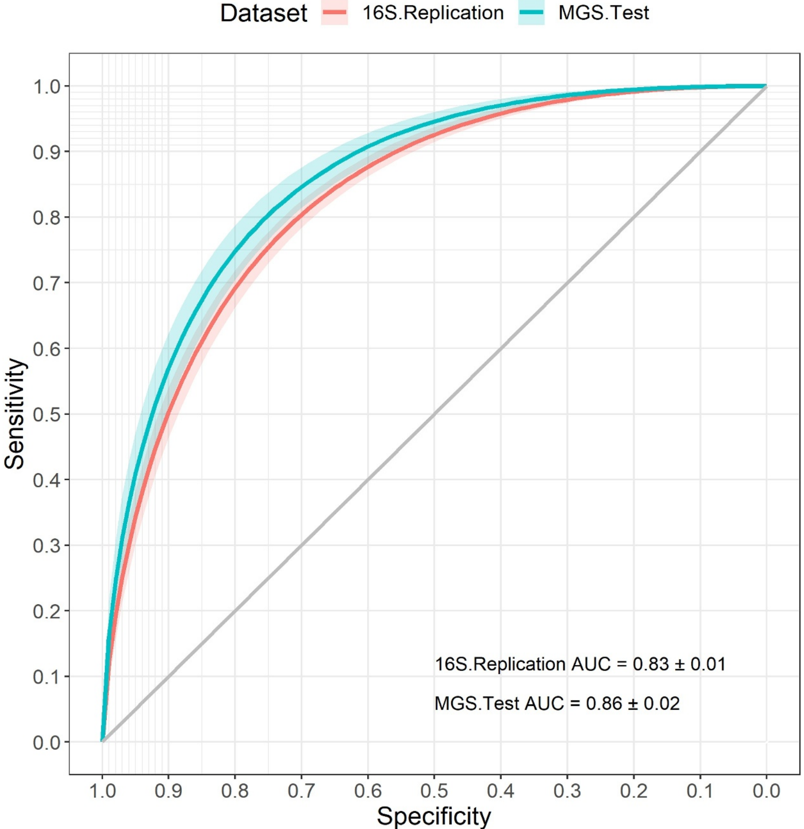

Figure 4. Performance of 16S data on the MGS model of IBD. Comparison of ROC curves of IBD prediction models trained on bacterial genera profiled using MGS sequencing of fecal samples of 447 IBD patients and 1044 population controls (green) to ROC curves constructed by testing the MGS model on the 16S-sequencing generated profile of the same cohort (red).

Noninvasive diagnosis of IBD in patients with bowel complaints and monitoring of IBD disease activity are currently unsolved clinical problems. While colonoscopy performed by skilled medical practitioners is highly successful in both diagnosing IBD and identifying disease activity, it entails considerable patient burden and high healthcare costs.[2,5] The only biomarker currently available in clinical practice is the FCal test, which is based on the detection of calprotectin protein secreted by neutrophils in the inflamed gut.[9] This test, while rapid and inexpensive compared to colonoscopy, suffers from multiple limitations. Its performance is far from perfect, with a reported AUC in the range of 0.75,[3,10] which results in a high rate of false positives when the test is calibrated for high sensitivity. It is also not a good predictor of ileal inflammation as FCal is degraded in the gut and tends to generate false positives in patients with colonic polyps, diverticulitis, infectious diarrhea, and colorectal neoplasia.[38] Furthermore, a comparison of different commercial FCal kits has found up to 5-fold differences in the reported levels of FCal, leading to concerns about the between-assay stability of FCal test results.[39–41] Given these limitations, supplementation of the FCal test with other independent biomarkers is a potential strategy to increase the diagnostic power of noninvasive diagnosis of IBD.

In this study, we focussed on HBD2, CgA, and the gut microbiome as potential biomarkers for enhancing the FCal test based on previously described relations of these biomarkers to IBD,[42] availability of commercial assays for CgA and HBD2, and our previous research on the gut microbiome, which identified striking changes in the gut microbiota of patients with IBD[3] and, independently, a strong association of CgA with the composition of the gut microbiome in a population-based cohort.[12]

HBD2 is a potential novel fecal biomarker for diagnosis of IBD

HBD2 is an antibiotic peptide expressed in response to inflammation and infection. Its basal

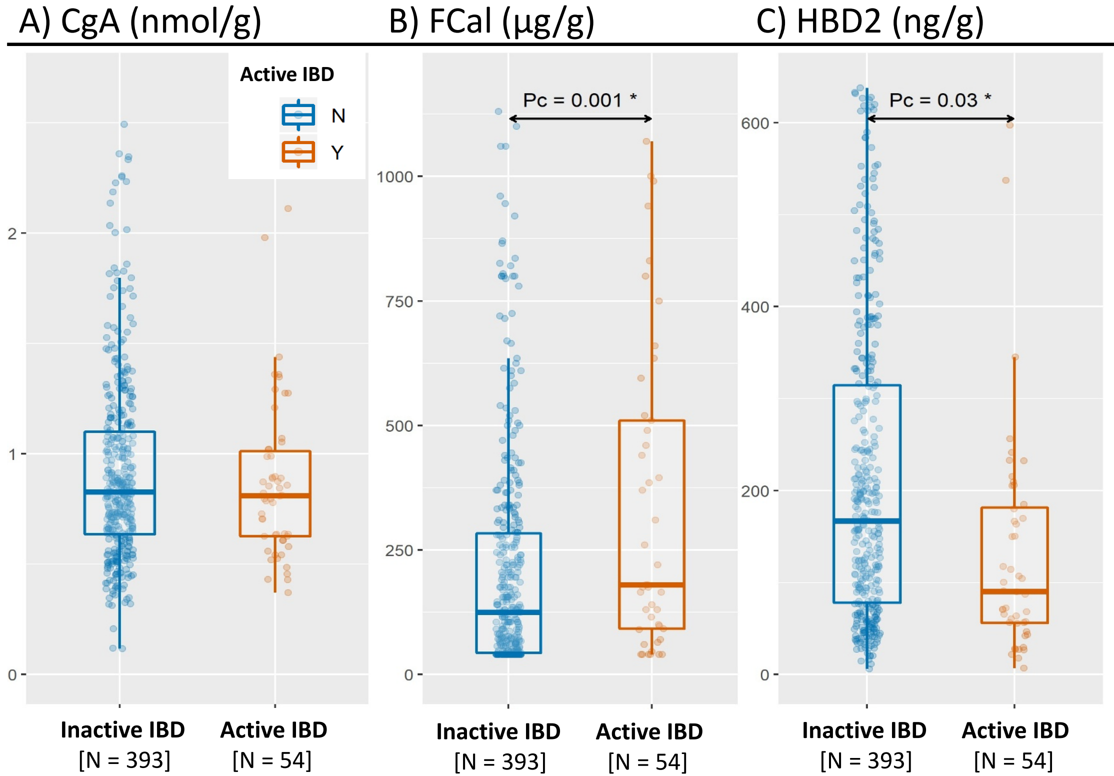

Figure 5. Fecal biomarkers in patients with active and inactive IBD. Biomarker measurements in fecal samples from IBD patients with active disease (red) and patients with inactive IBD (blue). Statistically significant differences (Mann–Whitney U test, adjusted for multiple testing by Bonferroni correction) are shown.

expression level is very low in the colon, but it was found to be induced during inflammation of the gastrointestinal epithelium, which makes it a potential biomarker for IBD.[42] We identified that IBD patients had significantly increased fecal concentrations of HBD2 compared to healthy controls or patients with clinically diagnosed IBS (Figure 1), and this increase was observed for both CD and UC patients (Table 2). These results are consistent with previous studies that show induction of colonic expression of HBD2 in the gut of patients with IBD.[43] Increased levels of HBD2 have also been observed in fecal samples of pediatric patients with IBD[44] and in colonic biopsies of adults with IBD.[43] Notably, HBD2 is not increased in the serum of IBD patients,[45,46] possibly because it is degraded before reaching systemic circulation at levels sufficient for detection.

When tested for predictive power, HBD2 showed high sensitivity and specificity for IBD in our cross-validation and test sets (Supplement S2, Figure 2), with predictive power further increased when HBD2 was combined with FCal (Figure 2), suggesting that HBD2 is a promising candidate biomarker for IBD. Furthermore, these results showed an additive value of HBD2 on top of FCal, indicating that the HBD2 signal is independent of the FCal signal. In our cohort, HBD2 calibrated at HBD2 ≥ 41 ng/g for IBD classification outperformed the FCal test in both sensitivity (0.9 for HBD2 vs 0.86 for FCal) and specificity (0.64 for HBD2 vs 0.58 for FCal), while the logistic regression model using both biomarkers further improved these values to a sensitivity of 0.86 and a specificity of 0.83. While there are no minimal predictive power values defined for medical biomarkers, the ROC values obtained in this study can be considered "acceptable" for FCal (ROC > 0.7), "excellent" for HBD2 (ROC > 0.8) and "outstanding" for the combination of biomarkers (ROC > 0.9).[47]

When translated to potential clinical benefits, our results imply that combining HBD2 and FCal has the potential to considerably (≈40%) decrease proportion of false-positive test results, overcoming what is currently one of the major weaknesses of

the FCal test. As patients with suspected IBD who have high FCal levels (>200 considered positive) require colonoscopy to confirm the IBD diagnosis, this reduction in false positives would lead to a ≈40% reduction in unnecessary colonoscopies. This could result in reduced patient burden and healthcare costs. For test comparison, we assumed that 20% of the tested population (patients with GI complaints suspected of IBD or IBS) have IBD, that all patients who test positive undergo colonoscopy, commercial FCal and HBD2 test cost of ≈€50 and colonoscopy cost of ≈€1,000.[48] Based on these assumptions, the FCal test from our models (Sensitivity = 0.79, Specificity = 0.44) results in 406 unnecessary colonoscopies per 1000 FCal tests and the average cost of ≈€656 per patient due to the high rate of false positives in the tested population. In comparison, our proposed test utilizing HBD2 and FCal would result in ≈167 unnecessary colonoscopies per 1000 tests and an average cost of ≈€466 per patient, resulting in considerable reduction in patient burden and healthcare costs (detailed cost and benefit calculations and assumptions are provided in Supplement S2/C). It should be noted, though, that commercial HBD2 kits are currently not available for medical use and our proposed tests need further standardization, testing, and validation.

No significant change in fecal CgA levels is observed in IBD patients

CgA is a member of the granin family of neuroendocrine secretory proteins that is used as a serum biomarker for diagnosis and monitoring of carcinoid tumors and other neuroendocrine tumors.[49] Previous studies have suggested that interactions between the enteric nervous system and the immune system play important roles in the pathophysiology of IBD, and that CgA could potentially be used as a biomarker for IBD.[50] Additionally, CgA levels in serum,[51] plasma,[52] and fecal samples[53] show mixed values in patients with IBD. These values have been observed to be elevated in patients with IBD, especially in patients with active disease, but there are other studies that have shown no relation between CgA levels and patients with IBD.[53] Furthermore, our previous work identified that CgA has a strong association with the composition of the gut microbiota.[12] In this study, we observed a significant increase in CgA levels in fecal samples of clinically diagnosed IBS patients, but not in IBD patients (Table 2).

The gut microbiome has potential to supplement biomarkers for diagnosis of IBD

Gut microbiome changes have been associated with IBD in numerous studies, with strong microbiome signals reported in both major subtypes of IBD (UC and CD), in pediatric[54] as well as adult[3] patient groups, and in treatment-naïve[55] as well as in treated patients of different ethnicities.[37] We previously reported that the gut microbiome has the potential to be used as a biomarker for distinguishing patients with non-IBD gut complaints, such as IBS, from patients with IBD.[3] Here, we expand this research by building predictive models using multiple fecal biomarkers, integrating these biomarkers with gut microbiome data, and replicating the results using microbiome profiles obtained using the 16S sequencing of fecal samples.

Our results suggest that inclusion of a limited number of microbiome signals into a diagnostic model based on biomarkers has the potential to increase the power of biomarker tests for IBD. In our cohort, inclusion of five bacterial genera into a biomarker model using FCal and HBD2 increased the specificity of the test from 0.89 (biomarker test) to 0.92 and the sensitivity from 0.76 to 0.87 (Supplement S2). This increase in predictive power over FCal and HBD2 test would translate into approximately a doubling of the true positive rate of the test, potentially leading to a reduction in unnecessary colonoscopies. Assuming the 20% prevalence of IBD in the test group and healthcare costs listed above, the inclusion of bacterial genera into HBD+FCal test would reduce the number of unnecessary colonoscopies from ≈167 per 1000 patients (HBD+FCal test) to ≈90 per 1000 patients, considerably reducing the patient burden. The microbiome test would, however, lead to an ≈10% increase in average cost per patient (Supplement S2/C) and potential delays in test results due to the need for microbiome sequencing. While the shotgun metagenomic sequencing (MGS) is unlikely to be implemented in healthcare in the near future due to the added costs and the need for specialized bioinformatics infrastructure expertise, our results show that 16S rRNA sequencing

provides a feasible alternative to MGS (Figure 4) at a fraction of the MGS sequencing and data processing costs. Quantitative PCR test for limited number of bacterial taxa (for example, genera *Collinsella, Coprococcus, Ruminococcus, Methanobrevibacter*, and *Alistipes*, which were found to be strongest predictors of IBD in our data) would provide another option for fast, low cost and high precision microbiome test, but such test would have to be rigorously tested in clinical environment to ensure precision and reproducibility.

Monitoring disease activity in IBD with fecal biomarkers

We also identified that FCal and HBD2 have predictive potential for monitoring disease activity in patients with IBD (Supplement 2). While these results suggest that microbiome, FCal and HBD2 could be used for the identification of active IBD, our study was limited by the small number of patients with active IBD at the time of sampling (54 patients with active IBD). Larger training and test sets will therefore be necessary to achieve the full predictive potential of these biomarkers. In our previous work, we identified that the biochemical functionality of the gut microbiome changes before the onset of the IBD flare,[20] which suggests that longitudinal sampling of the microbiota should increase the predictive power of microbiome-based models for prediction of IBD exacerbations before the onset.

Study limitations

The nature of our study does pose some limitations. First, we were not able to perform replication analyses on an independent cohort because the combination of gut microbiome data and biomarkers derived from fecal samples were not available for any other cohort. Even though we did use separate training and test sets in our prediction models, with no overlapping samples, and replicated the results using 16S sequencing technology of our cohorts, it is important to replicate our findings in an independent cohort. This would ascertain whether differences in ethnicity, diet, geography, or the different techniques used for determining these biomarkers influence the results, thereby providing more insight into the possibility for widespread use. This is especially notable for our results for HBD2 – while this biomarker shows very high diagnostic yield in our study, especially when combined with FCal, this is the first time the fecal HBD2 was evaluated for the prediction of IBD, and potential differences in this biomarker in different populations and potential impacts of laboratory kits for measurement of HBD2 should be evaluated before it can be implemented in the clinic. In addition to the lack of replication cohorts, a limitation introduced by our splitting of data into a training and test sets is the limited sample size of the test set, which might have been insufficient to establish if models combining biomarkers and microbiome have higher predictive power than biomarker-only models. Combined models showed a significant increase in performance on cross-validation sets, but while they showed an increase in AUC, specificity, and sensitivity in test sets, these differences were not statistically significant in test sets. Therefore, larger test sets will be needed to evaluate whether combining the microbiome and biomarkers warrants the cost of adding microbiome sequencing and the expertise required to generate these and analyze these data.

Second, cohorts containing patients with IBD had all been previously diagnosed and treated for their IBD. However, we do have detailed information about these previous treatments and current medication use. To better validate our models for diagnosing IBD, new-onset patients with IBD should be included as they are treatment-naive and the target group for diagnostic testing.

Third, due to the cross-sectional nature of this study, we were not able to determine the stability of the measured biomarkers and microbiome. Longitudinal profiling of the gut microbiome and biomarkers by collecting multiple fecal samples for each individual to capture the microbiome dynamics before, during and after the onset of IBD flare-up is required.

Finally, the use of the microbiome as a biomarker requires there be a standardized protocol (DNA isolation, sample collection, sample storage, and sequencing platform or quantification of bacterial taxa) because variations in methodology are known to affect the readout of the fecal microbiome.[56] While our study demonstrates that 16S sequencing is a viable

alternative to shotgun metagenomics for use in models for the diagnosis of IBD, a clinical fecal microbiome test would ideally use a quantitative approach rather than the currently used relative abundance measurements produced by 16S and metagenomic sequencing. This could be achieved using qPCR specific for limited number of bacterial taxa. These novel qPCR tests will require new testing and replication studies before clinical implementation.

Conclusions

We evaluated the diagnostic potential of noninvasive fecal FCal, HBD2, and CgA, as well as the gut microbiome profiles, for the diagnosis of IBD in patients with gut complaints. HBD2 is a promising novel biomarker for IBD, with a predictive power comparable to FCal test. Combining the HBD2 with FCal test has potential to considerably increase the noninvasive diagnostic yield of IBD tests. The gut microbiome has limited predictive power in comparison to FCal and HBD2, but could potentially supplement biomarker tests to provide a decrease in false-positive rates.

Author contributions:

R.G. conducted the study, interpreted the data, and drafted the manuscript. A.V.V., V.C., A.K., M.D.V., E.A.M.F. assisted in statistical analyses, interpretation of data and drafting of the manuscript. Z.M., C.W., D.M.A.E.J., G.D., J.F., A.Z. collected data, assisted in study planning and critically reviewed the manuscript. F.I. and R.K.W. planned, coordinated, and supported the study. All authors approved the manuscript.

Acknowledgments

We would like to thank B.H. Jansen from the Department of Gastroenterology and Hepatology, UMCG, for technical assistance in sample preparation and processing. We would like to thank Lifelines biobank and study participants for providing data and supporting the study, the Center for Information Technology of the University of Groningen (RUG) for their support and for providing access to the Peregrine high-performance computing cluster, and the Genomic Coordination Center (UMCG and RUG) for their support and for providing access to Calculon and Boxy high performance computing clusters. We also thank K. Mc Intyre for English and content editing.

Funding

This study has been supported by a Diagnostics Grant (D16-14) from the Dutch Digestive Foundation. R.K.W. is supported by the Seerave Foundation and Diagnostics Grant (D16-14) from the Dutch Digestive Foundation. A.Z. is supported by the ERC Starting Grant 715772, Netherlands Organization for Scientific Research NWO-VIDI grant 016.178.056, the Netherlands Heart Foundation CVON grant 2018-27, and the NWO Gravitation grant ExposomeNL 024.004.017. J.F. is supported by the NWO Gravitation Netherlands Organ-on-Chip Initiative (024.003.001), the ERC Consolidator grant 101001678, and the Netherlands Heart Foundation CVON grant 2018-27. C.W. is supported by NWO Gravitation grant 024.003.001 and NWO Spinoza Prize SPI 92-266. Z.M. holds a Niels Stensen fellowship (from Amsterdam, the Netherlands).

ORCID

A. Vich Vila http://orcid.org/0000-0003-4691-5583
V. Collij http://orcid.org/0000-0003-3743-1544
A. Kurilshikov http://orcid.org/0000-0003-2541-5627
R.K. Weersma http://orcid.org/0000-0001-7928-7371

Data availability:

The raw metagenomic data that support these findings are openly available in European Genome-phenome Archive (EGA) at https://ega-archive.org/studies/EGAS00001002702 for IBD samples and at https://www.ebi.ac.uk/ega/datasets/EGAD00001001991 for healthy controls. Summary statistics and other supporting data, with the exception of participant personal data, are available within the article and/or its supplementary materials and on 1000IBD data portal (https://1000ibd.org/menu/main/home). Software and codes are publicly available at https://github.com/GRONINGEN-MICROBIOME-CENTRE/Groningen-Microbiome/tree/master/Projects/IBD_Predict.

The IBD patient metadata that supports the findings of this study are available on request from the corresponding author F.I. The patient data are not publicly available due to their containing information that could compromise the privacy of research participants. The metadata for healthy controls are available from Lifelines Biobank. Restrictions apply to the availability of these data due to privacy of research participants and data were used with the permission of Lifelines for this study. These data are available from R.K.W. with the permission of Lifelines.

Conflicts of interest:

The authors declare no conflicts of interest.

References

1. Abraham C, Cho JH. Inflammatory bowel disease. N Engl J Med. 2009;361(21):2066–2078. doi:10.1056/NEJMra0804647.
2. Chey WD, Kurlander J, Eswaran S. Irritable bowel syndrome: a clinical review. JAMA - J Am Med Assoc. 2015;313(9):949–958. doi:10.1001/jama.2015.0954.
3. Vich Vila A, Imhann F, Collij V, Jankipersadsing SA, Gurry T, Mujagic Z, Kurilshikov A, Bonder MJ, Jiang X, Tigchelaar EF, et al. Gut microbiota composition and functional changes in inflammatory bowel disease and irritable bowel syndrome. Sci Transl Med. 2018;10(472):eaap8914. doi:10.1126/scitranslmed.aap8914.
4. Palsson OS, Whitehead WE, Van Tilburg MAL, Chang L, Chey W, Crowell MD, Keefer L, Lembo AJ, Parkman HP, Rao SSC, et al. Development and validation of the Rome IV diagnostic questionnaire for adults. Gastroenterology. 2016;150(6):1481–1491. doi:10.1053/j.gastro.2016.02.014.
5. Birk J, Bath RK. Is the anesthesiologist necessary in the endoscopy suite? A review of patients, payers and safety. Expert Rev Gastroenterol Hepatol. 2015;9(7):883–885. doi:10.1586/17474124.2015.1041508.
6. Jørgensen LGM, Fredholm L, Hyltoft Petersen P, Hey H, Munkholm P, Brandslund I. How accurate are clinical activity indices for scoring of disease activity in inflammatory bowel disease (IBD)? Clin Chem Lab Med. 2005;43(4):403–411. doi:10.1515/CCLM.2005.073.
7. Miranda-García P, Chaparro M, Gisbert JP. Correlation between serological biomarkers and endoscopic activity in patients with inflammatory bowel disease. Gastroenterol Hepatol. 2016;39(8):508–515. doi:10.1016/j.gastrohep.2016.01.015.
8. Sipponen T, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. Inflamm Bowel Dis. 2008;14(1):40–46. doi:10.1002/ibd.20312.
9. Van Rheenen PF, Van de Vijver E, Fidler V. Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis. Bmj. 2010;341(jul15 1):c3369–c3369. doi:10.1136/bmj.c3369.
10. Lin JF, Chen JM, Zuo JH, Yu A, Xiao ZJ, Deng FH, Nie B, Jiang B. Meta-analysis: fecal calprotectin for assessment of inflammatory bowel disease activity. Inflamm Bowel Dis. 2014;20(8):1407–1415. doi:10.1097/MIB.0000000000000057.
11. Gecse KB, Brandse JF, Van Wilpe S, Löwenberg M, Ponsioen C, Van Den Brink G, D'Haens G. Impact of disease location on fecal calprotectin levels in Crohn's disease. Scand J Gastroenterol. 2015;50(7):841–847. doi:10.3109/00365521.2015.1008035.
12. Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, Mujagic Z, Vila AV, Falony G, Vieira-Silva S, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science (80-). 2016;352(6285):565–569. doi:10.1126/science.aad3369.
13. Öhman L, Stridsberg M, Isaksson S, Jerlstad P, Simrén M. Altered levels of fecal chromogranins and secretogranins in IBS: relevance for pathophysiology and symptoms. Am J Gastroenterol. 2012;107(3):440–447. doi:10.1038/ajg.2011.458.
14. Strid H, Simrén M, Lasson A, Isaksson S, Stridsberg M, Öhman L. Fecal chromogranins and secretogranins are increased in patients with ulcerative colitis but are not associated with disease activity. J Crohn's Colitis. 2013;7(12):e615–22. doi:10.1016/j.crohns.2013.04.019.
15. Mujagic Z, Tigchelaar EF, Zhernakova A, Ludwig T, Ramiro-Garcia J, Baranska A, Swertz MA, Masclee AAM, Wijmenga C, van Schooten FJ, et al. A novel biomarker panel for irritable bowel syndrome and the application in the general population. Sci Rep. 2016;6(April):1–10. doi:10.1038/srep26420.
16. Langhorst J, Junge A, Rueffer A, Wehkamp J, Foell D, Michalsen A, Musial F, Dobos GJ. Elevated human β-defensin-2 levels indicate an activation of the innate immune system in patients with irritable bowel syndrome. Am J Gastroenterol. 2009;104(2):404–410. doi:10.1038/ajg.2008.86.
17. Imhann F, Vich Vila A, Bonder MJ, Fu J, Gevers D, Visschedijk MC, Spekhorst LM, Alberts R, Franke L, Van Dullemen HM, et al. Interplay of host genetics and gut microbiota underlying the onset and clinical presentation of inflammatory bowel disease. Gut 2018;67:108–119. doi:10.1136/gutjnl-2016-312135
18. Imhann F, KJ V, Van D, Barbieri R, Alberts R, Voskuil MD, Vila AV, Khalili H, Ananthakrishnan AN. The 1000IBD project: multi-omics data of 1000 inflammatory bowel disease patients; data release 1. BMC Gastroenterol. 2019;19(5):1–10. doi:10.1186/s12876-018-0917-5.
19. Tigchelaar EF, Zhernakova A, Dekens JAM, Hermes G, Baranska A, Mujagic Z, Swertz MA, Muñoz AM, Deelen P, Cénit MC, et al. Cohort profile: lifeLines DEEP, a prospective, general population cohort study in the northern Netherlands: study design and baseline characteristics. BMJ Open. 2015;5(8):e006772. doi:10.1136/bmjopen-2014-006772.
20. Klaassen MAY, Imhann F, Collij V, Fu J, Wijmenga C, Zhernakova A, Dijkstra G, Festen EAM, Gacesa R, Vich Vila A, et al. Anti-inflammatory Gut microbial pathways are decreased during crohn's disease exacerbations. J Crohns Colitis. 2019;13(11):1439–1449. doi:10.1093/ecco-jcc/jjz077.
21. Zittan E, Kabakchiev B, Kelly OB, Milgrom R, Nguyen GC, Croitoru K, Steinhart AH, Silverberg MS. Development of the Harvey-Bradshaw Index-pro (HBI-PRO) score to assess endoscopic disease activity in crohn's disease. J Crohn's Colitis. 2016;11(5):jjw200. doi:10.1093/ecco-jcc/jjw200.

22. Walmsley RS, Ayres RCS, Pounder RE, Allan RN. A simple clinical colitis activity index. Gut. 1998;43 (1):29–32. doi:10.1136/gut.43.1.29.
23. Gevers D, Kugathasan S, Denson LA, Vázquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song S, Yassour M, et al. The treatment-naïve microbiome in new-onset Crohn ' s disease. Cell Host Microbe. 2014;15 (3):382–392. doi:10.1016/j.chom.2014.02.005.
24. Clausen PTLC, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. BMC Bioinform. 2018;19(1):1–8. doi:10.1186/s12859-018-2336-6.
25. Andrews S, Krueger F, Seconds-Pichon A, Biggins F, FastQC WS. A quality control tool for high throughput sequence data. Babraham bioinformatics. Babraham Institute. Available at: https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed January 10, 2019.
26. Truong DT, Franzosa EA, Tickle TL, Scholz M, Weingart G, Pasolli E, Tett A, Huttenhower C, Segata N. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. Nat Methods. 2015;12 (10):902–903. doi:10.1038/nmeth.3589.
27. Franzosa EA, McIver LJ, Rahnavard G, Thompson LR, Schirmer M, Weingart G, Lipson KS, Knight R, Caporaso JG, Segata N, et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods. 2018;15(11):962–968. doi:10.1038/s41592-018-0176-y.
28. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2014;12(1):59–60. doi:10.1038/nmeth.3176.
29. Suzek BE, Huang H, McGarvey P, Mazumder R, Wu CH. UniRef: comprehensive and non-redundant UniProt reference clusters. Bioinformatics. 2007;23(10):1282–1288. doi:10.1093/bioinformatics/btm098.
30. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537–7541. doi:10.1128/AEM.01541-09.
31. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T, Peplies J, Ludwig W, Glöckner FO, et al. The SILVA and "All-species Living Tree Project (LTP)" taxonomic frameworks. Nucleic Acids Res. 2014;42(Database issue):D643–8. doi:10.1093/nar/gkt1209.
32. Halfvarson J, Brislawn CJ, Lamendella R, Vázquez-Baeza Y, Walters WA, Bramer LM, D'Amato M, Bonfiglio F, McDonald D, Gonzalez A, et al. Dynamics of the human gut microbiome in inflammatory bowel disease. Nat Microbiol. 2017;2(5). doi:10.1038/nmicrobiol.2017.4
33. Evaluation: PD. From precision, recall and f-measure to roc., informedness, markedness & correlation. J Mach Learn Technol. 2011;2:37–63.
34. Ambroise C, McLachlan GJ. Selection bias in gene extraction on the basis of microarray gene-expression data. Proc Natl Acad Sci U S A. 2002;99(10):6562–6566. doi:10.1073/pnas.102102699.
35. Eck A, De Groot EFJ, De Meij TGJ, Welling M, Savelkoul PHM, Budding AE Robust microbiota-based diagnostics for inflammatory bowel disease 2017. Doi:10.1128/JCM.
36. Franzosa EA, Sirota-Madi A, Avila-Pacheco J, Fornelos N, Haiser HJ, Reinker S, Vatanen T, Hall AB, Mallick H, McIver LJ, et al. Gut microbiome structure and metabolic activity in inflammatory bowel disease. Nat Microbiol. 2019;4(2):293–305. doi:10.1038/s41564-018-0306-4.
37. Zhou Y, Xu ZZ, He Y, Yang Y, Liu L, Lin Q, Nie Y, Li M, Zhi F, Liu S, et al. Gut microbiota offers universal biomarkers across ethnicity in inflammatory bowel disease diagnosis and infliximab response prediction. MSystems. 2018;3(1):188–205. doi:10.1128/msystems.00188-17.
38. Brookes MJ, Whitehead S, Gaya DR, Hawthorne AB. Practical guidance on the use of faecal calprotectin. Frontline Gastroenterol. 2018;9(2):87–91. doi:10.1136/flgastro-2016-100762.
39. Lasson A, Stotzer PO, Isakssonc ÖL, Sapnara S, Strid H. M. The intra-individual variability of faecal calprotectin: a prospective study in patients with active ulcerative colitis. J Crohn's Colitis. 2015;9(1):26–32. doi:10.1016/j.crohns.2014.06.002.
40. Whitehead SJ, French J, Brookes MJ, Ford C, Gama R. Between-assay variability of faecal calprotectin enzyme-linked immunosorbent assay kits. Ann Clin Biochem. 2013;50(1):53–61. doi:10.1258/acb.2012.011272.
41. Kristensen V, Malmstrøm GH, Skar V, Røseth A, Moum B. Clinical importance of faecal calprotectin variability in inflammatory bowel disease: intra-individual variability and standardisation of sampling procedure. Scand J Gastroenterol. 2016;51 (5):548–555. doi:10.3109/00365521.2015.1117650.
42. Wehkamp J, Fellermann K, Herrlinger KR, Baxmann S, Schmidt K, Schwind B, Duchrow M, Wohlschläger C, Feller AC, Stange EF, et al. Human β-defensin 2 but not β-defensin 1 is expressed preferentially in colonic mucosa of inflammatory bowel disease. Eur J Gastroenterol Hepatol. 2002;14(7):745–752. doi:10.1097/00042737-200207000-00006.
43. Wehkamp J, Schmid M, Stange EF. Defensins and other antimicrobial peptides in inflammatory bowel disease. Curr Opin Gastroenterol. 2007;23(4):370–378. doi:10.1097/MOG.0b013e328136c580.
44. Kapel N, Benahmed N, Morali A, Svahn J, Canioni D, Goulet O, Ruemmele FM. Fecal β-Defensin-2 in children with inflammatory bowel diseases. J Pediatr

Gastroenterol Nutr. 2009;48(1):117–120. doi:10.1097/MPG.0b013e318174e872.

45. Yamaguchi N, Isomoto H, Mukae H, Ishimoto H, Ohnita K, Shikuwa S, Mizuta Y, Nakazato M, Kohno S. Concentrations of α- and β-defensins in plasma of patients with inflammatory bowel disease. Inflamm Res. 2009;58(4):192–197. doi:10.1007/s00011-008-8120-8.
46. Chang C, Lleo A, Kananurak A, Grizzi F, Tsuneyama K, Invernizzi P, Bevins CL, Bowlus CL. Human β-defensin 2 in primary sclerosing cholangitis. Clin Transl Gastroenterol. 2017;8(3):e80. doi:10.1038/ctg.2017.8.
47. Hosmer DW, Lemeshow S, Sturdivant RX. Applied logistic regression. New York (NY): Wiley; 2013.
48. Peterse EFP, Meester RGS, de Jonge L, Omidvari A-H, Alarid-Escudero F, Knudsen AB, Zauber AG, Lansdorp-Vogelaar I. Comparing the cost-effectiveness of innovative colorectal cancer screening tests. JNCI J Natl Cancer Inst. 2021;113(2):154–161. doi:10.1093/jnci/djaa103.
49. Singh S, Law C. Chromogranin A: a sensitive biomarker for the detection and post-treatment monitoring of gastroenteropancreatic neuroendocrine tumors. Expert Rev Gastroenterol Hepatol. 2012;6(3):313–334. doi:10.1586/egh.12.15.
50. Wagner M, Stridsberg M, Peterson CGB, Sangfelt P, Lampinen M, Carlson M Increased fecal levels of Chromogranin A, Chromogranin B, and secretoneurin in collagenous colitis n.d. Doi: 10.1007/s10753-013-9612-4.
51. Zissimopoulos A, Vradelis S, Konialis M, Chadolias D, Bampali A, Constantinidis T, Efremidou E, Kouklakis G. Chromogranin A as a biomarker of disease activity and biologic therapy in inflammatory bowel disease: a prospective observational study. Scand J Gastroenterol. 2014;49(8):942–949. doi:10.3109/00365521.2014.920910.
52. Sciola V, Massironi S, Conte D, Caprioli F, Ferrero S, Ciafardini C, Peracchi M, Bardella MT, Piodi L. Plasma chromogranin a in patients with inflammatory bowel disease. Inflamm Bowel Dis. 2009;15(6):867–871. doi:10.1002/ibd.20851.
53. Massironi S, Zilli A, Cavalcoli F, Conte D, Peracchi M. Chromogranin A and other enteroendocrine markers in inflammatory bowel disease. Neuropeptides. 2016;58:127–134. doi:10.1016/j.npep.2016.01.002.
54. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, Giannoukos G, Ciulla D, Tabbaa D, Ingram J, et al. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. PLoS One. 2012;7(6):e39242. doi:10.1371/journal.pone.0039242.
55. Shaw KA, Bertha M, Hofmekler T, Chopra P, Vatanen T, Srivatsa A, Prince J, Kumar A, Sauer C, Zwick ME, et al. Dysbiosis, inflammation, and response to treatment: a longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease. Genome Med. 2016;8(1):75. doi:10.1186/s13073-016-0331-y.
56. Fricker AM, Podlesny D, Fricke WF. What is new and relevant for sequencing-based microbiome research? A mini-review. J Adv Res. 2019;19:105–112. doi:10.1016/j.jare.2019.03.006.