

Fecal microbiota signatures are associated with response to Ustekinumab therapy among Crohn's Disease patients

Running title: Microbiota of Ustekinumab-treated Crohn's subjects.

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Abstract

The fecal microbiota is a rich source of biomarkers that have previously been shown to be predictive of numerous disease states. Less well studied is the effect of immunomodulatory therapy on the microbiota and its role in response to therapy. This study explored associations between the fecal microbiota and therapeutic response of ustekinumab (UST; STELARA[®]) treated Crohn's disease (CD) patients in the phase 2 CERTIFI study. Using stool samples collected over the course of 22 weeks, the composition of these subjects' fecal bacterial communities was characterized by sequencing the 16S rRNA gene. Subjects in remission could be distinguished from those with active disease 6 weeks after treatment using Random Forest models trained on subjects' baseline microbiota and clinical data (AUC = 0.844, specificity = 0.831, sensitivity = 0.774). The most predictive OTUs that were ubiquitous among subjects were affiliated with *Faecalibacterium* and *Escherichia/Shigella*. Among subjects in remission 6 weeks after treatment, the median baseline community diversity was 1.7 times higher than treated subjects with active disease ($p = 0.020$). Their baseline community structures were also significantly different ($p = 0.017$). Two OTUs affiliated with *Faecalibacterium* ($p = 0.003$) and *Bacteroides* ($p = 0.022$) were significantly more abundant at baseline in subjects who were in remission 6 weeks after treatment than those with active CD. The diversity of UST treated clinical responders increased over the 22 weeks of the study, in contrast to nonresponsive subjects ($p = 0.012$). The observed baseline differences in fecal microbiota and changes due to therapeutic response support the potential for the microbiota as a response biomarker. (word count= 246/250, TextWrangler)

Importance: CD is a global health concern, with increasing incidence and prevalence, causing large economic and health care impacts. Finding prognostic biomarkers that give clinicians the ability to identify patients more likely to respond to CD treatment at diagnosis will reduce the time subjects receive drugs that are unlikely to be beneficial. OTUs associated with remission after treatment induction, especially *Faecalibacterium*, could be biomarkers for successful UST treatment of anti-TNF- α refractory CD patients. More broadly, these results suggest the fecal

37 microbiota could be a useful non-invasive biomarker for directing or monitoring the treatment of
38 gastrointestinal diseases. (word count =98/150, TextWrangler)

39 **Keywords: IBD, microbiome, biologics, prediction, biomarkers, remission, Stelara, ma-**
40 **chine learning**

41 Introduction

42 The microbiome has been correlated with a variety of diseases and has shown promise as a
43 predictive tool for disease outcome for gingivitis (1), cardiovascular disease (2), *Clostridium*
44 *difficile* infection (3, 4), and colorectal cancer (5, 6). Additionally, the microbiome has been
45 shown to alter the efficacy of vaginal microbicides in African women (7), as well as cardiac drugs
46 (8) and cancer treatments (9, 10) in murine models of disease. These results demonstrate that it
47 is possible to use biomarkers from within the microbiome to predict response to therapeutics. In
48 relation to inflammatory bowel disease (IBD), previous studies have shown that the bacterial gut
49 microbiota correlates with disease severity in new-onset, pediatric Crohn's disease (CD) patients
50 (11, 12). Additionally, recent studies suggest the gut microbiota could be used to predict clinical
51 response to treatment in adult patients with IBD, including anti-integrin biologics (13, 14) and
52 treatment in pediatric IBD with anti-TNF- α or immunomodulators (15, 16). It remains to be
53 determined, however, whether the composition of the fecal gut microbiota can predict and monitor
54 response to biologic CD therapy directed at other targets, such as interleukin (IL-) 23. Considering
55 the involvement of the immune system and previous evidence for involvement of the microbiome,
56 we hypothesize that response to anti-IL-23 CD therapy can be predicted using microbiome data.

57 CD is a global health concern causing large economic and health care impacts (17, 18). The
58 disease is characterized by patches of ulceration and inflammation along the entire gastrointestinal
59 tract, with most cases involving the ileum and colon. Currently, individuals with CD are treated
60 based on disease location and risk of complications using escalating immunosuppressive treatment,
61 and/or surgery, with the goal of achieving and sustaining remission (19, 20). Faster induction
62 of remission following diagnosis reduces the risk of irreversible intestinal damage and disability
63 (20–22). Ideally, clinicians would be able to determine personalized treatment options for CD
64 patients at diagnosis that would result in faster achievement of remission (23). Therefore, recent
65 research has been focused on identifying noninvasive biomarkers to monitor CD severity and
66 predict therapeutic response (24–26).

The precise etiology of CD remains unknown, but host genetics, environmental exposure, and the gut microbiome appear to be involved (17, 27). Individuals with CD have reduced microbial diversity in their guts, compared to healthy individuals, with a lower relative abundance of *Firmicutes* and an increased relative abundance of *Enterobacteriaceae* and *Bacteroides* (11, 28–31). Additionally, genome-wide association studies of individuals with CD identified several susceptibility loci including loci involved in the IL-23 signaling pathway, which could impact the gut microbiota composition and function (19, 28, 32–35). If the fecal microbiota can be used to monitor disease severity and predict response to specific treatment modalities, then clinicians could use it as a noninvasive tool for prescribing therapies that may result in faster remission (36).

The FDA recently approved ustekinumab (UST; STELARA[®]), a monoclonal antibody directed against the shared p40 subunit of IL-12 and IL-23, for the treatment of CD (20, 37–39). Given the potential impact of IL-23 on the microbiota (32–35), we hypothesized that response to UST could be influenced by differences in subjects' gut microbiota and that UST treatment may alter the fecal microbiota. The effects of biologic treatment of IBD on the microbiota are not yet well described, but are hypothesized to be indirect, as these drugs act on host factors (19). We analyzed the fecal microbiota of subjects who participated in a double-blinded, placebo-controlled Phase II clinical trial that demonstrated the safety and efficacy of UST for treating subjects with CD refractory to anti-TNF agents (37). The original study found that UST induction treatment had an increased rate of response as well as increased rates of response and remission with UST maintenance therapy, compared to placebo. We quantified the association between the fecal microbiota and disease severity, tested whether clinical responders had a microbiota that was distinct from non-responders, and determined whether the fecal microbiota changed in subjects treated with UST using 16S rRNA gene sequence data from these subjects' stool samples. Our study demonstrates that these associations may be useful in predicting and monitoring UST treatment outcome and suggest the fecal microbiota may be a broadly useful source of biomarkers for predicting response to treatment.

Results

Study design

We characterized the fecal microbiota in a subset of anti-TNF- α refractory CD patients, with moderate to severe CD, who took part in a randomized, double-blinded, placebo-controlled phase 2b clinical trial that demonstrated the efficacy of UST in treating CD (37). Demographic and baseline disease characteristics of this subset are summarized in Table 1. Subjects were randomly assigned to a treatment group in the induction phase of the study and were re-randomized into maintenance therapy groups 8 weeks after induction based on their response (Figure 1A). In the current study, response was defined as a decrease in a subject's initial Crohn's Disease Activity Index (CDAI) greater than 100 points or remission. Remission was defined as a CDAI below 150 points. The CDAI is the standard instrument for evaluating clinical symptoms and disease activity in CD (40, 41). The CDAI weights patient reported stool frequency, abdominal pain, and general well being over a week, in combination with weight change, hematocrit, opiate usage for diarrhea, and the presence of abdominal masses or other complications to determine the disease severity score (40, 41). Subjects provided stool samples at baseline (screening) and at 4, 6, and 22 weeks after induction for analysis using 16S rRNA gene sequencing (Figure 1B). The number of subjects in each treatment group at the primary and secondary endpoints are summarized in Table 2 by their treatment outcome.

Association of baseline microbial signatures with treatment remission

We investigated whether the composition of the baseline fecal microbiota could predict therapeutic remission (CDAI < 150) 6 weeks after induction. To test this hypothesis, we generated Random Forest (RF) models to predict which subjects would be in remission 6 weeks after induction based on the relative abundance of the fecal microbiota at baseline, clinical metadata at baseline, and the combination of microbiota and clinical data. We determined the optimal model based the largest area under the curve (AUC) of the receiver operating characteristic (ROC) curve for the RF model (6, 42). Clinical data included components of the CDAI, biomarkers for inflammation,

and subject metadata described further in the methods section. We trained these models using 232 baseline stool samples from subjects induced with UST; 31 of which achieved remission (Table 2). Clinical data alone resulted in an AUC of 0.616 (specificity = 0.801, sensitivity = 0.452) (Figure 2A). Using only fecal microbiota data the model had an AUC of 0.838 (specificity = 0.766, sensitivity = 0.806). Finally, when combining clinical metadata with the microbiota we achieved an AUC of 0.844 (specificity = 0.831, sensitivity = 0.774) for remission 6 weeks after induction. Prediction with clinical metadata alone did not perform as well as models using the baseline fecal microbiome ($p = 0.001$) or the combined model ($p = 0.001$); however, there was not a significant difference between the baseline fecal microbiota model and the combined model ($p = 0.841$).

Optimal predictors were determined based on their mean decrease in accuracy (MDA) in the ability of the model to classify remission from active CD (Figure 2B). The majority of OTUs identified as optimal predictors in our model for remission had low abundance. However, two OTUs were differentially abundant for subjects in remission 6 weeks after induction. The relative abundance of *Escherichia/Shigella* (OTU1) was lower in subjects in remission 6 weeks after induction (median = 1.07%, IQR = 0.033-3.70) compared to subjects with active CD (median = 4.13%, IQR = 0.667-15.4). Also, the relative abundance of *Faecalibacterium* (OTU7) was not only higher in subjects in remission 6 weeks after induction (median = 7.43%, IQR = 1.43-11.9) than subjects with active CD (median = 0.167%, IQR = 0.00-5.10), but it was also present prior to the start of UST treatment in every subject who was in remission 6 weeks after induction.

Association of baseline microbial signatures with treatment response

To test whether the composition of the baseline fecal microbiota could predict therapeutic response (CDAI decrease ≥ 100 points or remission) 6 weeks after induction, we again used RF models to classify responders from non-responders 6 weeks after induction (Table 2). Clinical data alone resulted in an AUC of 0.651 (specificity = 0.545, sensitivity = 0.724) (Figure 2C). Using only microbiota data, the model predicted response with an AUC of 0.762 (specificity =

0.558, sensitivity = 0.882). When combining clinical metadata with the microbiome, the model predicted response with an AUC of 0.733 (specificity = 0.724, sensitivity = 0.684).

The microbiota model was significantly better able to predict response than the metadata alone ($p = 0.017$), whereas this was not true for the combined model ($p = 0.069$). Additionally, the combined model and the fecal microbiota model were not significantly different in their ability to predict response ($p = 0.263$). Optimal predictors were again determined based on their MDA in the ability of the model to classify response (Figure 2D). Also, the baseline combined model was significantly better at classifying remission compared to response ($p = 0.036$), whereas this was not true for the fecal microbiota model ($p = 0.117$).

Comparison of baseline microbiota based on clinical outcome

As the RF models identified OTUs abundant across this cohort that were important in classification of outcome, we further investigated differences in the baseline microbiota to assess whether they could serve as potential biomarkers for successful UST treatment. We compared the baseline microbiota of all 306 subjects who provided a baseline sample based on treatment group and treatment outcome 6 weeks after induction to assess diversity measures (Table 2). There was no significant difference in diversity based on response 6 weeks after induction, however the baseline β -diversity was significantly different by response ($p = 0.018$). No phyla were significantly different by treatment and response (Fig. S1) and no OTUs were significantly different based on UST response or among subjects receiving placebo for induction, regardless of response and remission status.

Subjects in remission 6 weeks after induction with UST had significantly higher baseline α -diversity based on the inverse Simpson diversity index than subjects with active CD (respective median values = 11.6 (IQR = 4.84-13.4), 6.95 (IQR = 4.25-11.8), $p = 0.020$). The baseline community structure was also significantly different based on remission status in subjects 6 weeks after induction ($p = 0.017$). Finally, 2 OTUs were significantly more abundant in subjects in remission 6 weeks after induction compared to subjects with active CD: *Bacteroides* (OTU19) ($p = 0.022$)

and *Faecalibacterium* (OTU7) ($p = 0.003$) (Figure 3).

Variation in the baseline microbiota is associated with variation in clinical phenotypes

Based on the associations we identified between baseline microbial diversity and response, we hypothesized that there were associations between the microbiota and clinical variables at baseline that could support the use of the microbiota as a non-invasive biomarker for disease activity (36). To test this hypothesis, we compared the baseline microbiota with clinical data at baseline for all 306 samples provided at baseline (Supplemental Table 1). We observed small, but significant correlations for lower α -diversity correlating with higher CDAI ($\rho = -0.161$, $p = 0.014$), higher frequency of loose stools per week ($\rho = -0.193$, $p = 0.003$), and longer disease duration ($\rho = -0.225$, $p = 0.001$). Corticosteroid use was associated with 1.45 times higher α -diversity ($p = 0.001$). No significant associations were observed between α -diversity and CRP, fecal calprotectin, or fecal lactoferrin. However, the β -diversity was significantly different based on CRP ($p = 0.033$), fecal calprotectin ($p = 0.006$), and fecal lactoferrin ($p = 0.004$). The β -diversity was also significantly different based on weekly loose stool frequency ($p = 0.024$), age ($p = 0.033$), the tissue affected ($p = 0.004$), corticosteroid use ($p = 0.010$), and disease duration ($p = 0.004$). No significant differences in α or β diversity were observed for BMI, weight, or sex.

The diversity of the microbiota changes following UST therapy

We tested whether treatment with UST altered the microbiota by performing a Friedman test comparing α -diversity, based on the inverse Simpson diversity index, at each time point within each treatment group based on the subject's response 22 weeks after induction. We included 48 subjects induced and maintained with UST (18 responders, 30 non-responders) and 14 subjects induced and maintained with placebo (8 responders, 6 non-responders), who provided samples at every time point (Figure 1). We saw no significant difference in the α -diversity over time in subjects who did not respond 22 weeks after induction, regardless of treatment, and in subjects who responded to placebo (Figure 4). However, the median α -diversity of responders 22 weeks after UST induction significantly changed over time ($p = 0.012$) having increased from baseline

(median = 6.65, IQR = 4.60 - 9.24) to 4 weeks after UST induction (median = 9.33, IQR = 6.54 - 16.7), decreased from 4 to 6 weeks after induction (median = 8.42, IQR = 4.93 - 17.5), and was significantly higher than baseline ($p < 0.05$) at 22 weeks after induction (median = 10.7, IQR = 5.49 - 14.6).

The microbiota after induction can distinguish between treatment outcomes

Having demonstrated the microbiome changes in subjects who responded to UST treatment, we hypothesized that the microbiota could be used to monitor response to UST therapy by classifying subjects based on disease activity (36). We again constructed RF classification models to distinguish between subjects by UST treatment outcome based on their fecal microbiota 6 weeks after induction (6, 42). The study design resulted in only 75 stool samples week 22 from subjects induced and maintained with UST, so we focused our analysis on the 220 stool samples collected at week 6 from subjects induced with UST. We were again better able to distinguish subjects in remission from subjects with active CD than subjects in clinical response versus non-response ($p = 0.005$; Figure 5A). Our model could classify response 6 weeks after induction using week 6 stool samples from subjects treated with UST with an AUC of 0.720 (sensitivity = 0.563, specificity = 0.812). For classifying subjects in remission from subjects with active CD 6 weeks after UST induction using week 6 stool samples, the model had an AUC of 0.866 (sensitivity = 0.833, specificity = 0.832). OTUs that were important for these classifications again included *Faecalibacterium* (OTU7), as well as *Blautia* (OTU124), *Clostridium XIVa* (OTU73), *Ruminococcaceae* (OTU53), and *Roseburia* (OTU12). These all had higher median relative abundance in subjects in remission 6 weeks after induction than those with active disease (Figure 5B).

Discussion

This study sought to determine whether fecal microbiota can be used to identify patients who will respond to UST therapy and to gain a more detailed understanding of how UST treatment

may affect the microbiota. We demonstrated that the microbiota could identify patients more likely to achieve remission following UST therapy, compared to clinical metadata alone, in this unique cohort. If this can be validated in future studies with independent cohorts, than it may lead to a clinically useful prognostic tool. We also found the fecal microbiota to be associated with CD severity metrics and treatment outcomes. Finally, we found that the microbiota of treated responders changed over time. These results helped further our understanding of the interaction between the human gut microbiota and CD in adult subjects with moderate-to-severe CD refractory to anti-TNF- α therapies.

The development of predictive models for disease or treatment outcome is anticipated to have a significant impact on clinical decision-making in health care (43). These models may help clinicians decide on the correct course of disease treatment or interventions for disease prevention with their patients. Additionally, patients may benefit with more individualized care that may potentially reduce adverse effects and result in faster recovery, reduce expenses from ineffective therapies, or increase quality of life by preventing disease in patients at high risk.

Our predictive model revealed potential microbial biomarkers indicative of successful UST therapy and allowed us to generate hypotheses about the biology of CD as it relates to the microbiome and UST response. *Faecalibacterium* frequently occurred in our models. It is associated with health, comprising up to 5% of the relative abundance in healthy individuals, and is generally rare in CD patients (28, 30, 44, 45). Each subject in remission 6 weeks after UST induction had measurable *Faecalibacterium* present at baseline. This supports the hypothesis that *Faecalibacterium* impacts CD pathogenesis. It may even be beneficial to administer *Faecalibacterium* as a probiotic during therapy. *Escherichia/Shigella* also occurred frequently in our models. This OTU is associated with inflammation and has been shown to be associated with CD (45). Many other taxa observed in our analysis had low abundance or were absent in the majority of subjects. However, in many cases these taxa are related and may serve similar ecologic and metabolic roles in the gut environment. We hypothesize that these microbes may have genes that perform redundant metabolic functions.

248 Performing metagenomics on stool samples in future studies, especially in patients who achieve
249 remission, could reveal these functions, which could be further developed into a clinically useful
250 predictive tool.

251 We were better able to predict whether a subject would achieve clinical remission rather than
252 clinical response, as determined by CDAI score. We hypothesize that this was due to the relative
253 nature of the response criteria compared to the threshold used to determine remission status.
254 While the field appears to be moving away from CDAI and towards patient reported outcomes
255 and more objectively quantifiable measures such as endoscopic verification of mucosal healing
256 (21, 46), research is ongoing to discover less invasive and more quantifiable biomarkers (36, 47,
257 48).

258 We identified several associations between the microbiota and clinical variables that could impact
259 how CD is monitored and treated in the future. Serum CRP, fecal calprotectin, and fecal lacto-
260 ferrin are widely used as biomarkers to measure inflammation and CD severity. In this study,
261 the microbial community structure was different among subjects based on these markers. These
262 results support the hypothesis that the fecal microbiota could function as a biomarker for mea-
263 suring disease activity in patients, especially in concert with established inflammatory biomarkers
264 (36, 47, 48). Higher CDAI scores were also associated with lower microbial diversity. This is
265 consistent with other studies on the microbiota in individuals with CD compared to healthy in-
266 dividuals and studies looking at active disease compared to remission (11, 36, 49). However,
267 the CDAI sub score of weekly stool frequency may have driven these differences (Supplementary
268 Table 1), which is consistent with previous studies (50). We also observed differences in the
269 microbial community structure based on disease localization, which is consistent with a study by
270 Naftali et al (44). Our study also showed that corticosteroid use impacts the composition of the
271 human fecal microbiota, which is consistent with observations in mouse models (51). We also
272 observed that longer disease duration is associated with a reduction in fecal microbial diversity.
273 We hypothesize that prolonged disease duration and the associated inflammation results in the

observed decrease in diversity.

Further research into fecal microbiota as a source of biomarkers for predicting therapeutic response could eventually allow for the screening of patients using stool samples at diagnosis to better inform treatment decisions for a wide range of diseases. For CD specifically, using the microbiota to predict response to specific treatment modalities could result in more personalized treatment and faster achievement of remission, thereby increasing patients' quality of life and reducing economic and health care impacts for CD patients. Our results showing that the α -diversity of clinical UST responders increased over time, in contrast to non-responsive subjects, and our ability to classify subjects in remission from those with active disease following UST treatment are again consistent with other studies suggesting the microbiota could be a useful biomarker for predicting or monitoring response to treatment (36). These predictive biomarkers will need to be validated using independent cohorts in future studies. Additionally, the positive and negative associations between the microbiota and CD allow us to predict the types of mechanisms most likely to alter the microbiota in order to increase the likelihood of achieving a therapeutic response or to monitor disease severity. Prior to the initiation of therapy, patients could have their fecal microbiome analyzed. Then the microbial community data could be used to direct the modification of a patient's microbiota prior to or during treatment with the goal of improving treatment outcomes. Since it has been shown experimentally that the microbiome can alter the efficacy of treatments for a variety of diseases (7–10), if fecal microbiota can be validated as biomarkers to non-invasively predict response to therapy, then patients and clinicians will be able to more rapidly ascertain effective therapies that result in increased patient quality of life.

Methods

Study Design and Sample Collection

Previously, a randomized, double-blinded, placebo-controlled phase II clinical study of approximately 500 subjects assessed the safety and efficacy of UST for treating anti-TNF- α refractory, moderate to severe CD subjects (37) (Figure 1). Institutional review board approval was acquired at each participating study center and subjects provided written informed consent (37). Subject data was de-identified for our study. Participants provided a stool sample prior to the initiation of the study and were then divided into treatment groups. Additional stool samples were provided 4 weeks after induction. At 6 weeks after induction an additional stool sample was collected, subjects were scored for their response to UST based on CDAI, and then divided into groups receiving either subcutaneous injection of UST or placebo at weeks 8 and 16 as maintenance therapy. Clinical response was defined as a reduction from baseline CDAI score of 100 or more points or as remission in subjects with a baseline CDAI score between 220 to 248 points (37). Remission was defined as a CDAI below the threshold of 150. Finally, at 22 weeks subjects provided an additional stool sample and were then scored using CDAI for their response to therapy. Frozen fecal samples were shipped to the University of Michigan and stored at -80°C prior to DNA extraction.

DNA extraction and 16S rRNA gene sequencing

Microbial genomic DNA was extracted using the PowerSoil-htp 96 Well Soil DNA Isolation Kit (MoBio Laboratories) and an EPMotion 5075 pipetting system (5, 6). The V4 region of the 16S rRNA gene from each sample was amplified and sequenced using the Illumina MiSeq™ platform (48). Sequences were curated as described previously using the mothur software package (v.1.34.4) (52, 53). Briefly, we curated the sequences to reduce sequencing and PCR errors (54), aligned the resulting sequences to the SILVA 16S rRNA sequence database (55), and used UCHIME to remove any chimeric sequences (56). Sequences were clustered into operational

taxonomic units (OTU) at a 97% similarity cutoff using the average neighbor algorithm (57). All sequences were classified using a naive Bayesian classifier trained against the RDP training set (version 14) and OTUs were assigned a classification based on which taxonomy had the majority consensus of sequences within a given OTU (58).

Following sequence curation using the mothur software package (52), we obtained a median of 13,732 sequences per sample (IQR = 7,863-21,978). Parallel sequencing of a mock community had an error rate of 0.017%. To limit effects of uneven sampling, we rarefied the dataset to 3,000 sequences per sample. Samples from subjects that completed the clinical trial and had complete clinical metadata were included in our analysis. Of these samples, 306 were provided prior to treatment as well as 258 provided at week 4, 289 at week 6, and 205 at week 22 after treatment, for a total of 1,058 samples. All raw sequence files and a MIMARKS spreadsheet with de-identified clinical metadata have been uploaded into the NCBI Sequence Read Archive (SRP125127) and are available at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA418765>. Additionally, detailed and reproducible descriptions of how the data were processed and analyzed can be found at https://github.com/SchlossLab/Doherty_CDprediction_mBio_2017.

Gut microbiota biomarker discovery and statistical analysis

R v.3.3.2 (2016-10-31) and mothur were used to analyze the data (59). To assess α -diversity, the inverse Simpson index was calculated for each sample in the dataset. Spearman correlation tests were performed to compare the inverse Simpson index and continuous clinical data. Wilcoxon rank sum tests were performed for pairwise comparisons and Kruskal-Wallis rank sum tests for comparisons with more than two groups (60, 61). To measure β -diversity, the distance between samples was calculated using the θ YC metric, which takes into account the types of bacteria and their abundance to calculate the differences between the communities (62). These distance matrices were assessed for overlap between sets of communities using the non-parametric analysis of molecular variance (AMOVA) test as implemented in the adonis function from the vegan R package (v.2.4.4) (63). Changes in α -diversity over time based on week 22 response was assessed

using a Friedman test on subjects who provided a sample at each time point (64). The Friedman test is a function in the stats R package (v.3.4.2). Multiple comparisons following a Friedman test were performed using the friedmanmc function in the pgirmess package (v.1.6.7) (65). Changes in β -diversity over time by treatment group and response were assessed using the adonis function in vegan stratified by subject. We used the relative abundance of each OTU, α -diversity, age, sex, current medications, BMI, disease duration, disease location, fecal calprotectin, fecal lactoferrin, C-reactive protein, bowel stricture, and CDAI sub scores as input into our RF models constructed with the AUCRF R package (v.1.1) (66), to identify phylotypes or clinical variables that distinguish between various treatment and response groups, as well as to predict or determine response outcome (67). Optimal predictors were determined based on their mean decrease in accuracy (MDA) of the model to classify subjects. Differentially abundant OTUs and phyla were selected through comparison of clinical groups using Kruskal-Wallis and Wilcox tests, where appropriate, to identify OTUs/phyla where there was a p-value less than 0.05 following a Benjamini-Hochberg correction for multiple comparisons (68). Other R packages used in our analysis included ggplot2 v.2.2.1 (69), dplyr v.0.7.4 (70), pROC v.1.10.0 (71), knitr v.1.17 (72), gridExtra v.2.3 (73), devtools v.1.13.3 (74), knitr v.1.0.8 (75), scales v.0.5.0 (76), tidyr v.0.7.2 (77), Hmisc v.4.0.3 (78), and cowplot v.0.8.0 (79). A reproducible version of this analysis and manuscript are available at https://github.com/SchlossLab/Doherty_CDprediction_mBio_2017.

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Tables

Table 1: Summary of clinical metadata of cohort at baseline

Clinical Variable	Treated	Placebo	Total
	n = 232	n = 74	n = 306
Age (years)	38 ± 13	40 ± 14	39 ± 13
Sex (% Male)	36.6	43.2	38.2
Race (% Caucasian)	91.8	93.2	92.2
Corticosteroid Use (%)	40.1	52.7	43.1
BMI (kg/m^2)	26 ± 6.7	25 ± 4.9	25 ± 6.3
Disease Duration (years)	12 ± 8.4	13 ± 10	12 ± 8.8
CDAI	330 ± 62	310 ± 69	320 ± 64
Bowel Stricture (%)	12.5	10.8	12.1
Tissue Involvement (%) Colon/Ileocolic/Ileal	28.9/51.3/19.8	24.3/39.2/36.5	27.8/48.4/23.9

369 **Table 2: Summary of subjects in each treatment group by endpoint and outcome**

Clinical Variable	Treated (n)	Placebo (n)
Week 6 Response (No, Yes)	156, 76	48, 26
Week 6 Remission (No, Yes)	201, 31	62, 12
Week 22 Response (No, Yes)	77, 43	14, 11
Week 22 Remission (No, Yes)	96, 24	18, 7

370

Supplemental Table 1: Diversity differences based on clinical metadata of cohort at baseline

Clinical Variable	Correlation	Alpha-Diversity (p-value)	Beta-Diversity (p-value)
CDAI	$\rho = -0.2$	0.014	0.324
Loose Stool Frequency (per week)	$\rho = -0.2$	0.003	0.024
C-Reactive Protein (mg/L serum)	$\rho = 0.06$	0.394	0.033
Fecal Calprotectin ($\mu\text{g/g}$)	$\rho = 0.08$	0.254	0.006
Fecal Lactoferrin ($\mu\text{g/g}$)	$\rho = 0.1$	0.070	0.004
BMI	$\rho = 0.07$	0.299	0.277
Weight (kg)	$\rho = 0.07$	0.299	0.112
Age (years)	$\rho = -0.05$	0.472	0.033
Sex (F/M)	-	0.539	0.277
Corticosteroid Use (Y/N)	-	0.001	0.010
Disease Duration (years)	$\rho = -0.2$	0.001	0.004
Tissue Involvement	-	0.190	0.004

Figures

Figure 1: Experimental design as adapted from Sandborn et al 2012. (A) Participants were divided into treatment groups receiving placebo or UST by IV for induction. At week 8, subjects were divided into groups receiving either subcutaneous injection of UST or placebo at weeks 8 and 16 as maintenance therapy, based on response at week 6. Finally, at 22 weeks subjects were scored using CDAI for their response to therapy. (B) Stool sampling, treatment, and response evaluation time line. ↑, treatment administration; IV, intravenous; PE, primary endpoint; R, randomization; RR, re-randomization (only for subjects receiving UST induction therapy); SC, subcutaneous.

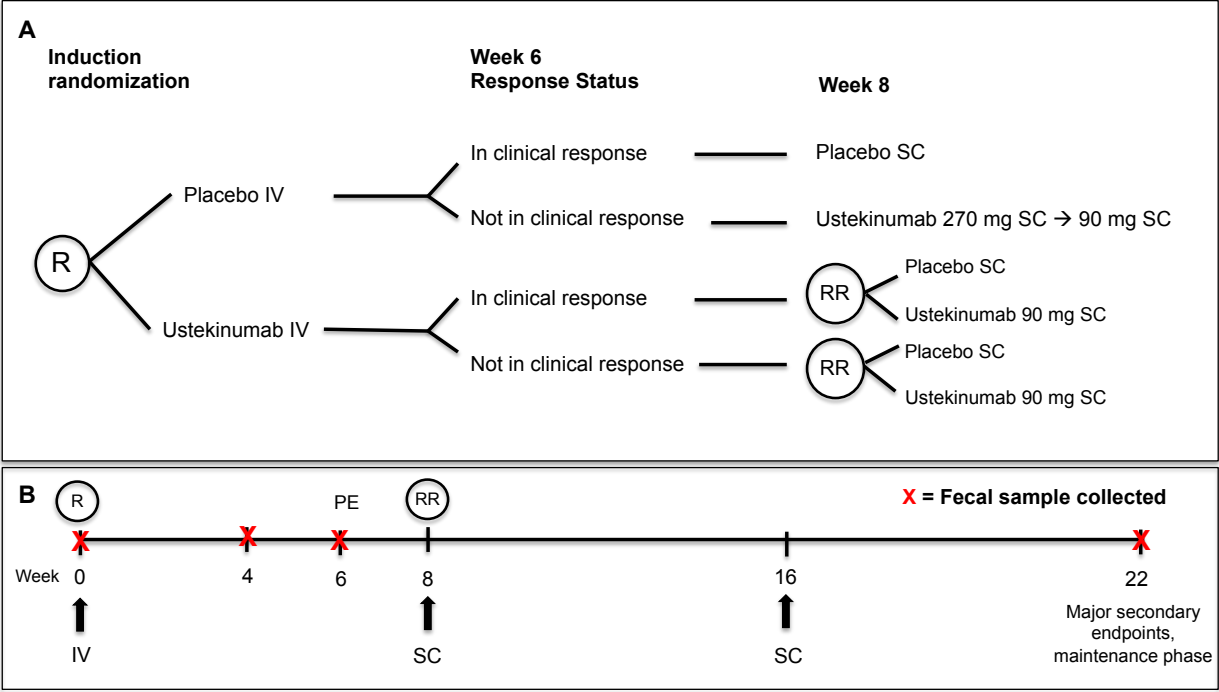


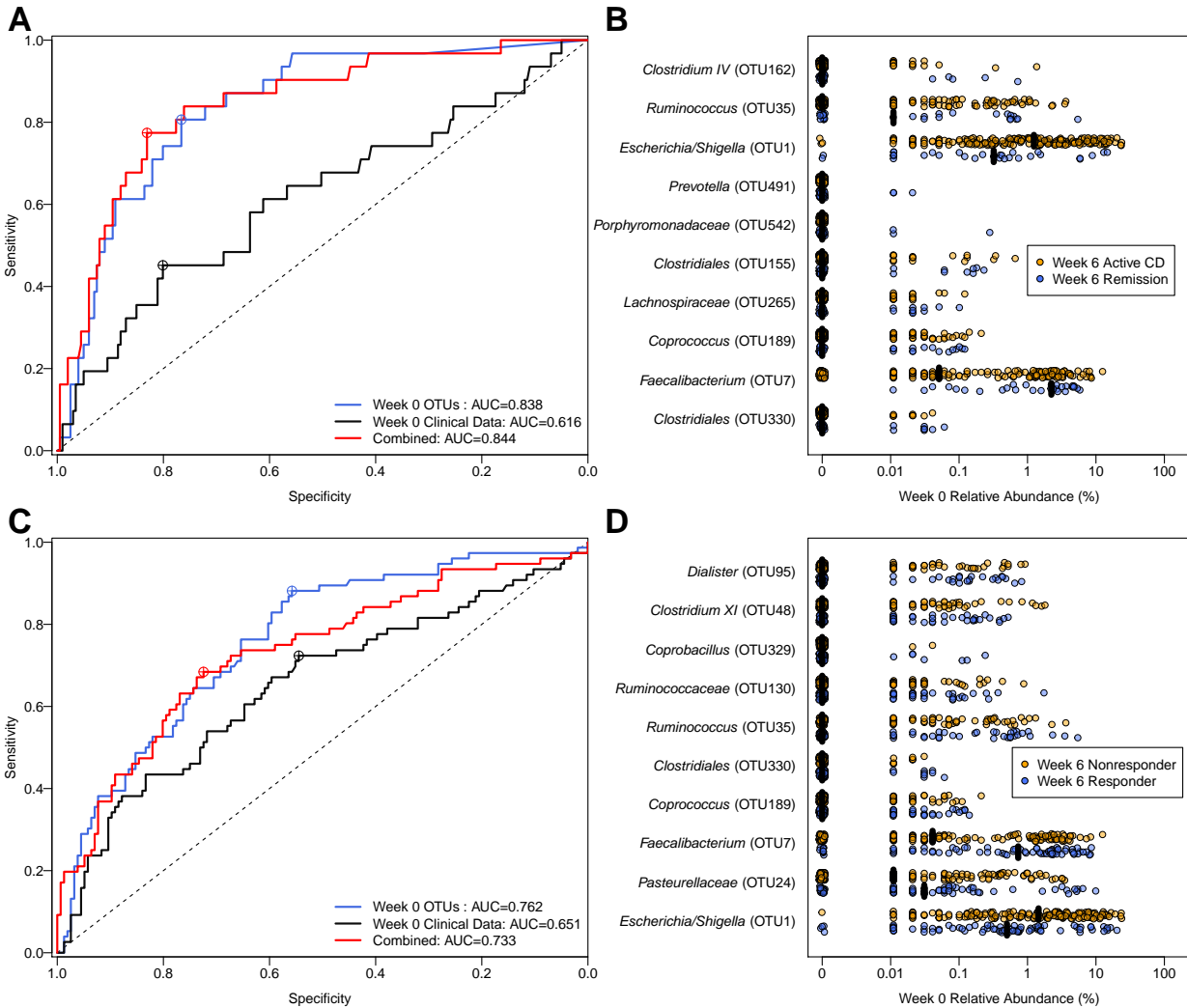
Figure 2: Prediction of week 6 treatment outcome in subjects treated with UST,

using baseline samples Receiver operating characteristic (ROC) curves for (A) response and

(C) remission using microbiota data (blue), clinical metadata (black), and a combined model (red).

Top predictive OTUs for the microbiota model based on mean decrease in accuracy (MDA) for

(B) response and (D) remission. Black bars represent the median relative abundance.



Supplemental Figure 1: Phyla from baseline stool samples in subjects treated with

UST by week six outcome The relative abundance of each phylum in UST treated subjects

were compared based on (A) response and (B) remission status using a Wilcoxon rank sum test

and to identify phyla where there was a p-value less than 0.05 following a Benjamini-Hochberg

correction for multiple comparisons. No comparisons were significant. Whiskers represent the

range and boxes represent the 25-75% interquartile range of the median (black bar).

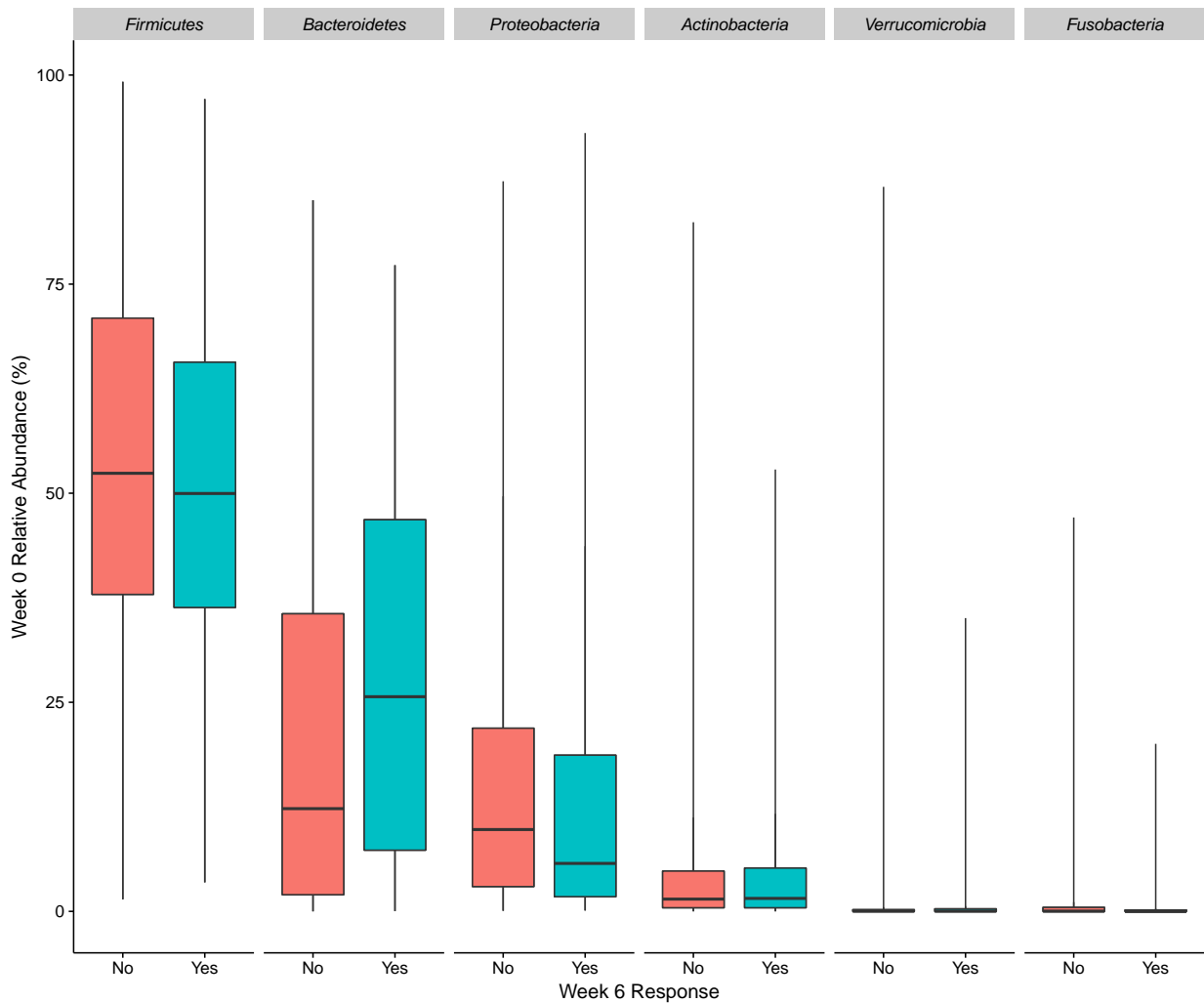


Figure 3: Differential taxa in baseline stool samples from subjects treated with UST,

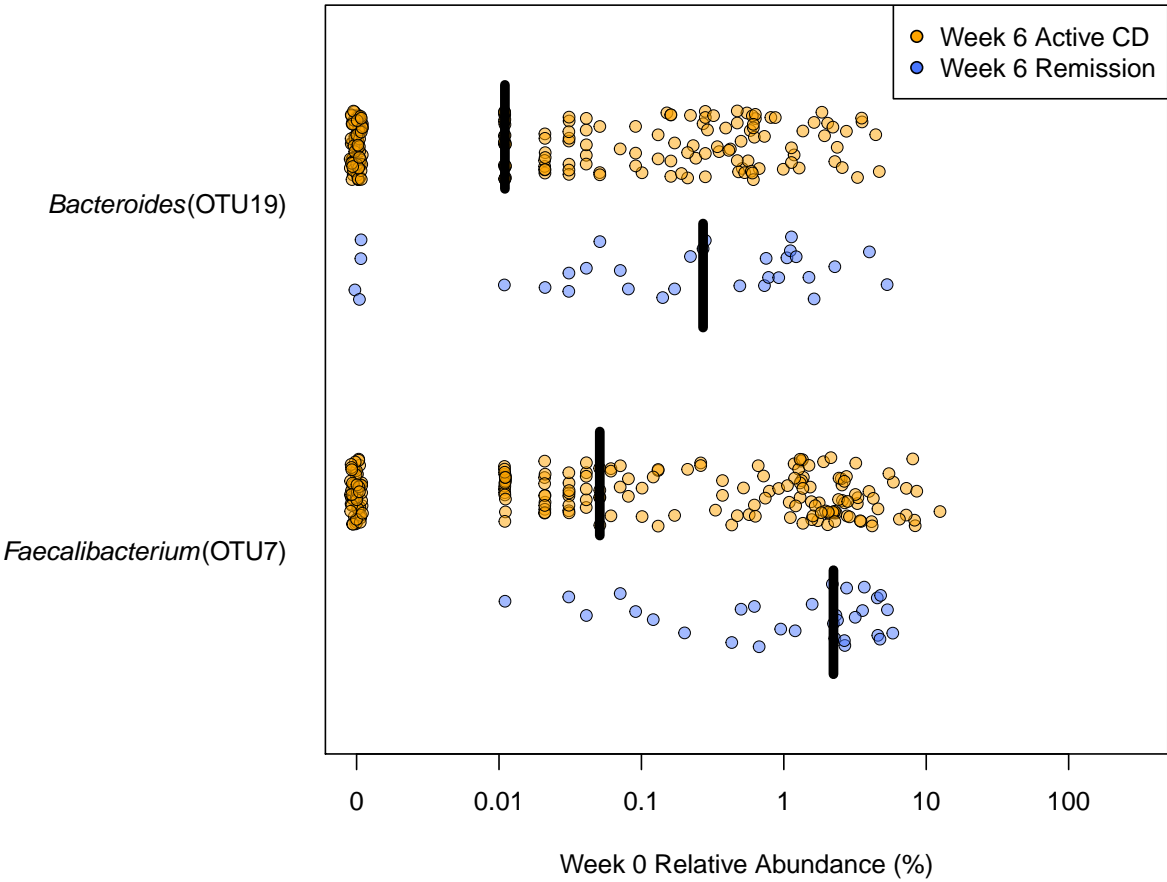
based on week six remission status The baseline relative abundance of each OTU was com-

pared between subjects in remission and those with active CD 6 weeks after induction using a

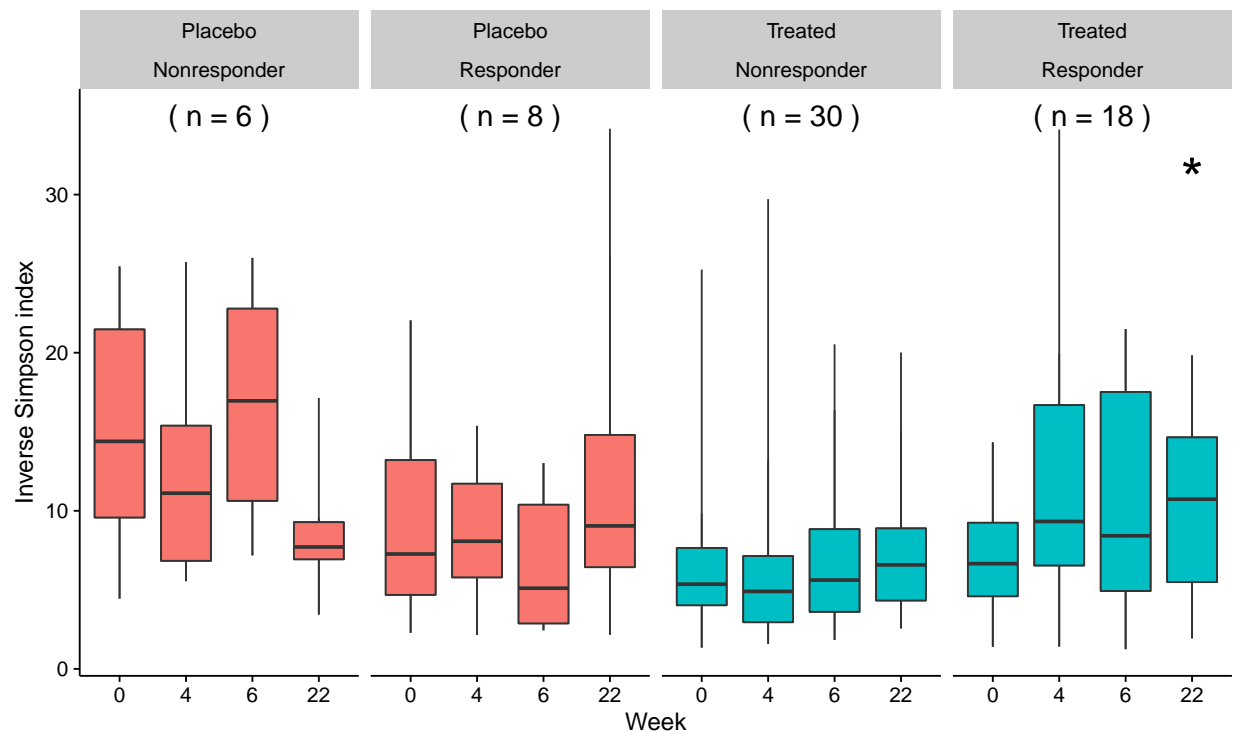
Wilcoxon rank sum test followed by a Benjamini-Hochberg correction for multiple comparisons.

This identified 2 OTUs with significantly different relative abundance at baseline ($p < 0.05$).

Black bars represent the median relative abundance.

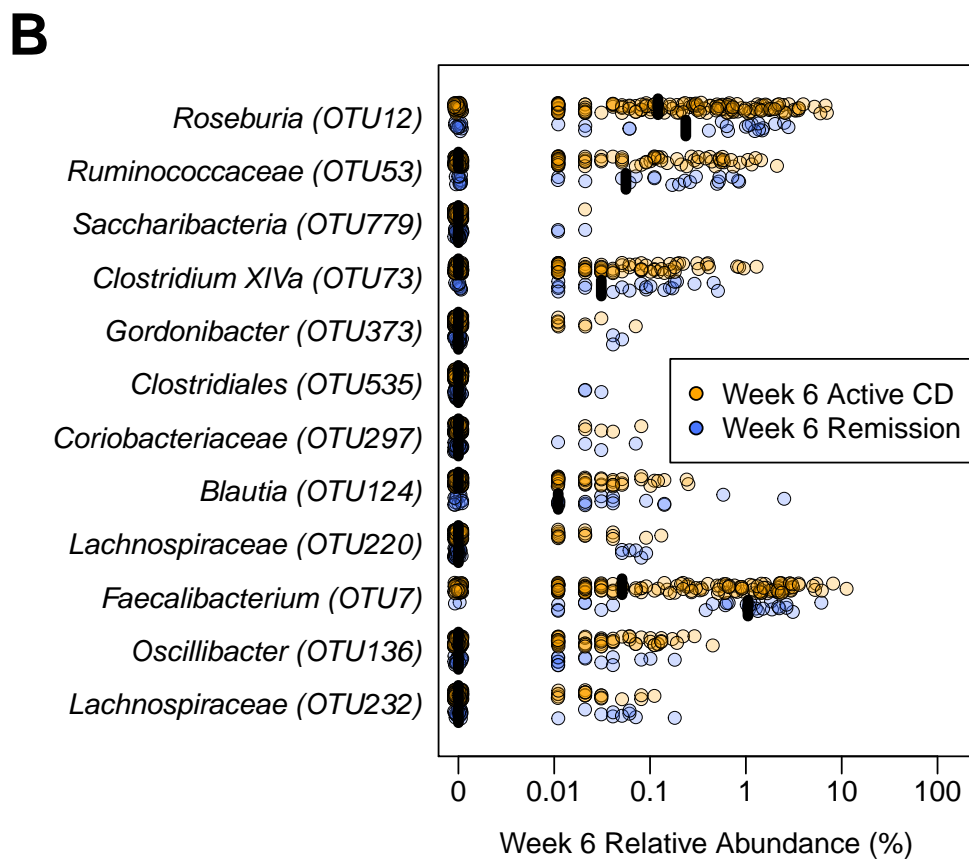
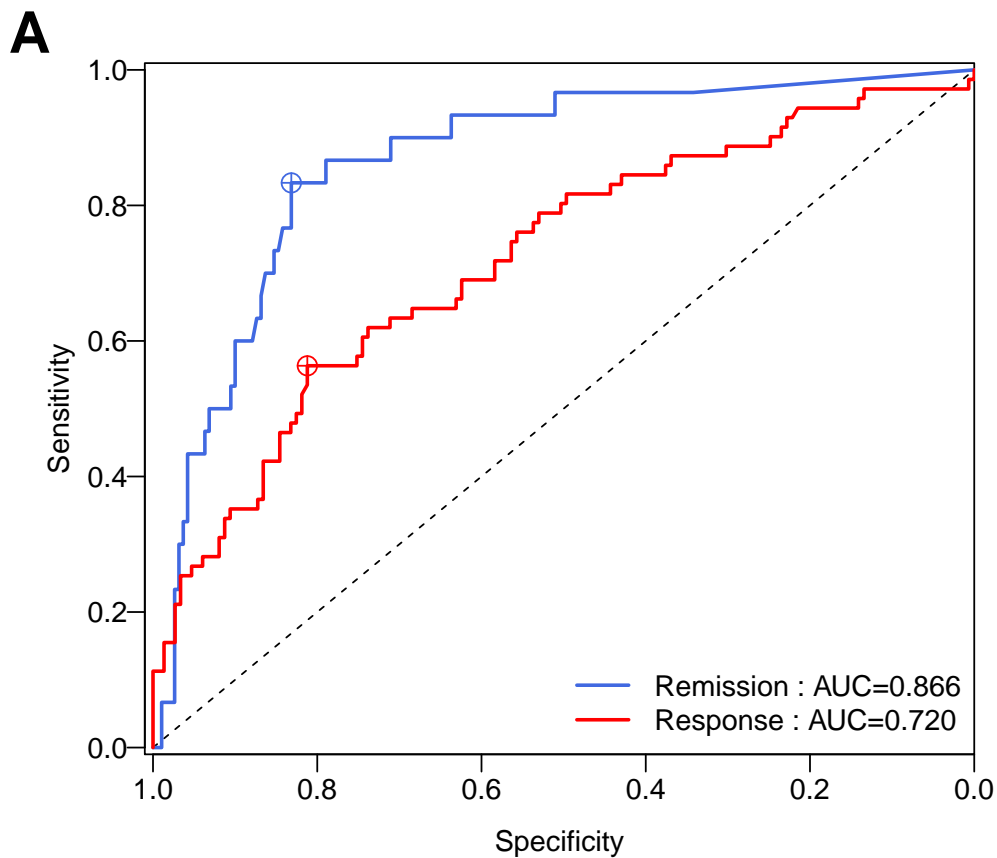


404 **Figure 4: Change in alpha diversity over time by induction treatment and week 22**
 405 **response status.** The α -diversity of 48 subjects induced and maintained with UST and 14
 406 subjects induced and maintained with placebo was assessed at each time point. Friedman test
 407 were performed within each treatment and responder group. Whiskers represent the range and
 408 boxes represent the 25-75% interquartile range of the median (black bar). * indicates week 22 is
 409 significantly different from baseline ($p < 0.05$).



410

411 **Figure 5: Classification of week 6 response or remission status using week 6 stool**
412 **samples from subjects treated with UST** (A) ROC curves for week 6 outcome based on
413 the week 6 microbiota. (B) Predictive OTUs from week 6 stool for remission status at 6 weeks
414 after induction, based on mean decrease in accuracy. Black bars represent the median relative
415 abundance.



References

1. Huang S, Li R, Zeng X, He T, Zhao H, Chang A, Bo C, Chen J, Yang F, Knight R, Liu J, Davis C, Xu J. 2014. Predictive modeling of gingivitis severity and susceptibility via oral microbiota. *ISME J* 8:1768–80.
2. Wang Y, Ames NP, Tun HM, Tosh SM, Jones PJ, Khafipour E. 2016. High molecular weight barley -glucan alters gut microbiota toward reduced cardiovascular disease risk. *Front Microbiol* 7.
3. Schubert AM, Sinani H, Schloss PD. 2015. Antibiotic-induced alterations of the murine gut microbiota and subsequent effects on colonization resistance against *clostridium difficile*. *MBio* 6:e00974.
4. Seekatz AM, Rao K, Santhosh K, Young VB. 2016. Dynamics of the fecal microbiome in patients with recurrent and nonrecurrent *clostridium difficile* infection. *Genome Med* 8.
5. Zackular JP, Rogers MA, Ruffin MT th, Schloss PD. 2014. The human gut microbiome as a screening tool for colorectal cancer. *Cancer Prev Res (Phila)* 7:1112–21.
6. Baxter NT, Ruffin MT th, Rogers MA, Schloss PD. 2016. Microbiota-based model improves the sensitivity of fecal immunochemical test for detecting colonic lesions. *Genome Med* 8:37.
7. Klatt NR, Cheu R, Birse K, Zevin AS, Perner M, Noel-Romas L, Grobler A, Westmacott G, Xie IY, Butler J, Mansoor L, McKinnon LR, Passmore JS, Abdool Karim Q, Abdool Karim SS, Burgener AD. 2017. Vaginal bacteria modify hiv tenofovir microbicide efficacy in african women. *Science* 356:938–945.
8. Haiser HJ, Gootenberg DB, Chatman K, Sirasani G, Balskus EP, Turnbaugh PJ. 2013. Predicting and manipulating cardiac drug inactivation by the human gut bacterium *eggerthella lenta*. *Science* 341:295–8.
9. Sivan A, Corrales L, Hubert N, Williams JB, Aquino-Michaels K, Earley ZM, Benyamin FW, Lei

- YM, Jabri B, Alegre ML, Chang EB, Gajewski TF. 2015. Commensal bifidobacterium promotes antitumor immunity and facilitates anti-pd-l1 efficacy. *Science* 350:1084–9.
10. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, Rusakiewicz S, Routy B, Roberti MP, Duong CP, Poirier-Colame V, Roux A, Becharef S, Formenti S, Golden E, Cording S, Eberl G, Schlitzer A, Ginhoux F, Mani S, Yamazaki T, Jacquelot N, Enot DP, Berard M, Nigou J, Opolon P, Eggermont A, Woerther PL, Chachaty E, Chaput N, Robert C, Mateus C, Kroemer G, Raoult D, Boneca IG, Carbonnel F, Chamaillard M, Zitvogel L. 2015. Anticancer immunotherapy by ctla-4 blockade relies on the gut microbiota. *Science* 350:1079–84.
11. Gevers D, Kugathasan S, Denson LA, Vazquez-Baeza Y, Van Treuren W, Ren B, Schwager E, Knights D, Song SJ, Yassour M, Morgan XC, Kostic AD, Luo C, Gonzalez A, McDonald D, Haberman Y, Walters T, Baker S, Rosh J, Stephens M, Heyman M, Markowitz J, Baldassano R, Griffiths A, Sylvester F, Mack D, Kim S, Crandall W, Hyams J, Huttenhower C, Knight R, Xavier RJ. 2014. The treatment-naive microbiome in new-onset crohn's disease. *Cell Host Microbe* 15:382–92.
12. Wang F, Kaplan JL, Gold BD, Bhasin MK, Ward NL, Kellermayer R, Kirschner BS, Heyman MB, Dowd SE, Cox SB, Dogan H, Steven B, Ferry GD, Cohen SA, Baldassano RN, Moran CJ, Garnett EA, Drake L, Otu HH, Mirny LA, Libermann TA, Winter HS, Korolev KS. 2016. Detecting microbial dysbiosis associated with pediatric crohn disease despite the high variability of the gut microbiota. *Cell Rep*.
13. Tew GW, Hackney JA, Gibbons D, Lamb CA, Luca D, Egen JG, Diehl L, Eastham Anderson J, Vermeire S, Mansfield JC, Feagan BG, Panes J, Baumgart DC, Schreiber S, Dotan I, Sandborn WJ, Kirby JA, Irving PM, De Hertogh G, Van Assche GA, Rutgeerts P, O'Byrne S, Hayday A, Keir ME. 2016. Association between response to etrolizumab and expression of integrin alphaE and granzyme a in colon biopsies of patients with ulcerative colitis. *Gastroenterology* 150:477–87.e9.
14. Ananthakrishnan AN, Luo C, Yajnik V, Khalili H, Garber JJ, Stevens BW, Cleland T, Xavier RJ. 2017. Gut microbiome function predicts response to anti-integrin biologic therapy in inflam-

467 matory bowel diseases. *Cell Host Microbe* 21:603–610.e3.

468 15. Kolho KL, Korpela K, Jaakkola T, Pichai MV, Zoetendal EG, Salonen A, Vos WM de. 2015.
 469 Fecal microbiota in pediatric inflammatory bowel disease and its relation to inflammation. *Am J*
 470 *Gastroenterol* 110:921–30.

471 16. Shaw KA, Bertha M, Hofmekler T, Chopra P, Vatanen T, Srivatsa A, Prince J, Kumar A,
 472 Sauer C, Zwick ME, Satten GA, Kostic AD, Mulle JG, Xavier RJ, Kugathasan S. 2016. Dysbiosis,
 473 inflammation, and response to treatment: A longitudinal study of pediatric subjects with newly
 474 diagnosed inflammatory bowel disease. *Genome Med* 8:75.

475 17. Ananthakrishnan AN. 2015. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol*
 476 *Hepatol* 12:205–217.

477 18. Floyd DN, Langham S, Severac HC, Levesque BG. 2015. The economic and quality-of-life
 478 burden of crohn's disease in europe and the united states, 2000 to 2013: A systematic review.
 479 *Dig Dis Sci* 60:299–312.

480 19. Randall CW, Vizuite JA, Martinez N, Alvarez JJ, Garapati KV, Malakouti M, Taboada CM.
 481 2015. From historical perspectives to modern therapy: A review of current and future biological
 482 treatments for crohn's disease. *Therap Adv Gastroenterol* 8:143–59.

483 20. Wils P, Bouhnik Y, Michetti P, Flourie B, Brixi H, Bourrier A, Allez M, Duclos B, Grimaud
 484 JC, Buisson A, Amiot A, Fumery M, Roblin X, Peyrin-Biroulet L, Filippi J, Bouguen G, Abitbol
 485 V, Coffin B, Simon M, Laharie D, Pariente B. 2015. Subcutaneous ustekinumab provides clinical
 486 benefit for two-thirds of patients with crohn's disease refractory to anti-tumor necrosis factor
 487 agents. *Clin Gastroenterol Hepatol*.

488 21. Colombel JF, Reinisch W, Mantzaris GJ, Kornbluth A, Rutgeerts P, Tang KL, Oortwijn A,
 489 Bevelander GS, Cornillie FJ, Sandborn WJ. 2015. Randomised clinical trial: Deep remission in
 490 biologic and immunomodulator naive patients with crohn's disease - a SONIC post hoc analysis.

491 Aliment Pharmacol Ther 41:734–46.

492 22. Baert F, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P,
493 Hommes D, Rutgeerts P, Vermeire S, D'Haens G. 2010. Mucosal healing predicts sustained
494 clinical remission in patients with early-stage crohn's disease. Gastroenterology 138:463–8; quiz
495 e10–1.

496 23. Lichtenstein GR. 2010. Emerging prognostic markers to determine crohn's disease natural
497 history and improve management strategies: A review of recent literature. Gastroenterol Hepatol
498 (N Y) 6:99–107.

499 24. Chang S, Malter L, Hudesman D. 2015. Disease monitoring in inflammatory bowel disease.
500 World J Gastroenterol 21:11246–59.

501 25. Boon GJ, Day AS, Mulder CJ, Garry RB. 2015. Are faecal markers good indicators of
502 mucosal healing in inflammatory bowel disease? World J Gastroenterol 21:11469–80.

503 26. Falvey JD, Hoskin T, Meijer B, Ashcroft A, Walmsley R, Day AS, Garry RB. 2015. Disease
504 activity assessment in ibd: Clinical indices and biomarkers fail to predict endoscopic remission.
505 Inflamm Bowel Dis 21:824–31.

506 27. Sartor RB. 2006. Mechanisms of disease: Pathogenesis of crohn's disease and ulcerative
507 colitis. Nat Clin Pract Gastroenterol Hepatol 3:390–407.

508 28. Wright EK, Kamm MA, Teo SM, Inouye M, Wagner J, Kirkwood CD. 2015. Recent advances
509 in characterizing the gastrointestinal microbiome in crohn's disease: A systematic review. Inflamm
510 Bowel Dis 21:1219–28.

511 29. Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin
512 C, Chardon P, Marteau P, Roca J, Dore J. 2006. Reduced diversity of faecal microbiota in crohn's
513 disease revealed by a metagenomic approach. Gut 55:205–11.

514 30. Hansen R, Russell RK, Reiff C, Louis P, McIntosh F, Berry SH, Mukhopadhy I, Bisset WM,

Barclay AR, Bishop J, Flynn DM, McGrogan P, Loganathan S, Mahdi G, Flint HJ, El-Omar EM, Hold GL. 2012. Microbiota of de-novo pediatric IBD: Increased faecalibacterium prausnitzii and reduced bacterial diversity in crohn's but not in ulcerative colitis. *Am J Gastroenterol* 107:1913–22.

31. Haberman Y, Tickle TL, Dexheimer PJ, Kim MO, Tang D, Karns R, Baldassano RN, Noe JD, Rosh J, Markowitz J, Heyman MB, Griffiths AM, Crandall WV, Mack DR, Baker SS, Huttenhower C, Keljo DJ, Hyams JS, Kugathasan S, Walters TD, Aronow B, Xavier RJ, Gevers D, Denson LA. 2014. Pediatric crohn disease patients exhibit specific ileal transcriptome and microbiome signature. *J Clin Invest* 124:3617–33.

32. Riol-Blanco L, Lazarevic V, Awasthi A, Mitsdoerffer M, Wilson BS, Croxford A, Waisman A, Kuchroo VK, Glimcher LH, Oukka M. 2010. IL-23 receptor regulates unconventional il-17-producing t cells that control infection¹. *J Immunol* 184:1710–20.

33. Round JL, Mazmanian SK. 2009. The gut microbiome shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 9:313–23.

34. Eken A, Singh AK, Oukka M. 2014. INTERLEUKIN 23 in crohn'S disease. *Inflamm Bowel Dis* 20:587–95.

35. Shih VFS, Cox J, Kljavin NM, Dengler HS, Reichelt M, Kumar P, Rangell L, Kolls JK, Diehl L, Ouyang W, Ghilardi N. 2014. Homeostatic il-23 receptor signaling limits th17 response through il-22-mediated containment of commensal microbiota. *Proc Natl Acad Sci U S A* 111:13942–7.

36. Tedjo DI, Smolinska A, Savelkoul PH, Masclee AA, Schooten FJ van, Pierik MJ, Penders J, Jonkers DMAE. 2016. The fecal microbiota as a biomarker for disease activity in crohn's disease. *Scientific Reports*, Published online: 13 October 2016; doi:101038/srep35216.

37. Sandborn WJ, Gasink C, Gao LL, Blank MA, Johanns J, Guzzo C, Sands BE, Hanauer SB, Targan S, Rutgeerts P, Ghosh S, Villiers WJ de, Panaccione R, Greenberg G, Schreiber S, Lichtiger S, Feagan BG. 2012. Ustekinumab induction and maintenance therapy in refractory

crohn's disease. *N Engl J Med* 367:1519–28.

38. Sandborn WJ, Feagan BG, Fedorak RN, Scherl E, Fleisher MR, Katz S, Johanns J, Blank M, Rutgeerts P. 2008. A randomized trial of ustekinumab, a human interleukin-12/23 monoclonal antibody, in patients with moderate-to-severe crohn's disease. *Gastroenterology* 135:1130–41.

39. Kopylov U, Afif W, Cohen A, Bitton A, Wild G, Bessissow T, Wyse J, Al-Taweel T, Szilagyi A, Seidman E. 2014. Subcutaneous ustekinumab for the treatment of anti-TNF resistant crohn's disease—the McGill experience. *J Crohns Colitis* 8:1516–22.

40. Peyrin-Biroulet L, Panes J, Sandborn WJ, Vermeire S, Danese S, Feagan BG, Colombel JF, Hanauer SB, Rycroft B. 2016. Defining disease severity in inflammatory bowel diseases: Current and future directions. *Clin Gastroenterol Hepatol* 14:348–354.e17.

41. Best WR, Becktel JM, Singleton JW, Kern J F. 1976. Development of a crohn's disease activity index. national cooperative crohn's disease study. *Gastroenterology* 70:439–44.

42. Calle ML, Urrea V, Boulesteix A-L, Malats N. 2011. AUC-RF: A new strategy for genomic profiling with random forest. *Human Heredity* 72:121–132.

43. Vogenberg FR. 2009. Predictive and prognostic models: Implications for healthcare decision-making in a modern recession. *Am Health Drug Benefits* 2:218–22.

44. Naftali T, Reshef L, Kovacs A, Porat R, Amir I, Konikoff FM, Gophna U. 2016. Distinct microbiotas are associated with ileum-restricted and colon-involving crohn's disease. *Inflamm Bowel Dis* 22:293–302.

45. Sartor RB, Wu GD. 2016. Roles for intestinal bacteria, viruses, and fungi in pathogenesis of inflammatory bowel diseases and therapeutic approaches. *Gastroenterology*.

46. Williet N, Sandborn WJ, Peyrin-Biroulet L. 2014. Patient-reported outcomes as primary end points in clinical trials of inflammatory bowel disease. *Clin Gastroenterol Hepatol* 12:1246–56.e6.

47. Boon GJ, Day AS, Mulder CJ, Gearry RB. 2015. Are faecal markers good indicators of

564 mucosal healing in inflammatory bowel disease? *World J Gastroenterol* 21:11469–80.

565 48. Chang S, Malter L, Hudesman D. 2015. Disease monitoring in inflammatory bowel disease.
566 *World J Gastroenterol* 21:11246–59.

567 49. Papa E, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, Giannoukos G, Ciulla D,
568 Tabbaa D, Ingram J, Schauer DB, Ward DV, Korzenik JR, Xavier RJ, Bousvaros A, Alm EJ. 2012.
569 Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory
570 bowel disease. *PLoS One* 7:e39242.

571 50. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J. 2016. Original
572 article: Stool consistency is strongly associated with gut microbiota richness and composition,
573 enterotypes and bacterial growth rates. *Gut* 65:57–62.

574 51. Huang EY, Inoue T, Leone VA, Dalal S, Touw K, Wang Y, Musch MW, Theriault B, Higuchi
575 K, Donovan S, Gilbert J, Chang EB. 2015. Using corticosteroids to reshape the gut microbiome:
576 Implications for inflammatory bowel diseases. *Inflamm Bowel Dis* 21:963–72.

577 52. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA,
578 Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.
579 2009. Introducing mothur: Open-source, platform-independent, community-supported software
580 for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–41.

581 53. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a
582 dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the
583 miseq illumina sequencing platform. *Appl Environ Microbiol* 79:5112–20.

584 54. Schloss PD, Gevers D, Westcott SL. 2011. Reducing the effects of PCR amplification and
585 sequencing artifacts on 16S rRNA-based studies. *PLoS One* 6:e27310.

586 55. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013.
587 The silva ribosomal rna gene database project: Improved data processing and web-based tools.

588 Nucleic Acids Res 41:D590–6.

589 56. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity
590 and speed of chimera detection. *Bioinformatics* 27:2194–200.

591 57. Schloss PD, Westcott SL. 2011. Assessing and improving methods used in operational
592 taxonomic unit-based approaches for 16S rRNA gene sequence analysis. *Appl Environ Microbiol*
593 77:3219–26.

594 58. Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naive bayesian classifier for rapid assignment
595 of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol* 73:5261–7.

596 59. R Core Team. 2016. R: A language and environment for statistical computing. R Foundation
597 for Statistical Computing, Vienna, Austria.

598 60. Sokal RR, Rohlf FJ. 1995. *Biometry: The principles and practice of statistics in biological*
599 *research*, 3rd ed. Freeman, New York.

600 61. Magurran AE. 2004. *Measuring biological diversity*. Blackwell Pub., Malden, Ma.

601 62. Yue JC, Clayton MK. 2005. A similarity measure based on species proportions. *Communica-*
602 *tions in Statistics-Theory and Methods* 34:2123–2131.

603 63. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara
604 RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2016. *Vegan: Community*
605 *ecology package*. r package version 2.4-1.

606 64. Friedman M. 1937. The use of ranks to avoid the assumption of normality implicit in the
607 analysis of variance. *Journal of the American Statistical Association* 32:675–701.

608 65. Giraudoux P. 2016. *Pgirmess: Data analysis in ecology*.

609 66. Urrea V, Calle M. 2012. AUCRF: Variable selection with random forest and the area under

610 the curve.

611 67. Breiman L. 2001. Random forests. *Machine Learning* 45:5–32.

612 68. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: A practical and powerful
613 approach to multiple testing. *Journal of the Royal Statistical Society Series B (Methodological)*
614 57:289–300.

615 69. Wickham H. 2009. *Ggplot2: Elegant graphics for data analysis*. Springer-Verlag New York.

616 70. Wickham H, Francois R. 2016. *Dplyr: A grammar of data manipulation*.

617 71. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, Müller M. 2011. PROC:
618 An open-source package for r and s+ to analyze and compare roc curves. *BMC Bioinformatics*
619 12:77.

620 72. Xie Y. 2015. *Dynamic documents with R and knitr*, 2nd ed. Chapman; Hall/CRC, Boca
621 Raton, Florida.

622 73. Auguie B. 2016. *GridExtra: Miscellaneous functions for “grid” graphics*.

623 74. Wickham H, Chang W. 2016. *Devtools: Tools to make developing r packages easier*.

624 75. Boettiger C. 2015. *Knitcitations: Citations for ‘knitr’ markdown files*.

625 76. Wickham H. 2016. *Scales: Scale functions for visualization*.

626 77. Wickham H. 2017. *Tidyr: Easily tidy data with ‘spread()’ and ‘gather()’ functions*.

627 78. Harrell Jr FE, Charles Dupont, others. 2016. *Hmisc: Harrell miscellaneous*.

628 79. Wilke CO. 2016. *Cowplot: Streamlined plot theme and plot annotations for ‘ggplot2’*.