Full Title: Microbiota and radiotherapy-induced gastrointestinal side-effects (MARS) study: a large pilot study of the microbiome in acute and late radiation enteropathy.

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Running Title:

Microbiota and radiotherapy gastrointestinal side-effects.

Abbreviations list:

ADT: Androgen deprivation therapy

CRO: Clinician-reported outcomes

IQR: Inter-quartile range

KEGG: Kyoto Encyclopaedia of Genes and Genomes

LENT-SOM: Late Effects of Normal Tissues

OTU: Operational taxonomic unit

PLN-IMRT: Intensity-modulated radiotherapy to the prostate and pelvic lymph nodes

PRO: Patient-reported outcomes

RE: Radiation enteropathy

RTOG: Radiation Therapy Oncology Group

SCFA: short-chain fatty acids

UCLA-PCI: University of California, Los Angeles Prostate Cancer Index

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interpretation, sample collection, literature search and manuscript design and review. LT was responsible for data collection and study management at the Bob Champion Unit and contributed with manuscript review. KM contributed with database design, statistical support and manuscript review. SGow was involved in sample processment, data collection and interpretation, and manuscript review. JVL contributed for study design and manuscript review. SGull was involved in data analysis and interpretation, manuscript design and review. JL was involved in study design, data analysis and interpretation, manuscript design and review. MF, HJNA and DD all participated in patient recruitment. JM was involved in study design, data collection, analysis and interpretation, literature search, manuscript design and review. DD is the Chief Investigator of the study and was involved with study design, data interpretation and manuscript design and review. All authors reviewed and approved the manuscript.

STATEMENT OF TRANSLATIONAL RELEVANCE

Clinical evidence shows that gut microbiota changes during radiotherapy and suggests associations with radiation enteropathy (RE), but this evidence is limited. Clinical studies often include patients receiving concurrent cytotoxic systemic therapies. Experiments in animal models indicate that gut microbiota are necessary for RE to occur and that an irradiated microbiota promotes enteropathy. However, animal models have different radioresistance and microbiota compared to humans and usually receive high-dose single-fraction radiation, limiting clinical translation. Moreover, all evidence focuses on acute RE and does not address dose-limiting late RE.

We report the largest clinical study to date into associations of the microbiota with acute and late RE. It is the only study where patients received homogeneous treatment and where no patients received cytotoxic systemic therapies. Our novel methodology allowed assessment of acute and late RE. We demonstrate that some bacteria producing short-chain fatty acids (SCFA) are associated with radiation-induced side-effects and that this relates to an altered intestinal micro-environment.

We demonstrate that an altered microbiota associates with early and late RE, with clinical implications for risk assessment, prevention and treatment of radiation-induced side-effects.

STATEMENT OF SIGNIFICANCE

We report the largest clinical study to date into associations of the microbiota with acute and late RE. An altered microbiota associates with early and late RE, with clinical implications for risk assessment, prevention and treatment of radiation-induced side-effects.

ABSTRACT

Purpose: Radiotherapy is important in managing pelvic cancers. However, radiation enteropathy (RE) may occur and can be dose-limiting. The gut microbiota may contribute to the pathogenesis of RE. We hypothesized that the microbiome differs between patients with and without RE.

Patients and Methods: Three cohorts of patients (n=134) were recruited. The early cohort (n=32) was followed sequentially up to 12 months post-radiotherapy to assess early RE. Linear mixed models were used to assess microbiota dynamics. The late cohort (n=87) was assessed cross-sectionally to assess late RE. The colonoscopy cohort compared the intestinal mucosa microenvironment in patients with RE (cases, n=9) with healthy controls (controls, n=6). Faecal samples were obtained from all cohorts. In the colonoscopy cohort, intestinal mucosa samples were taken. Metataxonomics (16S rRNA gene) and imputed metataxonomics (Piphillin) were used to characterise the microbiome. Clinician (CRO) and patient-reported (PRO) outcomes were used for clinical characterisation.

Results: In the acute cohort, we observed a trend for higher pre-radiotherapy diversity in patients with no self-reported symptoms (p=0.09). Dinamically, diversity decreased less over time in patients with rising RE (p=0.05). A consistent association between low bacterial diversity and late RE was also observed, albeit non-significantly. Higher counts of *Clostridium IV*, *Roseburia*, and *Phascolarctobacterium* significantly associated with RE. Homeostatic intestinal mucosa cytokines related to microbiota regulation and intestinal wall maintenance were significantly reduced in RE (IL-7 (p=0.05), IL-12/IL-23p40 (p=0.03), IL-15 (p=0.05), IL-16 (p=0.009)). IL-15 inversely correlated with counts of *Roseburia* and *Propionibacterium*.

Conclusions: The microbiota presents opportunities to predict, prevent or treat RE.

INTRODUCTION

Pelvic radiotherapy is an important curative treatment option for patients with pelvic cancers. However, acute (≤90 days of starting radiotherapy) and chronic (thereafter) gastrointestinal side-effects, collectively summarized by the term "radiation enteropathy" (RE), may develop. Indeed, risk of gastrointestinal toxicity limits the radiation dose that can be delivered.(1) RE can be defined as a progressive, ischaemic, profibrotic process occurring after abdominal or pelvic irradiation, driven by pathophysiological processes which are incompletely defined.(1,2) Mechanisms involving the microbiota may contribute to the spectrum of RE.(1) However, published research concentrates on acute RE, whereas it is late RE that is usually dose-limiting, and often uses animal models, which have limitations.(3–6)

To better understand the role of the microbiota in RE, we prospectively collected faecal samples from three complementary cohorts of patients, collectively assessing the whole spectrum of RE. We hypothesized that the microbiome differs between patients with and without radiation enteropathy after pelvic radiotherapy.

METHODS

The MARS study

The MARS study was an observational, non-interventional study. Three cohorts were recruited in parallel (figure SUPP-1). All patients attending relevant clinics were invited to participate during a 2-year period (see section 1.c in supplementary text for sample size justification).

The first (termed "early cohort") assessed the development of early RE in a group of patients recruited before undergoing high-dose intensity-modulated radiotherapy to the prostate and pelvic lymph nodes (PLN-IMRT) and followed longitudinally up to a year thereafter. Patients undergoing PLN-IMRT were chosen because they are at increased risk of RE when compared to prostate-only radiotherapy.(7) Clinical assessment and sampling was performed at baseline (pre-radiotherapy), at 2/3 weeks, 4/5 weeks, 12 weeks, 6 months and 12 months post- radiotherapy initiation (figure SUPP-2).

The second (termed "late cohort") explored late RE and included patients with ≥2 years of follow-up after PLN-IMRT who were evaluated cross-sectionally. Patients in this cohort were recruited from the population of a previously reported dose-escalation trial of PLN-IMRT.(7) Their radiotherapy followed an identical protocol to the longitudinal cohort.

The third (termed "colonoscopy cohort") assessed the intestinal mucosa immune environment in RE and its relationships with the microbiome. It included patients with ≥1 year of follow-up after radiotherapy for prostate cancer and attending a specialist clinical service for managing radiation-induced gastrointestinal symptoms who were undergoing colonoscopy for symptom investigation (termed "cases"), as well as non-irradiated control subjects ("controls"), undergoing colonoscopy for colon cancer screening and confirmed free of gastrointestinal diseases. We sampled anterior rectum (cases/controls) and distal sigmoid (cases only). The anterior rectum is the gastrointestinal location receiving maximal irradiation in radiotherapy for prostate cancer, while the distal sigmoid is less irradiated and was thus used as a self-control in cases.

All subjects provided written informed consent prior to entry into the study. The study was approved by the Committee for Clinical Research at the Royal Marsden (no.: 4010) and by the London-Bromley Research Ethics

Committee (no.: 13/LO/1527), and registered by the NHS Health Research Authority (ID: 130287). All study procedures were conducted in accordance to the Declaration of Helsinki.

Assessments

Clinician-reported outcomes (CRO) included items of the Radiation Therapy Oncology Group (RTOG) and Late Effects of Normal Tissues (LENT-SOM) scales with an impact on quality of life (bowel problem/distress measured with the University of California, Los Angeles Prostate Cancer Index (UCLA-PCI) scale).(8–10) The criteria used were RTOG diarrhoea and proctitis, and LENT-SOM sphincter control (subjective); tenesmus (subjective), bleeding (objective), pain (objective), and bleeding (management). Two summary figures (RTOG maximum and LENT/SOM maximum) were created from maximum toxicity scores. Both scales are graded 1-5, with increasing scores representing worse symptoms. Patient-reported outcomes (PRO) were analysed with the bowel subset of a gastrointestinal symptom score validated for radiation enteropathy and graded 1-7 for 10 items (table SUPP-1), with scores ranging 10 (very symptomatic) to 70 (no symptoms).(11)

In the late cohort, peak cumulative late toxicity scores (from 6 month after radiotherapy onwards) were available as per the IMRT for Prostate Cancer study protocol. CRO included RTOG diarrhoea and RTOG proctitis. PRO included UCLA-PCI bowel problem and distress. For convenience, we have termed prevalence data at the time of sampling “actual toxicity”, and peak cumulative data “historic toxicity”.

Patient comorbidity and diet, were also assessed (supplementary methods and table SUPP-2). Intestinal mucosa histology (colonoscopy cohort), was evaluated with a semi-quantitative histopathology score (table SUPP-3).(12)

Definition of symptom groups

Patients in the early cohort were divided in three groups, which were (1) No symptoms (**no** symptoms at **either** 4/5 weeks **or** 6 months); (2) Non-persistent symptoms (symptoms at **either** 4/5 weeks **or** 6 months); and (3) Persistent symptoms (symptoms at 4/5 weeks **and** 6 months). In order to not lose data, the CRO-based symptom classification was substituted for 13 patients (41%) where PRO data was missing at either of these timepoints, which were chosen as representative of maximal acute enteropathy (4/5 weeks) and early late

enteropathy (6 months).(7) This strategy enables identification of patients experiencing non-healing acute toxicity, which may be related to a consequential reaction and determines a higher risk of long-term RE.(13)

In the late cohort, CRO groups were defined by symptom grade. PRO-based groupings were based on the data, by dividing patients in quartiles defining increasing symptoms. For convenience, these categories were identified as no, mild, moderate, and severe symptoms (table SUPP-4).

In the colonoscopy cohort, cases were compared to controls.

Sampling procedures and processing

Sampling of stool

Sampling of stool was performed according to published guidance.(14) Details are given in supplementary methods.

Sampling of intestinal mucosa (colonoscopy cohort only)

Three biopsies were taken per site (figure SUPP-3) for metataxonomics, cytokine analysis, and pathology assessment. In cases and controls, samples were obtained from the anterior rectum, which is the part of the gastrointestinal tract which receives the greatest radiation dose during radiotherapy for prostate cancer.(15) In cases only, another three biopsies were obtained from a macroscopically unaffected region as close to affected areas as possible, which was in all the distal sigmoid, to be used as a self-control. Further details are given in supplementary methods.

Data acquisition

DNA extraction procedure, data acquisition and processing

Genomic DNA was extracted from faecal (250mg) and gut biopsy (whole biopsy) samples using the Qiagen Stool Kit (Qiagen, Crawley, UK) according to manufacturer instructions with an additional bead beating step for homogenisation of sample and lysis of bacterial cells. Library preparation and Illumina (MiSeq) sequencing of the V1-2 regions of the 16S-rRNA gene were performed at RTLGenomics (Lubbock, Texas, USA). Details are given in supplementary methods.

Cytokine detection

Total protein was extracted from mucosal samples and cytokine detection was carried out with the MSD® V-PLEX Human Cytokine 30-Plex Kit according to manufacturer instructions. The manufacturer states that all cytokine isoforms are detected. Details are given in supplementary methods.

Statistical considerations

Bioinformatic processing of 16S rRNA gene data

Sequences generated from Illumina (MiSeq) sequencing were analysed with MOTHUR (version 1.36.0) for identification of operational taxonomic units (OTU), taxonomic assignment, community comparison, and data cleaning by adapting its standard operational procedure.(16) Details are given in supplementary methods.

Inferred metagenomes were obtained by using the Piphillin web tool by Second Genome, using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) May 2017 database and a 97% identity cutoff.

Significance testing

The significance of taxonomic differences was assessed with one way ANOVA (≥3 group comparisons), or White's non-parametric two-sided t-test (two-group comparisons). The Benjamini-Hochberg method was used for false discovery rate correction. However, a pragmatic approach was taken, with uncorrected p-values taken into account given the exploratory context of this work.(17) Uncorrected p-values are termed "p*", while p-values after correction are termed "p". Statistically significant results explained by large peaks in <10% of a group were considered non-biologically relevant.

The Kruskal-Wallis H test was used to assess differences observed when comparing α-diversity indices, diet and histology scores (colonoscopy cohort).

Longitudinal dynamics in the early cohort were evaluated with linear mixed models. Linear mixed models use fixed and random effects in the same analysis. Unlike univariate or multivariate linear regression, one can assess individual variation by subject per timepoint by analysing the longitudinal change of a variable of

interest over time by symptom group.(18) Also, mixed models allow for missing observations, as other data endpoints can be still be used as long as the missing data meets the missing-at-random definition.(18) This analysis was performed in R using the "nlme" package and the following formulation:

$$Y_{ti} = \beta_0 + \beta_1(timepoint_{ti}) + \beta_2(symptom\ classifier_i) + \beta_3(symptom\ classifier_i)(timepoint_{ti}) + b_{0i} + b_{1i}(timepoint_{ti}) + \varepsilon_{ti}$$

Where β_0 is the population estimate of the intercept for the control group (no symptoms), β_1 is the population estimate of the linear slope of the control group, β_2 and β_3 capture the estimates of the mean difference in intercept and slope between symptom groups, b_{0i} and b_{1i} are random effects that allow the intercepts and slopes to vary across individuals, and ε_{ti} is a time-specific residual that expresses the difference between and individual's fitter linear trajectory and the observed data. Thus, β_3 represents the "symptom group by timepoint" interaction.(19) To assess significance, t-tests using Satterthwaite's method were implemented.

Variable transformations were used according to the data and are discussed with results. The Akaike Information Criterion was used to assess if models with transformed variables improved goodness of fit. Full results (including all effect estimates and significance) are provided in supplementary materials.

Multivariate analysis was performed with robust linear models in R using the "MASS" and "sfsmisc" packages with the following formulation:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2$$

Where β_0 is the mean intercept, and β_1 and β_2 are the coefficients for variables x_1 and x_2 respectively. Significance of coefficients was assessed with a robust F test (Wald test) using the f.robftest() function.

Comparison of cytokine levels and correlations with microbiome

The significance of differences between cytokine levels was assessed with the Kruskal-Wallis H test. A significance of $p<0.1$ (uncorrected for multiple comparisons) was defined for post-hoc (Mann-Whitney) testing. We report results of post-hoc tests. Correlations of cytokines with the microbiome were explored in bacterial genera where operational taxonomic unit (OTU) counts were >0 in ≥20% of subjects with Spearman's rank correlation coefficient.

RESULTS

Demographics and symptoms

One-hundred thirty-four men were enrolled between 18/03/2014 and 01/02/2016 (table 1): 32 in the early cohort, 87 in the late cohort, and 15 in the colonoscopy cohort (9 cases/6 controls). All patients in the early and late cohorts underwent prostate and pelvic radiotherapy following a previously published protocol.(7) In the colonoscopy cohort, 6 cases had undergone radiotherapy to the prostate and seminal vesicles, 1 had undergone radiotherapy to the prostate, seminal vesicles and pelvic lymph nodes, and 2 had undergone post-prostatectomy radiotherapy to the prostate bed and pelvic lymph nodes. Control subjects had not been treated with any radiotherapy.

In the early cohort, patients with non-persistent symptoms mostly experienced symptoms at 4/5 weeks (84%/PRO, 92%/CRO). Classification was concordant (i.e., patients classified in the same group with both PRO and CRO) in 21 patients (66%; table SUPP-5).

Symptoms and diet are described detail in supplementary text. We did not detect biologically-relevant dietary differences between groups.

As per our study design, cohorts were not compared to one another, but used to assess different aspects of RE. Therefore, we analysed if comorbidities (including BMI, smoking status/history, metabolic diseases, androgen deprivation therapy (ADT) and other comedications) were different between symptom groups to be analysed in each cohort. Overall, comorbidities were well balanced between groups (tables SUPP-6 to SUPP-8). No significant differences were found in the early cohort. In the late cohort, actual CRO-stratified groups showed significantly higher proportions of irritable bowel syndrome ($p=0.0004$) in patients with rising symptoms. In the colonoscopy cohort, proportions of controls under hypertensive medication were higher than in cases ($p=0.02$). The overall low proportions of patients with IBS in all cohorts may be attributed to eligibility criteria for pelvic radiotherapy, which is relatively contraindicated for patients with gastrointestinal conditions.

Comparison of stool and mucosal microbiome in the colonoscopy cohort

We did not find significant differences when comparing stool and intestinal mucosa microbiomes in the colonoscopy cohort, although there was a trend for higher α-diversity, measured with the Chao index, in stools compared with intestinal mucosal microbiome in cases (median (IQR): 77 (59.3-117.1) vs 54.1 (51.3-65.2)). However, no significant differences in β-diversity or in individual phyla or genera were found. Results are described in detail in supplementary text.

Low bacterial diversity associates with RE

Low bacterial diversity has been consistently associated with acute RE.(6,20–23) Relationships with late enteropathy have never been explored. We therefore assessed bacterial diversity between irradiated patients with and without gastrointestinal side-effects.

In the early cohort, we firstly explored associations between baseline diversity and RE in the acute cohort. We found a trend for higher diversity at baseline in patients with no self-reported symptoms (p=0.09; median (IQR) Chao richness for no RE: 89.1 (78.9-114.0); non-persistent RE: 55.2 (42.6-72.5); persistent RE: 68.6 (41.8-75.1)). This observation was recapitulated with CRO, albeit non-significantly (p=0.61; 76.3 (65-86.1); non-persistent RE: 55.2 (48.0-84.5); persistent RE: 65.3 (39.1-75.1)). We next examined dynamics of α-diversity over time with linear mixed models. The variable of interest was Chao abundance of bacterial species, with predictors of dynamics specified as timepoint and symptom group (figure 1A-B, table SUPP-9). When not stratified by symptom group, diversity appeared to decrease in the whole cohort over time, albeit non-significantly (effect of timepoint: -0.02, p=0.35, figure SUPP-5). With PRO stratification, diversity generally decreased over time (p=0.03). A positive effect of timepoint by symptom group indicates differential dynamics of diversity over time (p=0.05; figure 1A). This pattern was similar when an identical model based on CRO was used, although it did not reach statistical significance. We next examined differences in bacterial diversity between patients with and without late enteropathy in the late cohort (figure 1C-J). No significant differences were found with PRO or CRO in the late (figures 1C-J and SUPP-4) or colonoscopy (figure 1K-L) cohorts. However, a non-significant pattern of higher diversity in symptomatic patients was observed in both cohorts.

Patients with radiation enteropathy have higher counts of *Roseburia*, *Clostridium IV* and *Faecalibacterium*

Enrichment in specific microbial taxa has been described in patients with primary inflammatory bowel disease (IBD).(24) Similarly, associations between specific bacterial taxa and acute RE have been reported.(6,21,23,25) We therefore investigated whether specific bacterial taxa were associated with RE. We first compared proportions of phyla and genera between patients with and without RE at each timepoint in the early cohort. No microbial features showed statistically significant relationships. However, due to the limited power of this cohort for detecting differences based on direct comparisons per timepoint, we defined biologically plausible relationships as progressive changes in proportions of microbial features (i.e., either increasing or decreasing) with rising symptoms, irrespective of statistical significance. Results are summarized in table SUPP-10. We used linear mixed models to evaluate longitudinal dynamics of specific microbial taxa taking into account the results above. Bacterial taxa with biologically plausible relationships where uncorrected p values (p*) <0.05 were retained. They were *Clostridium IV*, *Roseburia*, and *Phascolarctobacterium* which are short chain fatty acid (SCFA) producers. *Sutterella* dynamics were also analysed in light of a biologically plausible relationship and a published evidence suggesting that a microbiome enriched in this taxon associates with acute radiation proctitis in an animal model.(23) Results are summarized in figure 2 and table SUPP-11. *Clostridium IV* proportions increased significantly with PRO (effect=0.4, p=0.007), with a trend towards a progressively more negative slope of proportions over time with increasing symptoms group (estimate=-0.04, p=0.11). This behaviour was reflected with CRO. A trend was also observed for increased *Roseburia* counts in direct proportion with patient-reported symptoms (effect=0.37, p=0.08), which were reflected with CRO. Plotting the models shows a comparatively steep decrease in *Roseburia* proportions in patients with persistent symptoms. A trend of higher proportions of *Phascolarctobacterium* in direct proportion to CRO was observed (effect=0.26, p=0.09) and reflected with PRO. Proportions of *Sutterella* appeared to increase with symptoms with minimal change over time, albeit non-significantly.

We next examined microbial taxa in the late cohort. It is noted that this analysis was completely independent of the early cohort, so all microbial taxa (and not only SCFA producers) were included, with ensuing FDR

correction. No significant differences were found at either phylum or genus levels when stratifying patients according to either actual or historical PROs. However, when stratifying patients according to CROs, *Roseburia* significantly associated with toxicity (figure 3 and table SUPP-12). Proportions of *Roseburia* rose with maximum actual ($p^*<0.000001$, $p<0.00001$) and historical ($p^*=0.001$, $p=0.06$) CRO symptom grade. No relevant differences at either phylum or genus level were detected for proctopathy. *Roseburia* significantly rose with both actual ($p<0.000001$) and historical ($p<0.00001$) clinician-reported diarrhoea grade. To test if significance was due to very high peaks in patients with grade 3 diarrhoea, all patients with grade 3 toxicity were removed and differences re-tested including only patients with grade 0 to 2 diarrhoea. Results with actual ($p=0.056$) and historical ($p=0.04$) diarrhoea remained significant. Proportions of *Roseburia* also rose with historical PRO-stratified symptoms, albeit non-significantly (table SUPP-12). As higher proportions of IBS were found in patients with CRO-stratified actual symptoms, we used robust linear regression to adjust for these parameters in two independent multivariate models. The model predicted actual CRO grade with IBS ($p=0.002$) and *Roseburia* ($p=0.02$) as significant variables. To further assess if a relationship between *Roseburia* and IBS was present, we also examined correlation between the two variables, which was not present (Spearman's Rho=0.09, $p=0.43$). Moreover, no significant differences in genus-level taxa were found between patients with and without IBS in the late cohort, including *Roseburia* ($p^*=0.60$; $p>0.1$) and Clostridium IV ($p^*=0.59$, $p>0.1$). Because ADT has been associated with a modified microbiota in a previous report, we also examined in the microbiota of the late cohort stratified by active ADT or testosterone recovery status.(26) No significant differences were found in α-diversity or in taxa (see supplementary text). We note that we did not carry out such analyses in the early cohort due to all patients being on active ADT since before baseline sampling and consequently having undetectable testosterone levels.

In the colonoscopy cohort, no significant differences were found when comparing cases and controls. However, the size of this cohort limited statistical power (see supplementary text).

We then hypothesized that metagenomic abundances of microbial SCFA metabolism pathways differed between patients with and without symptoms of RE where significant associations were detected. We combined community composition with annotated genomes from the KEGG catalogue and selected pathways related to microbial SCFA metabolism for analysis.(27) We again used linear mixed models to evaluate dynamics in the early cohort (figure SUPP-6, table SUPP-13). Abundances of SCFA-related microbial metabolic

pathways increased consistently with symptoms, most noticeably with PRO, although this effect only trended for significance for propionate metabolism (p=0.07). Propionate and other SCFA fuel colonocytes and upregulate colonic Treg lymphocytes, thereby promoting gut homeostasis.(28) Its benefits to gut health have been reviewed elsewhere.(29) The consistently negative effect of timepoint by symptom group suggests that microbial SCFA pathways may decrease more over time with rising symptoms. In the late cohort, only the abundance of fatty acid metabolism pathways decreased consistently with rising CRO diarrhoea grade (p<0.0001; figure SUPP-7, table SUPP-14).

Patients with radiation enteropathy have depletion of rectal mucosa cytokines regulating gut microbiota and homeostasis, correlating with higher counts of *Roseburia* and *Propionibacterium*

Cytokines are small molecules involved in cell signalling and have immunomodulatory, paracrine and autocrine functions with pathophysiological implications. However, gastrointestinal mucosal cytokine changes have never been studied in late RE. We therefore investigated differences in the concentrations of 29 cytokines, divided in 3 panels, between cases and controls in the colonoscopy cohort. A distinct general pattern of highest concentration in controls and lowest concentrations in the anterior rectum of cases was observed, except for pro-inflammatory cytokines, where no differences were found. When analysing differences between sample types by cytokine, IL-7 (p=0.05), IL-12/IL-23p40 (p=0.03), IL-15 (p=0.05), IL-16 (p=0.009) were significantly higher in control than in case rectal biopsies, while eotaxin (p=0.03) followed an inverse pattern (figure 4A-C). We did not find significant differences in pathology (including fibrosis) or in pro-inflammatory cytokines between cases and controls, which argues against the hypothesis of difficulty in tissue permeation in cases or sub-clinical inflammation in controls (table SUPP-15). Interestingly, cytokines observed to be lower in cases have intestinal homeostatic properties by regulating the microbiota and the intestinal barrier (table SUPP-16).

We then examined correlations between the microbiome of the anterior rectal mucosa and cytokine concentrations. Rectal *Roseburia* and *Propionibacterium*, which are SCFA producers, and *Streptococcus*, an acetate producer, were inversely correlated with IL-15 (decreased in patients with RE) in our dataset

(respectively: rho=-0.54 and -0.52; p=0.04 and 0.05, figure 5).(30) *Flavonifractor*, a butyrate-producing genus, correlated positively with eotaxin (increased in patients with RE).(31) These observations suggests an association between mucosal SCFA producers and RE in the anterior rectal mucosa, which is the gastrointestinal location receiving the highest levels of radiation in prostate radiotherapy. We observed similar correlations, albeit not so evidently, with sigmoid and stool microbiota.

DISCUSSION

In this study, we have shown that a modified microbiota is associated with radiation enteropathy and that key homeostatic intestinal mucosa cytokines related to microbiota regulation and intestinal wall maintenance are also significantly reduced in patients with RE. Our study confirms previous observations in small cohorts of patients where acute radiation injury was associated with an altered microbiota.(6,20,21,23,25) However, our data are not compromised by the delivery of concurrent cytotoxic systemic treatments which made the findings from these smaller studies difficult to interpret. In addition, we have shown for the first time that a modified microbiota is associated with late RE.

We previously reviewed the potential of the microbiota in the prediction and treatment of RE.(1) Although the importance of the gastrointestinal microbiota in radiation-induced intestinal toxicity is highlighted by the recognition of causes of enteropathy such as small intestinal bacterial overgrowth, the studies evaluating the microbiome in patients with radiation-induced gastrointestinal symptoms are limited by low patient numbers. Also, all previous authors focused only on acute RE. Although bacteriotherapy has been studied by administering probiotics or prebiotics, interventions were marred by insufficient knowledge of the microbiota in RE, which is reflected in modest and often conflicting results. Also, although preclinical studies provide useful information, they do not reflect the clinical reality of RE in the modern era of precision radiotherapy. We thus intended to provide a comprehensive characterisation of the microbiota in RE which provides a foundation for further studies in this field.

We acknowledge the limitations of our study. RE has multiple causes, which are likely to have differential contributions from the microbiota.(2) As yet, no objective markers of radiation enteropathy have been defined and there is no option but to rely on abnormal symptoms. However, symptom scales have known limitations for detecting RE, hence our approach of using both clinician and patient-reported outcomes for better characterisation of patients. Also, although our patients had comorbidities, as expected in the aged population of prostate cancer patients, they were globally well distributed between symptom groups. We nevertheless adjusted for their effect where significant differences in comorbidities could have an impact, and our results were robust to these analyses. The relatively younger age of patients in the early compared to the late cohort reflects that patients undergoing treatment are younger than patients on long-term follow-up. However, this is

unlikely to significantly affect the microbiota, given its overall stability with time.(1) Moreover, we did not directly compare these cohorts, which were used to analyse different phases of RE as per our study design.(32) We also acknowledge that, although we detected consistent results across all cohorts, high inter-subject variability of the microbiota is known to affect cohort studies and is the main conundrum bedevilling all microbiota research in humans.(33) Unfortunately, studies in animals are limited by administration of extreme (often lethal) radiation doses and very different radioresistance and microbiota when compared to humans. Although findings may appear more clear-cut, such models poorly represent clinical radiotherapy.(5,23) Furthermore, we acknowledge the limitation of not measuring diet longitudinally in the acute cohort, which was due to ethical concerns of study procedure-related patient exhaustion. We did not find, however, biologically-relevant dietary differences between any of the cohorts. Also, although radiation-induced gastrointestinal side-effects remain the main dose-limiting factor in modern prostate cancer radiotherapy, their severity has been much reduced by successful improvements in treatment delivery. Limitations of metataxonomics are also acknowledged, such as polymerase chain reaction bias and artificial over-representation of some species carrying multiple copies of 16S rRNA genes.(34)

We observed associations of microbiota endpoints with acute (mostly with PRO) and late (mostly with CRO) toxicity. Using both types of instruments is known to provide a full representation of toxicity and is the reason why radiotherapy trialists now report both separately.(35) We hypothesise that this discrepancy is due to three factors: (1) differences in perception of side-effects from the point of view of patients and clinicians; (2) limitations of both PRO and CRO instruments; and (3) the overall low grade of toxicity produced by modern radiotherapy. In the acute setting, where patients are naïve to radiotherapy, their perception of symptoms may be higher and therefore PROs may be more sensitive. In the late setting, both successful ongoing treatment of toxicity and increased patient tolerance to side-effects may make clinician-reported outcomes more sensitive. For example, a patient successfully using loperamide for diarrhoea may not report symptoms, but clinicians would classify such a patient as having diarrhoea. Furthermore, we acknowledge limitations in using PRO instruments. PROs in the acute cohort were analysed as difference to baseline, and will thus reflect better each patient's longitudinal evolution in terms of symptoms.(36) However, patients were assigned to groups of increasing late patient-reported toxicity (late and cohort) based on dividing them in 4 quartiles, as there are is no "normal threshold" in our validated PRO score. Given the small range in PRO scores in the late

cohort (described in supplementary materials), patients with different toxicity phenotypes may have been grouped together, therefore making results more difficult to interpret. Despite these limitations and in the absence of a reliable biomarker of RE, our approach provides the most comprehensive clinical characterisation of RE ever carried out in a study in this field.

Decreased bacterial diversity was consistently associated with RE in all three cohorts, and we conclude that this observation is not random, although results in two of our cohorts were non-significant. A less diverse microbiota associates with other forms of colitis, including IBD, IBS and infective colitis, as well as with diseases such as obesity and auto-immune diseases.(37,38) Associations between acute RE and reduced diversity have also been reported by other authors.(6,20–23) In animal models, a less diverse irradiated microbiota (which is enriched in *Sutterella* among other bacteria) is sufficient for the induction of higher susceptibility to intestinal inflammation, suggesting that reduced bacterial diversity may cause patients to be at risk of enteropathy in the short and long terms.(23) It is noteworthy that *Sutterella* was higher in patients with RE in the early cohort, albeit non-significantly.(23) Our results suggest that strategies for increasing bacterial diversity in patients at risk could be trialed to see if they modify the course of RE.

We found significant associations between some organisms producing SCFA and RE in all cohorts, again suggesting that this association is non-random. Imbalances in the microbiota, often termed dysbiosis, associate with many gastrointestinal diseases, including IBD, IBS and viral colitis. Generally, such imbalances are characterised by an increase in bacteria which are recognised to be pathogens, such as *Escherichia coli*, or the *Shigella* and *Klebsiella* genera.(1) However, SCFA producers promote intestinal homeostasis and their depletion has been associated with IBD, so increased proportions in patients with symptoms are surprising.(39) Mechanistic exploration is beyond the scope of our study, but some hypotheses can be suggested. These bacteria are part of intestinal mucosa-associated communities and it is possible that, in patients at risk of symptoms, increased competition by potentially pathogenic bacteria leads to increased shedding in the stools. This shedding would be consistent with differential dynamics observed between groups. An alternative hypothesis would be that chronic, subclinical pre-radiotherapy intestinal dysfunction may lead to a dependence on microbiota-derived nutrients for epithelial health.(2) Radiotherapy led to decreased SCFA production capacity, associating with symptom onset. The high counts of *Roseburia* associating with CRO-stratified but not PRO-stratified late symptoms support that higher proportions of these

bacteria relate to decreased symptom perception by patients in the presence of clinician-perceived disease. This hypothesis is consistent with the limited clinical effectiveness of oral or topical butyrate when treating RE.(40) Although we acknowledge the low comparative proportions of these bacteria when compared to other SCFA producers such as *Faecalibacterium*, the trend of patients with RE having higher, but dynamically decreasing, SCFA production capacity (early cohort) and significantly decreased levels of homeostatic rectal mucosa cytokines involved in mucosal barrier maintenance and microbiota regulation (colonoscopy cohort) would support this assumption. These hypotheses need to be further explored.

Our study provides evidence of structural and functional shifts in the microbiota in patients with RE. However, whether these changes are a cause or consequence of intestinal symptoms is a matter of considerable debate even in well researched fields of non-infectious colitis such as inflammatory bowel disease (IBD).(41) Also, unlike IBD, RE is characterised by non-inflammatory mechanisms, which is well illustrated by evidence of a recent placebo-controlled randomised trial where sulfasalazine, an anti-inflammatory drug often used to treat IBD, actually had a detrimental effect in terms of diarrhoea for patients undergoing RE.(42) We also did not find evidence of increased inflammatory cytokines in patients with late RE. Other authors have provided complementary mechanistic evidence which suggests a causative role for the microbiota in RE.(23) We provide a framework for further downstream studies assessing a causative role for the microbiota, which could provide further scope for microbial interventions such as faecal transplantation, which has recently been suggested as a successful treatment of immunotherapy-induced colitis.(43) Other bacteriotherapy interventions, such as the administration of probiotics (live organisms that, when consumed in an adequate amount, confer a health effect on the host) or prebiotics (non-digestible foods that promote the growth or activity of specific micro-organisms, promoting a health effect), have also been trialled in patients undergoing pelvic radiotherapy.(1) The mixed results observed may stem from the fact that many of these therapies modulate bacteria which do not have an impact in RE. However, Garcia-Peris and colleagues showed in a randomised trial that the delivery of a fiber mixture containing inulin, which promotes the growth of SCFA producers such as *Roseburia*, improves diarrhoea in patients undergoing pelvic radiotherapy, supporting our observations.(44,45)

We conclude that radiotherapy may upset the balance of microbiota which support intestinal health, by decreasing the influence of key micro-organisms, probably more susceptible to radiation effects. The

microbiota may be used to predict, prevent or treat clinical RE and our study provides an evidence base for developing pre-clinical and clinical studies.

Table 1: Demographics.

Item	Early cohort	Late cohort	Colonoscopy cohort	
			Cases	Controls
Median age at date of enrolment in years (IQR)	66 (63-72)	74 (68-79)	75 (71-76)	68 (57-69)
Median time in years between radiotherapy commencement and sampling	*NA*	6.05 (4.57-7.28)	4.2 (1.9-10.4)	*NA*
Radiotherapy details				
Patients treated with conventionally-fractionated radiotherapy†: 70-74Gy to prostate and seminal vesicles (35-37 fractions) or 64Gy to prostate bed (32 fractions); 50-60Gy to pelvic lymph nodes (35-37 fractions) – n (%)	31 (97%)	48 (55%)	3 (33%)	*NA*
Patients treated with hypofractionated radiotherapy†: 60Gy to prostate and seminal vesicles or 55Gy to prostate bed (20 fractions); 47Gy to pelvic lymph nodes) – n (%)	1 (3%)	39 (45%)	0 (0%)	*NA*
Patients treated with conventionally-fractionated radiotherapy to prostate and seminal vesicles only: 70-74Gy in 35-37 fractions	*NA*	*NA*	6 (67%)	*NA*
Prostate cancer details				
Median presenting PSA (IQR) in ng/mL	26.2 (13.4-47)	18.1 (11.05-34.50)	7.05 (5.43-13.40)	*NA*
Median PSA at time of sampling (IQR) in ng/mL	*NA*	*NA*	8.4 (5.7-14.6)	*NA*
Gleason 6 – n (%)	1 (3%)	3 (3%)	2 (22%)	*NA*
Gleason 7 – n (%)	12 (37%)	33 (38%)	6 (67%)	*NA*
Gleason 8 – n (%)	3 (9%)	14 (16%)	0 (0%)	*NA*
Gleason 9 – n (%)	16 (50%)	37 (43%)	1 (1%)	*NA*
N0 – n (%)	16 (50%)	62 (71%)	7 (78%)	*NA*
N1 – n (%)	16 (50%)	24 (28%)	2 (22%)	*NA*
NX – n (%)	0 (0%)	1 (1%)	0 (0%)	*NA*
T1 – n (%)	1 (3%)	1 (1%)	0 (0%)	*NA*
T2 – n (%)	7 (22%)	18 (21%)	2 (22%)	*NA*
T3 – n (%)	24 (75%)	65 (75%)	7 (78%)	*NA*
T4 – n (%)	0 (0%)	2 (2%)	0 (0%)	*NA*
TX – n (%)	0 (0%)	1 (1%)	0 (0%)	*NA*
Subjects on short-course anti-androgen and long-term LHRH analogues	22 (69%)	NA	1 (1%)	*NA*
Subjects on bicalutamide monotherapy	1 (3%)	NA	0 (0%)	*NA*
Subjects on maximum androgen blockade	9 (28%)	NA	0 (0%)	*NA*
Subjects with recurrent tumours at time of sampling – n (%) ϯ	*NA*	11 (13%)	1 (1%)	*NA*
Subjects on ADT at time of sampling – n (%) ϯ	32 (100%)*	10 (11%)	1 (1%)	*NA*
Subjects with recovered testosterone levels (≥6 nmol/L) – n (%) ϯ	*NA**	47 (54%)	5 (56%)	*NA*
Other comorbidities ϯ				
Subjects with history of abdominal or pelvic surgery – n (%)	19 (59%)	40 (46%)	6 (67%)	3 (50%)
Median body mass index (IQR)	27 (25-32)	26.5 (24.7-29.8)	26 (25-27)	24 (24-25)
Subjects with dyslipidemia and on statins – n (%)	10 (31%)	45 (52%)	4 (44%)	2 (33%)
Subjects with history of diabetes – n (%)	7 (22%)	15 (17%)	0 (0%)	0 (0%)
Subjects with history of hypertension and on medical treatment – n (%)	13 (41%)	49 (56%)	7 (78%)	1 (16%)
Subjects with history of irritable bowel syndrome – n (%)	0 (0%)	3 (3.4%)	2 (22%)	2 (33%)
Subjects with history of diverticular disease – n (%)	1 (3%)	10 (11%)	2 (22%)	0 (0%)
Non-smokers/ex-smokers/smokers – n (%)	19 (59%)/11 (34%)/2 (6%)	37 (42%)/38 (44%)/12 (14%)	4 (44%)/4 (44%)/1 (1%)	1 (17%)/ 5 (83%)/0 (0%)

ADT = Androgen Deprivation Therapy. NA = Not Applicable. IQR = Inter-quartile range.

The reader is reminded that cohorts were not directly compared, but independently assessed to investigate the microbiota of patients with early and late side-effects. ϯ: A detailed comparison of comorbidities between toxicity groups in each cohort is reported in the main text and in tables SUPP-6 to SUPP-8 in supplementary materials.

ϯ: Conventional and hypofractionated radiotherapy schedules used to treat patients were shown to produce comparable rates of tumour recurrence, as well as early and late toxicities in a phase II trial (see reference 7).

**: all subjects in the early cohort were under neo-adjuvant ADT from the time of recruitment, as per the protocol for treating high-risk prostate cancer (including ADT starting before radiotherapy and extending for 2-3 years in total) and their testosterone levels were therefore undetectable. Some patients in the late cohort (≥2 years after RT) were under long-term ADT for the same reason. ADT was not found to significantly impact the microbiome in this study (see supplementary text).*

FIGURE CAPTIONS

Figure 1: Bacterial diversity in the early (A-B), late (C-J) and colonoscopy (K-L) cohorts of the MARS study. A-B: Dynamics of Chao diversity over time in PRO (A) and CRO (B) stratified groups, where the effect of timepoint (p=0.03) and timepoint by symptom group (p=0.05) were significant in PRO-stratified groups. *Groups: 0 = no symptoms, 1 = non-persistent symptoms, and 2 = persistent symptoms. Timepoints: 1=baseline, 2=2/3 weeks, 3=4/5 weeks, 4=12 weeks, 5=6 months, and 6=12 months after radiotherapy initiation. A log transformation was used due to a positive skew of the data, which was confirmed to provide superior goodness of fit when compared to square-root transformations.* **C-J:** Chao diversity in the late cohort in groups stratified by C-H/ CRO actual/historical diarrhoea (C/F) proctitis (D/G) and maximum toxicity (E/H); and by I-J/PRO actual (I) and late (J) toxicity. p>0.05 in all comparisons. *The reader is reminded that scales for PRO stratification differed between actual and historical toxicity (see materials and methods).* **K-L:** Chao diversity in the colonoscopy cohort with stool (K) and intestinal mucosa (L) samples. p>0.05 in both comparisons.

Figure 2: Dynamics of proportions of Clostridium IV (A/B), Roseburia (C/D), Phascolarctobacterium (E/F) and Sutterella (G/H) over time in PRO (left) and CRO (right) stratified groups. The effect of PRO symptom group was significant for Clostridium IV (p=0.007). There was a trend for significance for the effect of PRO and CRO symptom group for *Roseburia* (p=0.08) and *Phascolarctobacterium* respectively. *Groups: 0 = no symptoms, 1 = non-persistent symptoms, and 2 = persistent symptoms. Timepoints: 1=baseline, 2=2/3 weeks, 3=4/5 weeks, 4=12 weeks, 5=6 months, and 6=12 months after radiotherapy initiation. A square root transformation was used due to a positive skew of the data, which was confirmed to provide superior goodness of fit when compared to a log transformation in all models.*

Figure 3: Proportions of *Roseburia* in actual (A-C) and historical (D-F) CRO-stratified groups of the late cohort by CRO grade. A/D: Maximum toxicity. **B/E:** Diarrhoea. **C/F:** Proctitis. Higher grades reflect more serious symptoms. *: p=0.06; **: p≤0.05; ***: p≤0.01; ****: p≤0.001. All p-values shown are corrected for FDR. The x axis shows CRO grade.

Figure 4: Mean absolute cytokine concentrations by sample group. Blue: controls (rectum), green: cases (sigmoid), red: cases (rectum). A: Chemokine panel. B: Cytokine panel. C: Pro-inflammatory panel. *For scaling*

*purposes, all concentrations are pg/mL except (A) TARC, IL-7, IL-12/IL-23p40, IL-17α (x10 pg/mL); and (B) IL-16 (x10 ng/mL); and IL-8, VEGFα, IFN-γ, IL-1β, IL-2 (x0.1ng/mL); and (C) IL-8, IL-13 (x0.01pg/mL). *: p≤0.05.*

Figure 5: Correlation matrices of microbiome of stools (A), rectal mucosa (B) and sigmoid mucosa (C) and concentrations of cytokines. *The size of circles represents significance and the colour code represents Spearman's correlation coefficient (rho). Only significant results (p≤0.05) are shown. Stools and rectal mucosa microbiomes were correlated with rectal cytokine levels, whereas sigmoid microbiome was correlated with sigmoid cytokine levels. Class is defined as either cases (coded 0) or controls (coded 1) and therefore a positive correlation denotes higher concentration/proportion in controls and vice-versa.*

DATA AVAILABILITY

All data generated or analysed during this study are included in the published article and its supplementary information files.

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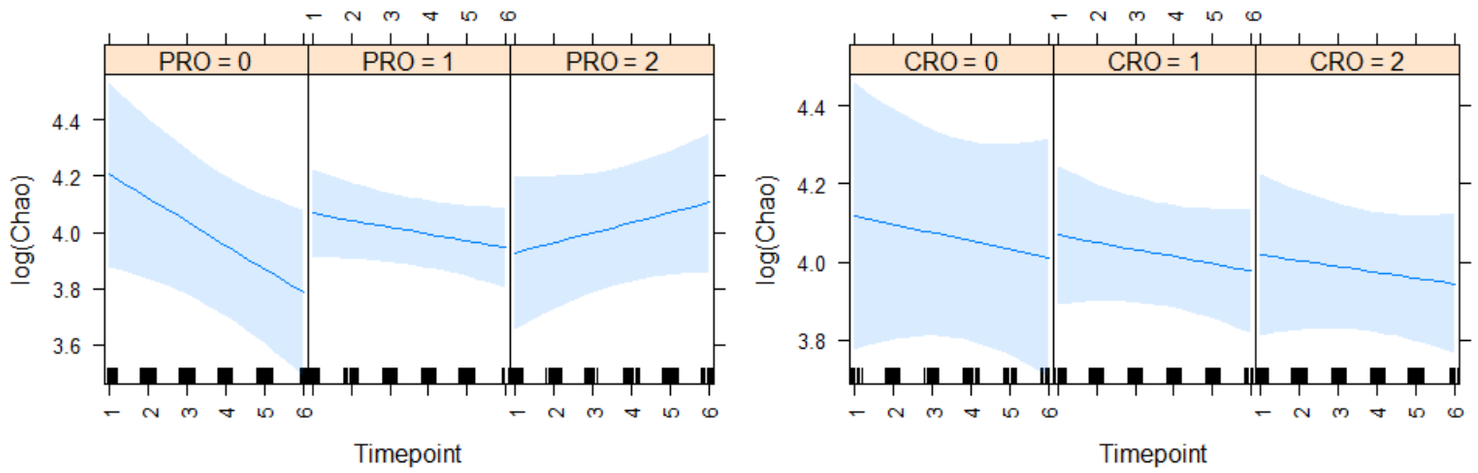
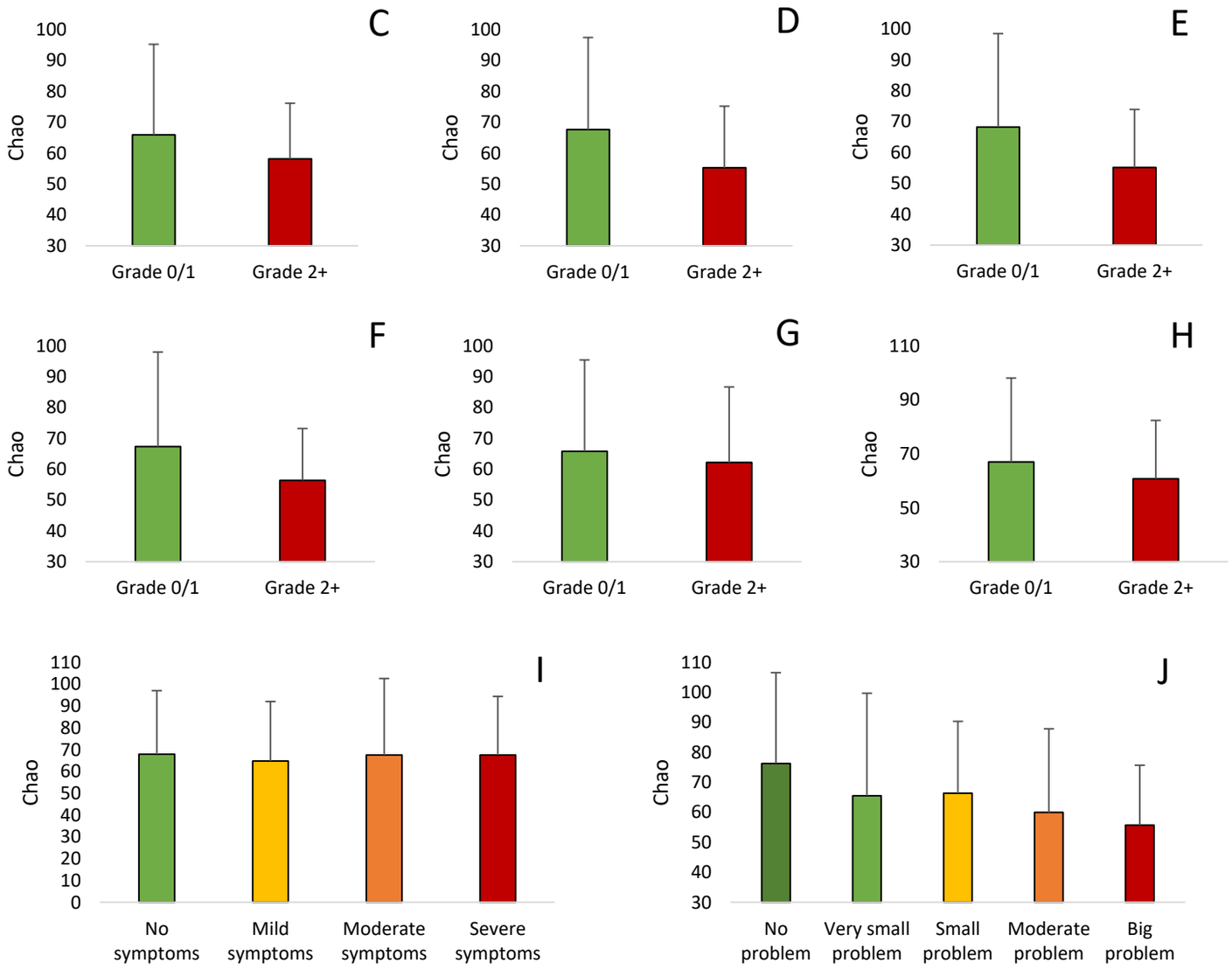
Figure 1

EARLY COHORT



LATE COHORT



COLONOSCOPY COHORT

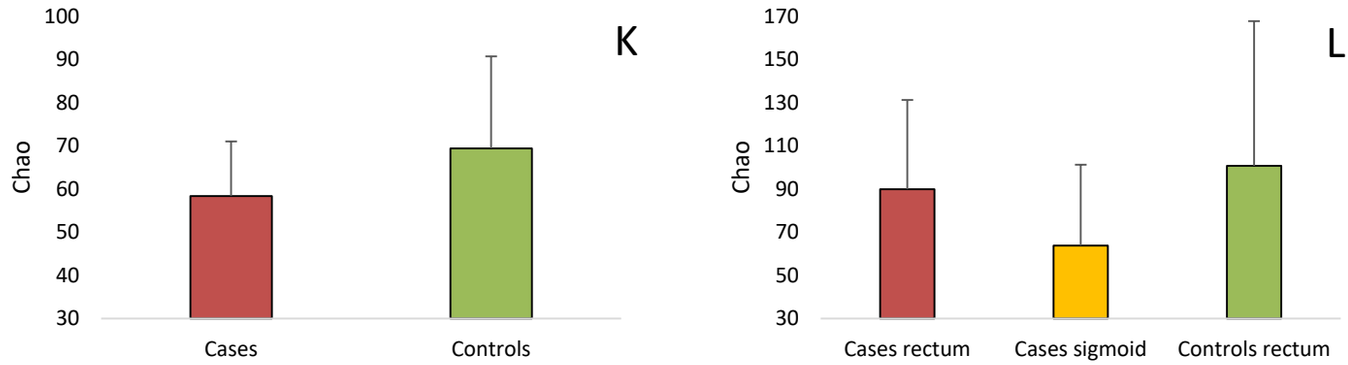


Figure 2

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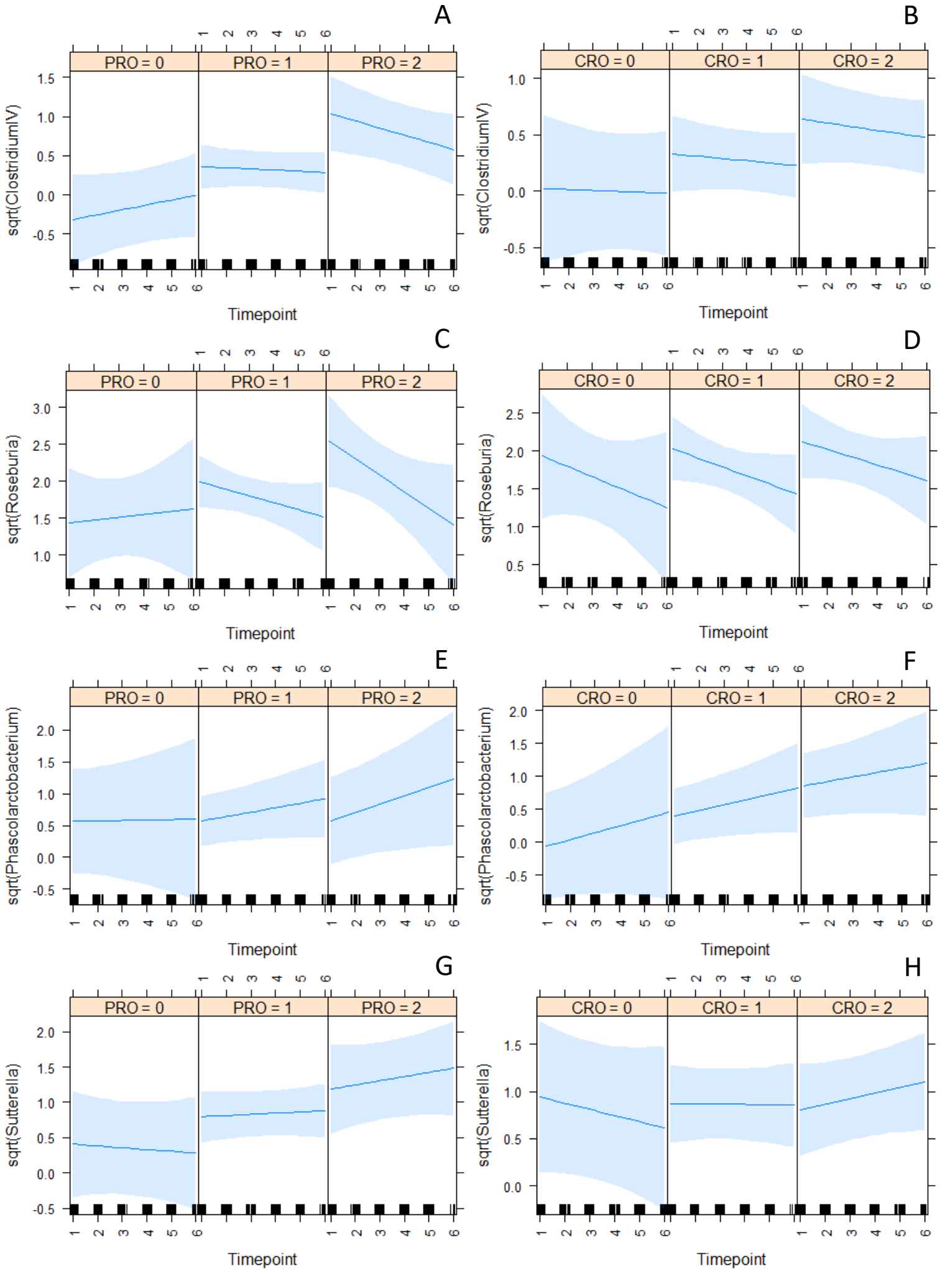

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

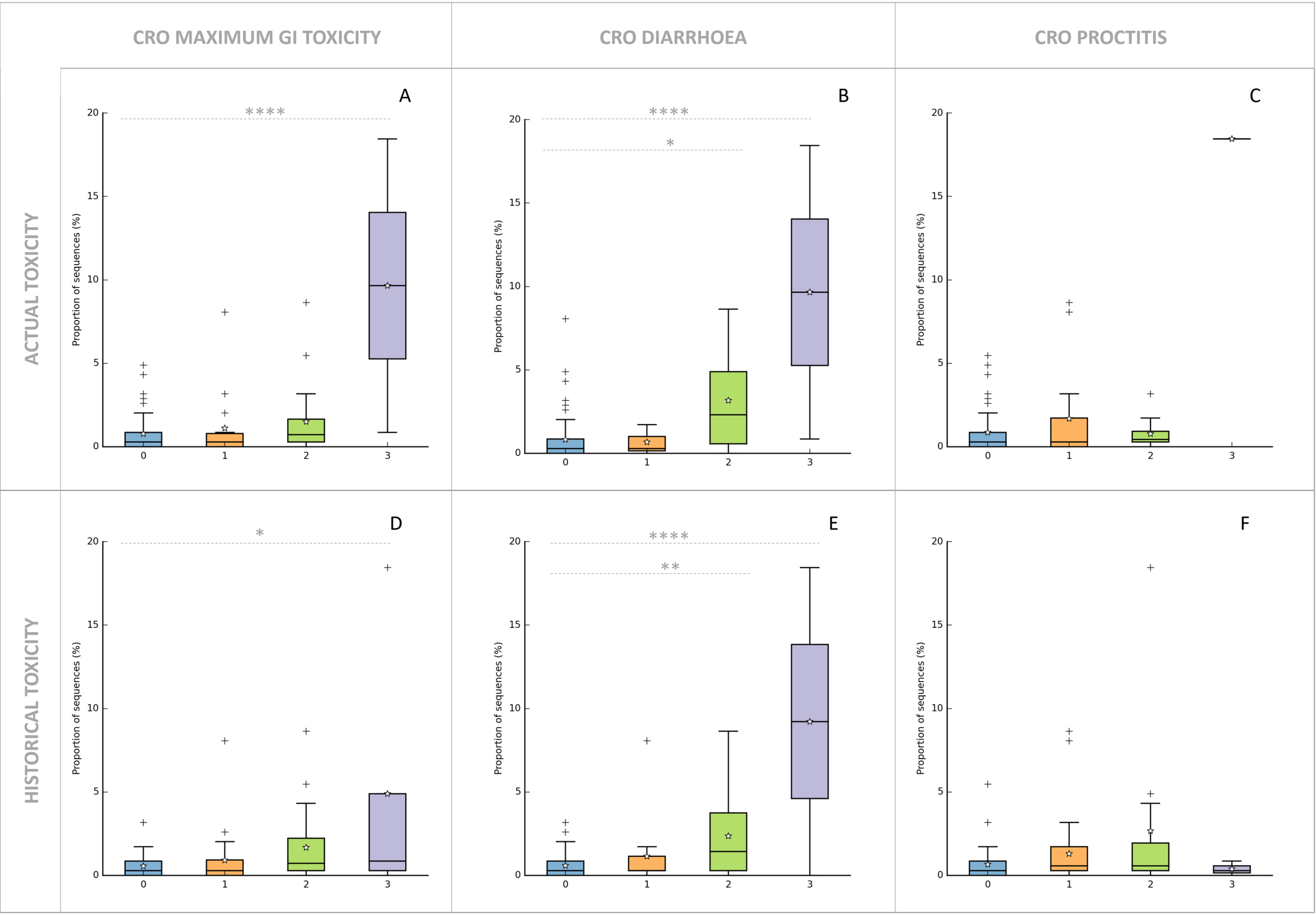


Figure 4

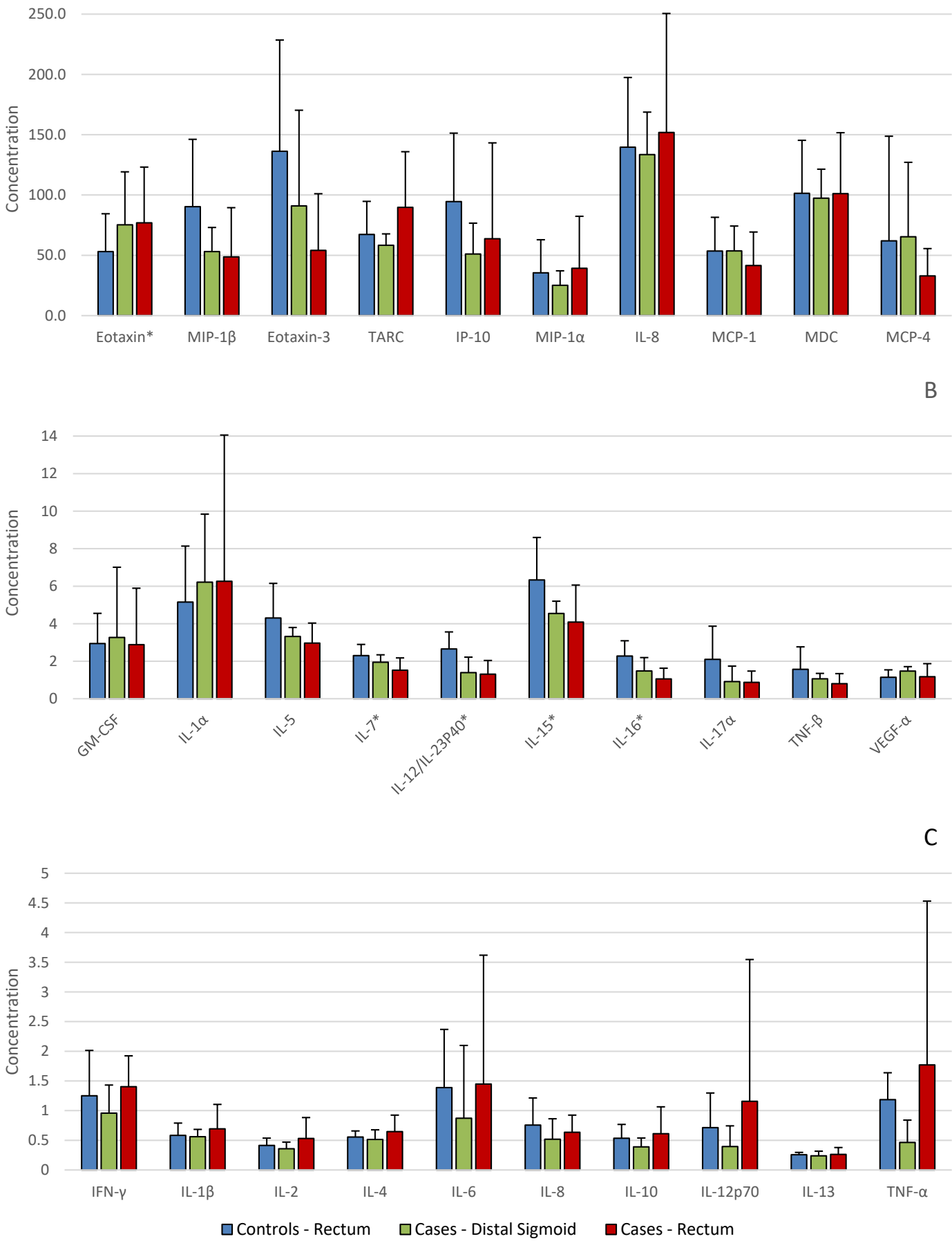
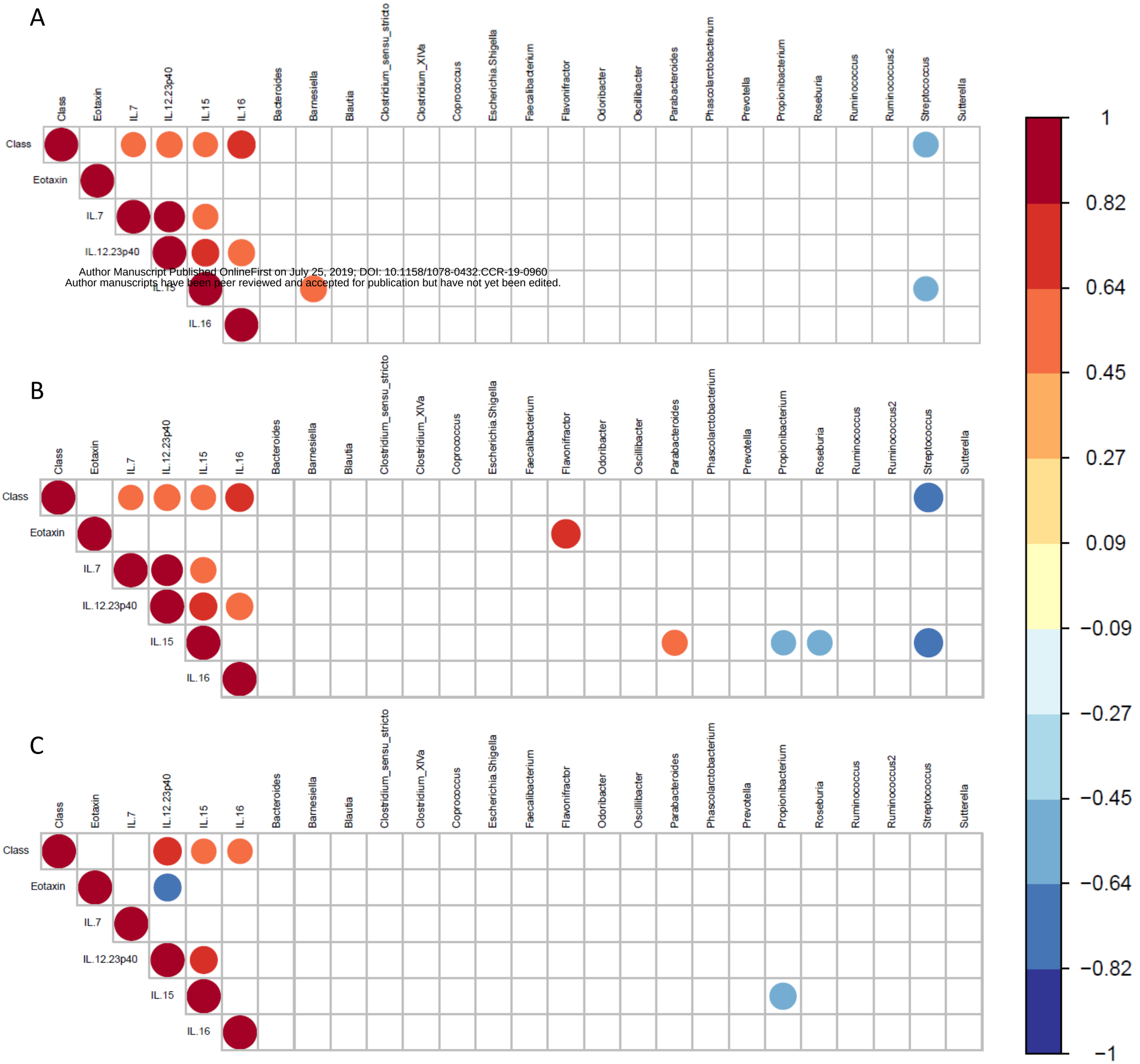


Figure 5



AACR American Association for Cancer Research

Clinical Cancer Research

Microbiota and radiotherapy-induced gastrointestinal side-effects (MARS) study: a large pilot study of the microbiome in acute and late radiation enteropathy.

Miguel Reis Ferreira, Jervoise Andreyev, Kabir Mohammed, et al.

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