Making Sense of the Noise: Leveraging Existing 16S rRNA Gene Surveys to Identify Key Community Members in Colorectal Cancer

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Abstract

Background. An increasing body of literature suggests that there is a crucial role for the microbiota in colorectal cancer (CRC) pathogenesis. Important drivers within this context have ranged from individual microbes to the whole community. Our study expands on a recent meta-analysis investigating microbial biomarkers for CRC by testing the hypothesis that the bacterial community has important associations to both early (adenoma) and late (carcinoma) stage disease. To test this hypothesis we examined both feces (n = 1737) and colon tissue (492 total samples from 350 individuals) across 14 previously published 16S rRNA gene sequencing studies on CRC and the microbiota.

Results. Fecal samples had a significant decrease for both Shannon diversity and evenness after correcting for study effect and variable region sequenced with more severe disease (P-value < 0.05). This reduction in evenness translated into small increases in relative risk for adenoma (P-value = 0.032) and carcinoma stages of CRC (P-value = 0.00034) while the reduction in Shannon diversity only translated into an increased relative risk for developing carcinomas (P-value = 0.0047). Increases in mouth-associated microbes were commonly in the top 5 most significantly increased relative risk of adenoma and carcinoma for both stool and tissue samples. A prediction model for adenoma and carcinoma was built using either the whole community or selected genera with highest and lowest relative risk from fecal and tissue samples. Both approaches resulted in similar classification success according to Area Under the Curve (AUC) regardless of whether genera or OTUs were used to build the model. The most important groups within the full community models consistently belonged to genera such as *Ruminococcus*, *Bacteroides*, and *Roseburia* across studies. Although a number of associations between the microbiota and CRC were identified, the majority of studies that we used in this meta-analysis were only individually adequately powered for large effect sizes.

Conclusions. These data provide support for the importance of the bacterial community to both adenoma and carcinoma genesis. The evidence collected within this study on the role of the microbiota in CRC identifies a number of correlations that may not have been detected because of the low power associated with the majority of studies that have been performed to date.

Keywords

microbiota; colorectal cancer; polyps; adenoma; meta-analysis.

Background

Colorectal cancer (CRC) is a growing world-wide health problem in which the microbiota has been purported to play an active role in disease pathogenesis [1,2]. Numerous studies have shown the importance of both individual microbes [3–7] and the overall community [8–10] in tumorgenesis using mouse models of CRC. There have also been numerous case/control studies investigating the microbiota in the formation of both adenoma and carcinoma. A recent meta-analysis investigated whether specific biomarkers could be consistently identified using multiple data sets [11]. This meta-analysis focused on identifying microbial signatures of CRC (biomarkers) but did so on a small total number of individuals and only investigated stool.

Although there has been an intense focus on microbiota-based biomarker discovery for CRC, the number of candidate genera seem to be endless. Some studies point towards mouth-associated genera such as *Fusobacterium*, *Peptostreptococcus*, *Parvimonas*, and *Porphyromonas* as key enriched genera [6,12–18]. Yet, even in these studies, mouth-associated genera are far from the only microbes identified to be associated with CRC. These other genera include, but are not limited to, *Providencia*, *Mogibacterium*, *Enterococcus*, *Escherichia/Shigella*, *Klebsiella*, and *Streptococcus* [14–16]. In fact, there is good *in vivo* evidence that *Escherichia/Shigella* and *Streptococcus* can be important in the pathogenesis of CRC [5,19,20]. Other studies have also identified *Akkermansia muciniphila* and *Bacteroides fragilis* as potential markers of CRC with good mechanistic studies for the latter [15,21,22]. A recent meta-analysis confirmed the correlations of certain mouth-associated genera and *Akkermansia muciniphila* with carcinoma [11]. However, the sample size (n = 509) is equal to or less than some more recent individual studies investigating the microbiota and CRC, making it hard to know how extrapolatable these findings are. That particular meta-analysis also added more potential microbial associations to both carcinoma (*Pantoea agglomerans Ruminococcus*, *Lactobacillus*) and adenoma

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(*Prevotella*, *Methanosphaera*, *Succinovibrio*, *Haemophilus parainfluenzae*, *Ruminococcus*, *Lactobacillus*) stages of CRC that need to be investigated further, since a number of these genera have been found to be enriched in controls and not disease [13,16,17]. There are also genera like *Roseburia* that some studies find to be increased in CRC but other studies report either decreased or no difference [15,18,23,24].

Most of these studies have focused on carcinoma but the adenoma observations are not any clearer at identifying candidate genera. Groups focusing on broad scale community metrics have found that metrics such as richness are decreased in the adenoma stage of CRC versus controls. Other studies have identified *Lactococcus*, *Pseudomonas*, *Acidovorax*, *Cloacibacterium*, *Helicobacter*, *Lactobacillus*, *Bilophila*, *Desulfovibrio*, and *Mogibacterium* to be increased in adenoma [25–27]. Based on the studies mentioned, there seems to be very little overlap between the genera identified to be associated with adenoma and carcinoma, with *Lactobacillus* being one of the few commonalities.

Targeting the identification of CRC microbial biomarkers within stool seems logical since it offers an easy and cost-effective way to stratify risk and the current gold standard for diagnosis, a colonoscopy, can be time-consuming and is not without risk of complications. Although stool represents an easy and less invasive way to assess risk, it is not clear how well this sample reflects adenoma- and carcinoma- associated microbial communities. Some studies have tried to assess this in health and disease but are limited by their sample size [18,28]. Sampling the microbiota directly associated with colon tissue directly may provide clearer answers but is not without limitations. The community present for sampling following the colonoscopy bowel prep may reflect the better adhered microbiota versus the resident community. Additionally, these samples contain more host DNA, potentially limiting the types of analysis that can be done. It is well known that low biomass samples can be very difficult to work with and results can be study dependent due to the randomness of contamination [29].

In comparison to the previous meta-analysis, this study significantly increases the total stool samples investigated, re-examines important genera across adenoma and carcinoma across study, and examines differences and similarities between stool and tissue microbiota in the context of CRC. Importantly, this analysis and approach could provide valuable insights into the common genera that are both protective and detrimental in CRC and whether broad bacterial community measurements can account for these changes that were not provided by earlier meta-analysis studies [11].

Using both feces (n = 1737) and colon tissues (492 samples from 350 individuals) totaling over 2229 total samples across 14 studies [12–18,21,23–27,30] [Table 1 & 2], we expand both the breadth and scope of the previous meta-analysis to investigate whether the bacterial community or specific members are more important risk factors for both adenoma and carcinoma stages of CRC. To accomplish this we first assessed whether bacterial diversity changes throughout disease (control to adenoma to carcinoma) and if it results in an increased relative risk (RR) for adenoma or carcinoma stages of CRC. We then assessed what genera, if any, increase or decrease the RR of adenoma or carcinoma stages of CRC. Next, using Random Forest models, we analyzed whether the full community or only the combined top 5 increased and top 5 decreased RR genera resulted in better model classification, based on the area under the curve (AUC). Finally, we also examined at what effect and sample size the studies used were powered for and the sample size needed to get to the traditionaly accepted 80% power. Our results from these analyses suggests that the bacterial community changes as disease severity worsens, that specific members are important for disease classification, and that many of the studies are underpowered for assessing small effect sizes.

Results

Lower Bacterial Diversity is Associated with Increased RR of Carcinomas: To
assess differences in broad scale community metrics as disease severity worsens
Operational Taxonomic Unit (OTU) richness, evenness, and Shannon diversity
measurements were power transformed and Z-score normalized. These metrics are
commonly used to assess the total number of OTUs, the equality of their abundance, and
the overall diversity, respectively. Using linear mixed-effect models to control for study
and variable region we assessed whether OTU richness, evenness, or Shannon diversity
changed in a step-wise manner with disease severity. In stool, there was a significant
decrease in both evenness and Shannon diversity as disease severity moved from control
to adenoma to carcinoma (P-value = 0.025 and 0.043, respectively) [Figure 1A]. We next
tested whether the detectable differences in community significantly increased in RR
of having an adenoma or carcinoma. For fecal samples, a decrease versus the overal
median in evenness resulted in a significantly increased RR for carcinoma (RR = 1.36
(1.15 - 1.61), P-value = 0.00034) and adenoma (RR = 1.16 (1.01 - 1.34), P-value =
0.032) while a decrease versus the overal median in Shannon diversity only increased
the RR for carcinoma (RR = 1.33 (1.09 - 1.62), P-value = 0.0047) [Figure 2]. Using the
Bray-Curtis distance metric and PERMANOVA, it was also possible to identify significant
bacterial community changes, in specific studies, for both carcinoma-associated and
adenoma-associated microbiota versus control [Table S1 & S2].

Using similar transformations for tissue samples, linear mixed-effect models were used
on the transformed combined data to control for study, re-sampling of the same individual,
and 16S variable region to test whether OTU richness, evenness, or Shannon diversity
changed in a step-wise manner as disease severity increased. For colon tissue, there
were no significant changes in OTU richness, evenness, or Shannon diversity as disease
severity progressed from control to adenoma to carcinoma (P-value > 0.05) [Figure 1B & C].

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134 We next analyzed the RR, for matched (unaffected tissue and an adenoma or carcinoma

135 from the same individual) and unmatched (control and adenoma or carcinoma tissue not

136 from the same individual) colon tissue samples. For individuals at either an adenoma or

137 carcinoma stage of disease there was no significant change in RR based on lower than

138 median values for OTU richness, evenness, and Shannon diversity [Table S3-S5]. Similar

139 to stool samples, significant differences in bacterial community, assessed by PERMANOVA,

140 were identified in unmatched tissue samples, for those at either adenoma or carcinoma

141 stage of CRC [Table S6 & S7]. For studies with matched samples no differences in bacterial

142 community were observed when assessed with PERMANOVA [Table S6 & S7]. These

143 tissue results suggest that the microbiota within an individual are similar to each other

144 regardless of disease status.

145 ***Mouth-Associated Genera are Associated with an Increased RR of CRC:*** Next we

146 asked if being higher than the median relative abundance, for any specific genera, resulted

147 in an altered RR for adenoma or carcinoma, in stool and colon tissue, due to our previous

148 observations of small increases in RR using OTU richness and Shannon diversity. To

149 investigate this we analyzed all common genera across each study, in colon tissue or stool,

150 and assessed whether a relative abundance higher than the median results in an increase

151 or decrease in RR. The top 5 most significantly increased RR were the same between

152 adenoma and carcinoma for both stool and colon tissue [Figure 3]. Mouth-associated

153 genera were commonly found in the top 5 genera associated with an increased RR of

154 having an adenoma (*Pyramidobacter* [Figure 3A] and *Rothia* [Figure 3C]) and carcinoma

155 (*Fusobacterium*, *Parvimonas*, *Porphyromonas*, and *Peptostreptococcus* [Figure 3B] and

156 *Fusobacterium* [Figure 3D]) for both stool and colon tissue samples. Conversely, genera

157 commonly associated with a normal gastrointestinal tract were correlated with a decreased

158 RR for both adenoma and carcinoma for both stool and colon tissue samples [Figure 3].

159 Even though mouth-associated genera were identified across disease stage, there was

160 little direct overlap of the top 5 increased or decreased RR genera between both stages

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161 and sample site.

162 When observing RRs with a P-value less than 0.05 there was almost no overlap between
163 genera from stool or colon tissue and when they were similar the RR was in opposite
164 directions (e.g. *Lactococcus*) [Table S8 & S9]. Many of the genera that had RRs with a
165 P-value under 0.05 for colon tissue are also highly prevalent in contamination, specifically,
166 *Novosphingobium*, *Selemonas*, and *Achromobacter* [Figure 3 & Table S8-S9]. For
167 carcinoma stage of CRC, certain mouth-associated genera (*Fusobacterium*, *Parvimonas*,
168 *Peptostreptococcus*) had a high RR for both colon tissue and stool samples [Table S10 &
169 S11]. Finally, these data suggest that the most significantly increased RR genera for tissue
170 was *Camplyobacter* while in stool it was *Peptostreptococcus* [Table S10 & S11].

171 **Select Genera Models can Recapitulate Whole Community Models:** Since specific
172 genera increased RR for carcinoma over diversity metrics we assessed whether the
173 bacterial community was better at classifying disease versus only a select group of genera
174 based on RR and P-value significance and tested this using two approaches. The first
175 approach used genus level data and tested whether there were any differences in AUC
176 between all genera and selected genera during training on one study or testing on all
177 other studies. The second approach used OTU level data and tested whether there was a
178 generalized decrease in the 10-fold cross validation (CV) model AUC across studies when
179 comparing all OTUs versus only OTUs that taxonomically classified to selected genera.

180 For the genera-based models the training set median AUC for model classification was
181 similar for select genera and full genera models, for both tissue and stool studies [Figure
182 S2-S3]. With respect to the test sets, comprised of genera data from other studies, both all
183 and select genera models had a similar ability to detect adenomas or carcinomas, with the
184 select genera models performing better in some instances [Figure S4-S6]. Conversely, the
185 OTU-based models showed a slight decrease in median AUC between the full and select
186 models, with one exception being carcinoma models for matched tissue [Figure 4 & 5].

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In stool, the most common genera in the full genera-based models belonged to genera such as *Ruminococcus*, *Bacteroides*, and *Roseburia* [Figure 6A & B]. Although all mouth-associated genera were present in carcinoma, none were present in the majority of studies and *Fusobacterium* was the only genus present in adenoma [Figure 6A & B]. For the full OTU-based models, *Ruminococcaceae* was present in the top 10 consistently for both adenoma and carcinoma models while *Roseburia* was only present in many adenoma models and *Bacteroides* was present in the overwhelming majority of the carcinoma models [Figure 6C & 6D].

Unlike stool, the tissue full genera-based Random Forest models showed no consistent representation of *Ruminococcaceae*, *Ruminococcus*, *Bacteroides*, and *Roseburia* in the top 10 across study [Figure S7]. The vast majority of the top 10 genera and OTUs occured in a study specific manner for these tissue based Random Forest models. For both the full genera and OTU Random Forest models of adenoma and carcinoma, there appears to be very little overlap in the top 10 most important variables between stool and tissue [Figure 6 & S7]. This discordance between stool and tissue also applies to the mouth-associated genera with one noticeable skew being that *Fusobacterium* and *Fusobacteriaceae* occur more often in the top 10 of Random Forest models using matched versus unmatched tissue samples [Figure S7B-C & S7E-F].

CRC Studies are Underpowered for Detecting Small Effect Sizes: Based on our reported results we assessed whether the studies analyzed were realistically powered to identify small, medium, and large scale differences between cases and controls. When assessing the power of each study at different effect sizes the majority of studies, for both adenoma and carcinoma, acheived 80% power to detect a 30% or greater difference between groups [Figure 7A & B]. No study that was analyzed had the standard 80% power to detect an effect size difference that was equal to or below 10% [Figure 7A & B]. In order to achieve a power of 80%, for small effect sizes, studies used in our meta-analysis would

213 need to recruit over 1000 individuals for each arm [Figure 7C].

Discussion

Our study identifies clear differences in diversity, both at the community level and for individual genera, that are present in patients with and without CRC [Figure 1-3]. Although there was a step-wise decrease in diversity from control to adenoma to carcinoma, this did not translate into large effect sizes for the RR of lesion. Even though mouth-associated genera increased the RR of carcinoma, they did not consistently increase the RR of adenoma. These mouth-associated genera are clearly important to carcinoma classification but our observations suggest that accounting for other community members can increase the ability of models to make predictions.

The data presented herein supports the importance of select genera for carcinoma but not necessarily adenoma formation. Our observations show that when only using the select genera to create models for carcinoma the AUC are similar to using all genera or OTUs [Figure 4 & 5]. This suggests that an interplay between a select number of potentially protective and exacerbating microbes, within the GI community, are crucial for carcinoma formation. Importantly, it suggests that there may be key members of the GI community that might be studied further to potentially reduce the risk of carcinoma. Conversely, using the present data, it is clear that new approaches may need to be used to identify members of the community that are associated with adenoma. Regardless of sample type and whether a full or select model was used our Random Forest models consistently performed poorly. Yet, the step-wise decrease in diversity suggests that the adenoma community is not normal but has changed subtly [Figure 1]. This change in diversity, at this early stage of disease, could be focal to the adenoma itself. One potential hypothesis from these observations is that at early stages of the disease, how the host interacts with these subtle changes is what ultimately leads to a thoroughly dysfunctional community that is supportive of CRC genesis.

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Within stool, common GI microbes were most consistently present in the top 10 genera or OTUs across study [Figure 6]. Changes in *Bacteroides*, *Ruminococcaceae*, *Ruminococcus*, and *Roseburia* were consistently found to be disciminative across the different studies for both adenoma and carcinoma [Figure 6]. This data would suggest that whether the non-resident bacterium is *Fusobacteria* or *Peptostreptococcus* is not as important as how these bacteria interact with the changing resident community. Based on these observations, it is possible to hypothesize that small changes in community structure lead to new niches in which any one of the mouth-associated genera can gain a foothold, exacerbating the initial changes in community and facilitating the transition from adenoma to carcinoma.

The tissue studies did not provide a clearer understanding of how the microbiota may be associated with lesion. Generally, the full OTU-based models of unmatched and matched [Figure S7E & F] tissue samples were concordant with stool showing that resident microbes were the most prevalent in the top 10 across study. Unlike in stool, *Fusobacterium* was the only mouth-associated bacteria consistently present in the top 10 of the full CRC models [Figure S7B-C & E-F]. The majority of the tissue results seem to be study specific with many top 10 taxa being present only in a single study. Additionally, the presence of genera associated with contamination, within the top 10 most important variables for the genera and OTU models is worrying. The low bacterial biomass of tissue samples coupled with potential contamination could ultimately explain why the these results seem to be more sporadic than the stool results.

One important caveat to this study is that even though specific genera associated with species such as *Bacteroides fragilis* and *Streptococcus gallolyticus* subsp. *gallolyticus* were not identified, it does not necessarily mean these specific species are not important in human CRC [20,22]. Since we are limited in our aggregation of the data, across study, to the genus level, it is not possible to clearly delineate what specific species are contributing to overall disease progression. Our observations are not inconsistent

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with the previous literature on either *Bacteroides fragilis* or *Streptococcus gallolyticus* subsp. *gallolyticus*. As an example, for stool, the full RF models consistently identified the genus *Bacteroides* as well as OTUs that classified as *Bacteroides* to be important model components across studies. Suggesting that even though *Bacteroides* may not increase the RR of lesion and may not vary in relative abundance, like *Fusobacterium*, it is still important in CRC. Additionaly, *Streptococcus gallolyticus* subsp. *gallolyticus* is a mouth-associated micobe and our study clearly shows that regardless of whether the sample is tissue or stool, mouth-associated genera are commonly associated with an increased RR of both adenoma and carcinoma.

The associations between the microbiota and adenoma are inconclusive, in part, because many studies may not be powered effectively to observe small effect sizes. None of the studies analyzed were properly powered to detect a 10% or lower change between cases and controls. A small effect size may well be the scope in which differences consistently occur in adenoma based on the results observed within our meta-analysis. Future studies investigating adenoma and the microbiota need to take power into consideration if we are to reproducibly study whether the microbiota contributes to polyp formation. In contrast to adenoma, our observations suggest that most studies analyzed have sufficient power to detect many changes in carcinoma because of large effect size differences between cases and controls [Figure 7].

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Conclusion

By aggregating together a large collection of studies from both feces and tissue, we are able to provide evidence in support of the importance of the bacterial community in CRC. Further, the data presented here suggests that mouth-associated microbes can gain a foothold within the colon and are are commonly associated with the greatest RR of carcinoma. Conversely, no conclusive signal with these mouth-associated micobes could be detected for adenoma. Our observations also highlight the importance of power and sample number considerations when undertaking investigations into the microbiota and adenoma due to the subtle changes in the community. Overall, the microbiota associations with carcinoma are much stronger than the associations with adenoma.

Methods

Obtaining Data Sets: The studies used for this meta-analysis were identified through the review articles written by Keku, *et al.* and Vogtmann, *et al.* [31,32] and additional studies not mentioned in the reviews were obtained based on the authors' knowledge of the literature. Studies that used tissue or feces as their sample source for 454 or Illumina 16S rRNA gene sequencing analysis were included and only data sets that had sequences available for analysis were included. Some studies were excluded because they did not have publicly available sequences or did not have metadata in which the authors were able to share and were excluded. After these filtering steps, the following studies remained: Ahn, *et al.* [12], Baxter, *et al.* [13], Brim, *et al.* [30], Burns, *et al.* [16], Chen, *et al.* [14], Dejea, *et al.* [24], Flemer, *et al.* [18], Geng, *et al.* [23], Hale, *et al.* [27], Kostic, *et al.* [33], Lu, *et al.* [26], Sanapareddy, *et al.* [25], Wang, *et al.* [15], Weir, *et al.* [21], and Zeller, *et al.* [17]. The Zackular [34] study was not included because the 90 individuals analyzed within the study are contained within the larger Baxter study [13]. After sequence processing all the case samples for the Kostic study had 100 or less sequences remaining and was not used which left a total of 14 studies that analysis could be completed on.

Data Set Breakdown: In total, there were seven studies with only fecal samples (Ahn, Baxter, Brim, Hale, Wang, Weir, and Zeller), five studies with only tissue samples (Burns, Dejea, Geng, Lu, Sanapareddy), and two studies with both fecal and tissue samples (Chen and Flemer). The total number of individuals that were analyzed after sequence processing for feces was 1737 [Table 1]. The total number of matched and unmatched tissue samples that were analyzed after sequence processing was 492 [Table 2].

Sequence Processing: For the majority of studies, raw sequences were downloaded from the Sequence Read Archive (SRA) (ftp://ftp-trace.ncbi.nih.gov/sra/sra-instant/reads/ ByStudy/sra/SRP/) and metadata was obtained by searching the respective accession

319 number of the study at the following website: http://www.ncbi.nlm.nih.gov/Traces/study/. Of

320 the studies that did not have sequences and metadata on the SRA, data was obtained from

321 DBGap (n = 1, [12]) and directly from the authors (n = 4, [18,21,25,27]). Each study was

322 processed using the mothur (v1.39.3) software program [35] and quality filtering utilized

323 the default methods, used in mothur, for either 454 or Illumina based sequencing. If it was

324 not possible to use the defaults, the stated quality cut-offs, from the study itself, were used

325 instead. Chimeras were identified and removed using VSEARCH [36] before *de novo* OTU

326 clustering at 97% similarity was completed using the OptiClust algorithm [37].

327 **_Statistical Analysis:_** All statistical analysis after sequence processing utilized the R

328 (v3.4.3) software package [38]. For the α-diversity analysis, values were power transformed

329 using the rcompanion (v1.11.1) package [39] and then Z-score normalized using the car

330 (v2.1.6) package [40]. Testing for α-diversity differences utilized linear mixed-effect models

331 created using the lme4 (v1.1.15) package [41] to correct for study, repeat sampling of

332 individuals (tissue only), and 16S hypervariable region used. Relative risk was analyzed

333 using both the epiR (v0.9.93) and metafor (v2.0.0) packages [42,43] by assessing how

334 many individuals with and without disease were above and below the overall median value

335 within each specific study. Relative risk significance testing utilized the chi-squared test.

336 β-diversity differences utilized a Bray-Curtis distance matrix and PERMANOVA executed

337 with the vegan (v2.4.5) package [44]. Random Forest models were built using both the caret

338 (v6.0.78) and randomForest (v4.6.12) packages [45,46]. Power analysis and estimations

339 were made using the pwr (v1.2.1) and statmod (v1.4.30) packages [47,48]. All figures were

340 created using both ggplot2 (v2.2.1) and gridExtra (v2.3) packages [49,50].

341 **_Study Analysis Overview:_** α-diversity was first assessed for differences between controls,

342 adenoma, and carcinoma using both linear mixed-effect models and RR. β-diversity was

343 then assessed for each individual study for differences between control-adenoma and

344 control-carcinoma. Next, all common genera were assessed for differences in RR for

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adenoma and carcinoma and ranked based on P-value. We then built Random Forest models based on all or a selected model, based on the top 5 increased and top 5 decreased RR based on P-value, and these models were trained on one study then tested on the remaining studies, for every study. A similar approach was then applied at the OTU level with the exception that a 10-fold CV over 100 different models, based on random 80/20 splitting of the data, was used to generate a range of expected AUCs. For these OTU-based models, the selected model included all OTUs that had a taxonomic classification to a variable in the top 5 increased and top 5 decreased RR based on P-value. Finally, the power of each study was assessed for an effect size ranging from 1% to 30% and an estimated sample size, for these effect sizes, was generated based on 80% power. For comparisons in which normal versus adenoma were made the carcinoma samples were excluded from each respective study. Similarly, for comparisons in which normal versus carcinoma were made the adenoma samples were excluded from each respective study. The data was split between feces and tissue samples. Within the tissue groups the data was further divided between samples from the same individual (matched) and those from different individuals (unmatched). Where applicable for each study, predictions for adenoma and carcinoma were then tested for feces, matched tissue, and unmatched tissue.

Obtaining Genera Relative Abundance and Selected Models: For the genera analysis of the RR, OTUs were added together based on the genus or lowest available taxonomic classification level and the total average counts, for 100 different subsamplings, were collected. The OTU based Random Forest Models using selected OTUS utilized a similar approach except that the OTUs were not aggregated together by taxonomic identity but kept as separate OTUs. OTU Random Forest models using the full community included all OTUs while those for the selected model included only those OTUs that had a taxonomic classification to a variable in the top 5 increased of top 5 decreased RR based on P-value.

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Matched versus Unmatched Tissue Samples: In general, tissue samples that had control and lesion samples, that did not belong to the same individual, were classified as unmatched while samples, that belonged to the same individual, were classified as matched. Studies with matched data included Burns, Dejea, Geng, and Lu while those with unmatched data were from Burns, Flemer, Chen, and Sanapareddy. For some studies samples became unmatched due to one of the corresponding matched samples not making it through sequence processing. All samples, from both tissue sample types, were analyzed together for the linear mixed-effect models with samples from the same individual corrected for. For all other analysis, not mentioned herein, matched and unmatched samples were analyzed separately using the statistical approaches mentioned in the Statistical Analysis section.

Assessing Important Random Forest Model Variables: Using Mean Decrease in Accuracy (MDA) the top 10 most important variables were obtained in two different ways depending on whether the model used genera or OTU data. For the genus based models, the number of times that a genus showed up in the top 10 of the training set across each study was counted while, for the OTU based models, the medians for each OTU across 100 different 80/20 splits of the data was generated and the top 10 OTUs then counted for each study. Common taxa, for the OTU based models, were identified by using the lowest classification within the RDP database for each of the specific OTUs obtained from the previous counts and the number of times this classification occurred in the top 10, in each study, was recorded. The two studies that had adenoma tissue were equally divided between matched and unmatched groups and were grouped together for the counting of the top 10 genera and OTUs.

Reproducible Methods: The code and analysis can be found here at https://github.com/ SchlossLab/Sze_CRCMetaAnalysis_Microbiome_2017. Unless otherwise mentioned, the accession number for the raw sequences for the studies used in this analysis can be found

397 directly in the respective batch file in the GitHub repository or in the original manuscript.

Declarations

Ethics approval and consent to participate

Ethics approval and informed consent for each of the studies used is mentioned in the respective manuscripts used in this meta-analysis.

Consent for publication

Not applicable.

Availability of data and material

A detailed and reproducible description of how the data were processed and analyzed for each study can be found at https://github.com/SchlossLab/Sze_CRCMetaAnalysis_Microbiome_2017. Raw sequences can be downloaded from the SRA in most cases and can be found in the respective study batch file in the GitHub repository or within the original publication. For instances when sequences are not publicly available, they may be accessed by contacting the corresponding authors from whence the data came.

Competing Interests

All authors declare that they do not have any relevant competing interests to report.

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Authors' contributions

All authors helped to design and conceptualize the study. MAS identified and analyzed the data. MAS and PDS interpreted the data. MAS wrote the first draft of the manuscript and both he and PDS reviewed and revised updated versions. All authors approved the final manuscript.

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Table 1: Total Individuals in each Study Included in the Stool Analysis

Study	Data Stored	16S Region	Control (n)	Adenoma (n)	Carcinoma (n)
Ahn	DBGap	V3-4	148	0	62
Baxter	SRA	V4	172	198	120
Brim	SRA	V1-3	6	6	0
Flemer	Author	V3-4	37	0	43
Hale	Author	V3-5	473	214	17
Wang	SRA	V3	56	0	46
Weir	Author	V4	4	0	7
Zeller	SRA	V4	50	37	41

Table 2: Studies with Tissue Samples Included in the Analysis

Study	Data Stored	16S Region	Control (n)	Adenoma (n)	Carcinoma (n)
Burns	SRA	V5-6	18	0	16
Chen	SRA	V1-V3	9	0	9
Dejea	SRA	V3-5	31	0	32
Flemer	Author	V3-4	103	37	94
Geng	SRA	V1-2	16	0	16
Lu	SRA	V3-4	20	20	0
Sanapareddy	Author	V1-2	38	0	33

Figure 1: α-Diversity Differences between Control, Adenoma, and Carcinoma Across Sampling Site. A) α-diversity metric differences by group in stool samples. B) α-diversity metric differences by group in unmatched tissue samples. C) α-diversity metric differences by group in matched tissue samples. The dashed line represents a Z-score of 0 or no difference from the median.

Figure 2: Relative Risk for Adenoma or Carcinoma based on α-Diversity Metrics in Stool. A) α-metric relative risk for adenoma. B) α-metric relative risk for carcinoma. Colors represent the different variable regions used within the respective study.

Figure 3: Top 5 Genera that Decrease and Increase Relative Risk for Lesion. A) Adenoma relative risk in stool. B) Carcinoma relative risk in stool. C) Adenoma relative risk in tissue. D) Carcinoma relative risk in tissue. For all panels the relative risk was also compared to whether one, two, three, or four of the CRC-associated genera were present.

Figure 4: Stool OTU Random Forest Model Across Studies. A) Adenoma random forest model between the full community and select genera OTUs only. B) Carcinoma random forest model between the full community and select genera OTUs only. The dotted line represents an AUC of 0.5 and the lines represent the range in which the AUC for the 100 different 80/20 runs fell between. The solid red line represents the median AUC of all the studies for either the full community or select genera OTUS only model.

Figure 5: Tissue OTU Random Forest Model Across Studies. A) Adenoma random forest model between the full community and select genera OTUs only. B) Carcinoma random forest model between the full community and select genera OTUs only. The dotted line represents an AUC of 0.5 and the lines represent the range in which the AUC for the 100 different 80/20 runs fell between. The solid red line represents the median AUC of all the studies for either the full community or select genera OTUS only model.

Figure 6: Most Common Genera Across Full Community Stool Study Models. A)

Common genera in the top 10 for adenoma Random Forest genus models. B) Common genera in the top 10 for carcinoma Random Forest genus models. C) Common genera in the top 10 for adenoma Random Forest OTU models. D) Common genera in the top 10 for carcinoma Random Forest OTU models.

Figure 7: Power and Effect Size Analysis of Studies Included. A) Power based on effect size for studies with adenoma individuals. B) Power based on effect size for studies with carcinoma individuals. C) The estimated sample number needed for each arm of each study to detect an effect size of 1-30%. The dotted red lines in A) and B) represent a power of 0.8.

Figure S1: Relative Risk for Adenoma or Carcinoma based on α-Diversity Metrics

in Tissue. A) α-metric relative risk for adenoma. B) α-metric relative risk for carcinoma. Colors represent the different variable regions used within the respective study.

Figure S2: Stool Random Forest Genus Model AUC for each Study. A) AUC of adenoma models using all genera or select genera only. B) AUC of carcinoma models using all genera or select genera only. The black line represents the median within each group.

Figure S3: Tissue Random Forest Genus Model AUC for each Study. A) AUC of adenoma models using all genera or only select genera divided between matched and unmatched tissue. B) AUC of carcinoma models using all genera or select genera only. The black line represents the median within each group divided between matched and unmatched tissue.

Figure S4: Stool Random Forest Prediction Success Using Genera Across Studies. A) AUC for prediction in adenoma using all genera or select genera only. B) AUC for prediction in carcinoma using all genera or select genera only. The dotted line represents an AUC of 0.5. The x-axis is the data set in which the model was initially trained on. The red lines represent the median AUC using that specific study as the training set.

Figure S5: Tissue Random Forest Prediction Success of Carcinoma Using Genera Across Studies. A) AUC for prediction in unmatched tissue for all genera or select genera only. B) AUC for prediction in matched tissue using all genera or select genera only. The dotted line represents an AUC of 0.5. The x-axis is the data set in which the model was initially trained on. The red lines represent the median AUC using that specific study as the training set.

Figure S6: Tissue Random Forest Prediction Success of Adenoma Using Genera Across Studies. The red lines represent the median AUC using that specific study as the

training set.

Figure S7: Most Common Genera Across Full Community Tissue Study Models. A)

Common genera in the top 10 for adenoma Random Forest genus models. B) Common

genera in the top 10 for unmatched carcinoma Random Forest genus models. B) Common

genera in the top 10 for matched carcinoma Random Forest genus models. D) Common

genera in the top 10 for adenoma Random Forest OTU models. E) Common genera in the

top 10 for unmatched carcinoma Random Forest OTU models. F) Common genera in the

top 10 for matched carcinoma Random Forest OTU models.