Characterizing the Role of the Human Virome in Colorectal Cancer

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Abstract

Colorectal cancer is the second leading cause of cancer-related death in the United States and is a primary cause of morbidity and mortality throughout the world. Although the cause of colorectal cancer remains unclear, it has been strongly linked to colon bacterial communities. Viruses are another important component of the colon microbial community that have yet to be studied in colorectal cancer, despite their oncogenic potential. We evaluated the colon virome (virus community) role in colorectal cancer using a cohort of 90 human subjects with either healthy, precancerous, or cancerous colons. We utilized 16S rRNA gene, whole shotgun metagenomic, and purified virus metagenomic methods to compare the virome's role to that of the bacterial community. We found that alpha and beta diversity metrics were insufficient for detecting an association between the virome and colorectal cancer, but virome-based classification models were highly associated with colorectal cancer. Bacteriophages, not eukaryotic viruses, made up the majority of the CRC-associated virome, suggesting the community was indirectly linked to colorectal cancer, modulating bacterial community structure and functionality. Phages with broader host ranges had a more significant role in cancer development. These results provide foundational evidence that phage communities are associated with colorectal cancer, and that broadly tropic phages play more significant roles. Because of its importance, the virome should be considered in future colorectal cancer studies, as well as other cancer types.

Word Count: 229

Significance Statement

Colorectal cancer is a leading cause of cancer-related death in the United States and worldwide. It's progression and severity is linked to colon bacterial communities. Little is known about the cancer-associated colon virus communities and their influence on bacteria. We characterize this influence of colon virus community structure on colorectal cancer in humans. The link between the colon virome and colorectal cancer was established by using virome-based machine learning models to accurately classify patient cancer status. The cancer-virus link was driven primarily by bacteriophages (bacterial viruses). Wider phage host ranges were more strongly linked to colorectal cancer. The results suggest an indirect role for the virome impacting colorectal cancer by modulating their associated bacterial community.

Word Count: 119

Introduction

Cancer remains a devastating and persistent plague on humanity. Although cancer is still a primary cause of morbidity and mortality worldwide, we have made considerable therapeutic progress in recent decades. Perhaps one of our most impactful advances has not been in cancer treatment, but rather in detecting cancers at early stages, thereby improving treatment efficacy. This has been evident in a variety of prominent cancer types, with one of the most notable being colorectal cancer.

Colorectal cancer is the second leading cause of cancer-related deaths in the United States (1). The US National Cancer Institute estimates over 1.5 million Americans have been diagnosed with colorectal cancer in 2016, and over 500,000 Americans will have died from the disease (1). Although it remains a major health problem, the impact of colorectal cancer has been reduced by improved screening and prevention efforts (1, 2).

Development of colorectal cancer is a stepwise process that begins when healthy tissue develops into a precancerous polyp (i.e. adenoma) in the large intestine (3). If left untreated, the adenoma will develop into a cancerous lesion that can invade and metastasize, leading to severe illness and death. Progression to cancer can be prevented when precancerous adenomas are detected and removed during routine screening (2, 4). Survival for colorectal cancer patients may exceed 90% when the lesions are detected early and removed (4). Screening methods are effective, but their invasiveness has created a lack of compliance, creating a need for accurate, non-invasive screening methods. One such method is screening associated colon microbial communities.

Although the cause of colorectal cancer remains unclear, it has been strongly associated with colon bacterial communities (5–8). This association has allowed researchers to leverage bacterial community signatures as biomarkers to provide accurate, noninvasive colorectal cancer detection from stool (7, 9). While an understanding of colorectal cancer bacterial communities has proven fruitful both for disease classification and understanding underlying etiology, bacteria are only a subset of the colon microbiome. Viruses are another important component of the colon microbial community that have yet to be studied in the context of colorectal cancer.

Due to their mutagenic abilities and their propensity for functional manipulation, human viruses are strongly associated with, and in many cases cause, cancer (10–13). Additionally, because bacteriophages are crucial for bacterial community stability and composition (14–16), and because bacteria have been implicated as oncogenic agents (7, 8, 17), bacteriophages are potentially indirectly linked to cancer. The colon virome (the virus community of the colon) has the potential to impact health and disease (e.g. cancer), and has been

associated with diseases including periodontal disease (18), HIV (19), antibiotic exposure (20, 21), urinary tract infections (22), and inflammatory bowel disease (23). We aim to take this field of research further by beginning to assess the role of the virome in colorectal cancer.

Here we present a study of the colorectal cancer virome, highlighting the viruses most associated with the cancer state and the virome's utility for prognosis and diagnosis. We report that, like the association between the bacterial community and colorectal cancer was driven primarily by *Fusobacterium*, the association between the virome and colorectal cancer was driven by broadly infectious, bacteriophage hubs within the phage-bacteria ecosystem. By creating effective classification models using virus community signatures, we were able to accurately classify stool samples as cancerous, precancerous, or healthy. The implications of these findings are threefold. *First*, this supports a biological role for the virome in colorectal cancer development and suggests that more than bacteria are involved in the process. *Second*, we present a supplementary, or even alternative, virus-based approach for classification modeling of colorectal cancer using stool samples. *Third*, we provide initial support for the importance of studying the virome as a component of the microbiome ecological network, especially in cancer. We expect this study will provide opportunities for continued research into the role of the virome in human cancer development.

Results

The Colorectal Cancer Virome Cohort

The study cohort consisted of 90 human subjects, 30 of which served as healthy controls, 30 of which had adenoma lesions consistent with a precancerous state, and 30 which had carcinoma lesions consistent with colorectal cancer **(Figure 1)**. Half of the stool was used to sequence the bacterial communities using both 16S rRNA gene and shotgun sequencing techniques. The 16S rRNA gene sequences were reported in a previous publication but re-analyzed here (7). The other half of the stool samples were purified for virus like particles (VLPs) and consequent genomic DNA extraction, followed by shotgun metagenomic sequencing. The VLP purification allowed us to observe the *active virome* because we only sequence those viruses that are encapsulated.

Virus DNA was purified by re-suspending the stool in buffer and removing contaminating cells (e.g. human, bacteria, etc) by filtration followed by contaminating cell lysis and degradation of the released genomic DNA **(Figure 1)**. The resulting genomic DNA was used to prepare a shotgun metagenomic sequencing library that was sequenced on the Illumina HiSeq4000 platform. Each run was performed with a blank control

to detect any contaminants from reagents. Only one of the nine viral controls detected DNA, which was of a minimal concentration, providing initial evidence of successful sequencing of VLP genomic DNA over potential contaminants **(Figure S1 A)**. As was expected, these controls were sparsely sequenced and were mostly removed while sub-sampling to even depths **(Figure S1 B)**.

The high quality phage and bacterial sequences were assembled into highly covered contigs longer than 1kb **(Figure S2)**. Because contigs only represent genome fragments, we further clustered related contigs into operational genomic units (OGUs) with the majority containing hundreds of related contigs **(Figure S2 - S3)**. These operational units, which are conceptually similar to the operational taxonomic units (OTUs) used in 16S analysis, allow us to study bacterial and phage entities as highly related genomic entities.

Diversity is Insufficient for Virome-Based Cancer Classification

Microbiome and disease associations are often described as being of an altered diversity (i.e. "dysbiosis"). We therefore initially evaluated the diversity of virome OGUs and their association with colorectal cancer. We utilized the Bray-Curtis dissimilarity metric to evaluate the differences in communities between disease states. To control for uneven sequencing depths, we subsampled to a minimum depth that maintained most samples while excluding the sparsely sequenced negative controls.

There was no statistically significant clustering of the disease groups, as visualized by NMDS ordination (Anosim p-value = 0.432, **Figure S4 A**). An Anosim test with a post hoc multivariate Tukey test was used to calculate the statistical significance of the differences between the disease groups based on the variance around the cluster centroids **(Figure S4 B)**. There were no significant differences between the disease groups, although there was a strongly significant difference between the blank controls (those few that remained after sub-sampling) and the rest of the study groups, further supporting the quality of our sample set (Anosim p-value = $7.18 \times$ 10-28, **Figure S5)**.

In addition to beta diversity, we also calculated the differences in virus alpha diversity associated with colorectal cancer. Again we found no significant alterations in either Shannon entropy or richness of the virus communities **(Figure S4 C-D)**. Overall, standard diversity metrics were insufficient for capturing the differences in the virus communities between disease states. This suggested to us that a more sophisticated approach for understanding the microbial community may be required, such as machine learning classification.

Machine Learning Links the Virome to Colorectal Cancer

Previous work has shown that 16S rRNA community signatures are effective for classifying stool samples as originating from healthy, precancerous, or cancerous individuals (7, 9). This is valuable because it presents a potential alternative screening approach to an invasive and expensive colonoscopy. The exceptional performance of bacteria in these classification models also supports a role for bacteria in colorectal cancer. Here we built off of these findings by evaluating the ability of virus community signatures to classify stool samples and compared performance to models built using bacterial community signatures.

We built and tested random forest models to classify stool samples as belonging to either cancerous or healthy individuals. These models were based on virus metagenomic community signatures or bacterial 16S rRNA gene signatures. We also included a whole metagenomic sequence set to ensure viral metagenomic observations were not a trait of metagenomics in general. Each operational units' relative abundance was used in the feature set. The same model approach was used for all three datasets, and the only difference was the data used to learn the model. We confirmed that our model using bacterial 16S data replicated the performance of the original report which used logit models instead of random forest models **(Figure 2 A)** (7).

We compared the bacterial 16S rRNA gene model to a model built using the virome signatures. The viral model performed as well as the bacterial model (corrected p-value = 1) with the viral and bacterial models achieving mean AUC (area under the curve) values of 0.799 and 0.794, respectively, after fifteen random forest iterations **(Figure 2 A - B)**.

To confirm that this observation was due to the virome community itself, and was not a trait of metagenomic datasets in general, we built a model using whole metagenomic community signatures. This model performed poorly with a mean AUC of 0.536, lending support to our observation that classification performance is a trait unique to the virus metagenome **(Figure 2 A - B)**. Further investigation revealed that the bacterial 16S rRNA gene model was strongly driven by sparse and lowly abundant OTUs. Further filtration of OTUs with a median abundance of zero resulted in the removal of six OTUs, and a loss of model performance down to what was observed in the metagenome **(Figure S8 A)**. The majority of these OTUs had a relative abundance lower than 1% **(Figure S8 B)**.

To evaluate the synergistic capabilities of the bacterial and viral signatures within the model, we built a combinatory model using both bacteria community and virome data. The combination model yielded modest but significantly improved performance beyond the virome (corrected p-value = 0.00287) and bacterial (corrected p-value = 8.42 $\times$ 10-4) models, yielding an AUC of 0.826 **(Figure 2 A - B)**. This suggests

the virus and bacterial communities may have synergistic capabilities for classifying stool as belonging to cancerous individuals.

The association between the two communities and colorectal cancer was driven by a few important microbes, measured using the mean decrease in model accuracy when each was iteratively removed. *Fusobacterium* was the primary driver of the bacterial association with colorectal cancer, which is consistent with it's previously described oncogenic potential **(Figure 2 C)**(5). The virome signature was also driven primarily by a few operational genomic units, suggesting a role in cancer development **(Figure 2 D)**. The identified important viruses were bacteriophages, belonging to *Siphoviridae*, *Myoviridae*, and orphan phage taxa without taxonomic identifiers (denoted "unclassified"). Many of the important viruses were unidentifiable (denoted "unknown"), suggesting they are members of the abundant viral dark matter (uncharacterized virus genomic space) associated with the human virome. This is common in the virome, and studies can have as much as 95% of virus sequences belong to unknown genomic units (24, 25).

When the bacterial and viral community signatures were combined, two bacterial and two viral organisms primarily drove the community association with cancer **(Figure 2 E)**. The most important microbes were two unidentified viruses, followed by *Bacteroides* and *Fusobacterium*.

The colon Virome Allows for Cancerous, Adenomatous, and Healthy Classification

After evaluating our ability to classify samples as cancerous or healthy, we incorporated the precancerous adenoma samples into the model and evaluated our ability to classify all three states in our total sample set. We used three-class random forest models for the bacterial 16S and viral sample sets. The bacterial signature model yielded an AUC of 0.779 and outperformed the viral community model which yielded an overall AUC of 0.698 (p-value = $1.08 \times$ 10-5, **Figure 3 A-C**). Both models were best able to classify cancer samples from healthy or precancerous samples, but struggled to distinguish precancerous from healthy or cancerous **(Figure 3 A-B)**. The cancerous signal was the most discriminatory of the three sample types.

Many of the microbes important for the two-class (cancer vs healthy) bacteria and virus models were also important for the three-class model **(Figure 3 D-E)**. The most important bacterium was the same *Fusobacterium* between the two and three class models, supporting its significance to the association between cancer and the bacterial communities **(Figure 2 C, Figure 3 D)**. Unlike the two-class virome model, the viruses most important to the three-class model were identified bacteriophages **(Figure 2 D, Figure 3 E)**. A *Podoviridae* was most important, followed by two *Siphoviridae* phages.

The classification model determined cancer state by incorporating the relative abundance profiles of the microbes within each community. The signatures ranged from notably high abundance of some OGUs, low abundance of some OGUs, and an absence of other OGUs **(Figure 3 F)**. Not all important OGUs were of increased abundance. The viral classification model depended on the unique signatures of these different abundance profiles to accurately classify each sample.

Temperate Bacteriophages Drive Link Between Virome and Colorectal Cancer

The virome-based model was able to accurately classify stool samples as cancerous, precancerous, or healthy. Not only is this important for establishing an alternative diagnostic model, but it also suggests an underlying biological importance for viruses in colorectal cancer. Above we used our classification models to evaluate which virus OGUs were most highly linked to colorectal cancer. We were able to further characterize these important OGUs to better understand the underlying biological link between the virome and colorectal cancer.

The role of the virome in colorectal cancer could have been driven directly by eukaryotic viruses or indirectly by bacteriophages acting through their bacterial hosts. To better understand the types of viruses that are important for colorectal cancer, we utilized the longest sequences from the OGUs as the representative sequences to be used for taxonomic classification. These sequences were aligned to a set of all reference virus genomes, including bacteriophages and eukaryotic viruses. A strict e-value threshold of 1e-25 was used to improve our confidence in the matches between the genome sequences. The most important viruses to the classification model were identified as bacteriophages (**Figure 3 E)**. Overall we were able to identify 78.8% of the OGUs as known viruses, and 93.8% of those viral OGUs aligned to bacteriophage reference genomes.

We evaluated whether the phages in the community were primarily lytic (lyse their hosts) or temperate (lysogenic; can integrate into their host's genome, as well as lyse the cell). We accomplished this by identifying three markers for temperate phages in the OGU representative sequences: 1) presence of phage integrase genes, 2) presence of known prophage genes, according the the ACLAME (A CLAssification of Mobile genetic Elements) database, and 3) nucleotide similarity to regions of bacterial genomes. This approach was done as described in previous work (25, 26). We found that the majority of the colon phages were temperate, and that the overall fraction of temperate phages remained consistent throughout the healthy, adenomatous, and cancerous stages **(Figure 3 E)**. Thus the majority of the OGUs are temperate bacteriophages and not eukaryotic viruses, indicating the association between the virome and colorectal cancer is reliant on bacteriophage communities that can lie dormant in bacterial genomes. These findings are consistent with

previous reports suggesting the colon virome is primarily temperate phages (19, 23, 26, 27).

Influential Bacteria and Phages Associate Indirectly

Because the link between colorectal cancer and the virome was driven by bacteriophages, we hypothesized that the influential phages were predators of the influential bacteria, and thus influenced their relative abundance through predation (e.g. One of the important phages targets *Fusobacterium*). If this hypothesis was true, we would expect that the relative abundance between many important bacteria and phages to be correlated. We correlated the relative abundance values of every phage OGU and bacterial OTU and found strikingly low correlation between the bacterial and phage abundances **(Figure S7 A,C)**. There was an overall absence of correlations between the most influential phage OGUs and bacterial OTUs **(Figure S7 B)**. This evidence supported our null hypothesis that the influential phages are not predators of the influential bacterial.

We further investigated the infectious capabilities and potential host ranges of the influential phages by building a machine learning model to predict which phage OGUs infect which bacteria in the overall community. The predicted interactions were then used to build a network which was used for subsequent analysis. This approach was done as has been previously described (**Cite Network Preprint**). This analysis revealed a wide tropism range for the bacteriophages within the community **(Figure 4 A)**. We calculated the alpha centrality (measure of importance in the ecosystem network) of each phage OGU's connection to the rest of the network, and compared the centrality to the importance of each OGU in the colorectal cancer classification model. We found that phage OGU centrality is significantly positively correlated with importance to the disease model (p-value = 0.0173, rho = 0.14), indicating that phages important in driving colorectal cancer are also community hubs **(Figure 4 B)**.

Influential Phage Identities Shift During CRC Progression

Because our cohort sampled healthy, adenomatous, and cancerous colons, we are able to gain initial insight into virus community shifts during cancer progression. We evaluated the community changes at the two disease transitions (healthy to adenomatous and adenomatous to cancerous) by building individual random forest models to compare only those sample classes. We found that, while bacterial 16S rRNA models perform equally well for all disease class comparisons, the virome models performed worst when seperating healthy from adenomatous, better when seperating adenomatous from cancerous, and the best when seperating healthy from cancerous **(Figure S9 A-B)**. Like bacterial communities, the most important phage OGUs provide insight into which organisms are driving the transitions between disease states. Like bacteria **(Figure**

S9 F-H), different virome members drove the transitions from healthy to adenomatous and adenomatous to cancerous, with one phage exception **(Figure S9 C-E)**. A *Myoviridae* OGU (Cluster 188) was the seventh most important phage driving the transition from a healthy to adenomatous state, and was the most important phage driving the progression from an adenomatous to carcingenic state. Therefore, like bacteria, there are distinct phages assocaited with the transitions from a healthy to an adenomatous colon, and from an adenomatous to a cancerous colon.

Discussion

Because of their propensity for mutagenesis and capacity for modulating their host functionality, many viruses are oncogenic (10–13). Because some bacteria also have oncogenic properties, bacteriophages may play an indirect role in promoting carcinogenesis by altering bacterial communities (7, 8, 17). Despite their carcinogenic potential and the strong association between bacteria and colorectal cancer, the link between virus colon communities (the human colon virome) and colorectal cancer has yet to be evaluated. Here we show that, like colon bacterial communities, the colon virome is associated with colorectal cancer. Our findings support a working hypothesis for oncogenesis by phage-modulated bacterial community composition.

We found that the basic diversity metrics of alpha diversity (richness and Shannon entropy) and beta diversity (Bray-Curtis dissimilarity) were insufficient for identifying virome community changes between healthy and cancerous states. This led us to implement a more sophisticated machine learning approach (random forest classificaiton model) that allowed us to detect strong associations between the colon virus communities and colorectal cancer. Unlike other human cancer types that are often assocaited with human viruses, colorectal cancer is primarily assocaited with altered bacteriophage communities. These phage communities were not predators of the most influential bacteria (e.g. *Fusobacterium* linked to decreases in *Fusobacterium* phages), as demonstrated by the lack of correlation between the abundance of these entities. Additionally, we identified a correlation in which those phages strongly linked to colorectal cancer were also identified as influential hubs within the overall phage-bacteria network, linking the influence of a phage on the community as a whole to its importance to cancer progression. Our previous work has shown that modifying colon bacterial communities with antibiotics alters colorectal cancer progression and tumor burden in mice, and suggests phage-mediated community alterations may have a similar effect, especially if those phages were broadly infectious (6). Together this data supports a model in which the bacteriophage community modulates the bacterial community, and through those interactions indirectly influences the bacteria driving colorectal cancer progression **(Figure 5 A)**.

To better understand the potential mechanisms by which phages modulate the colon bacterial community, we built a working hypothesis that incorporated our findings into the Flynn model for colorectal cancer developement **(Figure 5 B)** (5). We hypothesized that the broadly infectious phages in the colon began lysing, and thereby disrupting, the bacterial communities to open a niche in which opportunistic bacteria were able to colonize, such as *Fusobacterium nucleatum*. Once the "driver" bacteria had established themselves in the epithelium, other opporunistic "passenger" bacteria were able to adhere to the driver, colonize, and begin establishing a biofilm. We feel it is likely that the phages played a role in biofilm dispersal and growth, by lysing bacteria within the biofilm, a process shown to be important for effective biofilm growth. The oncogenic bacteria were then able to transform the epithelial cells and disrupt tight junctions to infiltrate the epithelium, thereby initiating an inflammatory immune response. As the adenomatous polyps developed and progressed towards carcinogenesis, we observed a shift in the phages and bacteria important to our cancer classification model. As the bacteria entered their oncogenic synergy with the epithelium, we hypothesize the phages could continue mediating biofilm dispersal, as well as support the colonized oncogenic bacteria by lysing competing cells to maintain the niche and provide nutrients to the other bacteria. In addition to highlighting the most likely mechanisms by which the colorectal cancer virome is interacting with the bacterial communities, this outline will guide the future, mostly functional studies, by our group and others, into the role the virome plays in colorectal cancer.

Although we have evidence for the interaction between the bacteriophage and bacterial community, we are not able to address whether the phages are directly interacting with the human host **(Figure 5 A)**. Like most foreign entities, bacteriophages are known to be capable of acting as human antigens and thereby initiate or contribute to an immune response. In the context of colorectal cancer, some phages may have been induced out of their bacterial host (most phages were temperate) and interacted with the human host to promote the cancer-associated inflammatory response. Because we are not able to address this behavior with our dataset, we noted it as a known unknown, and another plausible mechanism by which some phages could be associated with colorectal cancer developement.

A notable observation from our analysis was the lack of performance observed using bacterial metagenomic methods compared to the performance of models using viral metagenomes or 16S rRNA gene sequences. This observation highlights the importance of high sequencing coverage in bacterial metagenomic studies, and the advantage of 16S rRNA over whole metagenomic shotgun sequencing. We found that there were six bacterial OTUs that drove the performance of the 16S rRNA classiciation model, and these OTUs were all sparsely present and lowly abundant. Filtration of OTUs with a median relative abundance of zero resulted in the removal of the six important OTUs and reduced model performance to being nearly random like the

bacterial metagenomic model. The bacterial metagenomic OGUs represented only the most abundant taxa, which was uninformative for this application. There has been some success in using shotgun metagenomic approaches for stool colorectal cancer classification, but these approaches did not utilize OGU clustering like we did here, and the models only performed **as well** as the 16S rRNA model. Thus the targeted 16S rRNA sequencing approach, which yielded only a fraction of the bacterial metagenomic sequences, was more effective for detecting colorectal cancer in stool samples. Despite a loss of enthusiasm in favor of shotgun metagenomic techniques, 16S rRNA gene sequencing is still a superior methodological approach for some important applications.

In addition to the therapeutic ramifications for understanding the colorectal cancer microbiome, our findings provide a proof-of-principle that viruses, while under-appreciated and understudied in the human microbiome, are an important contributer to human disease that has the potential to provide an abundance of information that supplements that of bacterial communities. Evidence has suggested that the virome is a crucial component to the microbiome and that bacteriophages are important players. Bacteriophage and bacterial communities cannot thrive without each other (15). Not only is the human virome an important part of human health and disease, but it appears to have a particular significance in cancer research.

Methods

Analysis Source Code & Availability

All associated source code and Makefile are available for review at the following GitHub repository: https://github.com/SchlossLab/Hannigan-2016-ColonCancerVirome.

Study Design and Patient Sampling

This study was approved by the University of Michigan Institutional Review Board and all subjects provided informed consent. Design and sampling of this sample set have been reported previously (7). Briefly, whole evacuated stool was collected from patients who were 18 years of age or older, able to provide informed consent, have had colonoscopy and histologically confirmed colonic disease status, had not had surgery, had not had chemotherapy or radiation, and were free of known co-morbidities including HIV, chronic viral hepatitis, HNPCC, FAP, and inflammatory bowel disease. Samples were collected from four locations: Toronto (Ontario, Canada), Boston (Massachusetts, USA), Houston (Texas, USA), and Ann Arbor (Michigan, USA).

Ninety patients were recruited to the study, thirty of which were designated healthy, thirty with detected adenomas, and thirty with detected carcinomas.

16S Data Acquisition & Processing

The 16S rRNA gene sequences associated with this study were previously reported (7). Sequence (fastq) and metadata files were downloaded from http://www.mothur.org/MicrobiomeBiomarkerCRC. The 16S rRNA gene sequences were analyzed as described previously, relying on the Mothur analytical toolkit (v1.37.0) (28, 29). Briefly, the sequences were de-replicated, screened for chimeras using UCHIME (30) and the SILVA database (31), and binned into operational taxonomic units (OTUS) using a 97% similarity threshold. Abundance was normalized for uneven sequencing depth by randomly sub-sampling to 10,000 sequences, as previously reported (9).

Whole Metagenomic Library Preparation & Sequencing

DNA was extracted from stool samples using the PowerSoil-htp 96 Well Soil DNA Isolation Kit (Mo Bio Laboratories) using an EPMotion 5075 pipetting system. Purified DNA was used to prepare a shotgun sequencing library using the Illumina Nextera XT library preparation kit according to the standard kit protocol. The tagmentation time was increased from five minutes to ten minutes to improve DNA fragment length distribution. The library was sequenced using one lane of the Illumina HiSeq4000 platform and yielded 125 bp paired end reads.

Virus Metagenomic Library Preparation & Sequencing

Genomic DNA was extracted from purified virus-like particles (VLPs) from stool samples, using a modified version of a previously published protocol (25). Briefly, an aliquot of stool (~0.1g) was resuspended in SM buffer and vortexed to facilitate resuspension. The resuspended stool was centrifuged to remove major particulate debris, followed by filtering through a 0.22µm filter to remove smaller contaminants. The filtered supernatant was treated with chloroform to lyse contaminating cells including bacteria, human, fungi, etc. The exposed genomic DNA from the lysed cells was degraded by treating the samples with DNase. The DNA was extracted from the purified VLPs using the Wizard PCR Purification Preparation Kit (Promega). Disease classes were staggered across purification runs to prevent run variation as a confounding factor. Purified DNA was used to prepare a shotgun sequencing library using the Illumina Nextera XT library preparation

kit according to the standard kit protocol. The tagmentation time was increased from five minutes to ten minutes to improve DNA fragment length distribution. The PCR cycle number was increased from twelve to eighteen cycles to address the low biomass of the samples, as has been described previously (25). The library was sequenced using one lane of the Illumina HiSeq4000 platform and yielded 125 bp paired end reads.

Metagenome Quality Control

Both the viral and whole metagenomic sample sets were subjected to the same quality control procedures. The sequences were obtained as de-multiplexed fastq files from the HiSeq platform and subjected to 5' and 3' adapter trimming using the CutAdapt program (v1.9.1) with an error rate of 0.1 and an overlap of 10 (32). The FastX toolkit (v0.0.14) was used to quality trim the reads to a minimum length of 75bp and a minimum quality score of 30 (33). Reads mapping to the human genome were removed using the DeconSeq algorithm (v0.4.3) and default parameters (34).

Contig Assembly & Abundance

Contigs were assembled using paired end read files that were purged of sequences without a corresponding pair (e.g. One read removed due to low quality). The Megahit program (v1.0.6) was used to assemble contigs for each sample using a minimum contig length of 1000 bp and iterating assemblies from 21-mers to 101-mers by 20 (35). Contigs from the virus and whole metagenomic sample sets were concatenated within their respective groups. Abundance of the contigs within each sample was calculated by aligning sequences back to the concatenated contig files using the bowtie2 global aligner (v2.2.1), with a 25 bp seed length and an allowance of one mismatch (36). Abundance was corrected for contig reference length and the number of contigs included in each operational genomic unit. Abundance was also corrected for uneven sampling depth by randomly sub-sampling virome and whole metagenomes to 1e6 and 5e5 reads, respectively, and removing samples with less total samples than the threshold. Thresholds were set for maximizing sequence information while minimizing numbers of lost samples.

Operational Genomic Unit Classification

Much like operational taxonomic units (OGUs) are used as an operational definition of similar 16S rRNA gene sequences in absence of taxonomic identification, we operationally defined closely related contig sequences as operational genomic units (OGUs) in the absence of taxonomic identity. OGUs were defined with the

CONCOCT algorithm (v0.4.0) which bins related contigs by similar tetra-mer and co-abundance profiles within samples using a variational Bayesian approach (37). CONCOCT was used with a length threshold of 1000 bp for virus contigs and 2000 bp for bacteria due to computational limitations.

Diversity

Alpha and beta diversity were calculated using the operational genomic unit abundance profiles for each sample. Sequences were sub-sampled down to 100,000 sequences. Samples with less than the cutoff were removed from the analysis. Alpha diversity was calculated using the Shannon Entropy and Richness metrics. Beta diversity was calculated using the Bray-Curtis metric (mean of 25 random sub-sampling iterations), and the statistical significance between the disease state clusters was assessed using an analysis of similarity (Anosim) with a post-hoc multivariate Tukey test. All diversity calculations were performed in R using the Vegan package [(38).

Classification Modeling

Classification modeling was performed in R using the Caret package (39). OTU and OGU abundance data was preprocessed by removing features (OTUs and OGUs) that were present in less than half of the samples. This served both as an effective feature reduction technique and made the calculations computationally feasible. The binary random forest model was trained using the Area Under the ROC Curve (AUC) and the three-class random forest model was trained using the mean AUC. Both were validated using five-fold cross validation. Each training set was repeated five times, and the model was tuned across five iterations of mtry values. For consistency and accurate comparison between feature groups (e.g. bacteria, virus), the sample model parameters were used for each group. The maximum AUC during training was recorded across 10 iterations of each group model creation to test the significance of the differences between feature set performance. Statistical significance was evaluated using a Wilcoxon test between two categories, or a pairwise Wilcoxon test with Bonferroni corrected p-values when comparing more than two categories.

Taxonomic Identification of Operational Genomic Units

Viral operational genomic units (OGUs) were identified using a reference database consisting of all bacteriophage and eukaryotic virus genomes present in the European Nucleotide Archives. The longest contiguous sequence in each operational genomic unit was used as a representative sequence for classification. Each

representative sequence was aligned to the reference genome database using the tblastx alignment algorithm (v2.2.27) and a strict similarity threshold (e-value < 1e-25) (40). Annotation was interpreted as phage, eukaryotic virus, or unknown.

Ecological Network Analysis & Correlations

The ecological network of the bacterial and phage operational genomic units were constructed and analyzed as previously described (cite network preprint here). Briefly, a random forest model was used to predict interactions between bacterial and phage genomic units, and those interactions were recorded in a graph database using *neo4j* graph databasing software (v2.3.1). The degree of phage centrality was quantified using the alpha centrality metric in the igraph CRAN package. A Spearman correlation was performed between model importance and phage centrality scores.

Phage Replication Style Identification

Phage OGU replication sytle was identified using methods described previously (25, 26, 41). Briefly, we identified lysogenic phage OGUs as representative contigs containing at least one of three genomic markers: 1) phage integrase genes, 2) prophage genes from the ACLAME database, 3) genomic similarity to bacterial reference genomes. Integrase genes were identified in phage OGU representative contigs by aligning the contigs to a reference database of all known phage integrase genes from the Uniprot database (Uniprot search term: "organism:phage gene:int NOT putative"). Prophage genes were identified in the same way, using the ACLAME set of reference prophage genes. In both cases, the blastx algorithm was used with an e-value of 10e-5. Representative contigs were also identified as potential lysogenic phages by having a high genomic similarity to bacterial genomes. To accomplish this, representative phage contigs were aligned to the European Nucleotide Archive bacterial genome reference set using the blastn algorithm (e-value < 10e-25).

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

We thank the members of the Schloss lab for their underlying contributions. GD Hannigan was supported in part by the Michigan Molecular Mechanisms of Microbial Pathogenesis Fellowship.

Figures

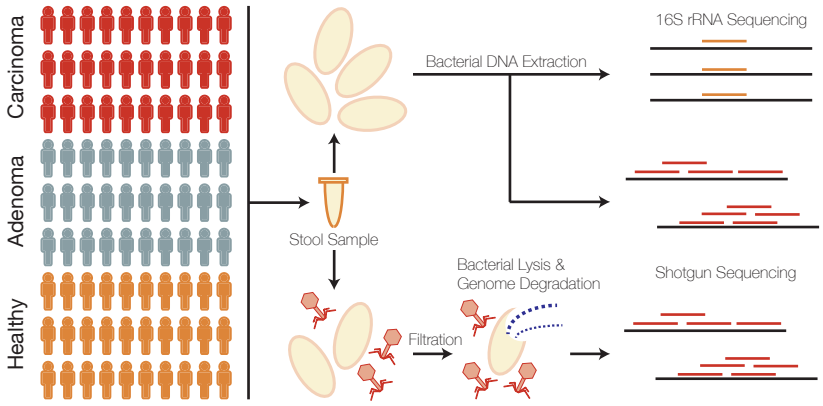

Figure 1: *Cohort and sample processing outline. Thirty subject stool samples were collected from healthy, adenoma (pre-cancer), and carcinoma (cancer) patients. Stool samples were split into two aliquots, the first of which was used for bacterial sequencing and the second which was used for virus sequencing. Bacterial sequencing was done using both 16S rRNA amplicon and whole metagenomic shotgun sequencing techniques. Virus samples were purified for viruses using filtration and a combination of chloroform (bacterial lysis) and DNase (exposed genomic DNA degradation). The resulting encapsulated virus DNA was sequenced using whole metagenomic shotgun sequencing.*

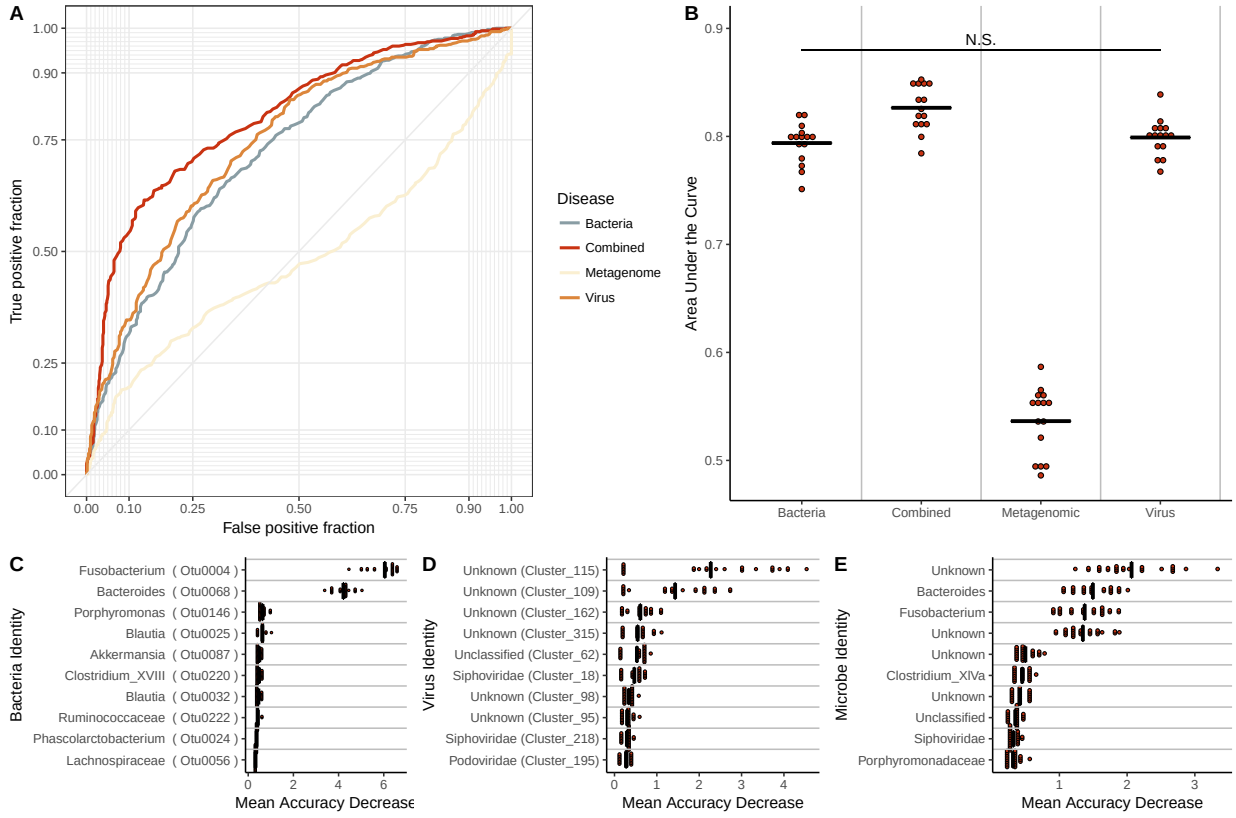

Figure 2: *Results from healthy vs cancer classification models built using virome signatures, bacterial 16S signatures, whole metagenomic signatures, and a combination of virome and 16S signatures. A) ROC curve for visualizing the performance of each of the models for classifying stool as coming from either a cancerous or healthy individual. B) Quantification of the AUC variation for each model, and how it compares to each of the other models based on 15 iterations. A pairwise Wilcoxon test with a Bonferroni multiple hypothesis correction demonstrated that all models are significantly different from each other (p-value < 0.01). C) Mean decrease in accuracy (measurement of importance) of each operational taxonomic unit within the 16S classification model when removed from the classification model. Results based on 25 iterations. OTU features are colored by taxonomic identity. D) Mean decrease in accuracy of each operational genomic unit in the virome classification model. E) Mean decrease in accuracy of each operational genomic unit and operational taxonomic unit in the model using both 16S and virome features.*

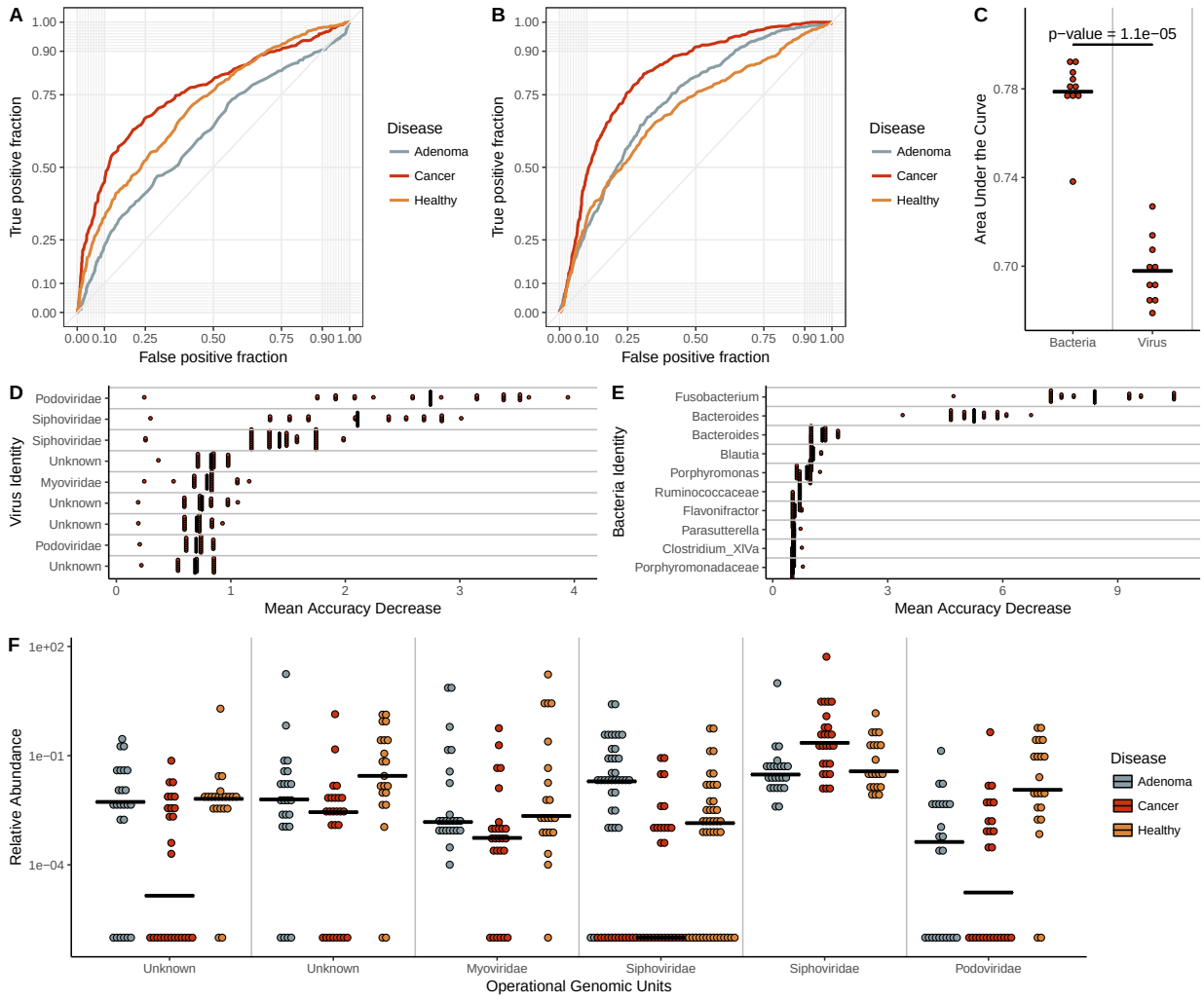

Figure 3: *ROC curves from A) virome and B) bacterial 16S three-class random forest models tuned on mean AUC. Each curve represents the ability of the specified class to be classified against the other two classes. C) Quantification of the mean AUC variation for each model based on 10 model iterations. A pairwise Wilcoxon test with a Bonferroni multiple hypothesis correction demonstrated that the models are significantly different (alpha = 0.01). D) Mean decrease in accuracy when virome operational genomic units and E) bacterial 16S OTUs are removed from the respective three-class classification models. Results based on 25 iterations. F) Relative abundance of the six most important virome OGUs in the model, with the most important on the right. Line indicates abundance mean.*

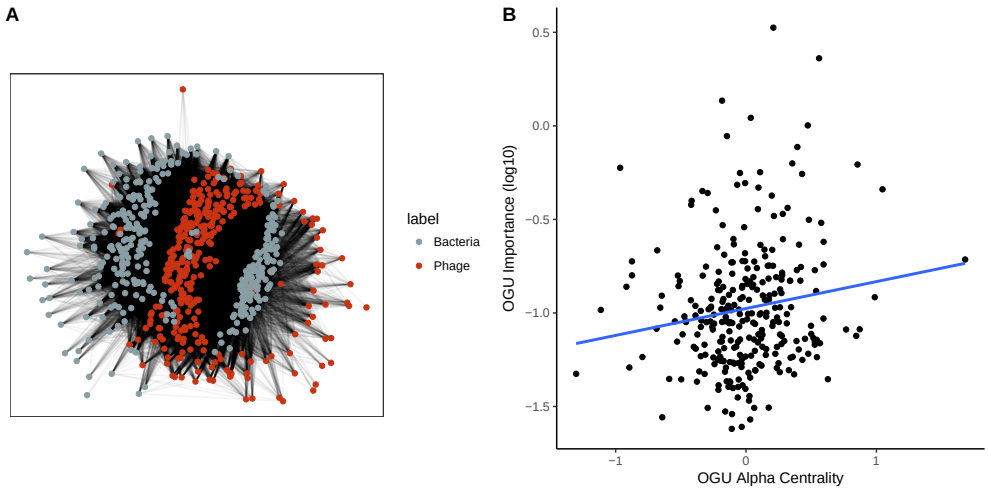

Figure 4: *Community network analysis utilizing predicted interactions between bacteria and phage operational genomic units. A) Visualization of the community network for our colorectal cancer cohort. B) Scatter plot illustrating the correlation between importance (mean decrease in accuracy) and the degree of centrality for each OGU. A linear regression line was fit to illustrate the correlation (blue) which was found to be statistically significantly and weakly correlated (p-value = 0.0173, rho = 0.14).*

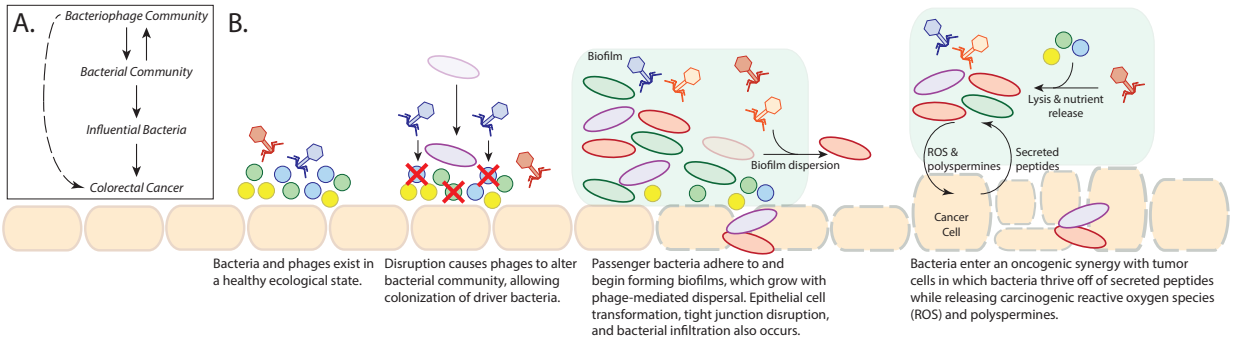

Figure 5: *Working hypothesis of how the bacteriophage community is associated with colorectal cancer and the associated bacterial community.*

Supplemental Figures

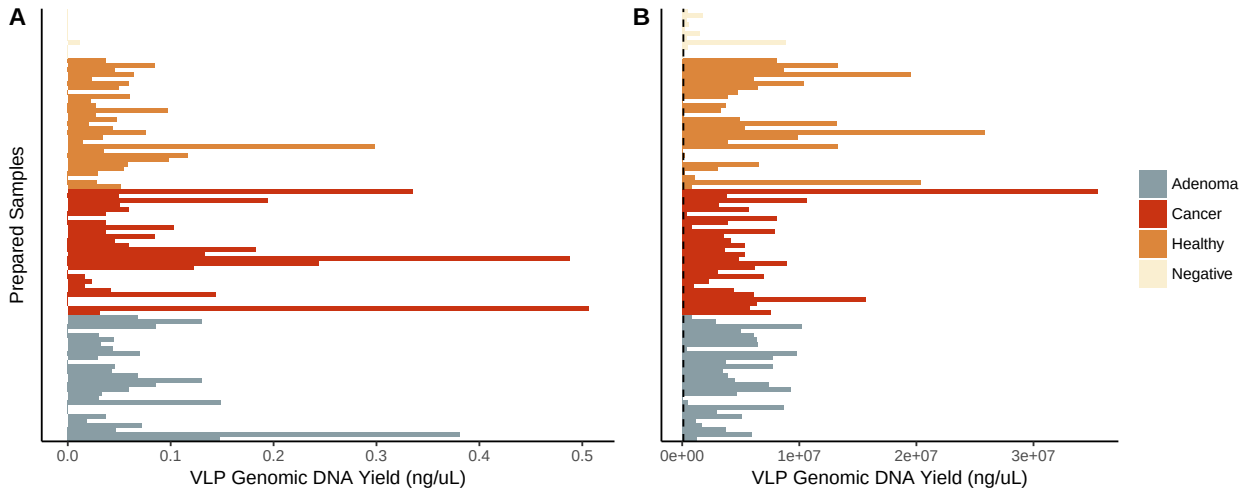

Figure S1: *Basic Quality Control Metrics. A) VLP genomic DNA yield from all sequenced samples. Each bar represents a sample which is grouped and colored by its associated disease group. B) Sequence yield following quality control including quality score filtering and human decontamination.*

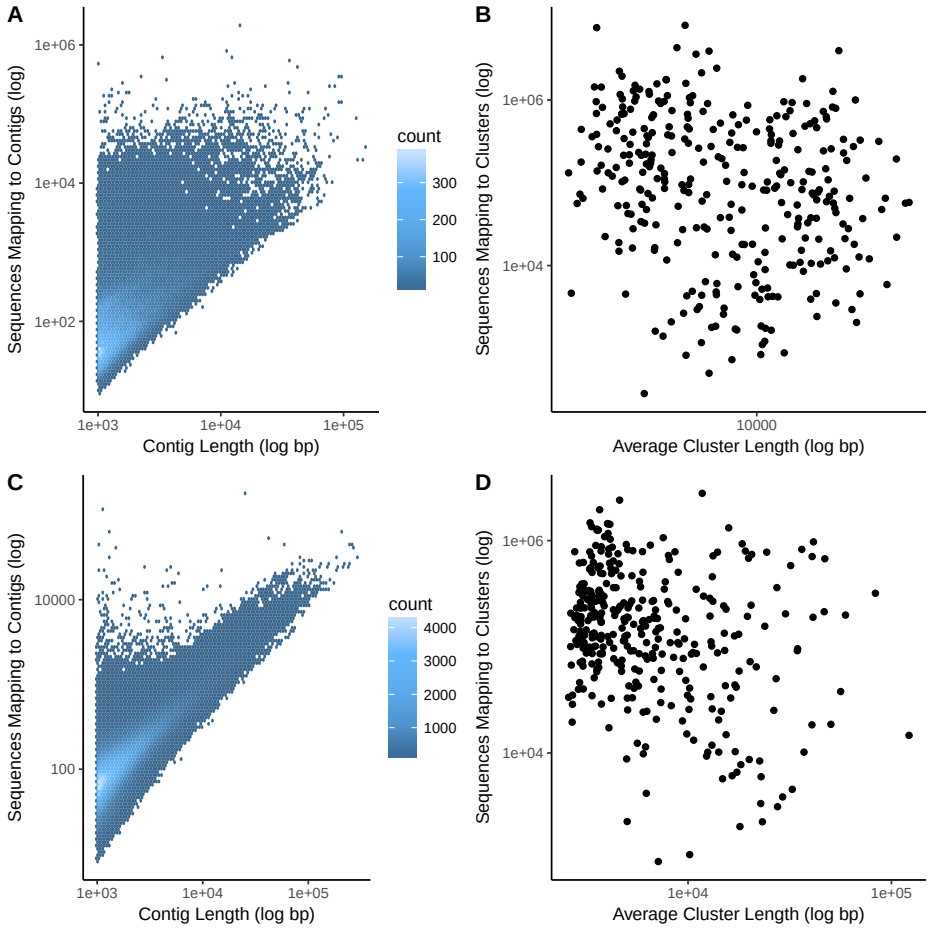

Figure S2: *Length and coverage statistics. A) Heated scatter plot demonstrating the distribution of contig coverage (number of sequences mapping to each contig) and contig length for the virus metagenomic sample set. B) Scatter plot illustrating the distribution of operational genomic unit (OGU) length and sequence coverage for the virus metagenomic sample set. C) Heated scatter plot demonstrating the distribution of contig coverage and length for the whole metagenomic sample set. D) Scatter plot illustrating the distribution of operational genomic unit (OGU) length and sequence coverage for the whole metagenomic sample set.*

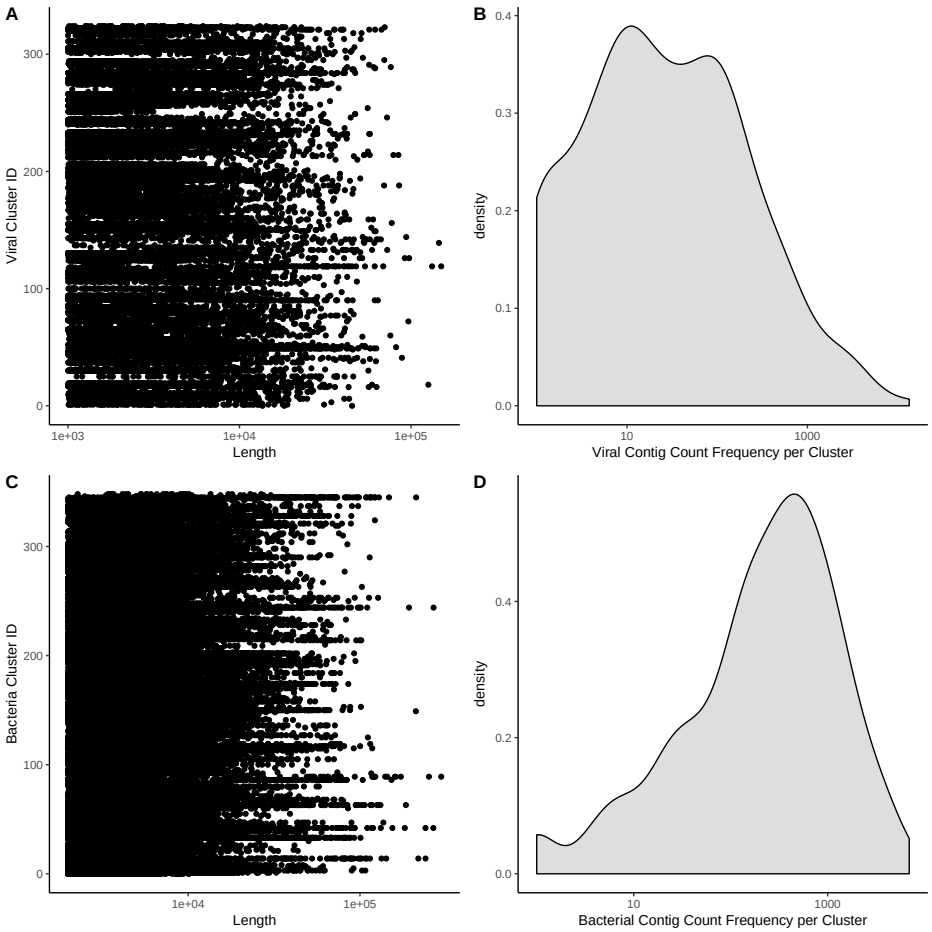

Figure S3: *Operational genomic unit composition stats. A) Strip chart demonstrating the length and frequency of contigs within each operational genomic unit of the virome sample set. The y-axis is the operational genomic unit identifier, and x-axis is the length of each contig, and each dot represents a contig found within the specified operational genomic unit. B) Density plot (analogous to histogram) of the number of virome operational genomic units containing the specific number of contigs, as indicated by the x-axis. C-D) Sample plots as panels C and D, but for the whole metagenomic sample set.*

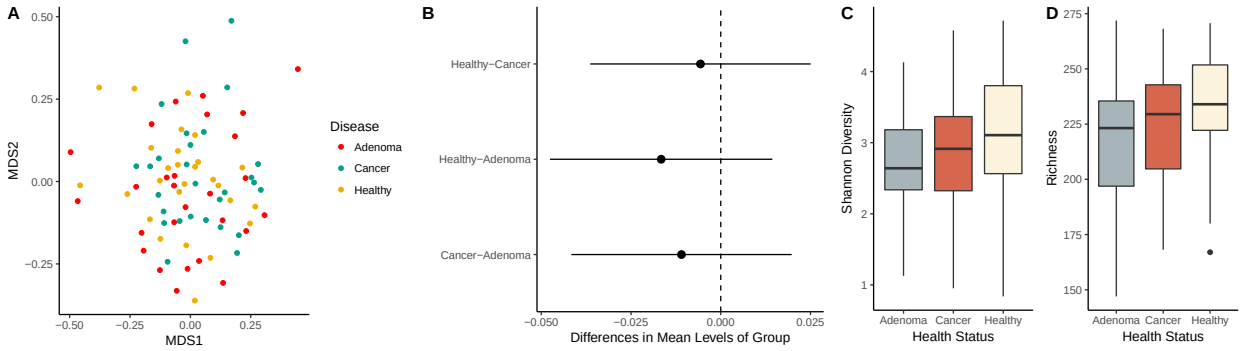

Figure S4: *Diversity calculations comparing cancer states of the colorectal virome, based on relative abundance of operational genomic units in each sample. A) NMDS ordination of community samples, colored for cancerous (green), pre-cancerous (red), and healthy (yellow). B) Differences in means between disease group centroids with 95% confidence intervals based on an Anosim test with a post hoc multivariate Tukey test. Comparisons (indicated on y-axis) in which the intervals cross the zero mean difference line (dashed line) were not significantly different. C) Shannon diversity and D) richness alpha diversity quantification comparing pre-cancerous (grey), cancerous (red), and healthy (tan) states.*

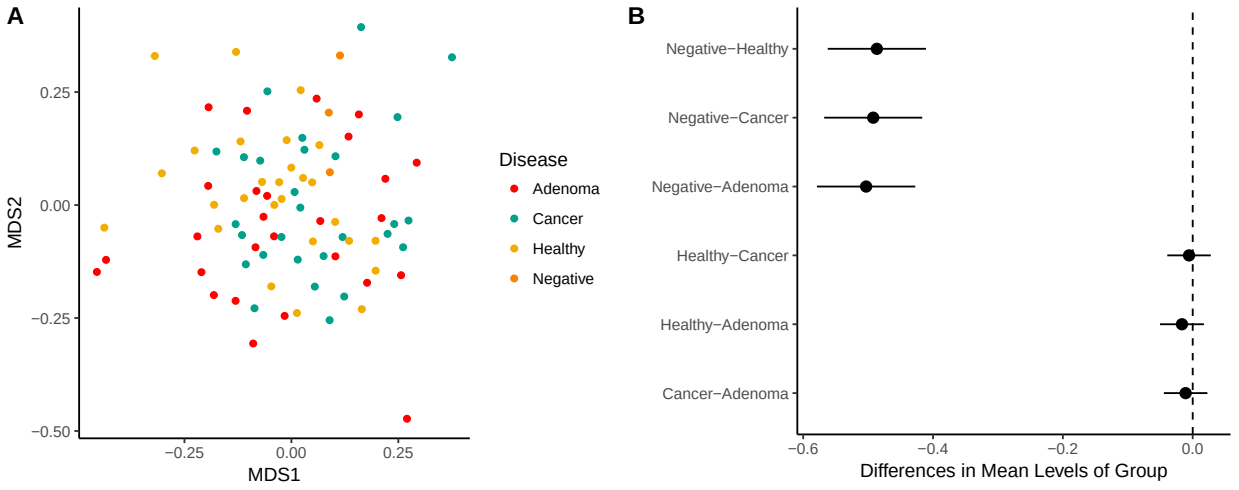

Figure S5: *Beta-diversity comparing disease states and the study negative controls. A) NMDS ordination of community samples, colored by disease state. B) Differences in means between disease group centroids with 95% confidence intervals based on an Anosim test with a post hoc multivariate Tukey test. Comparisons in which the intervals cross the zero mean difference line (dashed line) were not significantly different.*

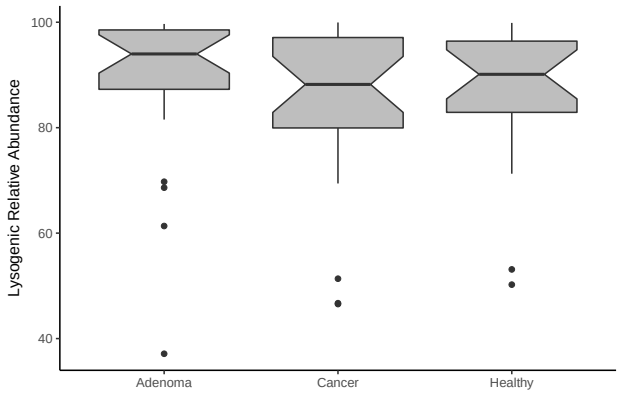

Figure S6: *Lysogenic phage relative abundance in disease states. Phage OGUs were predicted to be either lytic or lysogenic, and the relative abundance of lysogenic phages was quantified and represented as a boxplot. No disease groups were statistically significant.*

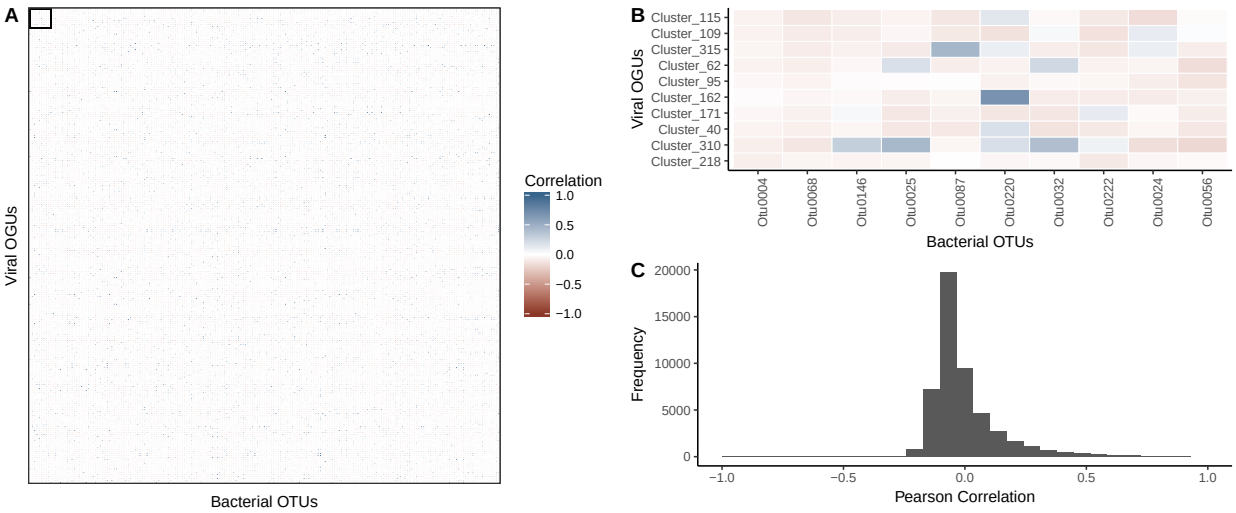

Figure S7: *Relative abundance correlations between bacterial OTUs and virome OGUs. A) Pearson correlation coefficient values between all bacterial OTUs (x-axis) and viral OGUs (y-axis) with blue being positively correlated and red being negatively correlated. Operational units are organized by importance in their colorectal cancer classification models, such that the most important units are in the top left corner. B) Magnification of the boxed region in pannel (A), highlighting the correlation between the most important bacterial OTUs and virome OGUs. The most important operational units are in the top left corner of the heatmap, and the correlation scale is the same as pannel (A). C) Histogram quantifying the frequencies of Pearson correlation coefficients between all bacterial OTUs and virome OGUs.*

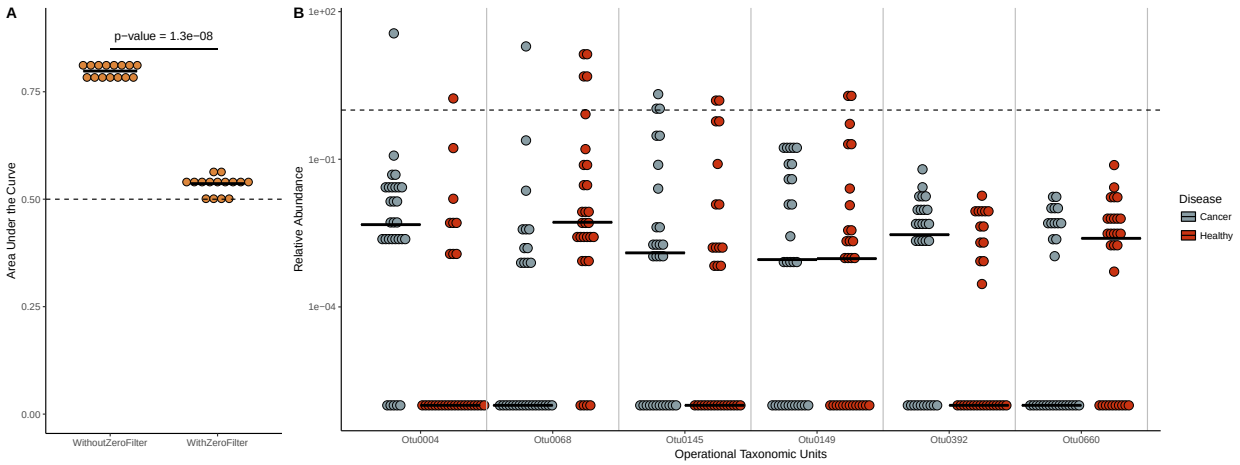

Figure S8: *Comparison of bacterial 16S rRNA classification models with and without OTUs whose median relative abundance are greater than zero. A) Classification model performance (measured as area under the curve) for bacteria models using 16S rRNA data both with and without filtering of samples whose median was zero. Significance was calculated using a Wilcoxon rank sum test, and the resulting p-value is shown. The random area under the curve (0.5) is marked with a dashed line. B) Relative abundance of the six bacterial OTUs removed when filtered for OTUs with median relative abundance of zero. OTU relative abundance is seperated by healthy (red) and cancerous (grey) samples. Relative abundance of 1% is marked by the dashed line.*

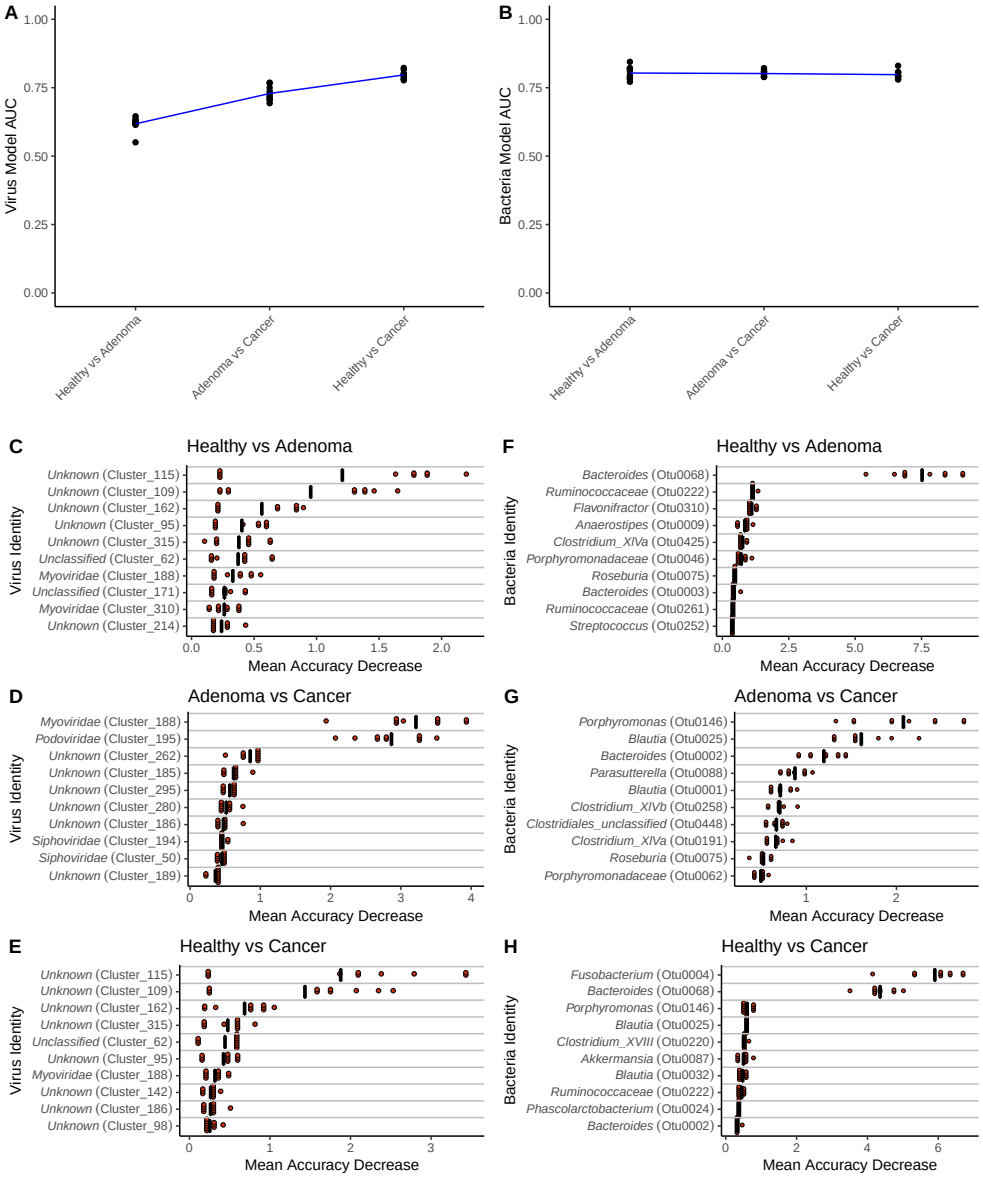

Figure S9: *Transition of colorectal cancer importance through disease progression.*

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