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<b>14. ABSTRACT</b> The <u>purpose</u> of this project is to prevent adverse patient responses to the cancer drug irinotecan by analyzing the gut microbiomes of patients. The <u>scope</u> of this project is to study irinotecan metabolism and the microbiome over time using fecal samples from healthy individuals and metastatic colorectal cancer patients. In this final report, we describe our accomplishments and the impact of our progress. Briefly, we have <b>1)</b> developed novel tools to aid in the discovery of connections between the microbiome and patient phenotypes such as drug metabolism; <b>2)</b> we have revealed novel chemical-chemical interactions in the gut that are relevant to drug metabolism; <b>3)</b> we have for the first time characterized temporal colorectal cancer patient microbiomes during treatment with therapeutic strategies that include the drug irinotecan and are beginning to unravel specific microbiome interactions with adverse patient responses to irinotecan. In <u>total</u> , our research has contributed substantially to the field's understanding of gut microbiome/disease/metabolic interactions in metastatic colorectal cancer patients. Regarding the <u>products</u> resulting from this funding we published an additional three original research papers this year which acknowledged DoD funding, bringing the total number of publications this grant has supported to 15.						
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## **1 INTRODUCTION:**

The microbiome shapes the metabolic and immunological landscape of individuals in health and disease. Its plasticity can be leveraged for therapeutic interventions and to improve therapeutic outcomes. Recent studies have implicated gut microbiome metabolism at the gene and species level in driving the variability in patient drug response and toxicity. One of few therapeutic drugs for which we have a mechanistic understanding of how the gut microbiome influences drug metabolism is the colorectal cancer chemotherapeutic and prodrug irinotecan (CPT-11). CPT-11, in combination with fluorouracil and leucovorin, is one of three first-line treatments for metastatic colorectal cancer. Reactivation of the drug by beta-glucuronidases (BGs) in the gut can lead to severe diarrhea in patients. We hypothesize that individuals with high gut-driven turnover of SN-38G are at heightened risk for ADRs and can be identified via microbiome-based pretherapy analysis. Our overall objective is to identify patients at high risk for adverse events by non-invasive fecal sampling. The results will provide a clinical forecast for therapy in high-risk patients.

## **2 KEYWORDS:**

Colorectal cancer, drug metabolism, microbiome, carbohydrate active enzymes, phase II drug metabolism, metabolomics, metagenomics

## **3 ACCOMPLISHMENTS:**

**What were the major goals of the project?**

**Major Task 1: Quantify CPT-11 metabolites in healthy and metastatic colorectal cancer Patients.**

**Subtasks 1-3**, obtaining IRB/HRPO approval for the studies in healthy and metastatic colorectal cancer patients, were 100% completed in the first three months of the project.

**Subtask 4:** Collect fecal samples from 20 healthy individuals and quantify metabolite production over time. We target 5 samples per individual. Samples will be used for both metabolite analysis and for metagenomic sequencing. Concentrations of SN-38G, SN-38 and the ISTD in the fecal extracts will be determined using the Agilent G6490 Triple Quadrupole Mass Spectrometer. We will examine our mass spectrometry data for any additional, closely structurally related, metabolites of CPT-11 that have not been previously described. We have now collected 46 individual fecal samples from six healthy individuals (~50% COMPLETE).

**Subtask 5:** Collect fecal samples from 20 metastatic colorectal cancer patients, targeting 5 samples per patient, and quantify metabolite production over time per Subtask 1 (~40% COMPLETE, we have collected 39 samples total from six metastatic colorectal cancer patients and have developed an in-house pipeline to quantify metabolite production which has been tested and validated on patient samples.)

**Subtask 6:** Correlate adverse responses in metastatic colorectal cancer patients with metabolite production. Parametric and nonparametric tests will be used to identify significant differences between adverse events quantified as continuous outcomes (number of instances of diarrhea, severity of diarrhea), and chi-square tests will be used to compare categorical outcomes (diarrhea/no diarrhea). All statistical tests will be two-tailed, and p values of less than 0.05 will be considered statistically significant. Analyses will be performed with the R statistical software package.

As of last year this subtask was 50% complete; we have developed and tested R scripts for the statistical analysis and we have developed an in-house pipeline to quantify metabolite production. This procedure was tested and validated on patient samples. Unfortunately over the past year we have not been able to obtain the specific tubes required for protein extraction from the remainder of our samples due to supply chain issues related to the COVID-19 pandemic from MilliporeSigma (Amicon Ultra #C18996237). While we were able to obtain some smaller tubes for testing, they were not appropriate for this complete this subtask. We have unfortunately been expecting to get these tubes in house for the past half year; for that reason we chose not to request a modification to this task.

## **Major Task 2: Quantify beta-glucuronidase abundance and taxonomy in colorectal cancer patients over time.**

**Subtask 1:** Sequence fecal metagenomes of 20 metastatic colorectal cancer patients (from Major Task 1) using Illumina NextSeq sequencing, with a target of 3.5 M paired end reads and 1 Gb sequence per sample (40% COMPLETE, we have sequenced 39 samples taken over time from six metastatic colorectal cancer patients). We were unable to recruit more patients due to the COVID-19 pandemic and the recent departure of our primary clinical collaborator, Dr. Sanjay Goel, to another institution. We will continue working with other clinicians at Einstein to collect more colorectal cancer patient samples.

**Subtask 2:** Correlate beta-glucuronidase abundance with adverse responses to CPT-11 by comparing all reads with our in-house database of beta-glucuronidases and comparing the relative abundance of specific beta-glucuronidases in patients who suffer adverse responses (diarrhea  $\geq$  grade 3) with those patients who do not suffer adverse responses (100% COMPLETE, we have collected adverse response data from patients for which we have DNA samples and our analysis of BGs in these patient samples is described below).

**Major Task 3: Activity-based protein profiling of functionally active human gut microbiome  $\beta$ -glucuronidases.** (25% COMPLETE). Our efforts to overcome the problems with this task were first hampered severely by COVID-19 and then by the death of the head of our Chemical Synthesis core, the wonderful Dr. John Blanchard, with whom we had been collaborating. We therefore shifted to a proteomics-based approach to identify active proteins in

our cohort of mCRC and healthy patient gut microbiomes to overcome these hurdles. We have preliminary data from our collaborator Jacob Waldbauer (U. Chicago) indicating that we can indeed successfully conduct proteomics analyses on our patient samples. However, due to our inability to obtain the MilliporeSigma spin columns (referenced also in Major Task 1), we have not made substantial further headway on this task this year.

**Major Task 4: Quantify microbiome gene expression during SN-38G exposure (25% COMPLETE)** Based on experiments with limited patient fecal protein preparations (before we ran out of spin columns) we know that the timescale for RNA extractions needs to be within one hour of experiment start and that sampling at 30 and 45 minutes will capture the time frame in which drug is metabolized by one individual. We will repeat these experiments for other patients once we have our protein preparation supplies in to ensure we are sampling for RNA sequencing at appropriate time points. At that point we are confident we will be able to complete the transcriptomics work.

### **What was accomplished under these goals?**

As this is the final report for this grant, I will focus on our overall accomplishments and the steps we will take to complete portions of the grant that we were not able to conclude during the funding period. I will describe future work that rests on the foundation of the work reported for this grant. I am very grateful that the DoD/PRCRP saw fit to fund our work and will show you how work from this grant will be utilized to improve outcomes for colorectal cancer patients.

*Per the reviewer comments*, I have substantially revised this report to summarize the entire project, to substantiate progress, and to provide interpretation of the data. I have used the SOW and labeled the progress for each task, I have further indicated where work is published and where it is unpublished. I thank the reviewer for their time and for improving the quality of this report.

### ***Significant accomplishments related to Major Task 1: Quantify CPT-11 metabolites in healthy and metastatic colorectal cancer patients***

#### *Significant published results*

#### Identifying potential food/drug interactions that modulate irinotecan therapy in colorectal cancer.

We have discovered foods and supplements that may interfere with irinotecan metabolism in the gut and have published this work in the journal eLife (Guthrie, et al, 2019). Briefly, we applied our enzyme/drug interaction tool MicrobeFDT to identify potential diet-derived substrates of BGs. We identified a microbial  $\beta$ -glucuronidase potential substrate pool of compounds structurally similar to SN-38G. Identifying the full substrate pool of  $\beta$ -glucuronidases is important for **1)** understanding how diet contributes to  $\beta$ -glucuronidase abundance and expression levels in the gut and **2)** to enable novel therapeutic strategies such as nutritional competition.

We hypothesized that some food compounds may be preferred substrates for microbiome  $\beta$ -glucuronidases which would otherwise deconjugate SN-38G. If true, one could potentially alleviate toxicity associated with the deconjugation of SN-38G via nutritional competition with a preferred substrate. Therefore, we scanned the chemical similarity module containing SN-38G for dietary compounds that may serve as alternative substrates for microbial  $\beta$ -glucuronidases. Most compounds identified as significantly similar to SN-38G were food derivatives or other constituents. Among these targets were flavonoids such as baicalin and scutellarin which are widely distributed in plants. We propose that these compounds may compete with SN-38G for turnover by microbial  $\beta$ -glucuronidases and are a potential avenue for decreasing the adverse drug responses associated with irinotecan administration.

As we note in this publication, a person consuming a large amount of the plant-based compound scutellarin as part of a supplement, for example, might be inadvertently modulating the effects of their cancer therapy. Once we have our backordered protein preparation supplies in house we will test microbiome deconjugation of baicalin and scutellarin and perform competition assays with these compounds and SN-38G.

#### Sulfide as a novel mechanism of microbiome drug metabolism.

Microbial biochemistry shapes a dynamic environment in the gut, yet how common bacterial metabolites such as hydrogen sulfide ( $H_2S$ ) mechanistically alter the gut chemical landscape is poorly understood. We recently demonstrated for the first time that microbially generated  $H_2S$  drives the reduction of azo ( $R-N=N-R'$ ) xenobiotics in bacterial culture, human fecal microbial communities, observations supported by an *in vivo* male mouse model. Changing dietary levels of the  $H_2S$  xenobiotic redox partner Red 40 transiently decreases mouse fecal sulfide, confirming that a xenobiotic can attenuate sulfide concentration *in vivo*. Cryptic  $H_2S$  redox thus can modulate sulfur homeostasis in the gut and at the same time introduces a new mechanism of azo food dye and drug metabolism. Non-enzymatic redox chemistry, here demonstrated via biogenic sulfide azoreduction, is an additional axis upon which the gastrointestinal chemical landscape can be structured. We therefore propose that chemical-chemical interactions derived from microbial community metabolism may be a key missing feature shaping metabolism in the gut. We note that in colorectal cancer, diets high in sulfur (which here we are demonstrating can be converted to sulfide by gut microbes), is associated with increased risk of early-onset colorectal cancer precursors.

Our future work in this project, directly related to the goals of this this grant, will **1)** ask whether sulfide can metabolize SN-38G; **2)** identify microbiome predictors of sulfide concentrations in human guts and; **3)** as we collect more samples from CRC patients, we will quantify sulfide concentrations using approaches described in this manuscript.

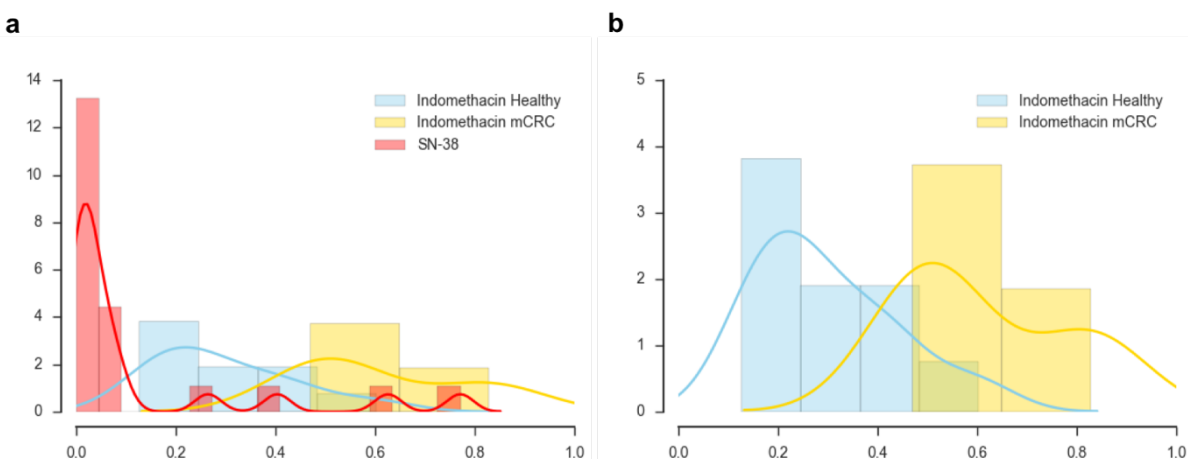
This paper was published in Nature Metabolism at the end of last year (Wolfson SJ, et al, Bacterial hydrogen sulfide drives cryptic redox chemistry in gut microbial communities. *Nat Metab.* 2022 Oct;4(10):1260-1270. doi: 10.1038/s42255-022-00656-z) and was the subject of a commentary by Kiran Patil and colleagues, highlighting the groundbreaking nature of this work and the community's interest in it (<https://www.nature.com/articles/s42255-022-00637-2>)

### Significant unpublished results

#### Preliminary analysis suggests different BG enzymes influence SN-38G metabolism and indomethacin glucuronide metabolism.

Other glucuronidated drugs, such as indomethacin, a nonsteroidal anti-inflammatory drug (NSAID) used to treat pain, can also cause serious dose-related adverse events in the GI tract caused by the inhibition of prostaglandin formation. Similar to the case of morphine, indomethacin also undergoes enterohepatic circulation after deconjugation from an inactivating glucuronide and this increase in drug AUC can lead to severe GI toxicity. It is unknown whether our work on SN-38G would also be relevant to a compound like indomethacin glucuronide, which is structurally distinct from SN-38G. We therefore also examined indomethacin glucuronide metabolism against the backdrop of SN-38G metabolism.

Indomethacin is converted into an inactive metabolite, indomethacin glucuronide, via UDP glucuronosyltransferases in the liver followed by hepatobiliary excretion into the gut, similar to irinotecan. Here we report the range of indomethacin glucuronide hydrolysis across healthy and metastatic colorectal cancer patient fecal microbiomes and discuss potential interpersonal variation in the exposure of GI cells to free indomethacin. Our preliminary findings indicate the metastatic colorectal cancer patient fecal microbiomes have significantly higher microbiota mediated turnover of indomethacin glucuronide (Fig. 1). Our data support the hypothesis that interpersonal variation in microbiota glucuronide hydrolysis activity is dependent on variations in therapeutic drug glucuronide fine structure.



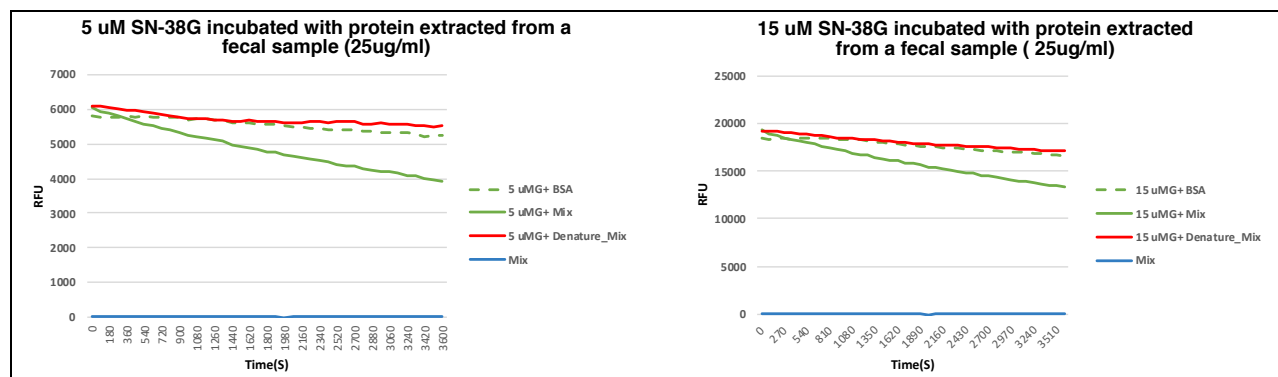
**Figure 1. Fecal microbiome based metabotypes for SN-38 and indomethacin formation**

**a** We previously identified two distinct metabolizer phenotypes or ‘metabotypes’ based on % SN-38 formation during a time course incubation of SN-38G with fecal samples from 20 individuals quantified by LC-MS/MS (Guthrie, et al, 2019). Participants were sub-grouped into low (n=16) and high (n=4) metabolizer phenotypes (red distribution). All samples were run in triplicate and values are the mean  $\pm$  sem. Subsequently, we quantified indomethacin formation during a time course incubation with indomethacin glucuronide with fecal samples following the same protocol with the following key differences: This study included 22 healthy individuals (blue distribution) and 3 time-points from a study participant with metastatic colorectal cancer (yellow distribution). **b** We identify two distinct metabotypes based on indomethacin formation between healthy individuals (n = 22) and the participant with mCRC

(n=3). All samples from the participant with mCRC were taken after the treatment regimen with irinotecan began.

### Development of a rapid, inexpensive, in-house, spectrophotometric approach to quantify microbiome metabolism of SN-38G.

Ruth Hauptman, an MSTP student supported on this grant, implemented a protocol from the Redinbo group (Jariwala et. al., *ACS Chemical Biology*, 2020) that allows us to record the concentration of SN-38G over time using a spectrophotometer. Because SN-38G is fluorogenic we can measure the relative fluorescent units (RFU) that it emits and compare it to the RFU of standards with a known concentration. At excitation 260 and emission 450 the RFU of SN-38G is ~200 fold greater than SN-38; this allows us to distinguish between the two metabolites using a spectrophotometer. When SN-38G is converted into SN-38 the RFU is decreased. We have assessed this protocol with two different concentrations of SN-38G incubated with protein extracted from fecal samples and get clear separation between the experiment (SN-38G+active fecal protein) and negative controls (SN-38G+denatured fecal protein, SN-38G+BSA, active protein alone). Based on these results we are confident that we can get reliable SN-38G concentrations using this method. It will both improve our throughput, as we can do it in house, and it is substantially cheaper than our former LC-MS/MS methods (**Fig. 2**).



**Figure 2. LCMS strategy for assessing SN-38G metabolism by fecal samples.** In the above graphs there are two concentrations of SN-38G (left, 5uM; right 15 uM) incubated with the same density of protein (25ug/ml) extracted from a fecal sample. The green solid line is SN-38G incubated with an intact protein mix extracted from a fecal sample (this is the experiment). The red line is the SN-38G incubated with a denatured protein extracted from a sample (negative control 1). The green dashed line is SN-38G incubated with BSA instead of intact protein (negative control 2). The blue line is intact protein mix without SN-38G (negative control 3).

### ***Significant accomplishments related to Major Task 2: Quantify beta-glucuronidase abundance and taxonomy in colorectal cancer patients over time.***

#### Significant published results

#### Identification of metastable states in timeseries microbiome data.

In anticipation of our sequencing data for the temporal microbiome samples from colorectal cancer patients, we developed a novel computational approach borrowed from physics,

topological data analysis, to identify metastable states and state transitions in microbiome data that are linked to patient outcomes (Chang, VanInsberghe, and Kelly, npj Biofilms and Microbiomes, 2020). In this publication, we show that gut disease processes in cholera are associated with transitions between community states, defined as topological features of the data density, in the microbiome. We find a reproducible two-state succession during recovery from cholera in the gut microbiomes of multiple patients and evidence of dynamic stability in the gut microbiome of a healthy human after experiencing diarrhea during travel. For our patient dataset we hypothesize that, despite the tremendous variability in individual patient guts (see below for details), we may be able to find compositions that indicate adverse event associated diarrhea.

### Oral microbes as a hallmark of diseases, including CRC, in the gut.

Case-control studies have identified human gut microbiome taxa associated with diseases including diabetes, inflammatory bowel, and colorectal cancer. However, many taxa are significantly associated with different diseases, confounding the utility of taxa as disease-specific diagnostics and potentially indicating underlying similarities in physiologically distinct diseases. Using a meta-analysis of 4446 gut metagenomic samples linked to 55 diseases across 21 datasets, we identify taxa consistently associated with health or disease and validate these results on external datasets. We find that taxa associated with disease in the gut are either oral microbiome commensals or are members of the polyphyletic class Clostridiales, whereas healthy taxa are gut specialists. Prevalence of organisms in the oral cavity correlates with gut disease association, revealing a novel, conserved relationship between disease and the gut microbiome. Our work highlights a major limitation of traditional metagenomics analysis methods for identifying disease-specific markers, a key goal of many microbiome studies, including our study in this grant which focuses on metastatic colorectal cancer patients undergoing treatment with irinotecan. With these newly identified oral microbes we will scan our mCRC patient microbiomes to see if oral microbes are a signature that link these very diverse temporal patient microbiomes. This paper is released as a preprint (<https://www.researchsquare.com/article/rs-1631596/v1>), we will submit it for peer review shortly.

### Significant unpublished results

In the past year we have conducted analyses of our metagenomic dataset of temporal whole community microbiomes sampled from patients undergoing treatment protocols for colorectal cancer that include the drug irinotecan. To our knowledge, this is the **first dataset of its kind** and I will discuss its utility in considering the role of the microbiome in adverse events related to irinotecan therapy.

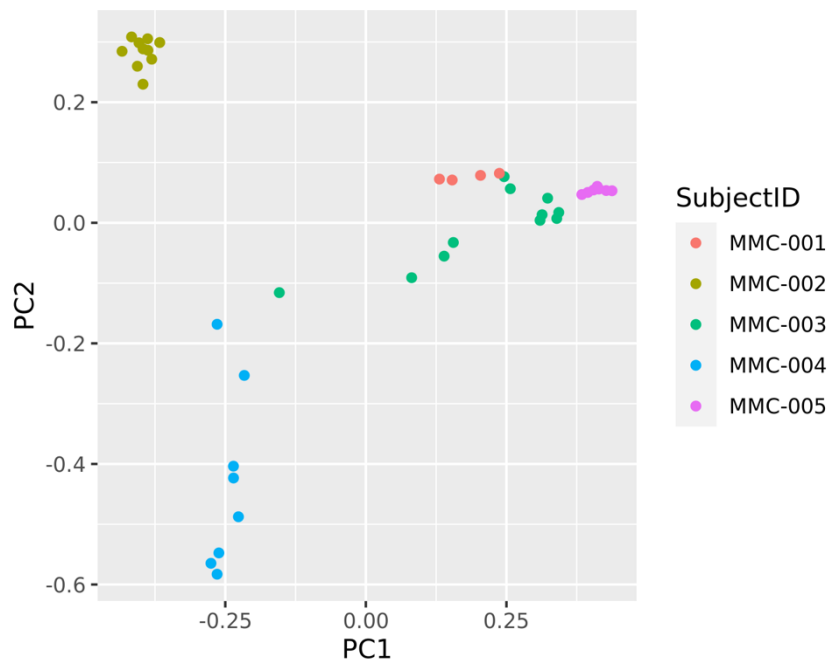
### Diversity in microbiomes reflects individual patients and not mCRC in our dataset.

Our work (Khan and Kelly, 2020) and that of many others demonstrates that CRC can be predicted from microbiome data. Therefore, in our CRC patients we first asked: are there similarities in CRC patient microbiomes that outweigh individual differences?

We analyzed 34 microbiome samples from 5 CRC patients. Sequence data was processed using the KneadData (<https://huttenhower.sph.harvard.edu/kneaddata/>), MetaPhlAn3

(<https://huttenhower.sph.harvard.edu/metaphlan/>) and HUMaAnN3 (<https://huttenhower.sph.harvard.edu/humann/>) pipelines from the Huttenhower lab. Briefly, these packages, considered state-of-the-art in the microbiome field, 1) clean sequence data (removing human reads, adaptor and barcoding sequences); 2) provide taxonomic profiles of each sample; 3) and provide functional profiles of each sample, respectively.

We find that, in most cases, microbiomes from an individual patient look more similar to each other than they do to other patient microbiomes, despite all patients having metastatic CRC (**Fig 3**). With the exception of patients 1 and 3 (red and green, respectively) who have samples that group very closely, other patient samples are spread out in Bray-Curtis distance space. We note that patient 2 (gold) and patient 5 (purple) each have samples that group very tightly together whereas patients 3 (green), 4 (blue), and 1 (red) are more spread out, indicating more diversity in their samples over time.



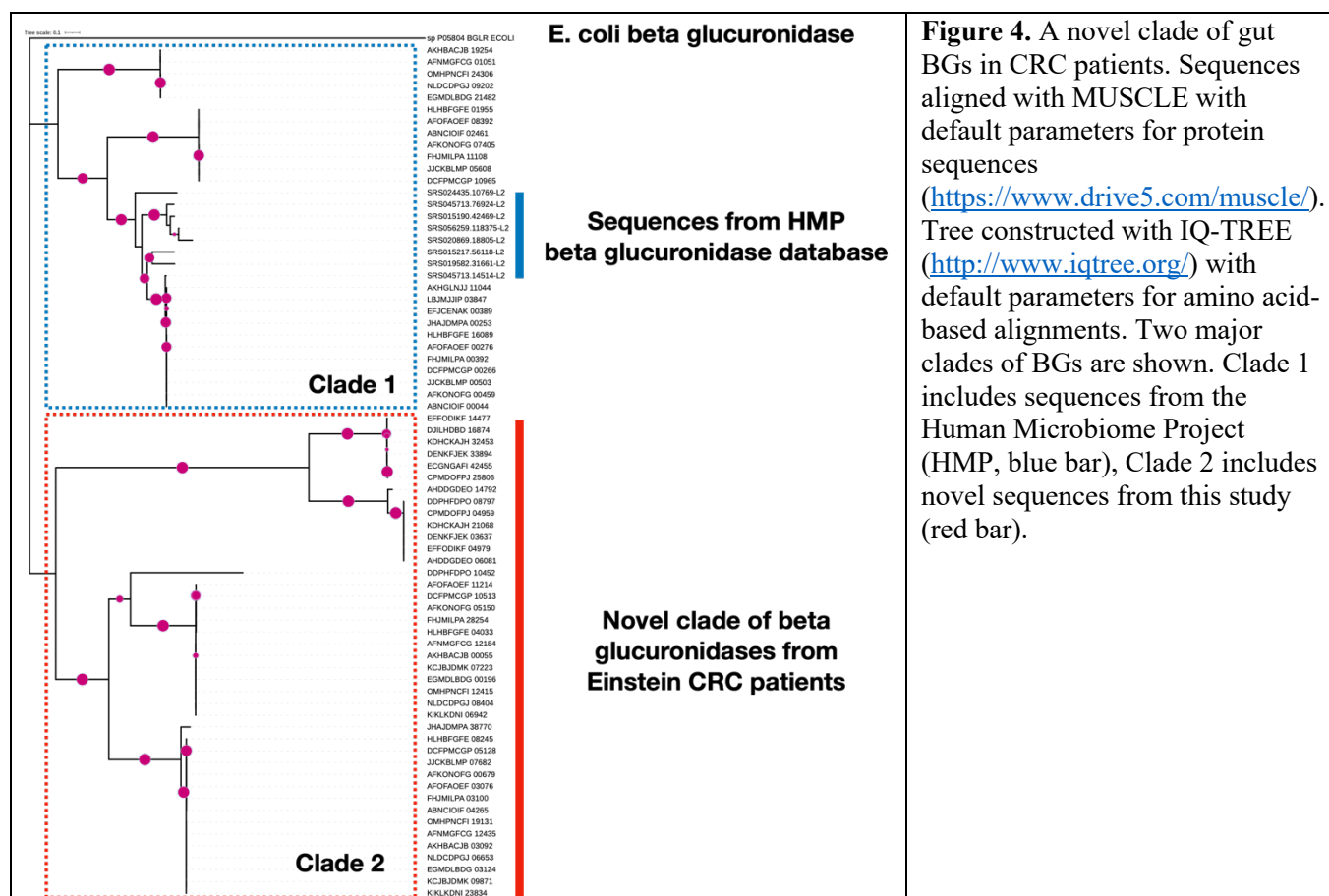
**Figure 3.** Bray-Curtis dissimilarity of microbiome samples from five metastatic CRC patients taken over the course of treatment.

### Novel BGs identified in Einstein CRC patient metagenomes expand previously identified microbiome BGs.

The Redinbo lab at UNC has done exemplary work characterizing the structural features of diverse BG sequences that associate with activity against irinotecan. They have identified seven primary BG structural classes. The five most common are referred to as NL (non-loop); L1 (loop 1); L2 (loop 2); mL1 (mini-loop 1); mL2 (mini-loop 2), per Pollet et al 2017 (<https://doi.org/10.1016/j.str.2017.05.003>). We downloaded the Redinbo lab's database of 279 human gut microbiome BGs and used blastp with a stringent cutoff of e-40 to identify all BG-like sequences in our CRC dataset.

We next assessed whether our patient metagenomes contained novel BG sequences. We first ensure that all sequences from our patients contained the NKG sequence motif, a requirement for BG function per Redinbo lab studies. We next generated a phylogenetic tree comparing our sequences to known BG sequences identified in the Human Microbiome Project (HMP) patient metagenomes. We identified a novel clade of BGs from our patients (Fig 4, red bar) that are sequence distinct from BGs characterized in the HMP.

These BGs were not limited to a single patient; rather, they formed subclades of sequences with high phylogenetic similarity across multiple patients, strongly suggesting that these sequences are conserved across individuals. Next steps will be to conduct similar analyses in metagenomic datasets collected from other colorectal cancer patients including ~300 samples available in the curatedMetagenomicDataset R package.

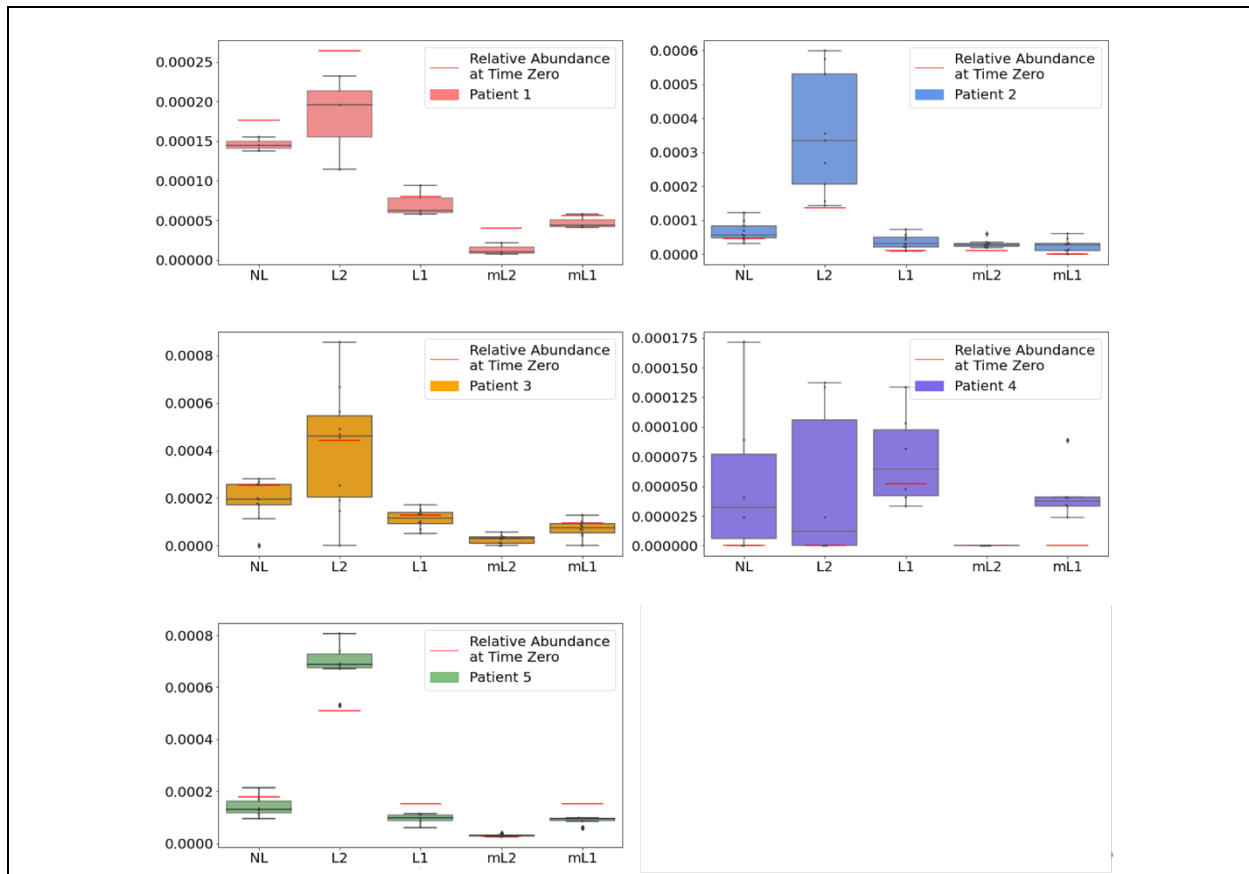


**Figure 4.** A novel clade of gut BGs in CRC patients. Sequences aligned with MUSCLE with default parameters for protein sequences (<https://www.drive5.com/muscle/>). Tree constructed with IQ-TREE (<http://www.iqtree.org/>) with default parameters for amino acid-based alignments. Two major clades of BGs are shown. Clade 1 includes sequences from the Human Microbiome Project (HMP, blue bar), Clade 2 includes novel sequences from this study (red bar).

Microbiome dynamics are an important, rarely studied, feature of colorectal cancer patient microbiomes.

We next asked what the distribution of each of the BG types across our CRC patients were over time. In some patients (for example, Pt 1), there was a major decrease in some BG types over the course of treatment. For this patient, we see a decrease in NL, L2, and mL2, which BG types L1 and mL1 remain similar over time (Fig. 5). Again, these results are not consistent between

patients; some patients have apparent expansions of particular BGs in their microbiomes but not other patients. Our work indicates that microbiome dynamics are an important, rarely studied, feature of colorectal cancer patient microbiomes.



**Figure 5. Distribution of BG types across CRC patients.** The relative abundance of BG types NL, L2, L1, mL2, and mL1 is displayed for all time points available for each individual patient. Relative abundance at time zero, the first collection, for each BG type in each patient is shown as a red line.

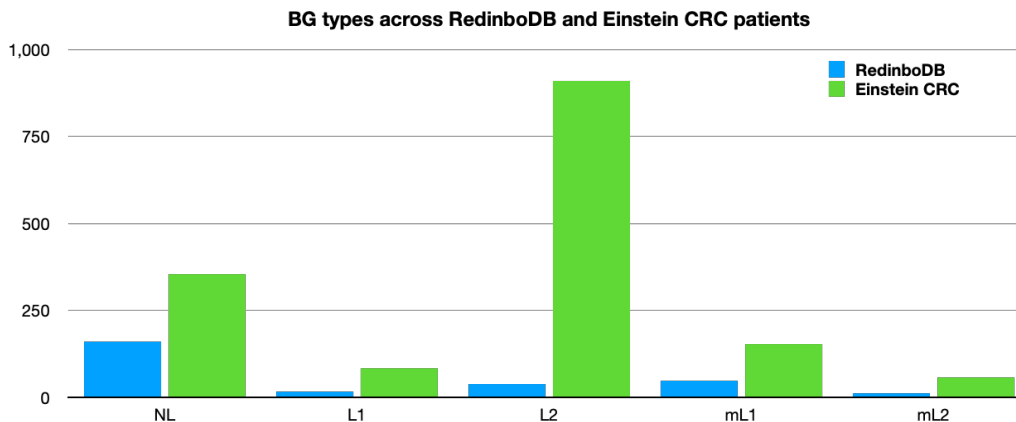
*Related to Major Tasks 3 and 4 (functional analysis of BGs in healthy and mCRC patients).*

Significant unpublished results

Differences in the comparative abundance of functionally active BG sequence types in Einstein CRC patients vs. healthy Human Microbiome Project patients.

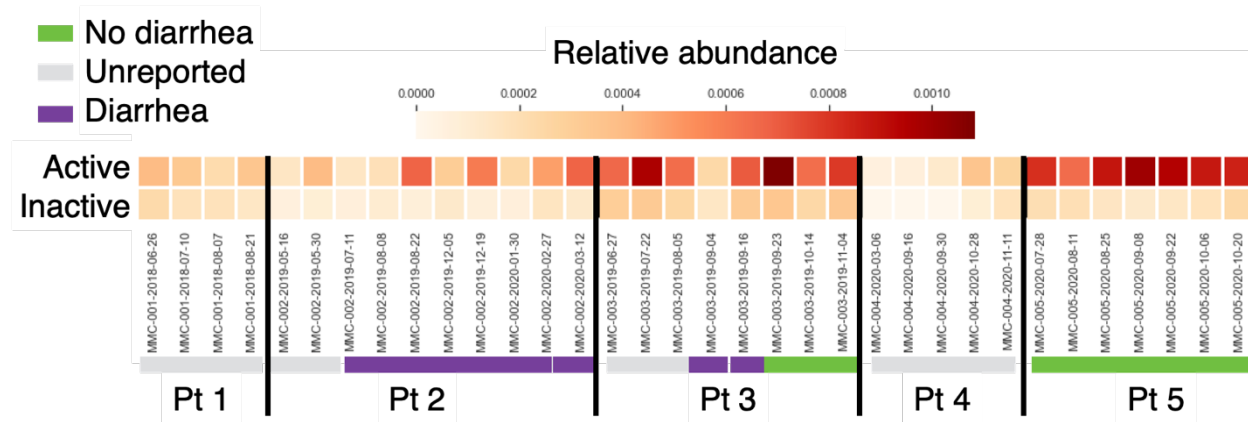
In Pollet et al, most identified human gut microbiome BG sequences were NL-type (Fig. 6). We were surprised to find that in our patients, L2, which represents ~12-14% of the sequences in human microbiomes studied by the Redinbo lab, represents ~58% of the BG sequences in our patient dataset. This is a **key finding** of this project because L2 BG sequences are known to metabolize SN38-G; for example, a representative sequence of the L2-type BG, in *Bacteroidetes uniformis*, has been shown to metabolize SN38-G (10.1073/pnas.1918095117). Coupled with our observation here that L2-type BGs are common in CRC patients (present in 34 of 39, or 87%, of

samples for which we have metagenomic sequencing) our work suggests that L2 enzymes could represent unrecognized, key players in SN38-G metabolism in patients receiving irinotecan.



**Figure 6. Beta-glucuronidase (BG) types identified in a comprehensive human gut microbiome database of BGs and in Einstein CRC patients from this grant.** Five different BG types are indicated: NL (non-loop); L1 (loop 1) ; L2 (loop 2); mL1 (mini-loop 1); and mL2 (mini-loop 2).

We next asked if drug active BG sequences as a group, defined by the Redinbo group as L1, L2, mL1, and mL2), were more or less abundant in patient samples than the inactive, NL sequences. We find that in our CRC patients, active BG sequences are more abundant compared to inactive BG sequences in almost every sample considered (**Fig. 7**). Of particular interest are Patients 2, 3, and 5, who all have abundant active BG sequences in their metagenomes. We hypothesize that these patients are the most likely to be high metabolizers and will test this hypothesis when we receive our spin columns. These preliminary results are relevant to **Major Task 3** where we will attempt to characterize functionally active human gut microbiome  $\beta$ -glucuronidases.



**Figure 7. SN38-G metabolizing, active, BGs are more abundant in individual CRC patient metagenomes than inactive BGs.** Active and Inactive BGs are identified per Pollet et al 2017. Individual CRC patient samples (Pt) are organized by time of sampling (earliest is furthest left).

Further work is necessary to determine whether L2-type BGs, or active BGs generally, are a common feature in CRC patients outside our dataset. If L2-type BGs are more abundant in CRC patients, these enzymes could represent a specific target to ameliorate adverse events associated

with irinotecan. Future efforts in our lab will conduct similar analyses in metagenomic datasets collected from other colorectal cancer patients including ~300 samples available in the curatedMetagenomicDataset R package.

### Complex interactions between active BG abundance and diarrhea in CRC patients.

A second key finding of our work is our data suggests that knowing the active BG composition of a CRC patient gut is not sufficient to predict diarrhea. While we hypothesized that BG composition alone might be a predictor of patient adverse events, here our data demonstrates in some patients, increased active BG abundances are seen during adverse events (**Fig. 7, Pt 2**); whereas in others, such as Patient 5 (**Fig. 7, Pt 5**), a patient can have prevalent active BGs without experiencing diarrhea.

### **Major review publications.**

We were invited to write a review for Annual Reviews in Pharmacology and Toxicology about microbiome contributions to adverse events and we discuss in part the potential for microbiome interventions to improve drug and treatment safety and efficacy in colorectal cancer (Khan, Hauptman, and Kelly, Ann Rev Pharm Tox, 2021). Since publication this review has already been cited 15 times, indicating community interest in approaches to target the microbiome to improve patient outcomes and treatment safety and efficacy for a variety of diseases.

We were invited to write a review to describe the difficulties and potential benefits of translating basic research on microbiome drug metabolism into the clinic and have published this work in the journal eBioMedicine (Guthrie and Kelly, 2019). This review has been cited 41 times, indicating substantial community interest in microbiome drug metabolism.

We were invited to write a review that gives pharmacologists a framework for understanding how microbial enzymes can metabolize drugs and have published this work in the journal Trends in Pharmacological Sciences (Hitchings and Kelly, 2019). This review has been cited 31 times and we hope that it is bringing microbiome research to pharmacologists to improve communication and collaboration between our fields.

### **Commentaries.**

We were invited to write a commentary on harnessing the microbiome to improve drug therapy (Kelly, Clin Pharmacol Ther, 2019). We were invited to write a commentary on a paper describing microbial metabolism of L-dopa for Cell Metabolism (Hitchings and Kelly, Cell Metab, 2019). We were invited to write a commentary on a paper on bioaccumulation as a mechanism of microbiome/drug interactions (Cohen and Kelly, Trends in Microbiology, 2022).

### **Additional research contributions.**

To better predict the likelihood of a patient suffering an adverse event based solely on his or her microbiome, we developed, tested, and validated three machine learning approaches to predict clinical outcomes based on microbiome data (Khan and Kelly, Pac Symp Biocomput, 2020).

In support of our patient population in the Bronx, NY our lab was involved in two studies that attempted to characterize the COVID-19 pandemic in the Bronx genomically and to predict patient outcomes based on clinical and demographic data to improve patient care. **1)** The Bronx was an early epicenter of the COVID-19 pandemic in the USA. We conducted temporal genomic surveillance of SARS-CoV-2 genomes across the Bronx from March-October 2020. Mapping the trajectories of variants, we found that while some have become ‘endemic’ to the Bronx, other, novel variants rose in prevalence in the late summer/early fall. Geographically resolved genomes enabled us to distinguish between a case of reinfection and a case of persistent infection. **2)** The clinical outcome of SARS-CoV-2 infection varies widely between individuals. Machine learning (ML) models can support decision making in healthcare by assessing fatality risk in patients that do not yet show severe signs of COVID-19. Most predictive models rely on static demographic features and clinical values obtained upon hospitalization. However, time-dependent biomarkers associated with severity, such as antibody titers, can contribute to the development of more accurate outcome models. ML models developed by my lab trained on immune biomarkers, longitudinally monitored throughout hospitalization, predicted mortality and were more accurate than models based on demographic and clinical data upon hospital admission.

Finally, I contributed genome sequence analysis to a paper from my mentor for this grant, Dr. Sridhar Mani, that demonstrated for the first time that swarming behavior in bacteria is enriched during intestinal stress and can ameliorate intestinal damage in mice (De, et al, Gastroenterology 2021).

### **Training opportunities and achievements.**

The career development opportunities provided by this grant have greatly improved my (Dr. Kelly) training and credentials as a translational microbiome scientist. ***All subtasks in the training goal were met or exceeded.***

#### *Subtask 1: Attend a scientific research workshop*

Dr. Kelly was one of 25 scientists selected to attend the National Cancer Institute’s Innovation Lab: Systems Biology for the Cancer Microbiome from April 29-May 3, 2019 at the Beaver Hollow Conference Center in Java Center, NY. This intensive, weeklong workshop that brought together experts from a wide variety of fields to form new collaborations, ideate and refine new projects, and identify opportunities to accelerate research on the influence of the microbiome in cancer using systems approaches. This innovation lab resulted in the team publication “The Cancer Microbiome: Distinguishing Direct and Indirect Effects Requires a Systemic View”, listed below, that has been cited 131 times (Google Scholar).

#### *Subtask 2: Present at the Albert Einstein Cancer Center seminar series*

In lieu of presenting at the Albert Einstein Cancer Center seminar series, Dr. Kelly presented her work for the Einstein Faculty work in progress seminar series which includes all members of the Albert Einstein College of Medicine Cancer Center faculty as well as the rest of the faculty at Albert Einstein College of Medicine in October, 2018.

*Subtask 3: Attend a national microbiology meeting*

*Subtask 4: Attend a national cancer research meeting*

Dr. Kelly has attended, presented at, and run symposia for numerous, competitive, international meetings during this grant. Dr. Kelly attended the International Society for Microbial Ecology's 2018 and 2022 meetings, the American Society for Microbiology's (ASM) 2018 and 2019 and 2021 annual meetings, and a 2019 Keystone microbiome meeting (Microbiome: Chemical Mechanisms and Biological Consequences), which had an emphasis on cancer drug metabolism by the microbiome. She gave invited talks at four of these meetings (Keystone, ASM 2018, ISME 2018, ISME 2022) and ran symposia at the other two (ASM 2019, ASM 2021).

*Subtask 5: Attend a translational research workshop*

Dr. Kelly attended the competitive American Association for Cancer Research Translational Cancer Research for Basic Scientists Workshop in November 2019. This training experience was phenomenal and instrumental in thinking about how we might translate our work on microbiome drug metabolism into the clinic.

*Subtask 6: Meet monthly with Dr. Mani*

Dr. Kelly and Dr. Mani have met monthly, Dr. Kelly was a co-author on a recent publication that Dr. Mani was senior author on titled "Bacterial Swarms Enriched During Intestinal Stress Ameliorate Damage." *Gastroenterology*. 2021 Jul;161(1):211-224. doi: 10.1053/j.gastro.2021.03.017.

**What do you plan to do during the next reporting period to accomplish the goals?**

This is the final report. Nothing to report

#### **4 Impact**

***What was the impact on the development of the principal discipline(s) of the project?***

The fundamental goal of this grant was to prevent adverse patient responses to cancer chemotherapeutics. In support of this goal we have **1)** developed novel tools to aid in the discovery of connections between the microbiome and patient phenotypes such as drug metabolism; **2)** we have revealed novel chemical-chemical interactions in the gut that are relevant to drug metabolism; **3)** we have for the first time characterized temporal colorectal cancer patient microbiomes during treatment with therapeutic strategies that include the drug irinotecan and are beginning to unravel specific microbiome interactions with adverse patient responses to irinotecan. **In total, our research has contributed substantially to the field's understanding of gut microbiome/disease/metabolic interactions in colorectal cancer**

In addition to our fundamental research contributions, we have contributed a number of reviews on gut microbiome drug metabolism and associations with adverse events that bring this

important field to other areas of medicine, including pharmacology, which traditionally has had little interaction with the microbiome. I describe the impact of this work below.

Our research demonstrating that machine learning/artificial intelligence approaches can reliably and robustly classify disease in a difficult, multi-class, classification setting lays the foundation for routine clinical use of the microbiome as a diagnostic (see Products; Khan and Kelly 2020)

Our novel, topological data analysis-based approach to analyzing microbiome time-series data identifies ‘states’, that is, microbiome compositions, and state transitions in microbiomes that can be linked to clinical outcomes such as adverse drug responses. As noted above, our approach overcomes several challenges in microbial time series analysis: 1) it makes minimal assumptions regarding biological mechanisms, and is applicable to systems as diverse as the human gut and the ocean; 2) it uses all available information regarding the similarity between samples of microbial communities; and 3) it inherently scales with the amount and dimensionality of data. We therefore anticipate that many other microbiome studies will benefit from this approach. All code for this project is publicly available at the Kelly Lab Github (<https://github.com/kellylab>). (see Products; Chang, VanIsenberghe, and Kelly 2020)

We developed the first network linking foods, drugs, microbiome enzymes, and patient outcomes, called MicrobeFDT. We used MicrobeFDT to identify compounds structurally similar to the conjugated, detoxified irinotecan metabolite SN-38G and found dietary substrates that may interact with similar BGs that this drug interacts with. Structurally similar compounds may act competitively - via inhibition of SN-38G turnover by higher priority BG substrates or synergistically - via substrate inducible transcriptional upregulation of BG enzymes. A person consuming a large amount of the plant-based compound scutellarin as part of a supplement, for example, might be inadvertently modulating the effects of their cancer therapy. This finding could impact on how patients are instructed in terms of diet while taking irinotecan; our work suggests that a novel method to decrease adverse drug responses may be to modify a patient’s diet. (see Products; Guthrie, Wolfson, and Kelly, 2019)

Our characterization of sulfide as a reducing agent in the gut is paradigm shifting for the field of microbial metabolism in the human body. Gut microbiome food, drug, and endogenous compound (collectively, ‘xenobiotic’) metabolism is a topic of intense interest. Work from our group and others demonstrates that microbes are nature’s biochemists, able to interact with an amazing variety of structurally diverse compounds. Here we report a novel mechanism of microbial xenobiotic metabolism. Currently, the paradigm for how microbes interact with xenobiotics is considered entirely enzymatic. For example, metabolism of the common food dye Red 40 was previously assumed to be enzymatic, carried out by azoreductase enzymes. In this work we reveal for the first time that microbially produced sulfide can reduce Red 40, a novel, non-enzymatic, mechanism for microbiome xenobiotic metabolism. Our experiments using abiotic, pure culture, fecal slurry, and *in vivo* mouse experiments, show that gut chemistry, derived from microbial community function, is a key missing feature that shapes xenobiotic metabolism in the gut. More broadly, our mouse models suggest that azo compounds can be used to tune sulfide concentrations. Altering sulfide homeostasis in the gut is a novel, completely unexplored, way to interface with diseases such as colorectal cancer, in which a high sulfur diet was associated with colorectal cancer risk (Wang, et al 2021, JAMA Netw Open.

2021;4(11):e2134308. doi:10.1001/jamanetworkopen.2021.34308). (see Products; Wolfson et al, 2022)

Our identification of oral microbes as hallmarks of gut disease has far reaching consequences for microbiome studies. Case-control studies have identified human gut microbiome taxa associated with diseases including diabetes, inflammatory bowel, and colorectal cancer. However, many taxa are significantly associated with different diseases, confounding the utility of taxa as disease-specific diagnostics and potentially indicating underlying similarities in physiologically distinct diseases. Using a meta-analysis of 4446 gut metagenomic samples linked to 55 diseases across 21 datasets, we identify taxa consistently associated with health or disease and validate these results on external datasets. We find that taxa associated with disease in the gut are either oral microbiome commensals or are members of the polyphyletic class Clostridiales, whereas healthy taxa are gut specialists. Prevalence of organisms in the oral cavity correlates with gut disease association, revealing a novel, conserved relationship between disease and the gut microbiome. Our work highlights a major limitation of traditional metagenomics analysis methods for identifying disease-specific markers, a key goal of many microbiome studies, and is a key step towards identifying the specific microbes that define colorectal cancer (see Products; Khan and Kelly, 2022).

Our temporal, metadata annotated, whole community metagenomic sequence data from colorectal cancer patients receiving treatment regimens that include irinotecan is a unique resource for both the colorectal cancer and the microbiome scientific communities. We anticipate that this dataset will be widely used and distributed and we hope that it will contribute substantially to our understanding of microbiome drug metabolism in colorectal cancer.

Our identification of novel, unreported BGs in colorectal cancer patients is impactful because it widens our understanding of what BGs look like in the human body. Although we do not know yet if these enzymes are functional or how widespread they are in colorectal cancer patients generally, this new group of enzymes expands the foundations from which we study and attempt to predict microbiome impacts on adverse drug responses in colorectal cancer patients taking irinotecan, the major goal of this grant.

### ***What was the impact on other disciplines?***

We anticipate that the MicrobeFDT database, discussed above, will be relevant to pharmacologists, clinical researchers, GI clinicians, and drug developers.

We have contributed a number of reviews on gut microbiome drug metabolism and associations with adverse events that bring this important field to other areas of medicine, including pharmacology, which traditionally has had little interaction with the microbiome. These reviews will help to connect areas of medicine and, we hope, spur novel research directions.

### ***What was the impact on technology transfer?***

There is no impact on technology transfer.

***What was the impact on society beyond science and technology?***

Nothing to report.

**5 Changes/Problems**

***Changes in approach and reasons for change***

Nothing to report.

***Actual or anticipated problems or delays and actions or plans to resolve them***

As noted above we anticipate finally getting in the needed supplies to complete our drug metabolism studies, which are ready to go. We have had success extracting RNA from test fecal samples and we understand the timescale in which to do our transcriptomics assays once we have drug metabolism data for all of our samples. We anticipate no further problems with these goals.

***Changes that had a significant impact on expenditures.***

No significant changes

**Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

No significant changes.

**6 Products**

We are proud to report that **15 publications from the Kelly lab acknowledge this grant**. Two additional publications are listed for which we were not allowed to acknowledge the grant but are relevant to microbiome drug metabolism and the microbiome. All papers are accessible via DOI links below and we have therefore not attached any individual papers to the appendix.

**Publications**

**Preprints**

Oral Microbes are a Signature of Disease in the Gut Saad Khan and Libusha Kelly, 24 May 2022, PREPRINT (Version 1) available at Research Square [<https://doi.org/10.21203/rs.3.rs-1631596/v1>] Federal support acknowledged? YES

## **Peer reviewed**

Wolfson SJ, Hitchings R, Peregrina K, Cohen Z, Khan S, Yilmaz T, Malena M, Goluch ED, Augenlicht L, Kelly L. Bacterial hydrogen sulfide drives cryptic redox chemistry in gut microbial communities. *Nat Metab.* 2022 Oct;4(10):1260-1270. doi: 10.1038/s42255-022-00656-z. Epub 2022 Oct 20. PMID: 36266544. Federal support acknowledged? YES

Genomic surveillance of SARS-CoV-2 during the first year of the pandemic in the Bronx enabled clinical and epidemiological inference. Fels JM, Khan S, Forster R, Skalina KA, Sirichand S, Fox AS, Bergman A, Mitchell WB, Wolgast LR, Szymczak WA, Bortz RH 3rd, Dieterle ME, Florez C, Haslwanter D, Jangra RK, Laudermilch E, Wirchnianski AS, Barnhill J, Goldman DL, Khine H, Goldstein DY, Daily JP, Chandran K, Kelly L. *Cold Spring Harb Mol Case Stud.* 2022 Jul 13:mcs.a006211. doi: 10.1101/mcs.a006211. Epub ahead of print. PMID: 35831070. Federal support acknowledged? YES

Longitudinally monitored immune biomarkers predict the timing of COVID-19 outcomes. Lasso G, Khan S, Allen SA, Mariano M, Florez C, Orner EP, Quiroz JA, Quevedo G, Massimi A, Hegde A, Wirchnianski AS, Bortz RH 3rd, Malonis RJ, Georgiev GI, Tong K, Herrera NG, Morano NC, Garforth SJ, Malaviya A, Khokhar A, Laudermilch E, Dieterle ME, Fels JM, Haslwanter D, Jangra RK, Barnhill J, Almo SC, Chandran K, Lai JR, Kelly L, Daily JP, Vergnolle O. *PLoS Comput Biol.* 2022 Jan 18;18(1):e1009778. doi: 10.1371/journal.pcbi.1009778. PMID: 35041647; PMCID: PMC8812869. Federal support acknowledged? YES

Bioaccumulation as a mechanism of microbiome/drug interactions. Cohen Z, Kelly L. *Trends Microbiol.* 2022 Feb;30(2):99-101. doi: 10.1016/j.tim.2021.12.003. Epub 2021 Dec 21. PMID: 34952771. Federal support acknowledged? YES

Degradation of host translational machinery drives tRNA acquisition in viruses. Yang JY, Fang W, Miranda-Sanchez F, Brown JM, Kauffman KM, Acevero CM, Bartel DP, Polz MF\*, Kelly L\*. *Cell Syst.* 2021 Jun 15:S2405-4712(21)00205-2. doi: 10.1016/j.cels.2021.05.019. PMID: 34143976. Federal support acknowledged: YES

Bacterial Swarms Enriched During Intestinal Stress Ameliorate Damage. De A, Chen W, Li H, Wright JR, Lamendella R, Lukin DJ, Szymczak WA, Sun K, Kelly L, Ghosh S, Kearns DB, He Z, Jobin C, Luo X, Byju A, Chatterjee S, San Yeoh B, Vijay-Kumar M, Tang JX, Prajapati M, Bartnikas TB, Mani S. *Gastroenterology.* 2021 Jul;161(1):211-224. doi: 10.1053/j.gastro.2021.03.017. PMID: 33741315. Federal support acknowledged: YES

Finding phenazine. Kelly L, Wolfson SJ. *Elife.* 2020 Oct 27;9:e62983. doi: 10.7554/eLife.62983. PMID: 33108273. Federal support acknowledged: NO

The Cancer Microbiome: Distinguishing Direct and Indirect Effects Requires a Systemic View. Xavier JB, Young VB, Skufca J, Ginty F, Testerman T, Pearson AT, Macklin P, Mitchell A, Shmulevich I, Xie L, Caporaso JG, Crandall KA, Simone NL, Godoy-

Vitorino F, Griffin TJ, Whiteson KL, Gustafson HH, Slade DJ, Schmidt TM, Walther-Antonio MRS, Korem T, Webb-Robertson BM, Styczynski MP, Johnson WE, Jobin C, Ridlon JM, Koh AY, Yu M, Kelly L, Wargo JA. Trends Cancer. 2020 Mar;6(3):192-204. doi: 10.1016/j.trecan.2020.01.004. Epub 2020 Feb 7. Review. PubMed PMID: 32101723; PubMed Central PMCID: PMC7098063. Federal support acknowledged: NO

Multiclass Disease Classification from Microbial Whole Community Metagenomes. Khan S, Kelly L. Pac Symp Biocomput. 2020;25:55-66. PubMed PMID: 31797586; PubMed Central PMCID: PMC7120658. Federal support acknowledged: YES

Drug Metabolism as a Community Effort. Hitchings R, Kelly L. Cell Metab. 2019 Aug 6;30(2):235-237. doi: 10.1016/j.cmet.2019.07.005. PubMed PMID: 31390549. Federal support acknowledged: YES

Harnessing the Microbiome to Improve Drug Therapy. Kelly L. Clin Pharmacol Ther. 2019 Aug;106(2):287-289. doi: 10.1002/cpt.1510. PubMed PMID: 3135545915 Federal support acknowledged: YES

Engineering the Microbiome to Prevent Adverse Events: Challenges and Opportunities. Khan S, Hauptman R, Kelly L. Annu Rev Pharmacol Toxicol. 2021 Jan 6;61:159-179. doi: 10.1146/annurev-pharmtox-031620-031509. Epub 2020 Oct 13. PMID: 33049161; PMCID: PMC9015100. Federal support acknowledged: YES

Topological analysis reveals state transitions in human gut and marine bacterial communities. Chang WK, VanInsberghe D, Kelly L. NPJ Biofilms Microbiomes. 2020 Oct 14;6(1):41. doi: 10.1038/s41522-020-00145-9. PMID: 33057043; PMCID: PMC7560872. Federal support acknowledged: YES

The human gut chemical landscape predicts microbe-mediated biotransformation of foods and drugs. Guthrie L, Wolfson S, Kelly L. Elife. 2019 Jun 11;8. pii: e42866. doi: 10.7554/eLife.42866. PubMed PMID: 31184303; PubMed Central PMCID: PMC6559788. acknowledgement of federal support: YES

Predicting and Understanding the Human Microbiome's Impact on Pharmacology. Hitchings R, Kelly L. Trends Pharmacol Sci. 2019 Jun 3. pii: S0165-6147(19)30091-4. doi: 10.1016/j.tips.2019.04.014. [Epub ahead of print] Review. PubMed PMID: 31171383. acknowledgement of federal support: YES

Bringing microbiome-drug interaction research into the clinic. Guthrie L, Kelly L. EBioMedicine. 2019 May 28. pii: S2352-3964(19)30310-X. doi: 10.1016/j.ebiom.2019.05.009. [Epub ahead of print] Review. PubMed PMID: 31151933. acknowledgement of federal support: YES

## **Presentations.**

I report here on highlights of presentations across the grant and include new presentations from this past year.

**Invited plenary speaker**, American Society for Microbiology (ASM) annual, June 2018

**Invited speaker**, Keystone Symposium: Microbiome: Chemical Mechanisms and Biological Consequences, March 2018

**Invited speaker**, American Society for Clinical Pharmacology & Therapeutics Annual Meeting. Science at Sunrise: Catching a glimpse of gut microbiome-drug interactions: what clinical pharmacologists need to know?, March 2019

**Keynote speaker**, University of Chicago Microbiome Symposium, April 2019

**Invited speaker**, American Society for Clinical Pharmacology & Therapeutics Annual Meeting. Science at Sunrise: Catching a glimpse of gut microbiome-drug interactions: what clinical pharmacologists need to know? March 2019

**Invited speaker**, Eleventh International Workshop on Pharmacodynamics of Anticancer Agents (Sept 8-12, 2019, Chateau des Vigiers, Monestier, France). External seminar and colloquia

**Invited speaker**, Pharmaceutical Sciences and Pharmacogenomics Seminar Series, University of California at San Francisco (Dec 4, 2019, San Francisco, California)

**Invited speaker**, SLAC National Accelerator Laboratory (Dec 2, 2019, Palo Alto, California)

**Invited speaker**, Pharmacology 2020, British Pharmacological Society, December 14-18, 2020 (Zoom)

**Symposium leader**, “What makes a microbiome ‘healthy’?” World Microbe Forum, American Society for Microbiology and the Federation of European Microbiological Societies, June 20-24, 2021 (Zoom)

**Invited speaker**, Cornell-Weill Institute for Computational Biology seminar series, Cornell Weill, New York, NY, October 2021

**Invited speaker**, Harvard Microbial Sciences Initiative 19<sup>th</sup> Annual Symposium, Harvard University, Cambridge, Massachusetts, April 2022

**Invited speaker**, ISME18: 18<sup>th</sup> International Symposium on Microbial Ecology, Lausanne, Switzerland, August 2022

***Website(s) or other Internet site(s)***

Kelly lab Github:

<https://github.com/kellylab>

This site is the repository for code and data used in all published analyses.

***Technologies or techniques***

Nothing to report.

***Inventions, patent applications, and/or licenses***

Nothing to report.

***Other Products***

Nothing to report.

**7 Participants & Other Collaborating Organizations**

*Participants on this grant over the full duration of the grant:*

**Name:** Libusha Kelly (PI)

**Name:** Ruth Hauptman (Graduate student)

**Name:** Leah Guthrie (Graduate student)

**Funding Support for Kelly:**

Irma T. Hirschl Career Scientist Award  
Kelly (PI)  
03/10/2022-03/10/2026

Elsa U. Pardee Foundation  
Kelly (PI)  
03/01/22-03/01/23

*Testing molecular features of the pathobiont flusobacterium nucleatum as an early diagnostic for colorectal cancer*

NIH NHLBI R01HL069438-21  
Kelly (PI)  
09/20/2021-09/20/2025

*In vivo mechanisms mediating sickle cell vaso-occlusion and organ damage*

## **8 Special Reporting Requirements**

*None to report.*

## **9 Appendices**

*None to report.*