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14. ABSTRACT

The purpose of this project is to prevent adverse patient responses to the cancer drug irinotecan by analyzing the gut microbiomes of patients. The scope of this project is to study irinotecan metabolism and the microbiome over time using fecal samples from healthy individuals and metastatic colorectal cancer patients. We have several major findings from the past year of work. We observe that beta-glucuronidase (BGs) enzyme distribution differs between healthy and a metastatic colorectal cancer patient gut, and we highlight colorectal cancer-specific BGs that may be good biomarkers or targets for predicting or altering metabolism of the colorectal cancer drug irinotecan. We have enrolled colorectal cancer patients and collected samples. We have demonstrated that irinotecan metabolism in fecal samples does not overlap with that of another glucuronidated drug, indomethacin. 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). We have written 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). We have written 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).

15. SUBJECT TERMS
Colorectal cancer, drug metabolism, microbiome, carbohydrate active enzymes, phase II drug metabolism, metabolomics, metagenomics

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1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   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:** *Provide a brief list of keywords (limit to 20 words).*

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

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

   What were the major goals of the project?

   For all major tasks I have included only subtasks that were designed to be completed within the first 12 months of the project.

   Major Task 1: Quantify CPT-11 metabolites in healthy and metatstatic colorectal cancer patients

   Subtask 1: Obtain secondary use protocol specific for analysis for the already approved IRB protocol (IRB#2013-2895). Secondary analysis of data collected retrospectively and prospectively under 2013-2985 would be a standalone submission and would fall under expedited category 5 **(COMPLETE)**

   Subtask 2: Obtain IRB approval to collect fecal samples from metastatic colorectal cancer patients taking irinotecan. Documents have been submitted to the Einstein IRB for approval **(COMPLETE)**

   Subtask 3: Obtain secondary use protocol specific for analysis for metastatic colorectal cancer patients taking irinotecan. Secondary analysis of data collected prospectively under the metastatic colorectal cancer patient IRB would be a standalone submission and would fall under expedited category 5 **(COMPLETE)**

Subtask 3.1: Obtain HRPO approval for the above secondary use protocols **(COMPLETE)**

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. For both Subtask 4 and Subtask 5, concentrations of SN-38G, SN-38 and the ISTD in the fecal extracts will be determined used, 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 **(~25% COMPLETE, we have collected 22 individual fecal samples from healthy individuals)**

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 **(~8 % COMPLETE, we have collected eight samples total from three metastatic colorectal cancer patients)**

*Milestone(s) Achieved: Characterization of variability in CPT-11 metabolite production in healthy individuals **(~10% COMPLETE)***

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 **(10% COMPLETE)**

Major Task 3: Activity-based protein profiling of functionally active human gut microbiome β-glucuronidases (0% COMPLETE, the problems with this task are discussed below)

Subtask 1: Optimize synthesis of a custom fluorescently labeled SN-38G probe in collaboration with the Einstein Chemical Synthesis core. We currently have low, impure yields that are not yet sufficient for our experiments

Subtask 2: Validate uptake of SN38-G labeled probe using positive and negative controls. Positive control: *E. coli* strain ATCC 25922 which can convert SN-38G to S38. Negative control: *E. coli* strain BW18812 (ΔuidA), which lacks the BG gene and thus should not convert SN-38G to S38G. Successfully sort labeled cells via flow cytometry

Subtask 3: Optimize flow sorting of fecal samples; specifically identify optimal sample concentrations and buffer conditions to reliably sort these very heterogeneous samples. Optimize sorting of cells that uptake the labeled probe by defining appropriate parameterizations for sorting and by quantifying the populations of cells that have taken up the labeled probe

Major Task 4: Quantify microbiome gene expression during SN-38G exposure. (0% COMPLETE, the problems with this task are discussed below)

Subtask 1: Amend fresh fecal samples from the same 6 healthy volunteers referenced in Major Task 3, 3 high and 3 low metabolizers, with SN-38G, extract RNA at timepoints corresponding to known metabolism of SN-38G. Sequence total RNA with a target of 12.5 M PE reads and 3.8 Gb of sequence per sample

What was accomplished under these goals?

Major activities

We have collected 31 fecal microbiome samples 22 from healthy individuals and eight from three metastatic colorectal cancer patients (three samples per patient for patients 1 and 2, one sample from patient 3).

We have published two invited reviews and one original research paper that acknowledge funding from the Department of Defense.

Specific objectives

Fecal microbiome samples from 22 healthy individuals, and three samples from one metastatic colorectal cancer patient were shotgun sequenced and processed for metabolite conversion assays. Preliminary computational analyses were carried out and are described in detail below.

Significant results

We have several significant results from the past year of work. We observe that beta-glucuronidase (BGs) enzyme distribution differs between healthy and metastatic colorectal cancer patient guts, and we highlight colorectal cancer-specific BGs that may be good biomarkers or targets for predicting or altering metabolism of the colorectal cancer drug irinotecan. 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). We have written 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). We have written 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). Finally, we have asked whether irinotecan metabolism by gut microbes is predictive of metabolism of other glucuronidated drug compounds, such as the commonly prescribed anti-inflammatory drug indomethacin. Our preliminary data suggests that deconjugation of one compound does not predict deconjugation of another compound, suggesting that there may in fact be specific microbial enzymes in the gut that can be targeted to modulate metabolism of a specific drug.

Differences in phylogenetic distribution and abundance of beta-glucuronidases (BGs) between healthy individuals and multiple samples from a metastatic colorectal cancer patient.

We sought to determine differences in the phylogenetic distribution and abundance of BGs. We identified 44 differentially abundant BGs of which two were elevated in metastatic colorectal cancer patient (mCRC) metagenomes (**Table 1**). One of the mCRC associated BGs was a predicted BG from *Lactobacillus gasseri*. The other was from an *Arthrobacter* spp; a functional screen identified the lateral transfer of this BG to fungal species. BGs that were elevated in healthy individuals were from species within diverse phyla including the Bacteroidetes, Firmicutes and Verrucomicrobia. The majority of BGs associated with either state, healthy or colorectal cancer, were intracellular localized BGs based on signal sequence predictions using SignalP; however some BGs contained signal sequences, suggesting differences in the types of glucuronides that these BGs may have access to and act on (**Figure 1, Table 1**).

Creation of the MicrobeFDT database, a resource linking drugs, foods, microbial enzymes, and toxicity data.

In the human gut, microbial biochemistry can be beneficial, for example vitamin production and complex carbohydrate breakdown; or detrimental, such as the reactivation of an inactive drug metabolite leading to patient toxicity as in the case of SN-38G. Identifying clinically relevant microbiome metabolism requires linking microbial biochemistry and ecology with patient outcomes. We developed and published a database resource, called MicrobeFDT, which clusters chemically similar drug and food compounds and links these compounds to microbial enzymes and known toxicities. We demonstrate that compound structural similarity can serve as a proxy for toxicity, enzyme sharing, and coarse-grained functional similarity. MicrobeFDT allows users to flexibly interrogate microbial metabolism, compounds of interest, and toxicity profiles to generate novel hypotheses of microbe-diet-drug-phenotype interactions that influence patient outcomes. We validate one such hypothesis experimentally, using MicrobeFDT to reveal unrecognized gut microbiome metabolism of the ovarian cancer drug altretamine. We used MicrobeFDT to ask whether there are potential food/drug interactions that may influence how SN-38G is metabolized by gut microbes. This work was published in the journal *eLife*.

MicrobeFDT identifies the diet-derived substrate pool for microbial BGs and candidates for nutritional competition with SN-38G

Some food compounds may be preferred substrates for microbiome BGs 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 BGs. Most compounds identified as significantly similar to SN-38G were food derivatives or other constituents (**Figure 2**). 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 BGs and are a potential avenue for decreasing the adverse drug responses associated with irinotecan administration.

Bringing basic research on microbiome/drug interactions into the clinic.

We wrote an invited review for the Lancet journal eBioMedicine providing an overview of microbiota chemistry that shapes drug efficacy and toxicity. We discuss experimental and computational approaches that attempt to bridge the gap between basic and clinical microbiome research. We highlight the current landscape of preclinical research focused on identifying microbiome-based biomarkers of patient drug response and we describe clinical trials investigating approaches to modulate the microbiome with the goal of improving drug efficacy and safety; we describes our work and the work of others attempting to identify biomarkers to predict SN-38G metabolism in gut microbiomes. With these goals in mind, we discuss approaches to aggregate clinical and experimental microbiome features into predictive models and review open questions and future directions toward utilizing the gut microbiome to improve drug safety and efficacy. Specifically we discuss one could use molecular data, such as microbial DNA and RNA sequencing, in combination clinical data such as patient serological profiling in hybrid COBRA-PBPK models to gain further predictive and mechanistic insight into drug pharmacokinetic profiles. The goal of this review is to bring basic research on microbiome drug metabolism to clinicians.

Predicting and understanding the human microbiome's impact on pharmacology

We wrote an invited review for the Cell Press journal Trends in Pharmacological Sciences that discusses how the microbiome alters the pharmacokinetic properties of drugs, and proposes a framework for pharmacologists interested in characterizing microbiome interactions with any drug of interest. SN-38G metabolism is discussed throughout the review with a focus on the toxification section where a gut microbiome-modified compound has a negative effect on host tissues. As part of this review, we noted that 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.

Preliminary data suggests that SN-38G metabolism is not predictive of indomethacin glucuronide metabolism.

Indomethacin is converted into an inactive metabolite, indomethacin glucuronide, via UDP glucuronosyltransferases in the liver followed by hepatobillary 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 (**Figure 3**). Our data support the hypothesis that interpersonal variation in microbiota glucuronide hydrolysis activity is dependent on variations in therapeutic drug glucuronide fine structure.

Time course *ex vivo* incubations of fecal samples with indomethacin acyl-β-D-glucuronide. To quantify the microbiome metabolism of indomethacin glucuronide we carried out *ex vivo* incubation of indomethacin acyl-β-D-glucuronide with each fecal sample as follows: To remove debris, 0.3 mg of each fecal sample was mixed with 3 ml of Dulbecco's phosphate-buffered saline, homogenized, centrifuged at 10,000g for 15 minutes at 4°C and the supernatant was collected for further processing. A final concentration of 200 ug/ml total protein per sample was prepared using the Bradford assay. Each sample was then incubated with 100 uM indomethacin acyl-β-D-glucuronide at 37°C. Reactions were terminated at 0, 1.5, and 3 minutes by removing a sample aliquot and adding a quenching solution containing the internal standard, 100 uM hydroxycampotothecin-d5 (ISTD), in 50% methanol. The ISTD is a compound similar in structure to indomethacin that is not metabolized by the gut microbiota. Samples were centrifuged at 12,000g for 10 minutes and 5 ul of supernatant was added to 45 ul of 10% methanol.

LC-MS/MS analysis. The concentrations of indomethacin glucuronide, indomethacin and the ISTD in the fecal extracts were determined by multiple reaction monitoring, focusing on selective ions for indomethacin (358.1 → 139), indomethacin acyl-β-D-glucuronide (565.1 → 358.1) and ISTD (371.1 → 327.1) (Table S1). The instrument used, the Agilent G6490 Triple Quadrupole Mass Spectrometer, was operated in the positive ionization mode and connected online to a 1290 Infinity series UHPLC. Mobile phase A was aqueous with 5% methanol and 0.1% formic acid to maintain the lactone form.

Mobile phase B was composed of 100 % acetonitrile. Each sample was run in triplicate at a flow rate of 0.350 ml/min with blanks consisting of sample buffer placed between each set of samples and the variance in triplicate points was determined. A calibration curve was established for each metabolite and the ISTD in both methanol and using a pooled fecal extract to determine the lower limit of detection (LLOD) and lower limit of quantitation (LLOQ). This work was carried out with the Albert Einstein College of Medicine Proteomics Core.

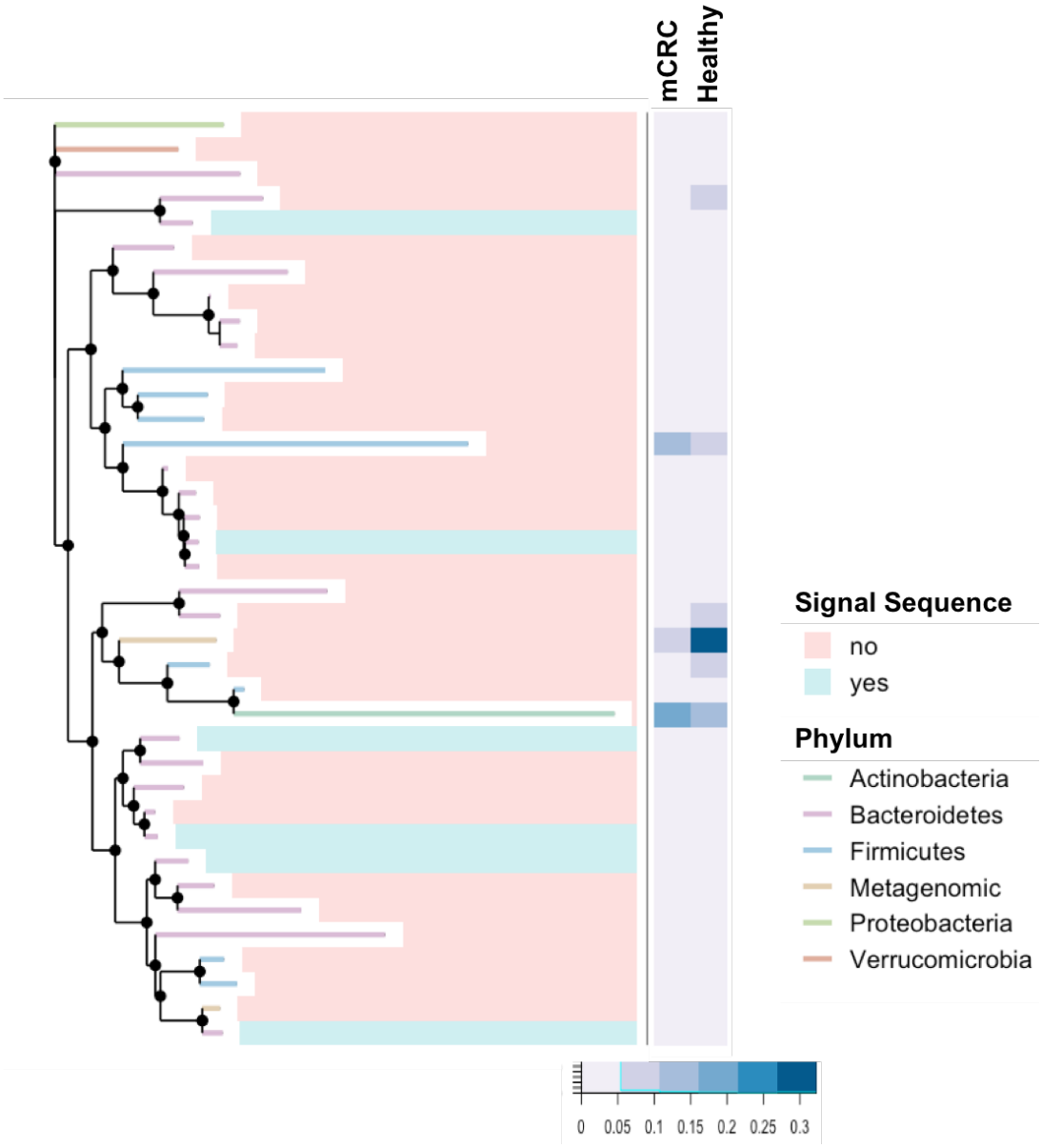

Figure 1. Phylogenetic distribution of differentially abundant extracellular and intracellular BGs between healthy and mCRC fecal metagenomes. The BG tree is rooted using the E. coli β-galactosidase sequence as an out-group. Phylum-level taxonomy is indicated by branch color. Salmon colored bars represent beta-glucuronidases that are maintained intracellularly given a lack of an identifiable signal sequence while light blue bars indicate the presence of a signal sequence that would enable the trafficking of the BG across the microbial membrane. The adjoining heat map displays the relative abundance of BG sequences represented in the tree with values normalized on a scale from 0, being least abundant to 1, being most abundant. (b) Differentially abundant BGs between the healthy and mCRC health status individuals were determined based on the Welch's t-test, two sided, with a Storey FDR, adjusted q-value 0.05 and followed by an effect size filter (ratio of proportions effect size 2.00).

Table 1. Differentially abundant BGs between healthy and mCRC fecal microbiomes

ID	HEALTHY: MEAN REL. FREQ. (%)	HEALTHY: STD. DEV. (%)	MCRC: MEAN REL. FREQ. (%)	MCRC: STD. DEV. (%)	P-VALUES (CORRECTED)
C1308780__GENE_159931	0.0034992	0.01603533	0.10467113	0.00389494	5.04E-09
C1737453__GENE_94517	0.03094699	0.05522687	0.10467113	0.00389494	4.24E-05
C1797245__GENE_94069	0.02202061	0.04139033	0.10467113	0.00389494	1.36E-07
C2166659__GENE_55777	0.02202061	0.04139033	0.10467113	0.00389494	1.36E-07
C2243869__GENE_76024	0.00571037	0.0261682	0.10467113	0.00389494	1.57E-10
C2254157__GENE_166555	0.03604325	0.04876862	0.10467113	0.00389494	2.41E-05
C2343857__GENE_64050	0.00762393	0.02419808	0.10467113	0.00389494	1.57E-10
C2386759__GENE_220704	0.36436393	0.20711468	0.17515422	0.05183113	0.01799697
C2422296__GENE_152951	0.01715712	0.03731643	0.10467113	0.00389494	1.73E-08
C2471972__GENE_111780	0.03695519	0.06572765	0.31442904	0.08551008	0.0478185
C2509540__GENE_239582	0.19696015	0.1068742	0.03377237	0.04776135	0.0249466
C2609297__GENE_246416	0.01788278	0.03822814	0.10467113	0.00389494	2.70E-08
C2637779__GENE_69527	0.37264513	0.23612134	0.06796041	0.04805796	0.00116867
C2699950__GENE_179059	0.02504477	0.04704078	0.10467113	0.00389494	1.68E-06
C2896810__GENE_94608	0.02155004	0.04022106	0.10467113	0.00389494	8.64E-08
C3035023__GENE_187903	0.01030537	0.03259548	0.10467113	0.00389494	1.28E-09
C3098817__GENE_230968	0.01830171	0.0398624	0.10467113	0.00389494	4.55E-08
C3110863__GENE_188545	0.01805084	0.03856072	0.10467113	0.00389494	2.94E-08
C3123204__GENE_116535	0.1246489	0.11769227	0.28024101	0.05565356	0.0394446
C3408260__GENE_247512	0.05385107	0.05919156	0.17263154	0.04420546	0.04987993
C4023951__GENE_190116	0.0161007	0.04074476	0.10467113	0.00389494	4.46E-08
C6681533__GENE_50920	0.01893529	0.04074756	0.10467113	0.00389494	6.97E-08

GI|12802351|GB|AAK07835.1|AF305888_2	0.52282738	0.34624965	1.32989063	0.1744867	0.01415856
GI|158445294|GB|EDP22297.1|	0.01835489	0.05031083	0.10467113	0.0038949	1.42E-06
GI|298266464|GB|EFI08122.1|	0.45468348	0.21333762	0.14138185	0.0557806	0.00226672
GI|371999664|GB|AEX64884.1|	0.02110084	0.04943374	0.10467113	0.0038949	1.68E-06
GI|396084163|GB|AFN84582.1|	1.77381257	0.53535534	0.76311626	0.1777846	0.00317315
GI|497944672|REF|WP_010258828.1|	0.17179133	0.15057467	0.41868451	0.0155797	3.32E-06
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GI|56474964|GB|AAV91791.1|	0.59397512	0.25665715	1.35526363	0.1019384	0.00198808
GI|649573538|REF|WP_026367225.1|	0.04339825	0.05548037	0.10467113	0.0038949	0.00040758
GI|738736031|REF|WP_036630120.1|	0.12793119	0.11255126	0.4212072	0.0998297	0.04987993
GI|916276276|REF|WP_051011322.1|	0.04260093	0.06514776	0.10467113	0.0038949	0.00155767
GI|982531322|GB|KWR63779.1|	0.03490591	0.10969416	0.10467113	0.0038949	0.01826248
SRS012902_BAYLOR_SCAFFOLD_103__GENE_276	0.01046307	0.03384379	0.10467113	0.0038949	1.62E-09
SRS013687_BAYLOR_SCAFFOLD_40049__GENE_71750	0.04789736	0.06068264	0.10467113	0.0038949	0.00177937
SRS015217_WUGC_SCAFFOLD_26749__GENE_56118	0.04319931	0.04837868	0.10467113	0.0038949	8.18E-05
SRS016018_WUGC_SCAFFOLD_15114__GENE_41825	0.01375675	0.03541067	0.10467113	0.0038949	4.64E-09
SRS019787_WUGC_SCAFFOLD_14153__GENE_27227	0.05031361	0.0679493	0.10467113	0.0038949	0.00519487
SRS022609_BAYLOR_SCAFFOLD_16101__GENE_23711	0.01301336	0.03381564	0.10467113	0.0038949	2.29E-09
SRS024331_LANL_SCAFFOLD_12638__GENE_19970	0.02151444	0.04015626	0.10467113	0.0038949	8.64E-08
SRS024549_LANL_SCAFFOLD_16933__GENE_45118	0.03594144	0.0711666	0.10467113	0.0038949	0.00135259
SRS049995_LANL_SCAFFOLD_12452__GENE_33736	0.04307686	0.07911883	0.17263154	0.0442054	0.0394446
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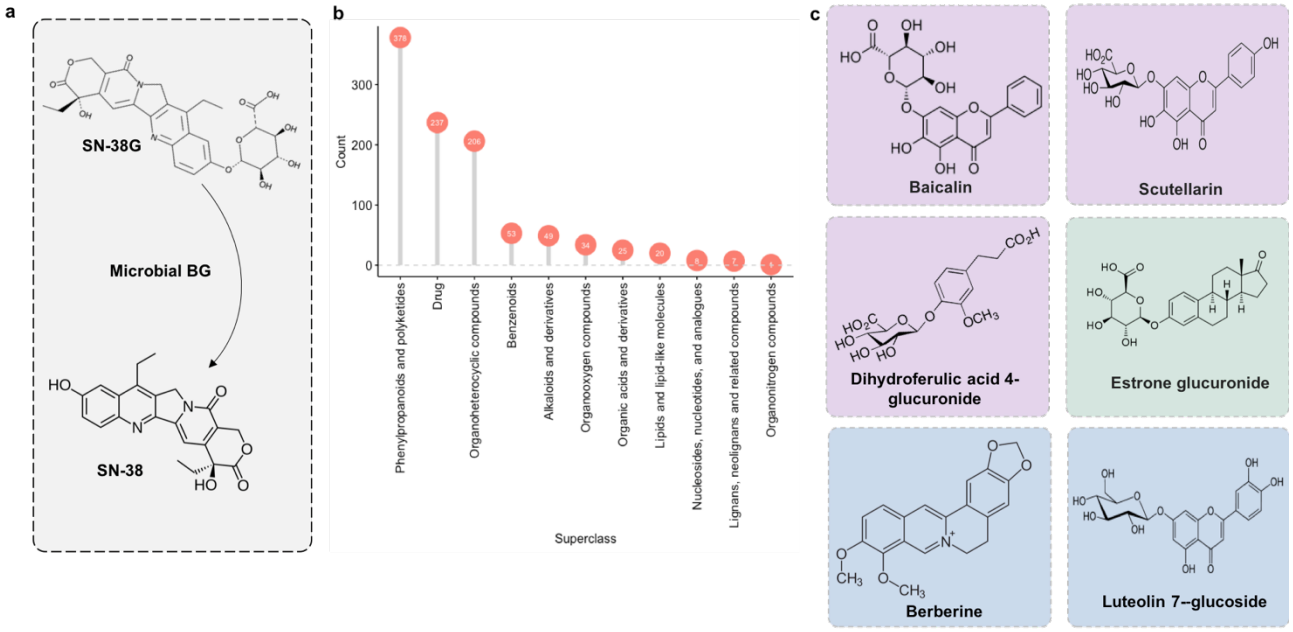

Figure 2. Microbial beta-glucuronidase potential substrate pool of compounds structurally similar to SN-38G. (a) SN-38G conversion to SN-38 in the gut is mediated by microbial beta-glucuronidases (b) The substrate pool for beta-glucuronidases with above threshold substructure overlap with SN-38G are members of a diverse range of chemical structure superclasses as defined by FooDB chemical ontology (Wishart, 2018). (c) These compounds include glucuronidated food-derived compounds (purple), endogenous glucuronides (tan) and other non-glucuronides (blue). Figure from Guthie et al, *eLife,* 2019.

Figure 3.

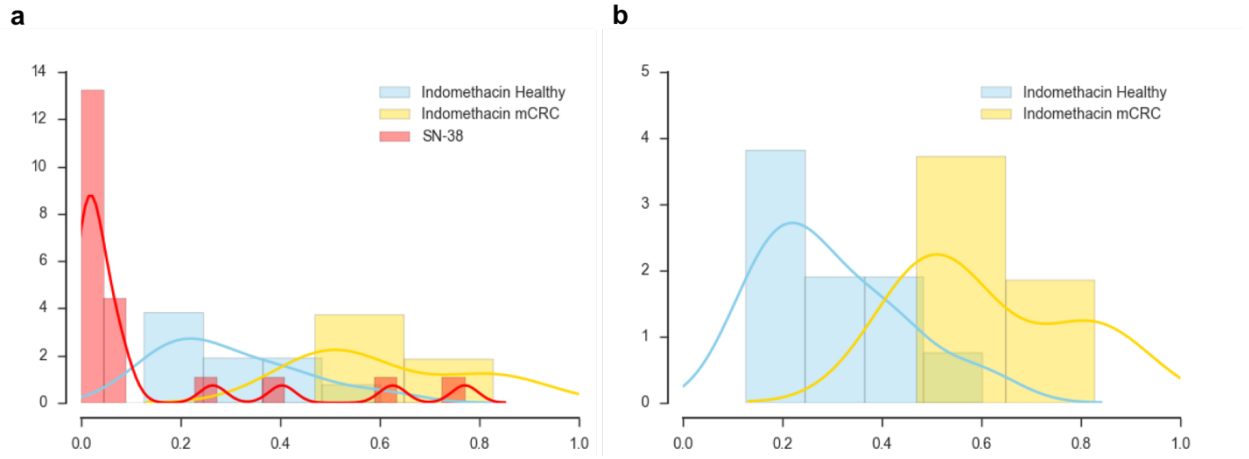

Figure 3. Fecal microbiome based metabotypes for SN-38 and indomethacin formation
a We previously identified two distinct metabolizer phenotypes or 'metabotypes' based on % SN-38 formation during a time course incubation of SN-38G with fecal samples from 20 individuals quantified by LC-MS/MS. Participants 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 ± 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.

Major findings/Developments

Our preliminary work points to the need for additional studies profiling patient fecal microbiomes pre- and post-drug treatment to gain greater insight the timescales and factors that control variation in microbiome function. We have identified BGs that are significantly more prevalent in metastatic colorectal cancer patient samples; these BGs may prove to be useful biomarkers for predicting SN-38G turnover in patients (Figure 1, Table 1). Using computational approaches, we have published the first network, MicrobeFDT, linking foods, drugs, microbiome enzymes, and patient outcomes. 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 (Figure 2). This finding could impact on how patients are instructed in terms of diet while taking irinotecan; our work suggests that one method to decrease adverse drug responses is to modify a patient's diet.

Goals not met

Major Task 3. We cannot get an appropriate SN38-G probe synthesized, we discuss potential pivots for this project below.

Major Task 4. We are having problems with our RNA extraction. We are confident that working with Genewiz (discussed below) will fix this problem.

What opportunities for training and professional development has the project provided?

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.

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 entire Einstein Faculty work in progress seminar series which includes all members

of the Albert Einstein College of Medicine Cancer Center faculty. Dr. Kelly presented her work Thursday, **October 11th 2017** at 12:00pm in the Forchheimer 3rd Floor Lecture Hall.

Subtask 3: Attend a national microbiology meeting

Dr. Kelly attended the International Society for Microbial Ecology's 2018 meeting, the American Society for Microbiology's (ASM) 2018 and 2019 annual meetings, and a 2019 Keystone microbiome meeting. ***She gave invited talks at two of these meetings (Keystone, ASM 2018) and ran a symposium at the third (ASM 2019)***. Her talks are detailed in the "How were results disseminated?" section.

Subtask 5: Attend a translational research workshop

Dr. Kelly was accepted for admission to and will attend the American Association for Cancer Research Translational Cancer Research for Basic Scientists Workshop in November 2019.

Subtask 6: Meet monthly with Dr. Mani

Dr. Kelly has met more than monthly with Dr. Mani and they are currently working on a manuscript together (Dr. Mani is senior author) as part of an unrelated project examining how the microbiome may mediate inflammatory responses in mouse models.

How were the results disseminated to communities of interest?

In addition to the publications and talks at meetings and other institutions described below Dr. Kelly was asked to participate in an article describing microbiome influences on drug metabolism for the online magazine Bustle (http://bustle.com/). This article, which is not yet published, will reach communities of interested individuals who are not scientists and will help them to understand how the microbiome plays a role in how patients respond to drugs. Dr. Kelly's work was also written up by the Albert Einstein College of Medicine press department: https://einstein.yu.edu/highlights/70/investigating-how-microbes-metabolize-drugs/

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

We will continue to recruit colorectal cancer patients and continue to collect samples from both our healthy patient recruits and our colorectal cancer patients. We will generate sequencing and metabolomics data and begin to build a picture of why some individuals have adverse responses to irinotecan. I have recruited a MD/PhD student, Ruth Hauptmann, who will take over the project from Leah Guthrie, who graduated this year. I will attend the AACR Translational Cancer Research for Basic Scientists Workshop from November 17-22, 2019. I have been invited to give talks on my work at UCSF and Stanford in Fall 2019. I was also asked by the journal *Cell Metabolism* to write a Preview article about a recently published paper on microbial drug metabolism and potential directions the field could go in; this article will be published in August.

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The papers my lab published this year will have a major impact on our understanding of how microbes living in the human body can influence how people respond to drugs. Specifically, we are starting to reveal the many different ways that microbes, including bacteria, archaea, small eukaryotes, fungi, and viruses, can impact how people's bodies process the drugs they take. Our published work, data, and analysis, will make it easier for clinicians and pharmacists to hone in on specific drugs that may be processed differently in different people and therefore eventually enable more targeted, personalized, safer treatment with drugs.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

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

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

There is no impact on technology transfer.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report.

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

1) We have been unable to synthesize stable fluorescent probes for SN38-G (MAJOR TASK 3). As an alternative we may utilize the following probe, which can be purchased, to study microbial uptake of glucuronidated substrates with similarity to SN-38G.

   https://www.carbosynth.com/carbosynth/website.nsf/(w-productdisplay)/6322D24BA12D2AFB80256E19004DFE27

2) We have had difficulty extracting RNA from fecal samples (MAJOR TASK 4). We will instead utilize Genewiz (http://www.genewiz/com) for mRNA extraction and purification from fecal samples. We have already sent unrelated fecal samples to Genewiz and they have successfully extracted mRNA and thus we are confident that we can overcome this hurdle for fecal samples from this project.

3) Our colorectal cancer patient recruitment has taken longer than expected. One reason for this is that many of our patients at Einstein/Montefiore speak Spanish as their primary language. We will therefore translate our informed consent documents and our description of the study into Spanish. We anticipate that this will expand our potential patient recruits.

4) Leah Guthrie, who was 75% on this project last year, successfully defended her thesis and graduated, she is now a postdoc at Stanford. I have recruited an MD/PhD student, Ruth Hauptmann, to continue work on this project. Ruth will start in the lab in August, 2019. This transition period has led to some delays in getting the work done as anticipated.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

No significant changes

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

No significant changes.

IRB 2018-9284 (Secondary analysis of date from study "human gut microbiome contributions to CPT-11 metabolism and adverse drug responses") approved through 5/15/20.

IRB 2018-9169 (Secondary analysis of date from study "defining the human microbiome in health and disease") approved through 4/30/20.

Significant changes in use or care of vertebrate animals

No vertebrate animals.

Significant changes in use of biohazards and/or select agents

No significant changes.

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

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

  2. Hitchings R, Kelly L. Predicting and Understanding the Human Microbiome's

Impact on Pharmacology. 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*

3. Guthrie L, Kelly L. Bringing microbiome-drug interaction research into the clinic. 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.

1. Invited speaker, American Society for Microbiology (ASM) annual meeting (plenary talk), June 2018

2. Invited seminar, Georgia Tech, November 2018

3. Invited seminar, Memorial Sloan Kettering Cancer Center, December 2018

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

5. 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

6. Keynote speaker, University of Chicago Microbiome Symposium, April 2019

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report.

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to report.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

  The Kelly lab Github page is a repository for data and analysis. The MicrobeFDT-neo4j dataset represents microbial enzyme-food-drug-side effect data in a neo4j format for easy searching and data retrieval:

  https://github.com/kellylab/microbeFDT-neo4j

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to report.

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

MicrobeFDT, published as part of Guthrie, Wolfson, and Kelly, is a publicly available database of potential microbial enzyme/drug interactions, overlaid with toxicity data. It is available here:

https://github.com/kellylab/microbeFDT-neo4j

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name:	*Libusha Kelly*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*3.6 (30%)*
Contribution to Project:	*Dr. Kelly oversees all work related to the project.*
Funding Support:	*No other support*
Name:	*Leah Guthrie*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*8.75 (75%)*
Contribution to Project:	*Dr. Guthrie was in charge of the sample collection, DNA extraction, metabolomics, and computational analyses.*
Funding Support:	*No other support.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Three manuscript reprints are attached.

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TOOLS AND RESOURCES

The human gut chemical landscape predicts microbe-mediated biotransformation of foods and drugs

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Abstract Microbes are nature's chemists, capable of producing and metabolizing a diverse array of compounds. In the human gut, microbial biochemistry can be beneficial, for example vitamin production and complex carbohydrate breakdown; or detrimental, such as the reactivation of an inactive drug metabolite leading to patient toxicity. Identifying clinically relevant microbiome metabolism requires linking microbial biochemistry and ecology with patient outcomes. Here we present MicrobeFDT, a resource which clusters chemically similar drug and food compounds and links these compounds to microbial enzymes and known toxicities. We demonstrate that compound structural similarity can serve as a proxy for toxicity, enzyme sharing, and coarse-grained functional similarity. MicrobeFDT allows users to flexibly interrogate microbial metabolism, compounds of interest, and toxicity profiles to generate novel hypotheses of microbe-diet-drug-phenotype interactions that influence patient outcomes. We validate one such hypothesis experimentally, using MicrobeFDT to reveal unrecognized gut microbiome metabolism of the ovarian cancer drug altretamine.

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Competing interests: The authors declare that no competing interests exist.

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Introduction

Complex gut microbiome phenotypes shape human nutrition (***Martens et al., 2014***; ***Sonnenburg et al., 2016***; ***Bretin et al., 2018***), therapeutic drug responses (***Guthrie et al., 2017***; ***Haiser et al., 2013***; ***Koppel et al., 2017***) and disease susceptibility (***Koeth et al., 2013***). Multi'omic studies suggest that the human gut microbiota can be discretized at the resolution of microbial enzymes (***Guthrie et al., 2017***; ***Tang and Hazen, 2014***), species (***Haiser et al., 2013***; ***Haiser et al., 2014***), guilds (***Joossens et al., 2011***; ***Wu et al., 2013***) or metabolites (***Clayton et al., 2009***) to characterize a range of human health and disease states. Gut microbial mediated biochemical transformations have consequences for drug treatment efficacy (***Koppel et al., 2017***; ***Spanogiannopoulos et al., 2016***; ***Alexander et al., 2017***; ***Wilson and Nicholson, 2017***) and the etiology of inflammatory gastrointestinal diseases (***Tilg et al., 2018***; ***Arthur et al., 2014***; ***Belcheva et al., 2014***; ***Brennan and Garrett, 2016***), however despite many examples there exist few unifying principles that govern microbiome impacts on human health.

Some microbiome/drug interactions have been characterized in detail. For example, the inactivation and decreased bioavailability of digoxin, a cardiac glycoside inhibitor, is linked to *cgr* operon expression levels in a single species, *E. lenta* (***Haiser et al., 2013***). Microbial β-glucuronidases mediate the reactivation of the key therapeutic metabolite of irinotecan, a chemotherapeutic prodrug used in the treatment of colorectal cancer, causing toxicity in some patients (***Guthrie et al., 2017***; ***Wallace et al., 2010***). Notably, diet-derived compounds that are conjugated to glucuronic acid in

eLife digest Microbes in the human gut can play helpful roles by producing vitamins or breaking down complex carbohydrates. Collectively, gut microbes carry out these roles using a large toolkit of enzymes that catalyze a diverse range of chemical reactions, some of which cannot be carried out by human enzymes. However, these microbial enzymes can also cause harm if they alter drugs in a way that makes them toxic or prevents them from working. Little is known about which microbial enzymes interact with which foods and drugs, or how these interactions affect human health.

Guthrie et al. have now developed and tested a tool called MicrobeFDT that can help researchers to understand these complex interactions. In MicrobeFDT, 10,000 compounds produced by the human body or found in food or drugs are grouped based on their structure. Compounds are linked to the microbial enzymes that interact with them and drugs are annotated with information on known toxicities. The result is a network where compounds with similar structure are linked to each other.

If a microbial enzyme interacts with one compound in a group, it may interact with related compounds as well, potentially causing similar effects on human health. The network makes it easier for researchers to work out which compounds are affected by particular gut microbes. For example, MicrobeFDT suggested how gut microbes might alter the structure of an ovarian cancer drug called altretamine, which can cause diarrhea and kidney damage as side effects. Experiments confirmed that the predicted structural change does occur in human feces.

MicrobeFDT may increase how quickly researchers can assess harmful interactions between gut microbes, food, and drugs. It also may help them to develop new strategies to improve human health based on how microbial enzymes interact with food and drugs.

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the human liver and excreted via the biliary route into the GI tract are known substrates for microbial β-glucuronidases (***O'Leary et al., 2003***; ***Sakurama et al., 2014***; ***Maathuis et al., 2012***).

Many other gastrointestinally-routed drugs share overlapping chemical properties with diet-derived compounds. We understand in detail species-specific metabolism of some discrete chemical structures in dietary compounds, particularly polysaccharides (***Martens et al., 2008***); however we know little about the potential spectrum of drug metabolism by the microbiome.

Beyond the role of the microbiome in therapeutic drug treatment efficacy and polysaccharide metabolism, we have some mechanistic insight into how microbial metabolism contributes to host immunity. Microbial enzymes mediate the conversion of tryptophan into indole (***Sasaki-Imamura et al., 2010***) and indole derivatives (***Arora and Bae, 2014***) that shape human host immune responses (***Levy et al., 2017***; ***Blacher et al., 2017***). Microbe produced indole 3-aldehyde functions as an activating ligand for human host aryl hydrocarbon receptors which are expressed by immune cells (***Zelante et al., 2013***). Indole binding induces IL-22 secretion by innate lymphoid cells, promoting the secretion of antimicrobial peptides that protects the host from pathogenic infection by *Candida albicans* (***Zelante et al., 2013***). Microbial production of short chain fatty acids (SCFAs) from dietary fiber also shapes host immunity, contributing to both innate and adaptive immune system functions (***Fukuda et al., 2011***; ***Donohoe et al., 2011***; ***Smith et al., 2013***).

Host-microbe interactions and phenotypes, ranging from host drug response to host immune response, are thus intimately connected to gut chemical signaling. Beyond these few well understood examples lie a vast space of uncharacterized microbe-drug-diet-phenotype interactions. We propose three key requirements to characterize the dynamics of the gut chemical space and its impact on health. The first is predicting which compounds microbes can metabolize, the second is connecting the chemistry of gut microbes to host phenotypes, and the third is linking gut chemistry to microbial ecology.

Towards the goal of systematically mapping the gut microbial chemistry that contributes to the metabolism of xenobiotics, including therapeutic drugs, recent efforts have used chemical structure-centric approaches to enable high-throughput computational predictions of gut microbe metabolism of drugs (***Sharma et al., 2017***; ***Mallory et al., 2018***). These tools represent an important first step

towards ecological and mechanistic insights into gut microbiota driven biotransformation of foods and drugs. The second requirement, which has not yet been achieved, is to connect the known and predicted chemistry of gut microbes to host phenotypes. To date, information on human responses to therapeutic drugs is available in disparate databases and formats including FDA Adverse Report System (FAERs) (***Burkhart et al., 2015***), the Side Effect Resource (SIDER) (***Kuhn et al., 2016***) and DrugBank (***Law et al., 2014***). The third requirement, also lacking, is to systematically link gut microbe chemistry to microbial ecology to understand how the distribution of enzymes in populations of microbes facilitates ecological interactions that structure the human gut.

Here, we develop MicrobeFDT, a resource encompassing this 3-step framework that connects compound structure, enzyme function, taxonomy, and toxicity to characterize microbe-diet-drug-phenotype interactions. We organize ~10,000 food, drug, and endogenous compounds by structural similarity. We then link toxicity, enzyme interactions, and the propensity for gut microbes to carry out metabolism on each compound to the structural similarity network. We validate MicrobeFDT computationally by demonstrating that structural similarity is a reasonable proxy for toxicity, enzyme sharing, and coarse-grained functional similarity. We propose, and experimentally validate, active gut microbiome demethylation of an ovarian cancer drug, altretamine, a metabolism that we propose may drive toxicity of this drug. All data is available in the MicrobeFDT database (MicrobeFDT; ***Guthrie, 2019***; copy archived at https://github.com/elifesciences-publications/microbeFDT-neo4j).

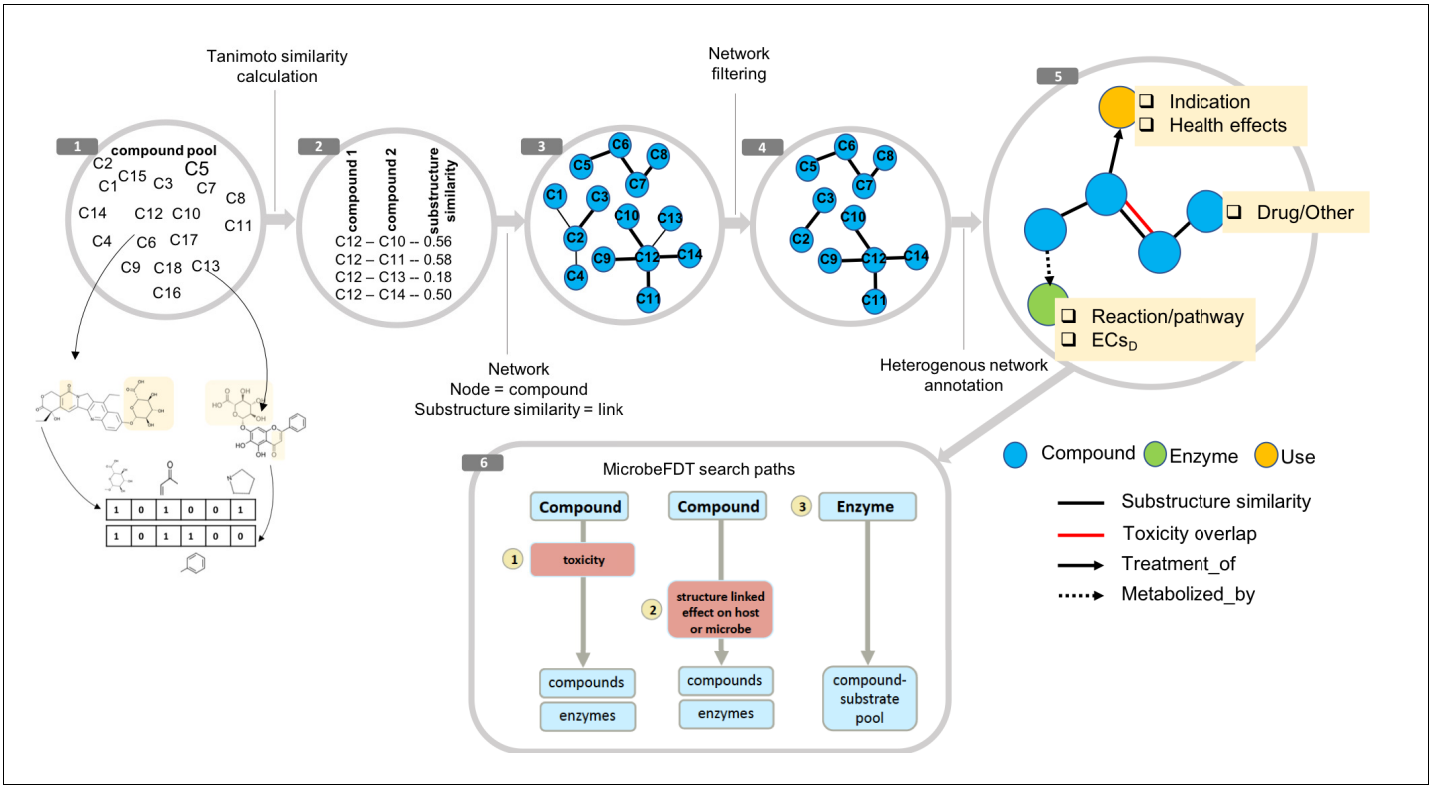

Figure 1. MicrobeFDT is a searchable resource of gut microbiome food and drug metabolism with associated toxicities. (1) Diet-derived, xenobiotic-derived and endogenous compounds were clustered based on the PubChem fingerprint system (***Kim et al., 2016***) and the Tanimoto coefficient (***Bajusz et al., 2015***). (2) The pairwise similarity matrix forms the basis of the (3) substructure similarity network in which nodes are compounds and links are weighted by substructure similarity. (4) A Z-score based threshold method was used to identify significant chemical similarity relationships between nodes (***Baldi and Nasr, 2010***). (5) The property graph model of nodes and relationships in the network highlights node-relationship pairs that can be queried. Node entities include compounds (blue), uses (orange) and enzymes (green). A compound node can have up to four types of directional relationships: compound pairwise substructure similarity, compound pairwise toxicity similarity, compound treatment use descriptor and compound microbial mediated metabolism descriptor.

DOI: https://doi.org/10.7554/eLife.42866.003

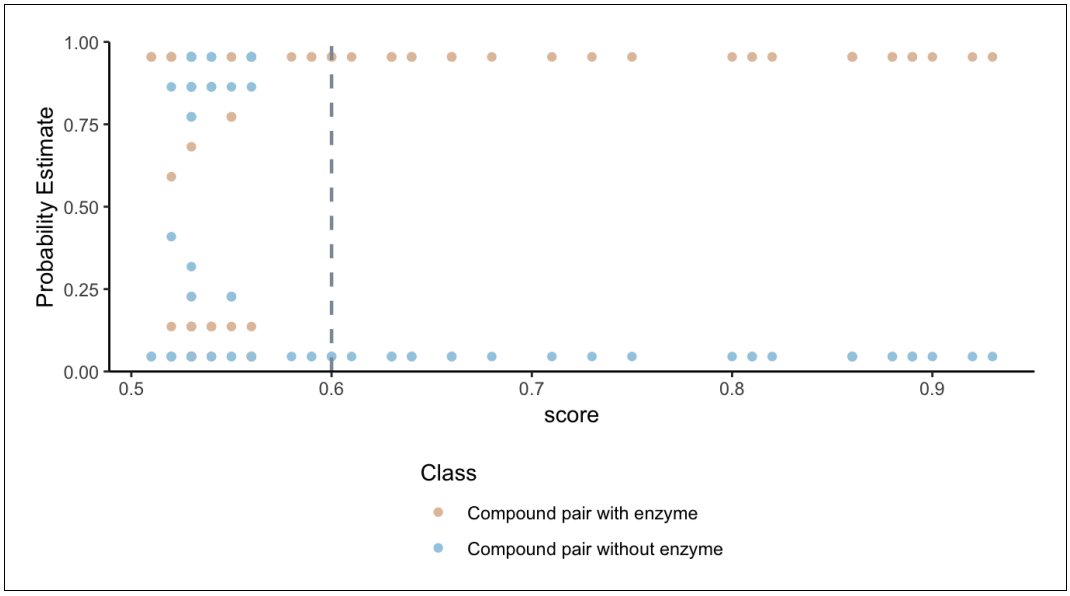

Figure 2. Higher substructure similarity scores between pairs of compounds are associated with higher probability of sharing an enzyme. Potential enzyme mediated metabolism of compound pairs is compared with substructure similarity to determine the probability that compounds have an experimentally determined shared enzyme (pink) or no known shared enzyme (blue). The gray vertical dashed line indicates the average cutoff for significance in substructure similarity neighborhood construction. Probability estimates are based on a Bayesian approach for support vector machines implemented in R using the probsvm package (***Zhang et al., 2013***).
DOI: https://doi.org/10.7554/eLife.42866.004

Results

Structural similarity as a metric to organize enzyme/taxonomy/toxicity links between compounds

The foundation of the MicrobeFDT resource is a chemical similarity network linking 10,822 food, drug, and endogenous compounds with PubChem compound identifier (CIDs) (***Kim et al., 2016***). In the network, nodes designate compounds and edges are weighted by pairwise chemical substructure similarity quantified by comparing PubChem fingerprints (***Kim et al., 2016***) using the Tanimoto score (***Bajusz et al., 2015***) (***Figure 1***). The Tanimoto score prioritizes overlap between compounds that share substructures over compounds with shared co-absences (***Bajusz et al., 2015***). We hypothesized that compounds with overlapping substructure and physiochemical properties, in which one compound is a known substrate of an enzyme, will be more likely to serve as substrates for the same enzyme. Recent *in silico* approaches to predict enzymatic reactions of drugs in the context of human enzyme catalyzed reactions also employ this hypothesis (***Niu et al., 2013***; ***Yu et al., 2018***). Substructure-based clustering thus serves as a first step towards synthesizing publicly available information on gut compound chemical diversity and gut microbiome biochemistry.

To validate that our network can identify shared metabolism, we developed an in silico prediction model to assign a probability of shared metabolism between compounds based on substructure overlap and the following physiochemical categories: geometry, functional groups, amino acid composition, polarity and hydrophobicity. We find that the probability estimates of compound-pairs sharing an enzyme based on substructure and physiochemical parameters, increase as the substructure overlap score between compound pairs increases (***Figure 2***). Weighting compound pair chemical similarity relationships based on substructure similarity is thus a reasonable filtering step to identify compounds that may share metabolism.

As an example of how the network can reveal shared metabolism we selected compounds in the network with substructure overlap with digoxin, a cardiac glycoside inhibitor. Reduction of digoxin by a human microbiome reductase inactivates the drug, contributing to poor bioavailability in some individuals (***Haiser et al., 2013***; ***Haiser et al., 2014***; ***Lindenbaum et al., 1981***). Koppel et al.,

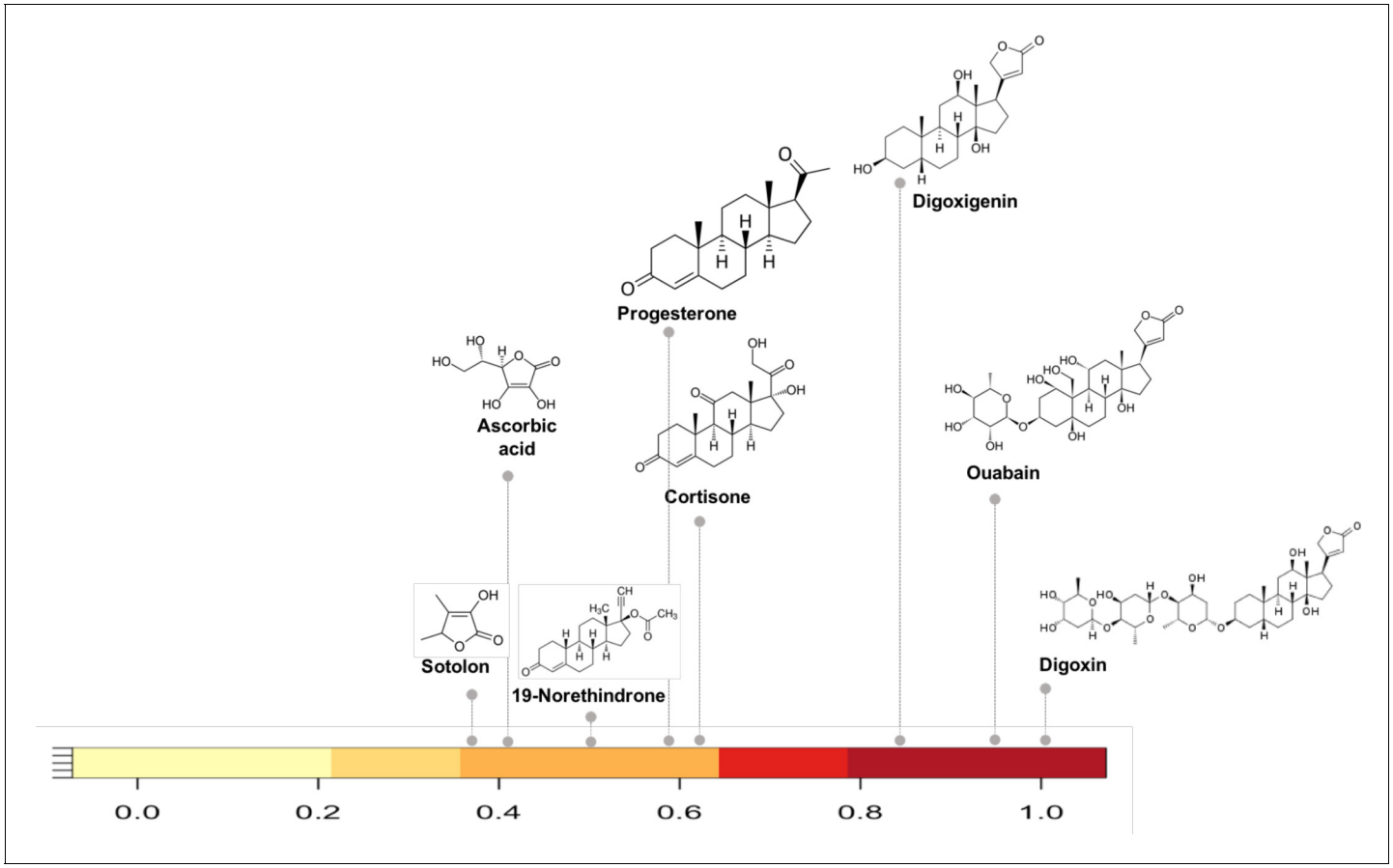

Figure 3. Substructure similarity range of Cgr2 enzyme susceptible compounds. Substructure based clustering distinguishes experimentally characterized substrates from non-substrates of the Cgr2 enzyme. Digoxin clusters with other cardenolides that are experimentally characterized substrates (***Koppel et al., 2018***) for Cgr2 at substructure similarity values greater than 0.8. Compounds that are not substrates of Cgr2 have lower substructure similarity with digoxin; compounds with minimal reduction (***Koppel et al., 2018***) include progesterone and cortisone (substructure similarity <= 0.63). Color bar intensity increases with compound overlap with digoxin.

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biochemically characterized the capacity of a single flavin- and [4Fe-4S] cluster-dependent reductase, *cgr2*, to reduce various substrates with a range of substructure similarity to digoxin (***Koppel et al., 2018***). We identified the substructure overlap between digoxin and compounds in the Koppel et al. study that were evaluated as substrates of Cgr2 enzyme. Among the biochemically assayed compounds (***Koppel et al., 2018***) that are present in the MicrobeFDT network, compounds with substructure similarity scores greater than 0.8 are also substrates for Cgr2. This assessment suggests that for the cgr enzyme substructure based clustering can distinguish experimentally characterized substrates from non-substrates (***Figure 3***).

Previous studies have found that structural similarity predicts both toxicity and drug target similarity (***Campillos et al., 2008***). To evaluate whether our network also recapitulates shared drug toxicity we fit a linear regression and computed the effect size to assess the association between substructure similarity and toxicity similarity for therapeutic drugs in our network. We find that structural similarity moderately positively predicts toxicity similarity for therapeutic drug pairs linked by structural similarity overall in the network ($r = 0.03116$, $p<2.2e-16$) (***Figure 4***).

Finally, we evaluated how well our compound clustering recapitulates structure-based chemical taxonomy as defined by the ClassyFire (***Djoumbou Feunang et al., 2016***) resource, a comprehensive chemical classification schema, at the level of superclass taxonomy. We found that substructure-based compound clustering, significantly groups compounds within a ClassyFire superclass based on a comparison of the MicrobeFDT network with a randomized network with the same number of

nodes and edges (p<8.06×10–15, Wilcoxon rank-sum test). Compound-pairs at higher substructure similarity share Superclass membership at higher substructure values and at a greater frequency than randomized pairs, indicating that the MicrobeFDT substructure similarity metric can capture established chemical classifications (***Figure 5***).

Overlapping structural diversity of food, drug, and endogenous compounds

In the network, therapeutic drug structural diversity is embedded within food-derived chemical diversity. For example, drugs share structural similarity with food-derived compounds from a diverse range of classes including benzenoids, lipids, nucleosides and phenylpropanoids (***Figure 6***). Food derived compounds also contributed significantly greater molecular structure diversity (***Figure 6—figure supplement 1***) and higher self-similarity than therapeutic drug compounds (two-sample K-S test 0.49, p value=4.7395e-06).

Assessing the distribution of enzymatic functions across taxonomic groups

Metabolic functions are not necessarily equally distributed across microbes in the microbiome. For example, as described above, inactivation of digoxin, a cardiac glycoside inhibitor, is linked to *cgr* operon expression levels in a single species, *E. lenta* (***Haiser et al., 2013***; ***Koppel et al., 2018***). In contrast, the deconjugation and resulting reactivation of SN-38, the active metabolite of the chemotherapeutic colorectal cancer drug irinotecan, is linked to a phylogenetically diverse guild of microbial β-glucuronidase carrying microbes (***Guthrie et al., 2017***; ***Pollet et al., 2017***; ***Wallace et al., 2015***).

The question arises, how many microbes can perform specific enzymatic functions? Knowing the taxonomic distribution of a function can guide approaches to validate hypotheses of microbiota driven modification of specific therapeutic drug or food compounds. More broadly, addressing this

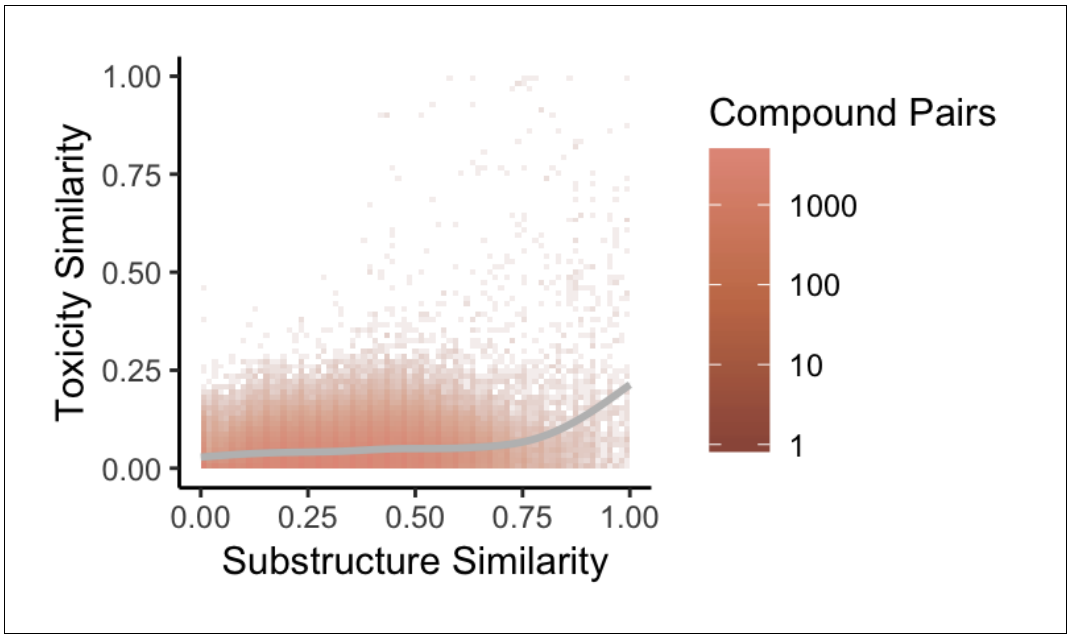

Figure 4. Substructure similarity is predictive of toxicity similarity. We evaluated the predictive power of substructure similarity to identify compounds with shared toxicity using a measure of pairwise toxicity defined by ***Campillos et al. (2008)*** and used a linear regression to determine the strength of the association. We find a modest positive correlation between substructure similarity and toxicity similarity that is stronger for more structurally similar compounds.

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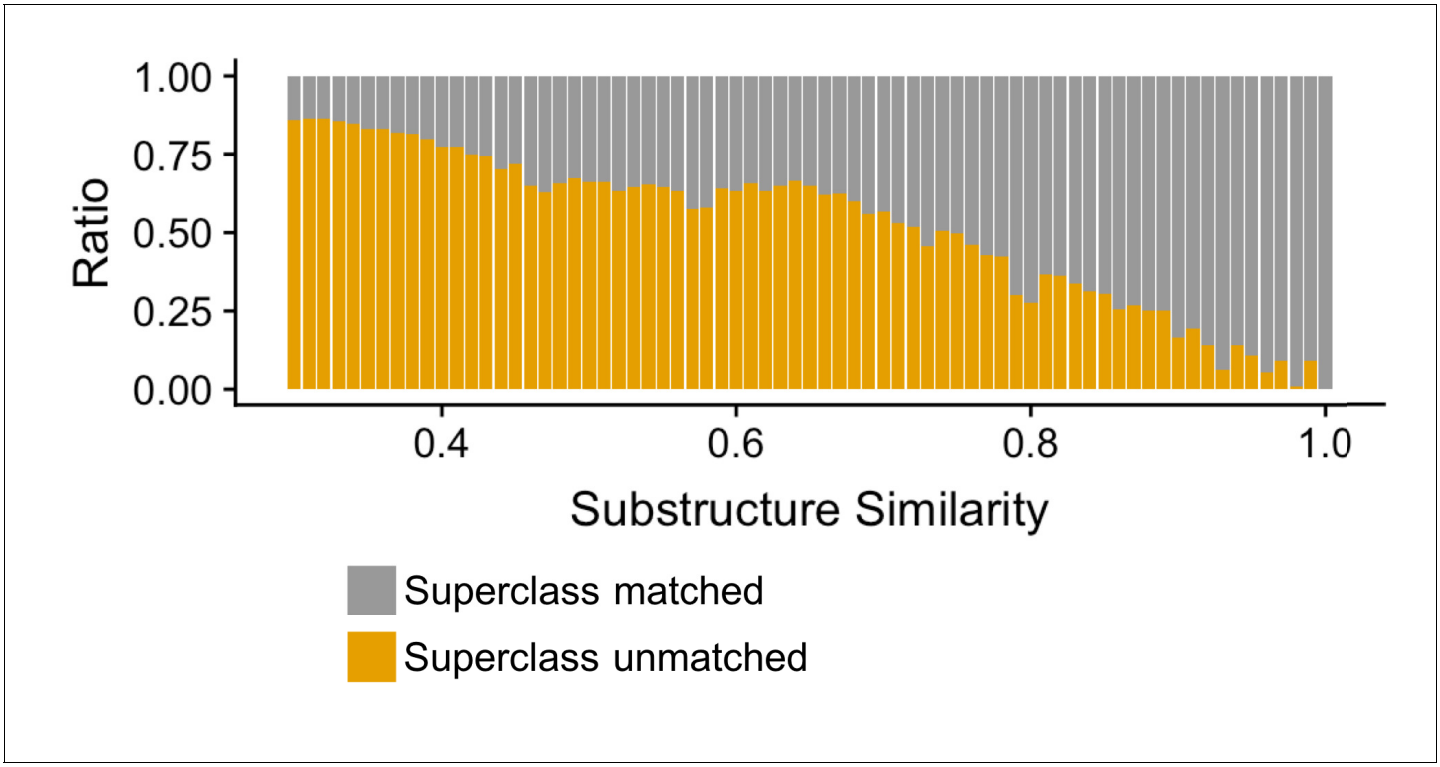

Figure 5. Compound-pairs share superclass annotation at a greater frequency as substructure similarity scores increase. Ratio of compound-pairs substructure similarity with matched and unmatched superclass annotation for all compound pairs represented in MicrobeFDT. Within the hierarchical ClassyFire classification schema, the superclass level annotation represents the second level and includes 31 different structure-based categories (***Djoumbou Feunang et al., 2016***).

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question informs therapeutic approaches for targeting specific enzymes to modulate patient responses to drugs and foods.

In MicrobeFDT, we quantify how many taxa have the capacity to carry out a specific function by applying a modified Simpson index function to compute an Enzyme Commission number-specific dominance (ECs_D) score for all enzymes present in the network. ECs_D scores are based on the abundance of enzymes annotated at the species level across healthy human metagenomes from the Integrative Human Microbiome Project (iHMP) (***Proctor et al., 2014***) and are normalized between 0 and 1. Functions carried out by small numbers of species have values closer to 0 while functions carried out by taxonomically diverse groups have functions closer to 1. Thus, the ECs_D indicates how broadly distributed a function is, a crucial metric for (1) understanding how to modify a function in the microbiome and (2) predicting how disruptive to the community modifying a function might be.

To validate ECs_D scores we first identified biochemical pathways containing enzymes with high and low taxonomic dominance in the literature. Bacterial synthesis of various B group vitamins including biotin, cobalamin and riboflavin vary in the number of potential producers at the Phylum level (***Magnúsdóttir et al., 2015***). The most commonly synthesized B vitamin across diverse microbial taxa is riboflavin while vitamin B12 is dominated by Fusobacteria (***Magnúsdóttir et al., 2015***). The ECs_D scores of cobalt-precorrin-2 C(20)-methyltransferase (0.305502) from the anaerobic Vitamin B12 synthesis pathway and riboflavin synthase (0.691618) from the riboflavin synthesis pathway in MicrobeFDT agree with the prior systematic genome assessment and experimental results of ***Magnúsdóttir et al. (2015)*** (***Figure 7***). While most bacteria do not synthesize sphingolipids, sphingolipid biosynthetic capacity has been identified in *Sphingomonas spp*, *Bacteroides* and human intestinal pathogens that synthesize and incorporate sphingolipids into their membranes or target host sphingolipids as a point of entry into host cell types (***Heaver et al., 2018***; ***Heung et al., 2006***; ***Olsen and Jantzen, 2001***). The low ECs_D score of phosphatidate phosphatase (0.007353), an

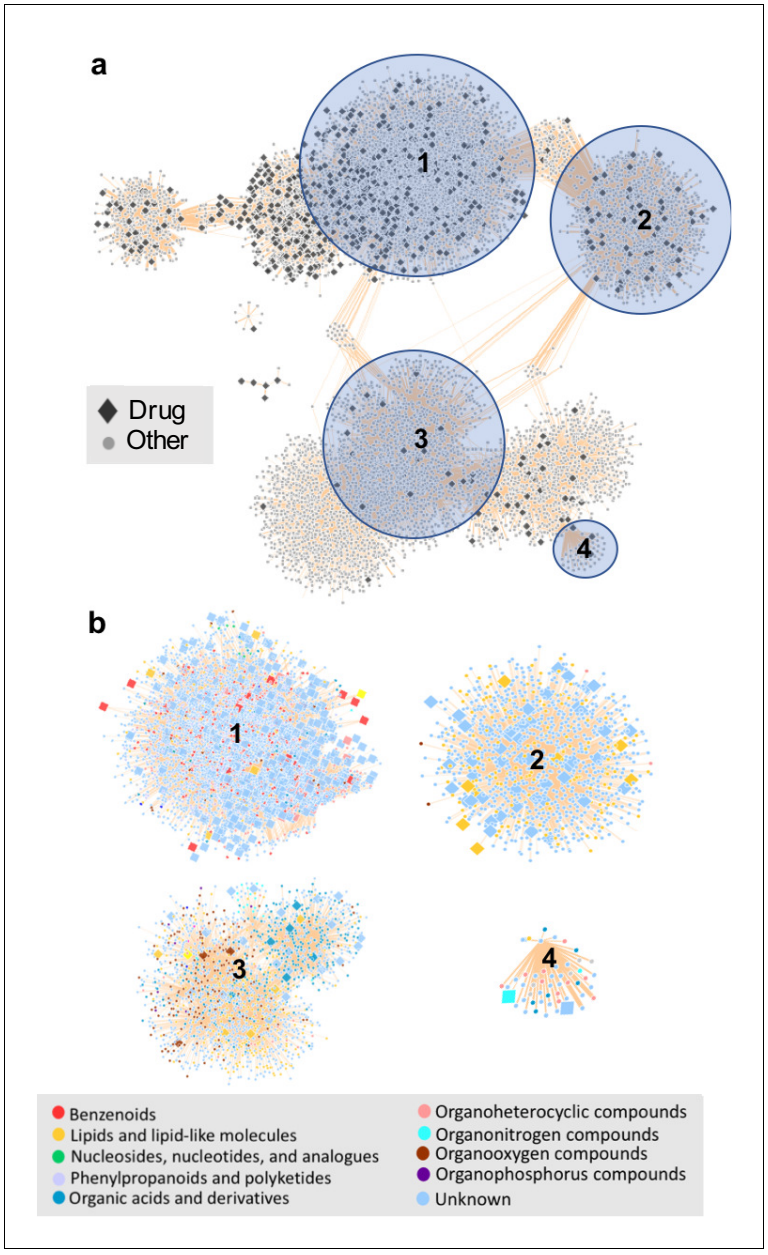

Figure 6. The chemical space of the gut microbiome. (**a**) Chemical similarity network of food-derived or endogenous compounds (gray circles, "Other") and therapeutic drugs (black diamonds, "Drug"). Tan edges are weighted by substructure similarity where thicker edges indicate higher substructure similarity. The distribution of compounds in chemical similarity space illuminates regions of low and high chemical substructure overlap between drugs and other compounds. (**b**) Compounds from selected regions of the network are colored by their superclass level taxonomy based on the FooDB chemical structure classification (***Wishart, 2012***). Food-derived or endogenously produced compounds are identified with blue circles, therapeutic drugs with red diamonds. Within high-drug density, highlighted regions **1** and **2**, drugs share substructure similarity with food-derived benzenoids, lipids, phenylpropanoids and polyketides. In the low-drug density highlighted region **3**, drugs overlap with organonitrogen compounds and nucleosides. Region **4** includes organonitrogen compounds and nucleosides in addition to lipid-like molecules which have minimal overlap with therapeutic drugs.

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The following source data and figure supplement are available for figure 6:

Source data 1. Chemical similarity scores for drug and non-drug compounds.

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Figure 6 continued on next page

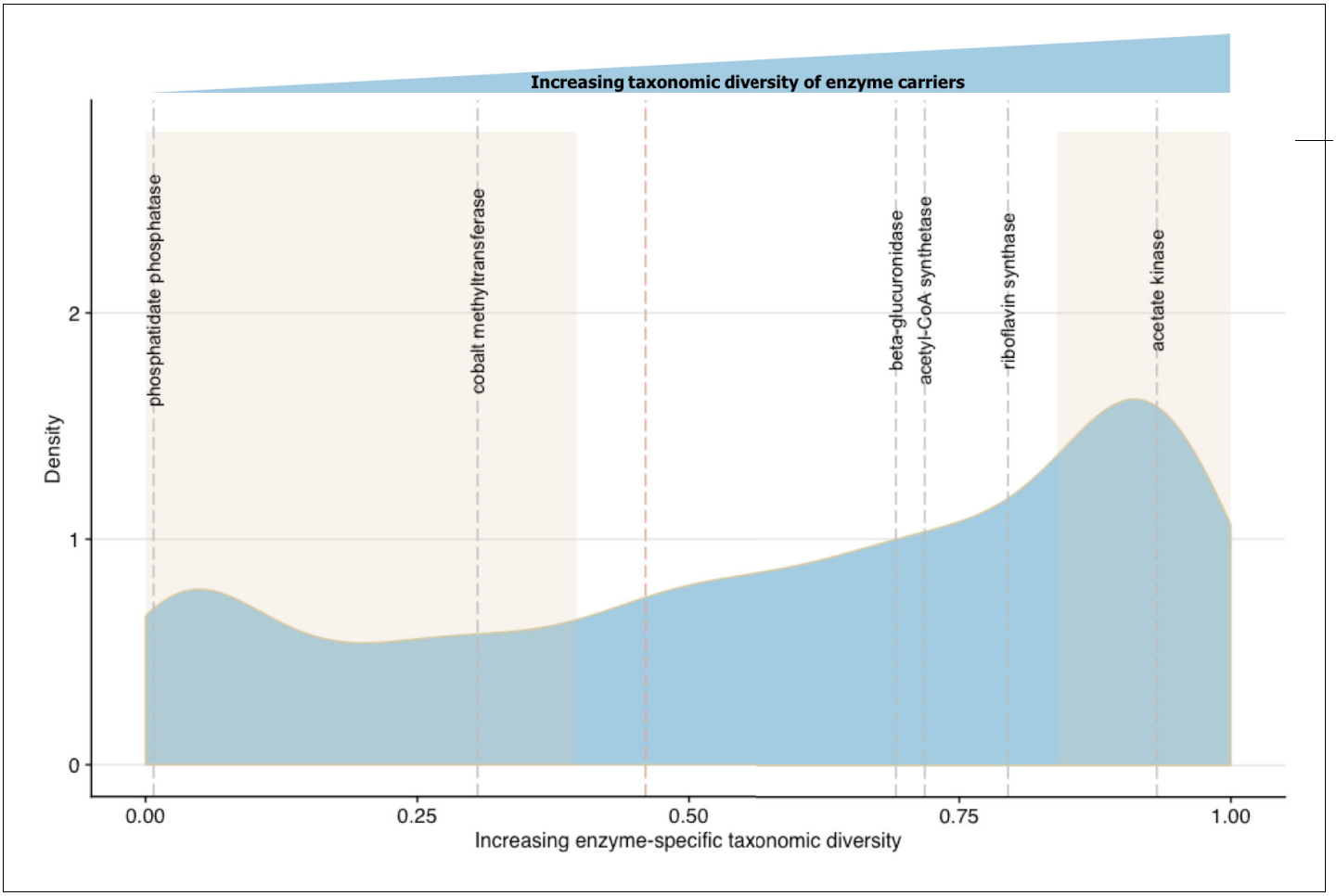

Figure 7. Linking enzymatic functions with taxonomic diversity. The Simpson index was adapted to describe enzyme-specific taxonomic dominance and diversity based on enzyme abundance in taxonomy-linked gene counts across healthy individuals in the Integrative Human Microbiome Project (***Proctor et al., 2014***). We define a microbial enzyme as high dominance and low taxonomic diversity if its Simpson index value falls below 0.46 (red dotted line), the mean value across all enzymes. Dominance-diversity values for gut microbiota functions that fall above or below the mean are highlighted by gray dashed lines and include the following enzymes and pathways: phosphatidate phosphatase (0.007353), cobalt-precorrin-2 C(20)-methyltransferase (0.305502) from the Vitamin B12 synthesis pathway, β-glucuronidase (0.691618), Acetyl-CoA synthase (0.718163) which is involved in the production of propionate from complex carbohydrates, riboflavin synthase (0.794781) from the riboflavin synthesis pathway and acetate kinase (0.931892) which is involved in acetate production. The shaded regions indicate the range of EDs_D values that are one standard deviation above and below the mean and reflect the most broadly distributed functions and most specialized functions.

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enzyme involved in sphingolipid biosynthesis and metabolism (***Olsen and Jantzen, 2001***), mirrors the limited distribution of the sphingolipid biosynthetic capacity across gut microbes.

Combining chemical and toxicity similarity to predict microbial N-demethylase contribution to drug metabolism and toxicity

To provide a practical example of using multiple features of MicrobeFDT to identify uninvestigated microbiota-driven drug toxicity, we searched the network for compounds with high structural and toxicity similarity. Among these compounds were the ovarian cancer drug altretamine (***Lee and Faulds, 1995***) and the environmental contaminant melamine (***Figure 8***). Both melamine and altretamine have toxicity profiles that include diarrhea and renal toxicity (***Rose et al., 1996***; ***Zheng et al., 2013***). Melamine, an industrial compound, has experimentally validated microbiome-mediated toxicity (***Zheng et al., 2013***). Altretamine toxicity, however, has not previously been linked to an

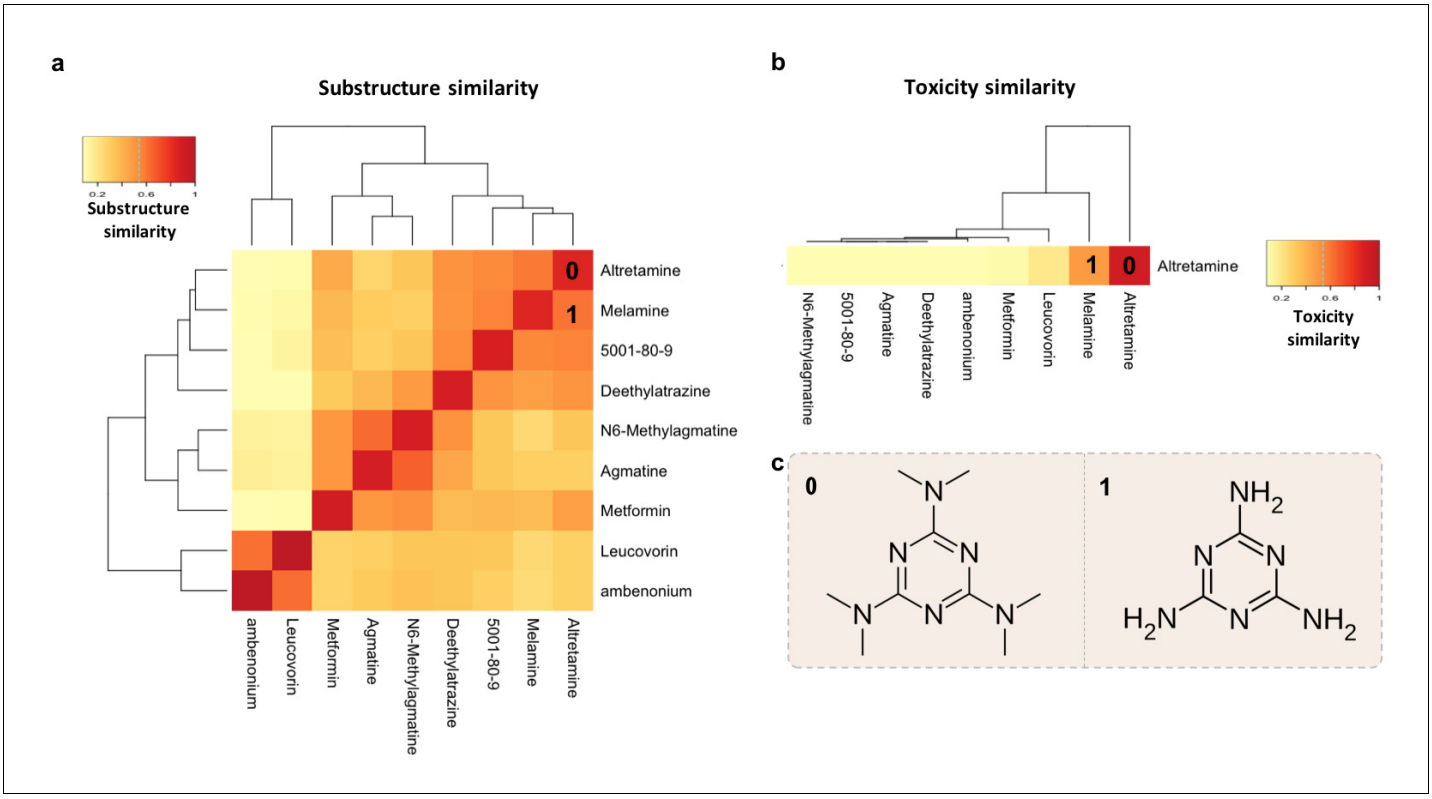

Figure 8. Structure-toxicity relationship between melamine and altretamine suggests a role for microbial N-demethylases in altretamine toxicity. (**a**) Substructure overlap between altretamine and its nearest neighbors in MicrobeFDT. A Z-score based threshold of significant overlap indicates that altretamine has both high substructure and (**b**) toxicity overlap with melamine. (**c**) The two compounds are distinguishable by the presence of N-methyl groups.

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The following figure supplement is available for figure 8:

Figure supplement 1. Phylogenetic distribution of N-demethylases in healthy human guts.

DOI: https://doi.org/10.7554/eLife.42866.013

individual's gut microbiota. Approximately half of patients taking altretamine orally experience various forms of gastrointestinal toxicity including diarrhea, nausea and/or vomiting (***Keldsen et al., 2003***).

Within the network altretamine is linked to microbial N-demethylase enzymes which may remove methyl groups from this compound, potentially leading to similar toxic effects as seen with melamine. We found no published experimental evidence of gut microbiota mediated conversion of altretamine. However, N-demethylases in *Pseudomonas putida CBB5* enable this microbe to grow on caffeine and other purine alkaloids as the sole carbon and nitrogen source; thus annotated N-demethylases in *P. putida CBB5* can act on compounds that are structurally similar to altretamine (***Summers et al., 2012***). Furthermore, we identify hypothetical proteins homologous to *Pseudomonas putida CBB5* N-demethylases in a subset of healthy human guts (***Figure 9—figure supplement 1***). We hypothesized that gut microbial N-demethylases may partially or completely N-demethylate altretamine, converting it into metabolites that contribute to patient toxicity.

A first step in validating this hypothesis is to demonstrate that the gut microbiome can demethylate altretamine. We incubated altretamine in a pooled fecal slurry generated from three healthy individuals and monitored altretamine and potential metabolites using LC-MS. We controlled for the formation of spontaneous N-demethylation of altretamine, which has been reported in the literature (***Damia and D'Incalci, 1995***), and found that a metabolite that is structurally identical to pentamethylmelamine, a demethylated altretamine metabolite, increases in active fecal microcosms over 48 hr (***Figure 9***). In active fecal biotic conditions the metabolite continually increased between

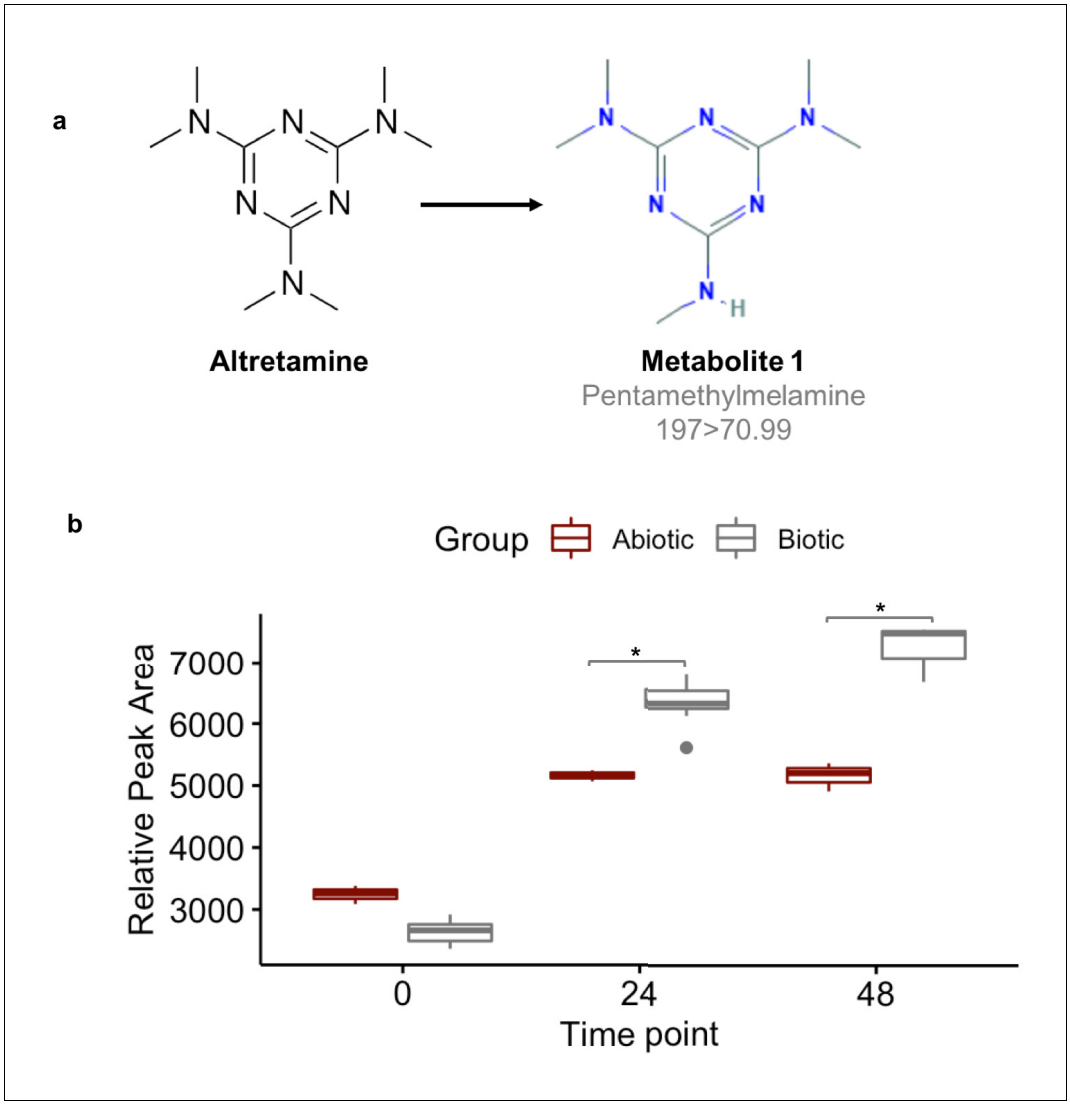

Figure 9. Fecal microbiomes actively demethylate altretamine. Liquid chromatography with tandem mass spectrometry (LC-MS) was used to quantify the formation of (**a**) pentamethylmelamine, an N-demethylated metabolite of altretamine identified in the pooled fecal microbiomes of three healthy unrelated individuals. (**b**) The formation of metabolite 1 at 24 and 48 hr was significantly increased under the experimental condition in comparison to the contribution of spontaneous N-demethylation by an unpaired two-sample Wilcoxon test (*=$P < 0.05$).

DOI: https://doi.org/10.7554/eLife.42866.014

The following figure supplement is available for figure 9:

Figure supplement 1. Experimental design and controls used to quantify fecal microbiome turnover of altretamine.

DOI: https://doi.org/10.7554/eLife.42866.015

time 0 and 48 hr. Killed controls demonstrated an increase in metabolite between 0 and 24 hr, though to a lesser extent than in active fecal microcosms. Notably there was little metabolite formation after 24 hr, indicating that in addition to abiotic N-demethylation, active gut microbes demethylate altretamine to the putative metabolite pentamethylmelamine.

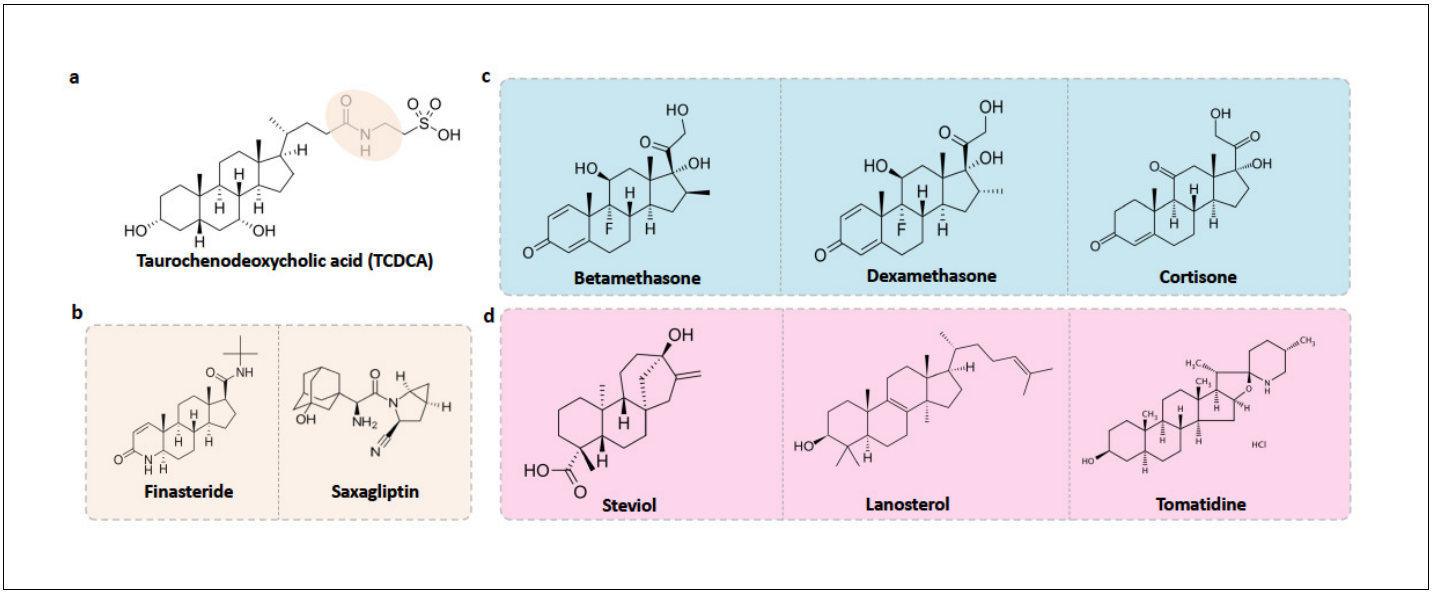

Figure 10. Food-drug compounds chemically similar to TCDCA are putative antimicrobials. (**a**) Chemical structure of taurochenodeoxycholic acid (TCDCA). (**b**) TCDCA-like therapeutic drugs that are susceptible to bile salt hydrolases include finasteride and saxagliptin. (**c**) Non-susceptible TCDCA-like therapeutic drugs include betamethasone, dexamethasone and cortisone. (**d**) TCDCA-like food derived compounds include steviol, lanosterol and tomatidine.

DOI: https://doi.org/10.7554/eLife.42866.016

The following figure supplement is available for figure 10:

Figure supplement 1. Phylogenetic distribution of bile salt hydrolases in healthy human guts.

DOI: https://doi.org/10.7554/eLife.42866.017

Food derived compounds and non-antibiotic therapeutic drugs with potential antimicrobial properties

MicrobeFDT suggests an unrecognized role for bile acid-like foods and drugs in altering the composition of the human gut. Conjugated primary bile acids (BA) function as potent detergents and antimicrobial agents capable of dissolving microbial membranes and causing intracellular acidification; bile acid function is linked to specific structural features of these compounds (***Jones et al., 2008***; ***Begley et al., 2006***). Taurochenodeoxycholic acid (TCDCA) is a taurine conjugated primary bile acid with a diet-tunable concentration in the gut (***Ridlon et al., 2016***). Energy drinks, animal protein and fish are rich sources of taurine while vegetarian and vegan diets dominated by fruits, vegetables, legumes and soy are poor sources (***Ridlon et al., 2016***). Taurine conjugated bile acids are hypothesized to contribute to the etiology of colorectal cancer by generating hydrogen sulfide during microbial mediated de-conjugation of taurine conjugates (***Ridlon et al., 2016***). Conjugated primary bile acids have demonstrated in vitro activity as antimicrobial compounds, for example glycocholic and taurocholic conjugated bile acids are bacteriostatic, inhibiting *S. aureus* growth by decreasing intracellular pH and disrupting the proton motor force (***Sannasiddappa et al., 2017***).

Using MicrobeFDT, we identified therapeutic drug and food compounds that are structurally similar to TCDCA; we propose these compounds might have similar antimicrobial effects on the microbiome and we discuss studies from other groups that support this hypothesis (***Figure 10a***).

Bile salt hydrolase (BSH) mediated bile salt deconjugation is one mechanism that gut microbes use to detoxify conjugated primary bile acids (***Begley et al., 2006***); thus BSH activity may support gut bacterial persistence in face of frequent contact with primary BAs. We first subdivided TCDCA-like antimicrobial compounds based on BSH enzyme susceptibility. BSH enzymes are phylogenetically diverse and abundant across healthy human fecal metagenomes (***Figure 10—figure supplement 1***). Among the BSH-susceptible therapeutic drug compounds, we identified known antibiotics such as clindamycin and lincomycin, as well as non-antibiotic prescribed therapeutics such as finasteride, which is used for the treatment of androgenetic alopecia (***Manabe et al., 2018***) and benign prostatic hyperplasia (***Chau et al., 2015***), and the oral antidiabetic drug saxagliptin (***Men et al.,***

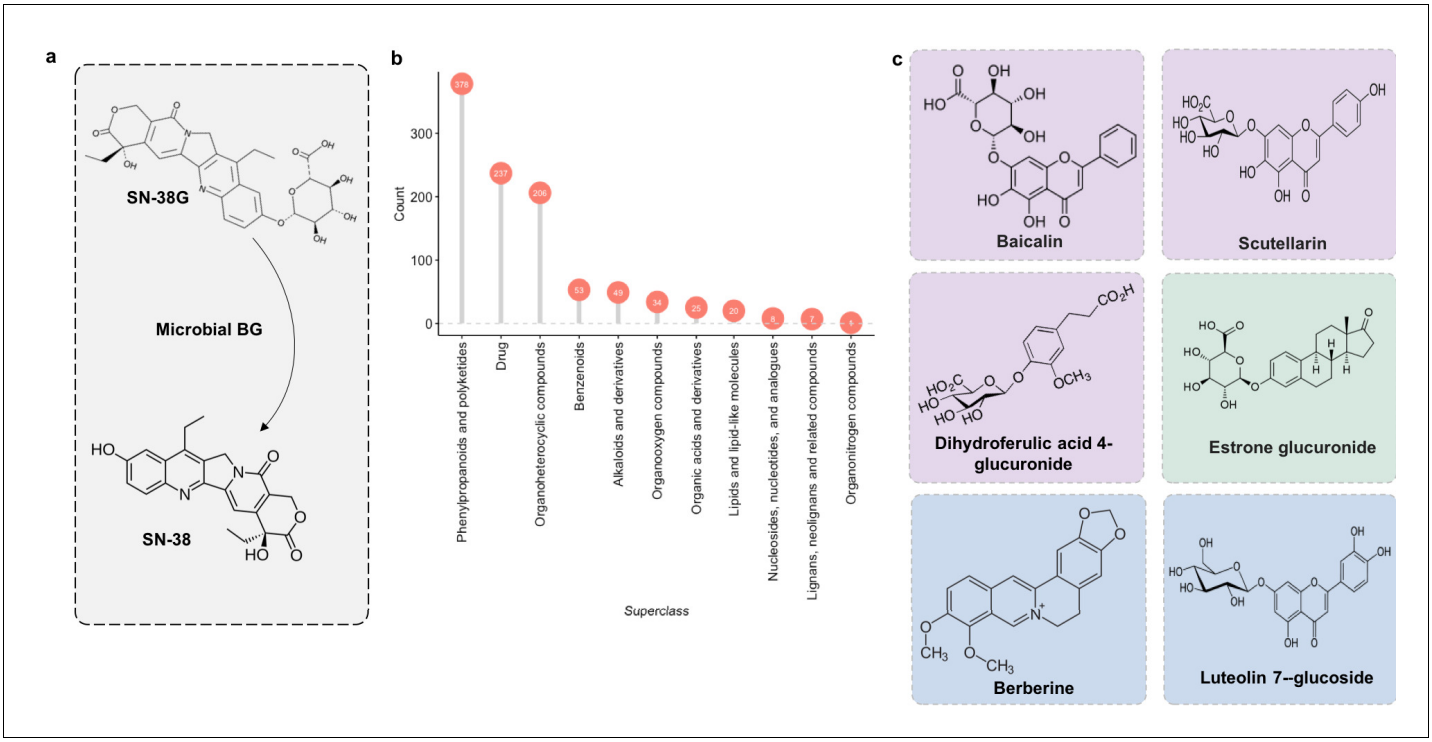

Figure 11. Microbial β-glucuronidase potential substrate pool of compounds structurally similar to SN-38G. (**a**) SN-38G conversion to SN-38 in the gut is mediated by microbial β-glucuronidases. (**b**) The substrate pool for β-glucuronidases with above threshold substructure overlap with SN-38G are members of a diverse range of chemical structure superclasses as defined by FooDB chemical ontology (***Wishart, 2018***). (**c**) These compounds include glucuronidated food-derived compounds (purple), endogenous glucuronides (tan) and other non-glucuronides (blue).
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2018) (***Figure 10b***). Notably, in a Wistar rat model of chronic bacterial prostatitis (CBP), finasteride reduces bacterial infection as a single agent and has a synergistic effect with ciprofloxacin through an unknown mechanism (***Lee et al., 2011***). Through in vitro studies, Chavex-Dozal and colleagues propose a role for finasteride in the prevention of *Candida albicans* biofilm formation and filamentation (***Chavez-Dozal et al., 2014***). These experimental results support the hypothesis that finasteride may have unrecognized off-target antibiotic effects.

Most TCDCA-like compounds in MicrobeFDT are non-BSH susceptible food-derived compounds. Among the TCDCA-like non-BSH susceptible compounds are oral steroid medications, including dexamethasone and betamethasone (***Figure 10c***). The immunomodulatory activities of glucocorticoids, including dexamethasone, involve the activation of genes related to anti-inflammatory cytokines such as IL-10 and proteins that inhibit the pro-inflammatory NFκB signaling pathway (***Coutinho and Chapman, 2011***; ***Huang et al., 2015***). Dexamethasone has known anti-microbial properties. For example, dexamethasone has dose-dependent anti-microbial activity against clinically isolated *Streptococcus milleri*, *Aspergillus flavus*, and *Aspergillus fumigatus* in culture, while not killing *Staphylococcus aureus* (***Neher et al., 2008***). *Pseudomonas aeruginosa* was found to be susceptible to dexamethasone at high concentrations (***Neher et al., 2008***). Cortisone, which also has significant structural overlap with TCDCA, has been linked to a variety of opportunistic infections by enteric bacterial pathogens, for example an increase in gastrointestinal parasites (***Nair et al., 1981***) and reactivation of *Chlamydia pneumoniae* (***Laitinen et al., 1996***).

Food-derived TCDCA-like compounds include steviol, lanosterol and tomatidine. Steviol is a component of stevia which has antimicrobial properties against *Borrelia burgdorferi in vitro* (***Theophilus et al., 2015***), and lanosterol derivatives have antifungal activities (***Shingate, 2013***). Tomatidine was recently identified as an antibiotic molecule that inhibits ATP synthesis against *Staphylococcus aureus* (***Lamontagne Boulet et al., 2018***); we hypothesize that the antimicrobial activity of this compound may include intracellular acidification given its structural overlap with

TCDCA (*Figure 10d*). The network thus identifies compounds with known anti-microbial properties in addition to proposing additional, structurally related compounds with uncharacterized effects. We propose that in addition to modulating immune responses, bile salt-like compounds may selectively alter human microbiomes, again, with unknown consequences for treatment outcomes and health.

MicrobeFDT identifies the diet-derived substrate pool for microbial BGs and candidates for nutritional competition with SN-38G

We next applied MicrobeFDT to identify diet-derived substrates of a gut carbohydrate active enzyme, β-glucuronidase. β-glucuronidases play a major role in the toxicity of the colorectal cancer chemotherapeutic prodrug irinotecan (CPT-11), whose active form, SN-38, is inactivated by hepatic glucuronidation and excreted into the gut as the inactive metabolite SN-38 glucuronide (SN-38G) (*Wallace et al., 2010*; *Sparreboom et al., 1998*). Microbial β-glucuronidases hydrolyze the glucuronide group, releasing the aglycone SN-38 into the intestinal environment (*Figure 11a*). Deconjugation promotes epithelial damage and severe diarrhea in some patients and in mouse models (*Wallace et al., 2010*; *Sparreboom et al., 1998*; *Slatter et al., 2000*).

We previously demonstrated that individual human fecal samples have variable capacities to deconjugate SN-38G (*Guthrie et al., 2017*). Identifying the full substrate pool of β-glucuronidases is thus important for 1) understanding how diet contributes to β-glucuronidase abundance and expression levels in the gut and 2) to enable novel therapeutic strategies such as nutritional competition.

Some food compounds may be preferred substrates for microbiome β-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 β-glucuronidases. Most compounds identified as significantly similar to SN-38G were food derivatives or other constituents (*Figure 11b*). Among these targets were flavonoids such as baicalin and scutellarin which are widely distributed in plants (*Kumar and Pandey, 2013*) (*Figure 11c*). We propose that these compounds may compete with SN-38G for turnover by microbial β-glucuronidases and are a potential avenue for decreasing the adverse drug responses associated with irinotecan administration.

Discussion

The chemical space of the human gastrointestinal tract ecosystem is shaped by host dietary intake, xenobiotic exposure, and host and gut microbiome derived products. In turn, diet shapes the composition and potential niches of organisms within human gut microbiomes. A combination of compound, host, and microbiome features influence potential microbial metabolism. Examining these features individually cannot reliably infer clinical phenotypes associated with microbiome/compound interactions. Two molecules may have the same toxicity profile but very different biochemistry, for example. Automated enzyme annotation may be incorrect, and compound structural similarity is often insufficient to predict substrate preferences. Finally, enzymes that carry out a reaction associated with a patient phenotype may be unevenly distributed across microbes and across human microbiomes. MicrobeFDT is designed to overcome some of these limitations by enabling a more holistic analysis of toxicity, structure, metabolism and ecology. We used a combination of network features to successfully predict the novel microbial metabolism of the cancer drug altretamine.

Metabolomics data indicate active demethylation of altretamine by fecal slurries but cannot propose a mechanism by which microbial activity metabolizes this compound. MicrobeFDT suggests that altretamine is a putative substrate of microbial N-demethylases. Microbe-mediated N-demethylation reactions, and the subsequent release of N-methyl groups, occur as a part of amino acid and nucleotide metabolism (*D'Mello and International, 2017*). Notably, diet is a source of amino acids which are derived in part from metabolism of dietary choline, carnitine and legumes, and have physiological functions for bacteria including osmoprotection and incorporation into bacterial flagellin proteins and lipid membranes (*Goldfine and Hagen, 1968*). Amino acid-specific bacterial N-demethylases have been identified but are poorly characterized (*Wargo, 2017*). Additionally, fecal and species specific N-demethylation has been observed for other therapeutic drugs and commonly ingested compounds such as caffeine, which clusters with altretamine in the network due to its structural similarity (*Summers et al., 2012*; *Caldwell and Hawksworth, 1973*; *Clark et al., 1983*;

Colombo et al., 1982). N-demethylases can act on chemically diverse substrates (*Wargo, 2017*; *Burnet et al., 2000*). Given this body of evidence, we propose N-demethylases may demethylate altretamine partially or completely, creating metabolites that are toxic to patients.

Human gut metagenomic data indicate that Rieske family oxidative N-demethylases are carried by a small, phylogenetically conserved set of gut taxa, with notable inter-personal variation. That these enzymes require oxygen may make them more relevant during disruptions to gut homeostasis when oxygen becomes available, such as colonic crypt hyperplasia caused by injuries to the intestinal epithelia (*Litvak et al., 2018*). Finally, we note that N-demethylation in the gut may be relevant for differences in individual metabolism of numerous other compounds such as the cancer drug tamoxifen, the widely used antihistamine diphenhydramine, and theobromine, a plant alkaloid found in foods. While is possible that N-demethylation is enzyme independent or that enzymes annotated with other functions are responsible for this activity, MicrobeFDT provides a clear path forward for mechanistic studies of N-demethylation in the gut.

Beyond predicting the toxicity or function of gut compounds, MicrobeFDT identifies the larger substrate pool for enzymes involved in drug metabolism. For example, shared conjugation patterns may represent a clinically relevant way to group compounds that share microbial enzymatic processing. As an example, compounds inactivated by glucuronidation are susceptible to microbial β-glucuronidase-mediated reactivation. 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 β-glucuronidases that this drug interacts with. Structurally similar compounds may act competitively – via inhibition of SN-38G turnover by higher priority β-glucuronidase substrates or synergistically – via substrate inducible transcriptional upregulation of β-glucuronidase 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.

Outside of drug metabolism, β-glucuronidases mediate deconjugation and enterohepatic circulation of estrogens, impacting the human host total estrogen burden (*Shapira et al., 2013*; *Kwa et al., 2016*). It has been hypothesized that β-glucuronidase deconjugation may result in greater absorption of estrogens and thus influence the development of estrogen-driven cancers including breast, ovarian and endometrial cancers (*Shapira et al., 2013*; *Kwa et al., 2016*). Our network is useful for developing mechanistic hypotheses targeting how diet and the microbiome jointly act as moderators of estrogen-driven cancers, and to suggest opportunities for diet-based modulation of total estrogen levels.

An important step towards characterizing the role of the gut microbiome in shaping individual responses to foods and drugs is identifying how gut microbiome metabolism varies from compound to compound and how this metabolism relates to inter-personal variation in diet or drug responses to specific compounds. To tackle this challenge, we add the context of taxonomic diversity to the predicted impact of microbial on specific targets by quantifying enzyme specific taxonomic dominance and diversity with a novel metric, the ECs_D score. This score distinguishes enzymatic activities carried out by single species or few taxa, such as N-demethylase activity, from those where many taxa may contribute, such as β-glucuronidase and bile salt hydrolase activity. The ECs_D score is a readout of potential substrate metabolism at the community level that can be linked to inter-personal variation in gut function and phenotypic outcomes.

The structural similarity network that underlies MicrobeFDT could be improved by using compound atom and bond connectivity information as an additional filtering step for compounds of interest, for example by using information from the SMARTS molecular pattern matching language (*Chepelev et al., 2012*). SMARTS can be used to specify sub-structural patterns in molecules; these patterns could be added to MicrobeFDT as an additional information source indicating potential active moieties in compounds.

MicrobeFDT does not predict substrate specificity for microbiome enzymes; available data and methods are not sufficient to achieve this goal. Enzyme promiscuity also shapes the probability that two chemically overlapping compounds will be processed by the same enzyme. A future improvement to our resource could extract data from resources like RetroRules (*Duigou et al., 2019*), which uses SMARTS strings to define reaction rules, or utilize the Promis server measure of enzyme multifunctionality (*Carbonell and Faulon, 2010*) to further support a user's ranking of hypothesized compound-enzyme interactions.

It must be noted that the set of diet-derived and xenobiotic compounds that form the basis of the network is a non-exhaustive representation of the gut chemical landscape. Efforts to characterize the gut chemical space using metabolomics approaches including mass spectrometry and nuclear magnetic resonance spectroscopy will play key roles in elucidating a fuller gut chemical landscape (***Vernocchi et al., 2016***; ***Wishart, 2012***). MicrobeFDT does not address the issue of compound concentrations in the gut, which are vital to assess likely physiological effects. Lastly, MicrobeFDT is limited to enzymes in KEGG, and does not address the many hypothetical enzyme sequences identified through metagenomic sequencing. Despite these limitations, MicrobeFDT highlights areas of known gut chemical space for which our understanding of microbial processing is limited and is a powerful tool to guide mechanistic investigations into diet-drug-microbiota interactions.

Materials and methods

Key resources table

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Biological sample (community microbiota, feces)	fecal sample	other		fecal sample obtained from three healthy adults
Chemical compound, drug (altretamine)	altretamine	Sigma	Pubchem_ID:329748966; CAS_No:645-05-6	prepared in DMSO, 0.1 mM final concentration in fecal slurry
Other	Brain Heart Infusion broth	Himedia	Himedia:M210I	
Chemical compound (Dimethyl sulfodixe)	DMSO	MP Biomedicals	MP:191418; CAS_No:67-68-5	
Chemical compound (Melamine-triamine-(15N3))	Melamine-triamine-($^{15}N_3$)	Sigma	Pubchem:329758619; CAS_No:287476-11-3	prepared in DMSO, 400 nM final concentration in analytical sample

MicrobeFDT pipeline

The MicrobeFDT graph database encodes heterogeneous information on the interactions between compounds and microbial enzymes in the gut chemical landscape, highlighting the following four relationships across 13,440 nodes (10,822 xenobiotic, diet-derived and human gut endogenous compounds, 2062 microbial enzymes and 525 therapeutic drug use labels) defined from publicly available data directly or computed: (1) compound-compound substructure similarity; (2) compound-compound toxicity similarity; (3) microbial enzyme-compound interactions; and (4) drug-indication associations. The database is implemented in Neo4j (https://neo4j.com/) and can be queried through the Cypher Query Language. Through graph-based searches users can query the network based on node or relationship features. MicrobeFDT can be accessed here (***Guthrie, 2019***).

Publicly available datasets and resources used as inputs for the network

The SIDER 4.1 side effect resource is a database of approved medicines and their known adverse reactions (***Kuhn et al., 2016***). Drugs from this database with pharmacokinetic profiles that involve entry into the gastrointestinal tract were identified through literature mining and manual curation and indexed by their PubChem CID identifier (***Kim et al., 2016***). Drug use annotations were based on the WHO Anatomical Classification System (***Skrbo et al., 2004***). FooDB (http://foodb.ca/) (***Wishart, 2018***), a database containing raw food component structures, biological interactions and chemical properties was the source of food components linked to PubChem CID identifiers. ClassyFire was used to annotate all xenobiotic and food derived compounds with a shared chemical taxonomy (***Djoumbou Feunang et al., 2016***).

To link microbial enzymes to the set of compounds they metabolize we used KEGGREST (v1.14.1) to retrieve KEGG compound identifiers with links to Enzyme Commission numbers, metabolic

modules and pathways, and presence in either organisms listed as microbial or *Homo sapiens* (**Tenenbaum, 2019**). Enzyme abundance data across human metagenomes were determined based on the total abundance of each enzyme in the healthy participants of the Human Microbiome Project. This data was extracted from the Integrated Microbial Genomes database (**Markowitz et al., 2012**). Enzyme specific dominance scores (ECs_D), which is a measure of the number of different species that carry a specific enzyme, were computed based on species-specific enzyme abundance data from healthy individuals from the Integrative Human Microbiome Project (**Proctor et al., 2014**).

Construction and assessment of the drug-food chemical similarity network

Chemical similarity calculation

To determine the pairwise chemical substructure similarity between all compounds we used the PubChem 2D molecular fingerprint (**Kim et al., 2016**). The fingerprint is an 881 dimension binary vector in which each bit represents a specific element, functional group, ring system or other discrete chemical entity (**Kim et al., 2016**). Similarity was defined by the Tanimoto coefficient of the molecular fingerprint representations present between two compounds (**Bajusz et al., 2015**).

Network construction

Similarity scores are percentages of substructure overlap between pairs of compounds and have values between 0 to 1. Similarity scores are filtered such that compound pairs with less than 0.3 substructure similarity were removed. These pairwise similarity scores formed the basis of the undirected chemical similarity network, where nodes represent compounds and edges represent substructure similarity score.

Network filtering

To cluster compounds in the network based on substructure similarity we used the Walktrap community detection method (**Pons and Latapy, 2006**) implemented in R/igraph v.1.1.1 (**R Development Core Team, 2016**). Within a community, significant similarity scores were defined as those with Z-scores of 1 standard deviation or greater away from the mean (**Baldi and Nasr, 2010**).

Assessment of compound substructure-based clustering recapitulation of chemical ontology

The MicrobeFDT substructure similarity network is defined by the Tanimoto coefficient of the PubChem 2D molecular fingerprint representations between two compounds (**Kim et al., 2016**; **Bajusz et al., 2015**). To assess how well compound substructure-based clustering recapitulates chemical ontology we compared network features between the MicrobeFDT substructure similarity network and randomized network with the same number of nodes, edges and labels. Each compound label includes a ClassyFire (**Djoumbou Feunang et al., 2016**) schema derived hierarchical set of chemical descriptors. The chemical similarity network was rendered in Cytoscape using NetworkRandomizer (**Martens et al., 2014**). Using the Wilcoxon rank-sum test we compared superclass level chemical descriptors across connected compounds between the real and random network. For the MicrobeFDT network, we also computed the ratio of compounds pairs with matched Superclass annotation to unmatched annotations for all pairs with the same substructure score to assess the relationship between substructure similarity and shared chemical ontology.

Predicting the probability of association of compound pairs both serving as substrates for an enzyme based on substructure and physiochemical parameters

Each compound pair was assigned one of two labels, associate or non-associated, based on whether both compounds are substrates for the same enzyme (associated) or not (non-associated), given compound-enzyme relationships in the KEGG database (**Kanehisa and Goto, 2000**). The DataWarrior program (**Sander et al., 2015**) was used to identify the following parameter categories for each compound: geometry, functional groups, aromaticity, amino acid composition, polarity and hydrophobicity. In order to translate compound pair substructure and physiochemical parameters into a

probability of overlapping metabolism we used a machine learning approach for generating probability estimates for multi-class classification problems (**Zhang et al., 2013**; **Wu et al., 2004**). Briefly, this approach builds a multi-class prediction model by using pair-wise coupling. We then implemented the prediction model using the probsvm package in R using a one-vs-one decomposition scheme (**Zhang et al., 2013**).

Assessing toxicity similarity

Toxicity similarity was computed as described by Campillos and colleagues (**Campillos et al., 2008**) with three key steps: (1) extraction and standardization of side effect concepts across drugs of interest; (2) weighting of unique side effect concepts based on frequency of occurrence and correlation with other side effects; and (3) computation of pair-wise toxicity similarity between drugs based on weighted side-effect concept values. Briefly, Campillos et al. curated a dictionary of side-effects based on the Concepts of the Coding Symbols for Thesaurus of Adverse Reaction Terms (COSTART) ontology (**US Food and Drug Administration, 1995**). Side-effect information on therapeutic drug package labels was identified from publicly available sources and searched against this dictionary such that all unique side effect concepts per drug were based on COSTART ontology. For our analysis we used the side effect labels for therapeutic drugs of interest that were extracted from the Medical Dictionary for Regulatory Activities (**Brown et al., 1999**), which is an updated replacement of COSTART, and made publicly available at the download page for SIDER 4.1 which can be found here.

In Campillos et al., each side effect concept was given a rarity score which is the frequency at which it is found across all drug side effect lists. To account for co-dependence between side effects Campillos and colleagues also determined the correlation between all side effects based, using the Tanimoto score between pairs of side effects. This measure is based on how many drugs share a given side effect relative to the number of drugs that have either. The resulting matrix was used as input for the Gerstein-Sonnhammer-Chothia Algorithm (**Gerstein et al., 1994**), to output a score for each concept that down weights concepts that are redundant. We used a publicly available implementation of this algorithm in R available here. Pair-wise toxicity similarity between drugs was computed based on summing the products of weights over all shared side effect concepts between drug pairs. We fit a linear regression to determine whether there is a linear relationship between compound pair substructure similarity and toxicity similarity.

Taxonomic signatures of microbial enzymes

For each enzyme, we computed an enzyme commission number-specific dominance (ECs_D) score. This score is an application of the Simpson's index, which is particularly sensitive to sample evenness (**DeJong, 1975**), and describes the dominance and diversity profile of species carrying the enzyme (**Ofaim et al., 2017**). The taxa-specific enzyme abundance information is based on data collected as a part of the integrative Human Microbiome Project (iHMP) (PRJNA306874) (**Proctor et al., 2014**). ECs_D scores are reported as Simpson index measure (**Simpson, 1949**) subtracted from one, as implemented in the phyloseq R package (**McMurdie and Holmes, 2013**). In this implementation, the Simpson dominance index per enzyme defined by its enzyme commission number (D(EC)) is computed such that n is number of individuals of each species that carry the enzyme and N is the total number of individuals of all species that carry the enzyme (1). For better interpretability, the dominance scores are subtracted from 1 (2).

$$D(EC) = \frac{\Sigma\, n(n-1)}{N(N-1)} \tag{1}$$

$$\mathrm{EC_{SD}} = 1 - D(EC) \tag{2}$$

Thus, enzyme functions carried out by small numbers of microbes have values closer to 0 while functions carried out by taxonomically diverse groups have functions closer to 1.

Altretamine microbiome turnover validation

Collection and preparation of fecal samples

Fresh fecal samples were provided by three healthy adult men aged 23–30 with no history of antibiotics for 6 months prior to the study. The study was approved by the Albert Einstein College of Medicine Institutional Review Board. Samples were deposited, immediately stored on ice, and processed within 1 hr. One gram stool from each donor was added to 300 mL BHI supplemented with 0.5% glucose (weight/volume) and homogenized. The final fecal slurry was thus comprised of the pooled feces of the three donors at 1% w/v.

Altretamine metabolism

Fecal slurry cultures were incubated at 37˚C in the dark under aerobic conditions. Altretamine stock was prepared in DMSO. Experimental cultures received a final concentration of 100 µM altretamine in DMSO and were prepared in triplicate. Triplicate heat-killed and denatured cultures were autoclaved three times on successive days and also received 100 µM altretamine in DMSO after the third autoclave. Background cultures received fecal slurry and DMSO but no altretamine. To determine matrix effects of altretamine in the media, a sterile media control was amended with 100 µM altretamine in DMSO. Cultures were sampled, immediately snap-frozen in liquid N_2 every 24 hr, and stored at −80˚C until analysis.

Altretamine and metabolite quantification

Samples were thawed, centrifuged, and 100 µL aliquots were added to 900 µL 80% methanol. Melamine-triamine-($^{15}N_3$) was used as internal standard. Altretamine and metabolites were identified using LC/MS (Waters Acquity LC system and Waters Xevo TQ MS). Liquid samples were diluted 1:50 in 80% methanol with melamine-triamine-($^{15}N_3$) as internal standard. Each sample was injected 3 times at 5 mL/injection. Separation was performed on an ACE2 C18 column set to 45˚C with 0.1% formic acid in 5% methanol (A) and 0.1% formic acid in methanol (B). Elution occurred at 0.35 ml/min with 100% A for 1 min, followed by a 1.5 min linear gradient from 100% A to 95% B, and finally 100% B for 1 min. The voltage was set to 0.044 kV.

Phylogenetic trees

N-demethylase phylogenetic tree

N-demethylases from *Pseudomonas putida* CBB5 (*ndmABCD*) (**Summers et al., 2012**) and *Sphingobium* sp. strain YBL2 (*pdmAB*) (**Gu et al., 2013**), both containing a Rieske non-heme iron oxygenase component, catalyze the N-demethylation of phenylurea herbicides and purine alkaloids, respectively; and range in size from 318 to 364 amino acids (**Summers et al., 2012**; **Gu et al., 2013**; **Sharma et al., 2018**). We clustered bacterial N-demethylase sequences described by Summers *et al.*, and Tao *et al.*, as well as protein sequences of >= 200 amino acids in length pulled based on text annotation from the RefSeq database (**Pruitt et al., 2005**) at 95% identity using the UCLUST algorithm (**Edgar, 2010**). The resulting 84 N-demethylase protein sequences served as a protein database which was mapped against the protein calls of healthy adult participants from the Human Microbiome Project (HMP) (PRJNA43021) using the UBLAST algorithm (**Edgar, 2010**) and e-value cutoff of e-40. N-demethylase hits of 200 amino acids or greater formed the basis of a phylogenetic tree which was constructed by aligning the protein sequences using MUSCLE with default parameters (**Edgar, 2004**). Aligned sequences were trimmed at 70% identity and phylogenetic trees were built with PhyML (**Guindon et al., 2010**) with 100 bootstrap replicates, a JTT model of substitution, and otherwise default parameters. The trees were visualized using the packages ggpplot2 (**Wickham, 2016**) and phyloseq (**McMurdie and Holmes, 2013**) in R (**R Development Core Team, 2016**). Each branch was colored based on the phylum level classification of the protein, marked by similarity to the experimentally characterized N-demethylase genes *ndmABCD* and *pdmAB* and by the normalized number of total hits found across individuals in the HMP. Black circles indicate bootstrap values of 80/100 or better.

Bile salt hydrolase phylogenetic tree

We identified bile salt hydrolase protein sequences based on text annotation from the RefSeq database (***Pruitt et al., 2005***) and developed a curated database of protein sequences that were clustered at 95% identity using the UCLUST algorithm (***Edgar, 2010***) resulting in 300 bile salt hydrolase protein sequences with a minimum amino acid length cutoff of 300. Bile salt hydrolase subunits can range in length up to 518 amino acids in the literature (***Breton et al., 2002***; ***Bron et al., 2006***; ***Schmid and Roth, 1987***). Sequence mapping against the HMP (***Human et al., 2012***), alignment and tree construction were carried out as described for the N-demethylases with the following exception: each branch representing a unique bile salt hydrolase sequence was marked by the presence or absence of reported activity in the literature.

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Data availability

Data to use or reproduce MicrobeFDT can be found at https://github.com/kellylab/microbeFDT-neo4j (copy archived at https://github.com/elifesciences-publications/microbeFDT-neo4j).

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Trends in Pharmacological Sciences

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REVIEWS

Review

Predicting and Understanding the Human Microbiome's Impact on Pharmacology

Reese Hitchings[1] and Libusha Kelly[1,2,*]

Our bodies each possess a unique and dynamic collection of microbes and viruses, collectively the 'microbiome', with distinct metabolic capacities from our human cells. Unforeseen modification of drugs by the microbiome can drastically alter their clinical effectiveness, with the most dramatic cases leading to fatal drug interactions. Pharmaceuticals can be activated, deactivated, toxified, or release metabolites that alter the 'canonical' pharmacokinetics of the drug. Thus, predicting and characterizing microbe–drug interactions is necessary to develop and implement personalized drug administration protocols and, more broadly, to improve drug safety and efficacy. In this review, we focus on microbiome-driven alterations to drug pharmacokinetics and provide a research framework for pharmacologists interested in characterizing microbiome interactions with any drug of interest.

Highlights

Microbiome–drug interactions have begun to be investigated mechanistically, and individual microbial gene products can now be identified as causing changes to drug pharmacokinetics. For example, the cgr operon of *Eggerthella lenta* inactivates digoxin, *Helicobacter pylori* absorbs and inactivates L-dopa, *Bacteroides* spp. toxify brivudine using a conserved purine nucleoside phosphorylase, and microbiome β-glucuronidases deconjugate the irinotecan metabolite SN-38G, regenerating the cytotoxic form of the drug.

Microbial alterations to human drug metabolism are complex and largely ignored by current pharmacological databases.

Microbiome metabolism is an underutilized opportunity for drug design.

The microbiome drastically alters the pharmacokinetic properties of many drugs, and there are likely many more unrecognized microbiome–drug interactions.

The *in silico* prediction of microbiome–drug interactions is in its infancy, but key early tools have been developed.

The Human Microbiome and Pharmacology

The human **microbiome** (see Glossary) is a major source of variability in the pharmacokinetics of many drugs [1]. A daunting challenge in drug design is to account for interindividual differences in drug response and to characterize the microbial communities that drive these differences. Over the past few decades, the field of **pharmacogenomics** has begun to identify the human gene variants that alter drug response, but the crucial role of the human gut microbiome in pharmacokinetic variance is still poorly understood mechanistically [2]. Individuals each have a unique microbiome with distinct metabolic capacities that vary over time [3]. Unforeseen modification of drugs by the gut microbiota can drastically alter their clinical effectiveness, with the most dramatic cases leading to fatal drug interactions [4]. Once in contact with the microbiome, pharmaceuticals may be activated, deactivated, toxified, or release metabolites that drastically alter the 'canonical' pharmacokinetics of drugs [5]. While traditional pharmacological approaches accurately describe the disposition of many drugs, more than 50 FDA-approved drugs have been reported in the literature to have a clinically recognized interaction with the gut microbiome [6], with individual phenotypes varying in extent. Clinical trials are generally not designed to study the role of the gut microbiome, limiting our ability to assess how widespread microbiome–drug pharmacokinetic interactions are. Predicting and characterizing microbe–drug interactions is a phenomenally difficult task but one that is necessary for the development and implementation of personalized medicine.

The gut microbiome is also a major player in human health [7], and can be better incorporated into the well-established traditions of pharmacology. When performing traditional **absorption, distribution, metabolism, excretion, and toxicity (ADMET)** experiments for an investigational new drug, it is not yet common to actively search for alterations to pharmacokinetic properties caused by the gut microbiome. Similarly, ADMET data are not always accessible, making it difficult to utilize existing studies to predict adverse events for new drugs or their metabolites [8]. Neglecting the diverse and complex human gut microbiome when evaluating the effectiveness of a drug may lead to ineffective dosing, avoidable adverse drug events [9], poor efficacy [10], and, in ex-

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treme cases, death [4]. In this review, we focus on microbiome-driven alterations to drug pharmacokinetics and provide a research framework for pharmacologists interested in characterizing potential microbiome interactions with any drug of interest.

Determining Drug Access to the Microbiota

The most direct route of access to drugs by the gut microbiome is through the digestive tract directly, via orally administered drugs not absorbed in the upper gastrointestinal (GI) tract. The small intestine is the site of most drug and nutrient absorption, although insoluble drugs, such as sulfasalazine (SSZ), and those in formulations designed to pass into the lower bowel, such as Pentasa®, arrive in the large intestine mostly unchanged [11] (Figure 1, Key Figure). Drug concentration in the gut will vary between individuals and compounds, but has been estimated to be as high as the low-millimolar range [12]. Upper GI tract drug absorption is difficult to predict by physicochemical properties alone because the thickness of mucus, pH, and tissue structure all determine the rate of absorption [13]. Likewise, the oral **bioavailability** of a drug can vary substantially even between related compounds, making prediction based on chemical properties alone problematic [14]. Drugs that are poorly absorbed have a direct interaction with the human gut microbiome, because they may remain in the large intestine for up to 4 days [15], while transit time through the upper GI tract is known to be on the scale of hours [16]. The contributions of the small-intestine microbiome to pharmacology have only recently started to be discovered, due to fecal sampling being the primary method for studying the gut microbiome. Bacterial tyrosine decarboxylases were recently shown to decarboxylate the Parkinson's drug levodopa (L-dopa) into dopamine in the jejunum, where the drug is absorbed [17].

The large intestine is considered the canonical site of most microbial modifications, as it is the host to 10^{11}–10^{13} colony-forming units (CFU)/g of contents [18] and more than nine million unique bacterial genes [19]. Drugs that enter the large intestine in this way include the 5-aminosalicylate (ASA) prodrugs and lovastatin, although many new delayed-release formulations of drugs have been designed to deliver otherwise soluble compounds directly to the large intestine [11]. These targeted drug formulations will deliver compounds to the gut that previously had been distributed and metabolized in other tissues [20], meaning that the same compound in different formulations may have a distinct interaction with the gut microbiome. All drugs and their metabolites must be evaluated for their impact on the gut microbiome and the likelihood of bacterial modification to their pharmacodynamics. For example, 5-ASA prodrugs, such as SSZ, rely entirely on azoreduction by the gut microbiota for their activation, with the 5-ASA released acting locally on the gut epithelia to treat inflammatory bowel disease (IBD) [21]. These drugs are poorly absorbed systemically and arrive in high concentrations to the colon [22]. Zheng *et al.* showed that IBD-model rats treated with SSZ had a microbiome more similar to healthy controls than their naïve counterparts; this difference may alter the efficacy of the drug [23].

Most orally administered drugs are absorbed in the stomach and small intestine, and pass through the hepatic portal vein to the liver, the major site of **first-pass metabolism** [24] (Figure 1). Here, as well as in the kidneys and other body tissues, drugs are generally transformed into more hydrophilic metabolites to aid clearance from the body [25]. This process often involves conjugation to an endogenous hydrophilic compound that facilitates excretion, most often glucuronidation. After conjugation, the drug metabolites are excreted into the urine or bile for clearance from the body [25]. The process of drug metabolism and excretion is complex, with many possible sources of interindividual variation [26]. The most common route of access for drugs to the microbiota is via biliary excretion into the large intestine [27]. Although individual drugs differ, the likelihood of a compound being excreted into the bile is generally increased by high polar surface area, low ΔG of solvation, the presence of carboxylic acids, and molecular weight >500 da [28]. There is an array of active transport proteins that function to move

Glossary

Absorption, distribution, metabolism, excretion, toxicity (ADMET): key criteria investigated for the pharmacology of a compound; each must be understood for drug approval.
Area under the curve (AUC): plasma concentration of drug over time; a higher AUC corresponds to more drug in the body.
Bioavailability: percentage of administered dose of a drug that reaches systemic circulation.
Enterohepatic circulation: regeneration of active form of a drug by bacteria after inactivation and excretion by human metabolism. The active molecule can then re-enter circulation and leads to an apparent increase in AUC.
First-pass metabolism: first biochemical changes made to a drug in the human body that often reduce the concentration of circulating drug compound. These modifications usually occur in the gut tissue or liver.
Microbiome: the consortia of bacteria, archaea, fungi, small eukaryotes, and viruses that reside within and on the human body.
Pharmacogenomics: the science of how human genes affect drug response, especially identifying human gene variants that alter the pharmacodynamics of a drug in an individual.
Probiotic: live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.

Key Figure

Routes of Access to the Gut Microbiome

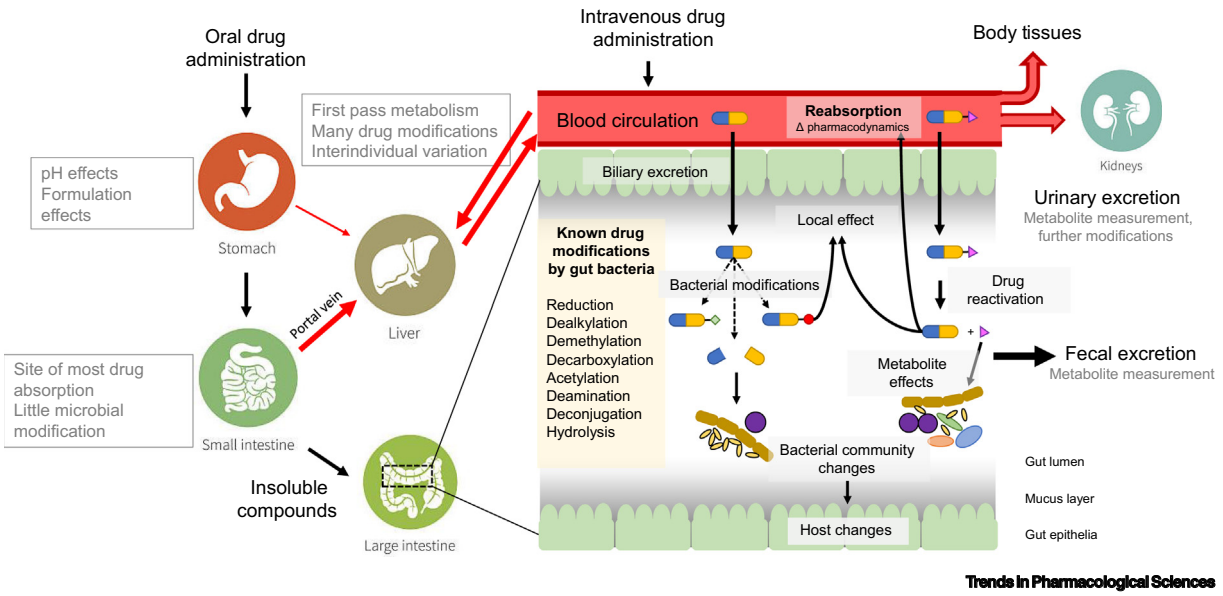


Figure 1. Drugs access the gut microbiome through direct transport through the gastrointestinal (GI) tract or biliary excretion into the intestinal tract. In the large intestine, drugs may be modified by microbial metabolism and exhibit altered pharmacological effects. Gut microbes may also generate compounds that are toxic and may alter the composition of the microbial community in the gut.

human-derived compounds and xenobiotics into the bile; individual differences in enzyme function can lead to drastic alterations of drug plasma concentration over time (**area under the curve**; **AUC**) [29,30]. Likewise, gut bacteria have a role in the observed AUC of drugs excreted into the bile. Several drugs, including morphine and the nonsteroidal anti-inflammatory drug (NSAID) indomethacin, undergo **enterohepatic circulation**, wherein the human gut microbiota removes excretory modifications to a drug compound, which is then reabsorbed to continue its effect and increase AUC [31]. In addition to the complexity of human variation in biliary excretion, the human gut microbiome has a reciprocal relationship with the excreted xenobiotics that enter the gut lumen. The human topoisomerase I inhibitor irinotecan is excreted into the gut as its glucuronidated metabolite SN-38G [29], where bacterial β-glucuronidases remove the glucuronide moiety, thus reconstituting the cytotoxic compound [32]. This microbiome-dependent alteration in drug toxicity varies between patients, and may be dependent on the presence of specific bacterial β-glucuronidase and transport genes rather than on specific species of bacteria [9].

Known Pharmacokinetic Modifications by the Microbiome

Although the specific modifications of drugs by the microbiome are diverse, the pharmacological outcomes of these modifications can be grouped into categories defined by their effect on the host (Table 1).

Activation and Reactivation

The gut microbiome produces the pharmacophore from which the drug derives its action or prevents the removal of the active compound. This type of modification causes an apparent increase in AUC or bioavailability. The best-known example of drug activation by the microbiome is the

Table 1. Selected Drug Modifications Made by Human Gut Microbiota

Phenotypic effect	Microbial modification	Subclass: drugs	Outcome	Host effect	Refs
Activation and reactivation	Reduction	Azoreduction: SSZ, balsalazide, ipsalazide, olsalazine	Prodrug activation: local 5-ASA release	Anti-inflammatory treatment	[6]
		Azoreduction: prontosil, neoprontosil	Antibiotic activation	Bacterial killing	[5]
	Dealkylation	*N*-dealkylation: Amiodarone	Increased bioavailability of active metabolite	Increased half-life, possible drug interactions	[6]
	Deconjugation	Deglucuronidation: morphine, codeine	Reformation of active metabolite	Increased AUC, enterohepatic circulation	[6]
	Other	Desulfation: sodium picosulfate	Solubility increase	Activation of laxative effect	[5]
Inactivation	Reduction	Nitroreduction: benzodiazepines: nitrazepam, clonazepam, bromazepam	Change to inactive metabolite	Inactivation of drug, a possible overdose intervention	[5,6]
		Lactone ring reduction: digoxin	Change to inactive metabolite	Narrow therapeutic window	[6]
	Dealkylation	*N*-Demethylation: methamphetamine	Change to inactive metabolite	Decreases therapeutic effect	[6]
	Dehydroxylation	*P*-Dehydroxylation: L-dopa	Decrease in L-dopa absorption, caused by *Helicobacter pylori*	Decreases therapeutic effect	[5,6]
	Proteolysis	Insulin, calcitonin	Breakdown of therapeutic protein	Decreases therapeutic effect	[5]
	Acetylation	*N*-Acetylation: 5-ASA	Change to inactive metabolite	Less efficacy, possible pancreatic toxicity	[6]
Toxification	Reduction	Nitroreduction: chloramphenicol	*p*-aminophenyl-2-amino-1,3-propanediol generation (speculated)	Bone marrow toxicity	[5]
		Nitroreduction: benzodiazepines: nitrazepam, clonazepam, bromazepam	Amino-metabolite generation, Inactivation	Teratogenicity	[5,6]
	Dealkylation	*N*-Dealkylation: brivudine, sorivudine	Generation of additional bromovinyluracil, drug AUC decrease, interaction with 5-fluorouracil (5-FU)	*Bacteroides*-mediated hepatotoxicity, potentially fatal 5-FU accumulation	[46]
	Deconjugation	Deglucuronidation: irinotecan, diclofenac, ketoprofen, indomethacin	Reformation of cytotoxic drug	Diarrhea, bowel distress, GI lesions	[5,6]

azoreduction of 5-ASA prodrugs, such as SSZ, and subsequent release of active 5-ASA from an inert carrier [21]. Matsukova *et al.* showed that, when the antiarrhythmic drug amiodarone was co-administered with a **probiotic** [33] strain of *Escherichia coli*, the AUC in the first 30 h increased by 1.4-fold [34]. This result was not found in rats that received other strains of *E. coli* [34] or the probiotic *Lactobacillus casei* [35], implying that changes in microbiome composition at the strain level have a profound impact on drug pharmacology. The gut microbiome has been described as often reversing the biotransformations performed by human cells, removing inactivating moieties and regenerating the active compound [1]. One such drug, morphine, is inactivated by glucuronidation to either morphine 3-glucuronide, which has no physiological effect, or morphine 6-glucuronide, which is ten times more potent than the parent compound [36]. Pharmacogenomics studies have identified several polymorphisms that associate with altered metabolite concentrations [37], including SNPs in the UDP-glucuronosyltransferase family 2

(UGT2), which cause differing production of the two metabolites [38]. Both glucuronidated metabolites are excreted into the bile by active transporters, where they travel to the large intestine and are accessed by the gut microbiome. Here, the microbiome can regenerate morphine, which crosses from the intestine into the blood and enterohepatic circulation (Figure 2) [39]. As with many drugs, the excretion of morphine into the gut shifts the composition of the gut microbiota [40]. Complicating the prediction of this effect, the responsible microbial β-glucuronidases are unknown, but are both phylogenetically diverse and horizontally transferred in the human gut [9].

Deactivation

The organisms in the gut decrease bioavailability or AUC of the active compound by modifying drugs such that they are cleared more rapidly by human metabolism or rendered inert by the change. The nitro-substituted subfamily of benzodiazepines, including clonazepam (Klonopin) and flunitrazepam (Rohypnol), among others, are sedative medications used to treat anxiety, insomnia, and other disorders. These psychoactive drugs can be converted to their inactive 7-amino metabolites by bacterial nitroreductases, a possible treatment for overdose [41]. In another well-characterized case, the cardiac drug digoxin can be inactivated via reduction of its lactone ring by the gut microbiome. In a rare case of strain-specific drug modification, patients who harbor strains of the species *Eggerthella lenta* that have the cardiac glycoside reductase (*cgr)* operon show a high rate of digoxin inactivation. Given that the mechanism of inactivation and responsible bacteria are known, successful dietary and antibiotic interventions to prevent digoxin inactivation are possible [42].

Toxification

The modified compound has a negative effect on host tissues. Irinotecan, secreted into the gut as the inert glucuronidated metabolite SN-38G, can be reactivated into its cytotoxic form in the gut,

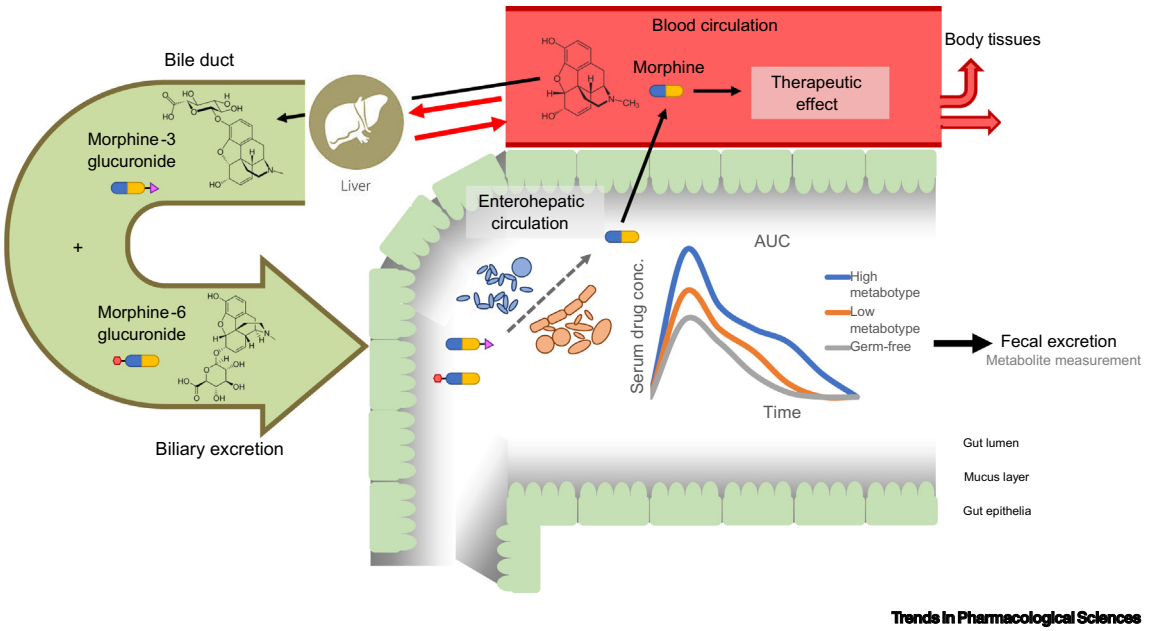


Figure 2. Enterohepatic Circulation of Morphine. Morphine is glucuronidated by host tissues to morphine-3 glucuronide (inactive) and morphine-6 glucuronide (ten times more active). After biliary excretion into the large intestine, microbial β-glucuronidases regenerate the parent compound, hypothetically increasing the apparent area under the curve (AUC) of the drug in individuals with high microbial β-glucuronidase activity.

causing GI distress [29]. Indomethacin is an NSAID used to treat pain and is known to cause serious dose-related adverse events in the GI tract that result from the inhibition of prostaglandin formation [43]. Similar to morphine, indomethacin also undergoes enterohepatic circulation after deconjugation from an inactivating glucuronide; however, this increase in drug AUC can lead to severe GI toxicity. Antibiotic-treated mice have significantly lower indomethacin plasma AUC and drug half-life compared with vehicle-treated controls, suggesting that the microbiome contributes to NSAID GI toxicity [44]. Furthermore, mice pretreated with an inhibitor of bacterial β-glucuronidases before dosage with indomethacin or another NSAID, ketoprofen, were protected from jejunal lesions seen in NSAID-only mice [45]. Recently, Zimmerman *et al.* elegantly demonstrated the mechanism by which the mammalian gut microbiome converts the nucleoside analog brivudine to the hepatotoxic metabolite bromovinyluracil (BVU). Using transposon mutagenesis, the group identified members of *Bacteroides* that most rapidly generate the toxic metabolite, and built a pharmacokinetic model to differentiate between human and microbiome metabolite generation [46]. This comprehensive approach unambiguously identified the key bacterial enzymes that contribute to microbiome-induced drug toxicity.

Community Change

One of the mechanisms of action of a drug may be to alter the microbiome, creating secondary effects incorrectly attributed to the drug molecule itself. Pharmaceutically driven changes to the gut microbiome are likely to be common; for example, Maier *et al.* demonstrated that nearly a quarter of 1000 nonantibiotic drugs inhibited the growth of gut-relevant bacteria *in vitro* [12]. The mechanisms of these alterations are only beginning to be elucidated. Metformin is the most prescribed medication for type 2 diabetes (T2D), characterized as reducing gluconeogenesis and opposing glucagon-mediated signaling in the liver [47]. In addition to these effects, Wu *et al.* demonstrated that the metformin-altered microbiota of mice was sufficient to significantly decrease blood glucose AUC after a meal in metformin-naïve mice [48]. Analysis of metagenomes from patients with T2D treated with metformin showed alterations in community structure and function, notably an increase in bacteria thought to produce the short-chain fatty acids butyrate and propionate [49]. Similarly, the acne drug Isotretinoin, marketed as Accutane in the USA, functions with a combined microbial and host effect. Isotretinoin is best known to decrease sebum production [50], a major carbon source for skin microbiota, and was recently shown to decrease the innate immune response to commensal bacteria on the skin [51]. These effects are likely synergistic, and prevent acne by altering host and microbiome metabolism [50].

The role of antibiotics in modifying the microbiome is beyond the scope of this review, but they can have a profound and long-lasting impact on the human gut microbiome [52]. Co-administration of antibiotics with drugs may radically alter the nature of microbial change to pharmacokinetics. As one example, *Helicobacter pylori* infection is prevalent in patients with Parkinson's disease [53], and has been shown to bind the Parkinson's drug L-dopa *in vitro* [54]. In a double-blind clinical study, antibiotic eradication of *H. pylori* significantly increased the AUC of L-dopa in patients with Parkinson's disease [10].

Drugs with Human and Microbiome Sources of Variability

The field of pharmacogenomics aims to understand the inherited determinants of drug metabolism and response in individuals [55]. Traditional pharmacogenomics stops at the point of excretion, neglecting the contributions of the human microbiome. The manually curated Pharmacogenomics Knowledgebase[i] (PharmGKB) identifies and diagrams key human genes involved in individualized drug response [56]. PharmGKB offers a concise and expert opinion of actionable human genes for further study and personalized medicine, but it does not incorporate microbial alterations to drugs. We identified drugs with known pharmacogenetic variation [57]

that also interact with the human gut microbiome (Table 2). For example, the widely prescribed anticoagulant drug and classical pharmacogenetic target warfarin may undergo interindividual variation at both the human and microbiome levels [58]. Warfarin functions by inhibiting vitamin K epoxide reductase (VKORC1), depleting the pool of reduced vitamin K needed as a cofactor for blood clotting [59]. Polymorphisms in *VKORC1* and the cytochrome *CYP2C9* are known to explain some of the variation in plasma concentration of warfarin, although its dosage still requires careful monitoring. To make warfarin dosing more consistent, dietary supplementation of vitamin K is often recommended, although it has not been shown to be effective [60], probably because vitamin K is produced by the gut microbiota and diet accounts for only a portion of the received vitamin [61,62]. 'Vitamin K' refers to a family of quinone compounds that differ depending on their derivation from dietary plants or bacteria [63]. Thus, the efficacy of warfarin may rely on human, dietary, and microbial sources of drug modulation. Microbe-produced and altered metabolites of drugs and other ingested compounds may also interact directly with human genes at the intestinal epithelia, with uncharacterized consequences. Individualized susceptibility to acetaminophen overdose-induced hepatotoxicity may also be explained by the interplay between host and microbial factors. SNPs have been identified in human sulfotransferase *SULT1A* gene family members that alter the rate of sulfoconjugation and clearance of acetaminophen [64]. Clayton *et al.* reported that volunteers with high levels of the bacterially produced *p*-cresol were more likely to have a lower acetaminophen sulfate:acetaminophen glucuronide ratio. Although it has not been demonstrated *in vitro,* it is proposed that *p*-cresol competitively competes with acetaminophen for sulfotransferase proteins [65]. Although these microbially driven shifts in metabolites are

Table 2. Drugs with Potential Human and Bacterial Sources of Variance

Drug	Human pharmacogene	Effect of polymorphism	Microbiome-associated metabolism	Effect of microbiome metabolism	Refs
Warfarin	*CYP2C9*	Altered activity of drug	Vitamin K production	Microbiomes produce variable concentrations of vitamin K. Alterations in vitamin K production by microbiome may alter warfarin metabolism	[60,58,76]
Irinotecan	*UGT1A1*28* 'Gilbert's syndrome'	Defect in glucuronidation, increased toxicity	Deglucuronidation of excreted SN-38G metabolite	Reformation of cytotoxic Irinotecan	[9,77]
Codeine	*CYP2D6*	Variant alleles may cause absent, decreased, or increased rate of biotransformation to morphine	Deglucuronidation of excreted morphine-glucuronide metabolite	Reformation of morphine, higher morphine AUC due to enterohepatic circulation	[78,79]
Morphine	*SLC22A1*, *OCT1*	Decreased clearance of morphine	Deglucuronidation of excreted morphine-glucuronide metabolite	Reformation of morphine, higher morphine AUC due to enterohepatic circulation, Induces virulence in some strains of *Pseudomonas aeruginosa*	[64,78,80]
Acetaminophen	*UGT1A*, *SULT1A3*	Increased rate of glucuronidation and decreased risk of liver failure due to unintentional overdose, decreased sulfation	Sulfonation	Increase in sulfonated metabolite, may be competitively inhibited by *p*-cresol sulfonation	[64,65,81]
Simvastatin	*SLCO1B1*	221% increase in simvastatin AUC for homozygotes	Unknown	Increased efficacy hypothesized to be due to microbial alteration of primary bile acids	[58,82–84]
Digoxin	*ABCB1*	Increased AUC may increase toxicity	Lactone ring reduction	Decreased AUC, narrow therapeutic window	[42,85]
Brivudine and sorivudine	*DYPD*	Increased drug–drug interactions with pyrimidine analogs	Generation of additional bromovinyluracil	Hepatotoxicity, bromovinyluracil prevents clearance of 5-FU	[46,86]

important, their impact on clinical efficacy is uncharacterized. In a murine model of high-dose acetaminophen hepatotoxicity, germ-free (GF) animals with no microbiota were not found to have significantly different levels of liver necrosis compared with their microbiome-containing counterparts [66]. We propose that a major advance in understanding pharmacokinetics will come from integrating the traditions of pharmacogenomics with microbiology and microbial ecology to generate more holistic models of individualized drug response.

Predicting and Identifying Pharmacokinetic Changes by the Microbiome

As part of the drug approval process, all lead compounds must undergo pharmacologic testing, often including a comprehensive tracing of the routes of excretion of the drug [67]. This testing is initially performed in animal models during preliminary ADME studies, followed by a limited set of human volunteers during Phase I clinical trials and up to several thousand volunteers during Phase III trials [68]. The SIDER[ii] database has been developed from publicly available adverse event data to aid the identification of cryptic host effects [69]. Although detecting interindividual variation is difficult with a small population, adverse event data can provide a wealth of insight into the major and minor modifications performed by the microbiome. Each microbially derived drug metabolite identified in feces or plasma has unique properties and bears investigation as a novel drug. To predict the phenotypic effect of these xenobiotics in the gut, computational tools have been developed to aid in hypothesis generation, reviewed in [70]. Although these tools can be used to identify the breadth of possible modifications and the likelihood that a certain xenobiotic would be altered, many are not freely available to the public and none but PathPred[iii] [71] have web interfaces. As interest in microbiome-altered pharmacology grows, so does the need for resources that collect, integrate, and model extant pharmacologic data, offer consensus expert opinion, and predict new drug–microbiome interactions.

In silico prediction of microbial modifications to drugs is complicated by the multiple layers of complexity that contribute to the physiological relevance of a modification. The accurate prediction of xenometabolism by the gut microbiome must contend with microbial variation at the enzyme, species, and community levels [70] before the proposed metabolites interact with human tissues. In addition to hypothesis generation, these *in silico* predictions may serve as benchmarks throughout the iterative process of drug development and investigation. For example, *in silico* modeling of human pharmacokinetics is incorporated into drug development pipelines by most pharmaceutical companies [72]. Similarly, the *in silico* prediction of drug toxicity in human tissues has benefited from recent advances in the field of quantitative systems toxicology (QST) by incorporating techniques including gene-set enrichment analysis, gene signature analysis, and co-expression network analysis [73]. These advances herald new opportunities in drug design that enable consortia such as transQST[iv] to incorporate existing large data sets, including transcriptomics, proteomics, and metabolomics data, to predict drug toxicity in humans at the preclinical stage. Despite the clear interest in leveraging computational tools for the prediction of pharmacological effects of drugs, most QST studies neglect the human microbiome as a source of variation. Recently, Thiele *et al.* used a constraint-based reconstruction and analysis (COBRA) methodology to generate a metabolic reconstruction of the human superorganism that includes the gut microbiome, coined the Virtual Metabolic Human[v] (VMH) [74]. Databases and models such as VMH serve as an important resource for pharmacokinetic and toxicological *in silico* investigations and lay the groundwork for a comprehensive understanding of the interaction of the microbiome with pharmacology.

Concluding Remarks

Microbiome research has grown rapidly with the advent of cheap and accessible sequencing technologies, allowing for mechanistic investigations into alterations to the pharmacology of drugs by the microbiome. Unfortunately, many microbiome studies are descriptive in nature

Outstanding Questions

How can characterized microbiome–drug interactions be used to predict microbiome interactions with new drugs?

How do we best use existing pharmacological data to discover novel microbiome–drug interactions?

How best can we aid communication between clinical and basic research pharmacologists and microbiologists?

How well do animal models used for early drug testing recapitulate human-relevant microbiome–drug interactions?

What changes should be made to the drug approval process to screen for microbiome–drug interactions?

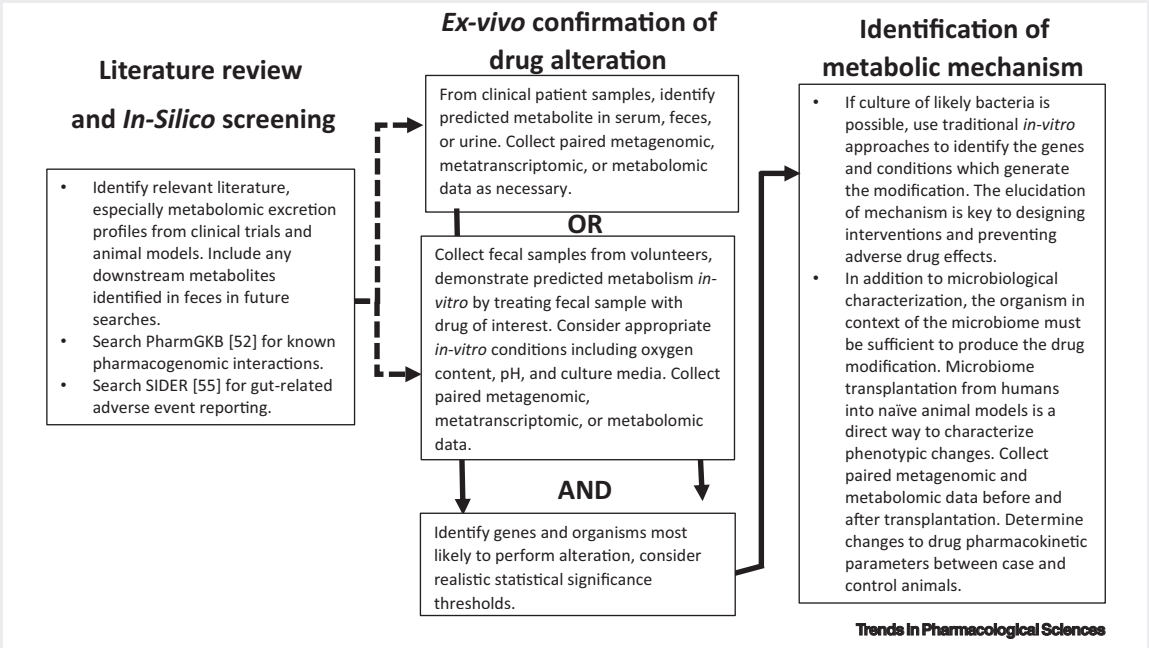
Are common GI-related adverse events caused by pharmaceutical alterations to the microbiome?

and rely heavily on 16S rDNA sequencing, which cannot always reliably probe the biochemical function of the community. Future investigations in microbiome pharmacology should be designed with the aid of pharmacologists to specifically and unambiguously identify the mechanism of the microbiome–drug interaction. We propose a series of recommendations for microbiome–pharmacology study design that guide in characterizing the role of the microbiome in altering drug pharmacokinetics (Box 1). Our recommendations focus on the rapid prediction of likely interactions between drugs and culturable members of the gut microbiome, followed by identification with broad *ex vivo* techniques, such as metabolite profiling and metagenomic sequencing. After identification of a suspected drug modification, we suggest a rigorous *in vitro* approach to identify the mechanism of pharmacological change using common techniques, such as transposon mutagenesis, knockout studies, and protein characterization, as well as observation of the microbiome dependence of the change in an animal model. Studies designed with this general model are often elegant and highly convincing [46,75].

The microbiome has many complex metabolic properties that can drastically alter the pharmacokinetics of drugs. Tools to predict microbiome-driven metabolic alterations to drugs are still in their infancy and require expert pharmacological opinion. Characterizing microbiome–drug interactions is interdisciplinary and critical to the future of drug design and testing. Despite recent discoveries, no integrated resource for predicting the interaction of the microbiome with a drug exists. However, necessary data sources, including but not limited to clinical adverse event data, enzyme biochemistry, and microbial diversity data are difficult to synthesize automatically (see Outstanding Questions).

Ultimately, the future of microbiome pharmacology relies on improving communication between basic and clinical research, and the development of experimental and computational resources to aid pharmacological investigations into the human gut microbiome.

Box 1. Considerations for Study Design in Microbiome Pharmacology



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Resources

[i]www.pharmgkb.org/

[ii]http://sideeffects.embl.de/

[iii]www.genome.jp/tools/pathpred/

[iv]http://transqst.org/

[v]www.vmh.life/

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Review

Bringing microbiome-drug interaction research into the clinic

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ABSTRACT

Our understanding of the scope and clinical relevance of gut microbiota metabolism of drugs is limited to relatively few biotransformations targeting a subset of therapeutics. Translating microbiome research into the clinic requires, in part, a mechanistic and predictive understanding of microbiome-drug interactions. This review provides an overview of microbiota chemistry that shapes drug efficacy and toxicity. We discuss experimental and computational approaches that attempt to bridge the gap between basic and clinical microbiome research. We highlight the current landscape of preclinical research focused on identifying microbiome-based biomarkers of patient drug response and we describe clinical trials investigating approaches to modulate the microbiome with the goal of improving drug efficacy and safety. We discuss approaches to aggregate clinical and experimental microbiome features into predictive models and review open questions and future directions toward utilizing the gut microbiome to improve drug safety and efficacy.

Contents

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1. Introduction

Microbiome research has reinvigorated an ecological and metabolic view of diseases, including, but not limited to, autoimmune and inflammatory diseases, metabolic diseases and cancers. Advances in culture-independent methodologies for high throughput analysis of microbial community composition and function, analytical chemistry techniques and gnotobiotic mouse models have expanded our understanding of gut microbiota-mediated biotransformations of exogenous compounds including diet-based chemicals, environmental toxins and therapeutic drugs [1,2]. In particular, recent studies provide mechanistic insight into the role of gut microbiota metabolism in drug bioavailability, efficacy and toxicity and suggest that the gut microbiome, in addition to human genetics and environmental variables, contributes to interpersonal variation in human drug responses [1,2].

However, we have limited insight into 1) the broader spectrum of human gastrointestinal tract microbial species and enzymes that can alter drug bioavailability and toxicity; and 2) the clinical relevance of microbiome metabolism. These gaps in our understanding of gut microbiome chemistry at both the community and individual gut strain level present a challenge to incorporating data from microbiome studies into accurate surrogate endpoints for clinical studies. Here, we describe human and microbial drivers of variability in drug response, and discuss current barriers and opportunities for translating basic research on microbial drug metabolism into clinical applications. We specifically focus on model systems, experimental approaches and computational techniques to characterize the microbiome and its interactions with drugs.

2. Connecting human and microbial drivers of variability in drug response

2.1. Human metabolism and individual variation in drug response

Advances in high throughput sequencing and analytical chemistry propel precision medicine initiatives that use genomic, gene expression, proteomic and metabolomic data to inform patient treatment and care [3]. Yet, using these diverse data types to systematically maximize drug efficacy and minimize toxicity remains an open challenge. Towards addressing this challenge, pharmacology subdisciplines, pharmacogenomics and pharmacometabolomics, aim to identify the impact of human genetics and metabolism on patient drug responses [4]. Among the early successes of pharmacogenomics research was the identification of genetic polymorphisms in the UDP-glucuronosyltransferase (UGT) enzyme family which catalyzes the glucuronidation of drug compounds, promoting their inactivation and elimination from the human body. Patients with specific UGT1A1 variants have lower glucuronidation rates, which impacts the detoxification of a number drugs, including the HIV drug atazanavir, nonsteroidal anti-inflammatory drugs (NSAIDs) and the chemotherapeutic irinotecan [5,6]. Clinical laboratories can thus use an UGT1A1 genotype assay to determine personalized patient toxicity risk [7]. Beyond UGT genotyping, pharmacogenomics tests that target other hepatic enzymes involved in drug metabolism, such as members of the cytochrome P450 (CYP) superfamily, may guide dosing decisions. For example, in the package insert for warfarin, a commonly prescribed drug with a narrow therapeutic range, the Food and Drug Administration (FDA) includes a dosing guide based on a patient's CYP2C9 genotype [8,9].

Early proponents of pharmacogenomics hypothesized that genetic polymorphism analysis in drug metabolizing enzymes and the human genome more broadly would substantially improve clinical practice to reduce poor efficacy and toxicity [10,11]. However, basic research advances characterizing how genome variants impact drug metabolism have not been broadly translated into the clinic. In part, this discrepancy relates to how drug metabolism has been traditionally characterized in the context of the human liver and intestinal mucosa. The gut microbiota is a third dimension in drug metabolism, providing a nonoverlapping enzymatic capacity that generates distinct metabolites from host enzymatic products and may also shape drug pharmacokinetics. Research focused on extending pharmacogenomics and pharmacometabolomics to include the impact of the microbiome on drugs falls under the umbrella of pharmacomicrobiomics [12].

2.2. Microbiome chemical mechanisms shape drug metabolism

The gut microbiota alters drugs by various mechanisms: degrading the drug [13,14]; activating the drug [13–15]; and modulating host enzymes that metabolize the drug [13,14] (Fig. 1). Known microbial reactions that shape drug metabolism have been reviewed extensively by Wilson et al., and Spanogiannopoulos et al., and highlight bacterial enzymes such as β-glucosidases, β-glucuronidases, aryl sulfatases, azoreductases and nitroreductases, which have prominent roles in xenobiotic metabolism and vary widely in activity, sequence similarity, and abundance across individuals [1,2]. Hydrolytic and reductive reactions are the primary chemical mechanisms of gut microbiota drug metabolism. These reactions reflect the physiochemical parameters of the distal intestine, which has a limited oxygen gradient. The gut microbiota is also the source of numerous other chemical reactions including acetylation, deamination, dehydroxylation, decarboxylation, demethylation, deconjugation and proteolysis [1,2]. To date, microbial strains and enzymes have been experimentally demonstrated to directly or indirectly impact the metabolism and efficacy of over 50 therapeutic drugs, driving inter-patient variability in drug activation, inactivation and toxicity [1,2].

2.3. Microbiome modulation of phase I and II drug metabolism enzymes

Microbial metabolism of dietary and endogenous compounds indirectly shapes key host hepatic enzymes that broadly contribute to drug metabolism. For example, Phase I hepatic enzymes account for 80% of oxidative metabolism of commonly used medications and include the cytochrome P450s (CYPs) superfamily and flavin-containing monooxygenases (FMOs) [16]. Phase II hepatic enzymes include glutathione S-transferases (GST), sulfotransferases (SULTs) and uridine diphosphate-glucuronosyltransferases (UGTs) and play key roles in drug detoxification and elimination from the body. The expression and activity of these enzymes is modulated by gut microbiota metabolism of uremic solutes, bile acids and steroid hormones; these microbiome-drug interactions can have adverse consequences for patients taking drugs that are substrates for these enzymes [17,18]. Microbiota produced uremic solute indoxyl sulfate decreases CYP3A4

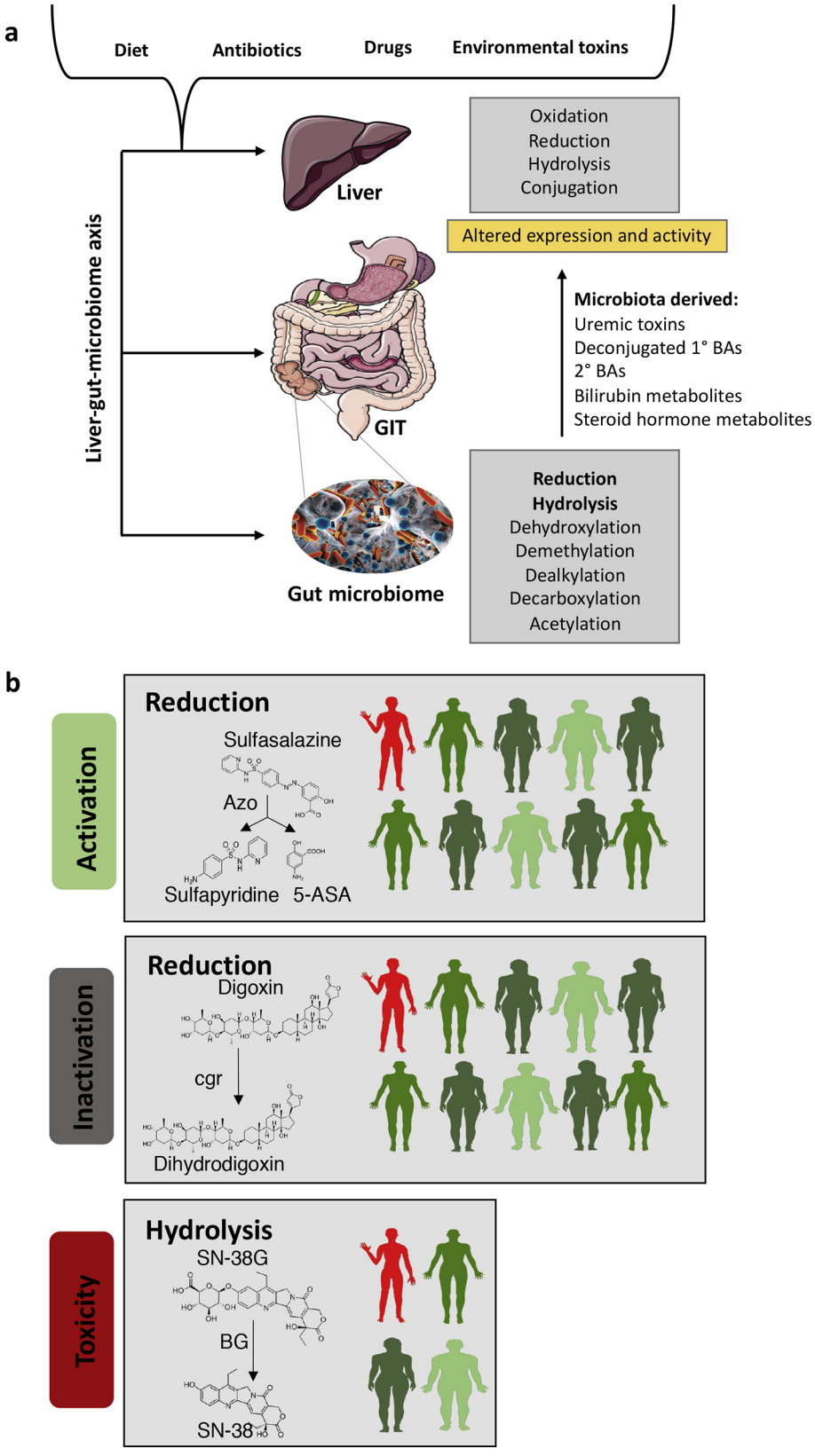

Fig. 1. Gut microbiota-host liver metabolic interactions drive variability in drug response. a Hepatic and gut microbiome enzymes co-metabolize chemically diverse exogenously derived substrates including foods, therapeutic drugs and environmental toxins. Key host hepatic enzymes include the cytochrome P450s (CYPs) superfamily and flavin-containing monooxygenases (FMOs) [16] which are involved in phase I metabolism. Phase II enzymes including glutathione S-transferases (GST), sulfotransferases (SULTs) and uridine diphosphate-glucuronosyltransferases (UGTs). Hydrophilic therapeutic drug and drug conjugates excreted from the liver into the gastrointestinal tract *via* the biliary route are chemically modified primarily by gut microbiota hydrolytic and reductive reactions into hydrophobic products that can be reabsorbed *via* enterohepatic circulation [72], modified or extensively degraded by the gut microbiota. Gut microbiota metabolism also indirectly regulates phase I and II hepatic enzymes by producing metabolites, including uremic toxins and secondary bile acids, that alter hepatic enzyme expression and activity. b Gut microbiota enzyme catalyzed reactions have been linked to variation in patient response phenotypes. For example, microbial mediated azoreduction transforms the anti-inflammatory drug, sulfasalazine, into bioactive products. 10% of healthy individuals are poor converters of sulfasalazine [73]. Microbial metabolism also negatively impacts host drug responses. Approximately 10% of patients given the cardiac glycoside, digoxin, excrete high levels of an inactive metabolite which is generated by microbial enzymes [74]. 25% of patients taking Irinotecan with 5-fluorouracil and leucovorin for the treatment of colorectal cancer experience grade 3–4 diarrhea which is mediated by microbial β-glucuronidase reactivation of a major inactive metabolite of the drug [75].

expression, reducing CYP3A4 mediated metabolism clearance of a diverse range of therapeutics including erythromycin, nimodipine and verapamil (Fig. 1) [19].

2.4. Therapeutic drug influences on the gut microbiome

Research studies defining how therapeutic drug moderation the gut microbiome can play a central role in a drug's mechanism of action are limited. Of note, metformin, an antidiabetic drug, has a poorly defined mechanism of action and is known to alter gut microbiome composition [20,21]. Recently Sun et al., used a combined metabolomics and shotgun metagenomic approach using human serum and feces to conclude that metformin decreases the abundance of *Bacteroides fragilis*, limiting its bile salt hydrolase activity and promoting an increase in glycoursodeoxycholic acid concentrations in the gut [20]. Sun et al., also used a mouse model to confirm that glycoursodeoxycholic acid suppresses intestinal farnesoid X receptor signaling and alleviates obesity-related metabolic disease [20]. Recent work by Maier et al., illustrates the off-target effects of therapeutic drugs on the microbiome. Maier identified non-antibiotic therapeutic drugs that inhibit the growth of specific gut relevant bacterial strains [22]. Additional work by Brochado et al., also highlights inter-species variation in sensitivity to therapeutic antibiotic and non-antibiotic compounds [23].

3. *Ex vivo* and animal models for microbiome drug metabolism research

3.1. Models of the human gastrointestinal tract

Experimental models for the study of human gastrointestinal tract (GIT) phenotypes reflect different features of the physiological complexity and biogeography of the human GIT. Defined anatomically, the GIT is a continuous tube, approximately 9 m in length in an adult human, that includes the pharynx, esophagus, stomach, duodenum, jejunum, ileum, colon, cecum and rectum [24]. Microbes populate the entire GIT, from the oral cavity to colon [25]. The activity of microbes in the small (duodenum, jejunum and ileum) intestine and colon, is of particular interest for human microbiome researchers, as these are the key sites for microbial activity. Choosing an appropriate study design, based in part on how anatomical and physiological features of the colon may impact its microbial ecology, may help address the challenge of reproducing findings from model systems to human biology.

3.1.1. Ex vivo colon models

Ex vivo colon models are a powerful approach to replicate the complexity and dynamics human gut microbial communities. Batch or continuous fermentation systems replicate the anaerobic condition of the colon and allow specification of physiological parameters such as pH and dissolved oxygen [26]. A human fecal sample prepared under anaerobic conditions serves as the initial inoculum into a multichambered bioreactor. Takagi and colleagues developed a single-batch fermentation system to evaluate the effect of prebiotics on the colonic microbiota and found that supplementation with prebiotic oligosaccharides increased the abundance of the genus *Bifidobacteria* and acetate production [27]. Fermentation systems can be manipulated through the introduction of substrates of interest, monitored and sampled at defined timepoints. However, there are concerns about how well the fecal microbiota approximates the activity of colon. Comparative intestinal and fecal sampling in a limited number of human and primate studies identified overlapping but distinct microbial communities between the small intestine, colon and fecal community [28,29].

A second class of *ex vivo* colon models, enteroids and organoid cultures, replicate key host physiological features. These cultures are generated from heterogenous cell populations that self-organize into three-dimensional structures that recapitulate features of the small intestinal epithelium [30]. These systems have been employed to gain insight into host-viral and host-bacteria interactions. For example, Finkbeiner et al. established an organoid model that supported rotavirus infection after inoculation with rotavirus infected stool [31]. Forbester et al. used an intestinal organoid model to assess interactions between the enteric pathogen, *Salmonella enterica serovar Typhimurium* with the intestinal mucosa [32]. Enteroids and organoids suffer from overlapping disadvantages with fermentation systems in that they can take months to stabilize for use [30]. It is also challenging to mimic and culture anaerobes under the conditions necessary to support organoid and enteroid systems. For example, in an organoid model of *Clostridioides difficile* (*C. difficile*) infection, the pathogen was viable for a maximum of 12 h [33].

3.2. Model systems in the study of microbiome-drug interactions

3.2.1. Rodent

Mouse models are considered the gold standard in terms of balancing tractability with approximating the anatomical, physiological and microbial features of human microbiomes. Both humans and mice are dominated by the microbial phyla Bacteroidetes and Firmicutes but vary at the genus level [34,35]. However, humans have a lower glandular pH stomach, a significantly thicker layer of mucin as a part of the epithelial barrier in the colon, an appendix and a segmented colon [34,35]. As highlighted in Section 2.4, mouse models play a powerful role in confirming mechanisms of microbiome-host-drug interactions that are identified through human studies.

3.2.2. Other whole organism model systems

Scott et al., used the worm *Caenorhabditis elegans* to investigate the role of host-microbe co-metabolism on the efficacy of fluoropyrimidine cancer drugs and identified several mechanisms by which microbial metabolic processes shape fluoropyrimidine efficacy. Bacteria convert the prodrug 5-fluorocytosine to 5-fluorouracil and the bacterial deoxyribonucleotide pool shapes 5-fluorouracil induced autophagy [36]. The advantages of using *C. elegans* include its short generation time and high tractability [37]. The zebrafish (*Danio rerio*) represents a vertebrate model for microbiome research. Zebrafish models have been employed to study the role of microbes in development [38]. Phelps et al., used a zebrafish model to uncover a role of microbial colonization in normal neurobehavioral development [39]. These systems, while less expensive than mice and more readily genetically tractable, recapitulate neither human physiology nor microbiome composition.

4. Community level analysis of microbiome function

4.1. High throughput sequencing

Our current knowledge of the microbial inhabitants of our gut is based primarily on community level analyses. A major unmet challenge is to design species level analyses that appropriately contextualize how individual species function within a larger community and to replicate the complexity of interactions in the gut environment. Towards addressing this challenge, the use of 16S ribosomal RNA (rRNA) sequencing and metagenomic shotgun sequencing of fecal samples can be employed to characterize the microbial community resolved at the level of species or strains and functional potential. The 16S rRNA gene has a region that is widely conserved across bacteria and a hypervariable region that allows classification of bacteria into closely related groups. Sequences that contain similar hyper-variable regions are clustered into Operational Taxonomic Units (OTUs) [40]. Recently, new methods have been developed to replace OTUs with Amplicon Sequence Variants (ASVs) as the “unit of analysis” [41].

4.1.1. Metagenomics and metatranscriptomics

Taxonomic studies are thus far limited in predicting human disease and health states [42,43]. Shotgun metagenomics, an alternative

approach, is the untargeted sequencing of the total DNA of a sample, providing insight into both phylogenetic diversity and the abundance of functional genes. Metatranscriptomics studies provide insight into community-level gene expression, a more direct measure of microbiome functional activity. These approaches are more expensive than 16S-based profiling and share technical and computational challenges. Assembling metagenomic and metatranscriptomics data and downstream statistical analyses to assess differences in microbial features are not standardized, contributing to variability in significant functional features between studies [42].

4.2. High throughput protein and metabolite analyses

4.2.1. Metaproteomics and metabolomics

While not yet used as a standard component of human microbiome research, metaproteomic and metabolomic analyses provide complementary and more direct insight into active functions of gut microbes than metatranscriptomic or metagenomic approaches. These analyses are based on the use of mass spectrometry coupled to a variety of front-end molecular separation approaches. Using combined and metagenomic and metaproteomic analysis Erickson et al. found significant differences in protein expression in the intestinal barrier between individuals in good health and those with Crohn's disease [44]. Microbiome studies including metabolomics have found that greater microbiota mediated p-cresol formation competitively reduced acetaminophen sulfonation and excretion in the urine, and is a key source of inter-personal variation in acetaminophen metabolism [45]. To date, targeted approaches, quantifying a defined set of metabolites, and untargeted approaches have been used to follow the fate and interplay between host and gut microbiota generated metabolites. For example, we used a combined shotgun metagenomic and targeted metabolomic approach to quantify inter-individual variability in microbiome metabolism of a glucuronidated metabolite of a chemotherapeutic drug and linked a high turnover phenotype to specific microbial β-glucuronidases [46]. There are notable bottlenecks that restrict the use of these approaches in microbiome studies including expense and the tradeoffs between efficient protein or metabolite extraction from fecal or intestinal samples while maintaining mass spectrometry sensitivity [47].

5. Computational approaches to pharmacokinetics in microbiome research

5.1. Computational approaches to pharmacokinetics in microbiome research

There are several computational approaches to model and predict drug pharmacokinetics and microbial metabolic processes that support the quantitative *in silico* assessment of microbiome-drug interactions. These approaches, which most notably include physiologically based pharmacokinetic (PBPK) models and constraint-based reconstruction and analysis (COBRA) methods, rely on data gathered from high throughput sequencing and analytical chemistry approaches.

5.1.1. Physiologically based pharmacokinetic (PBPK) models

PBPK models represent whole body drug kinetics with differential equations [48]. The model system is defined by compartments corresponding to specific tissues of the body such as the liver, kidney, gut, or lung. System-specific parameters are derived from experimental data such as enzyme and transporter expression, organ volumes and blood flow. Drug-specific parameters include drug physiochemical properties and tissue permeability. Traditionally these models exclusively modelled human metabolism; however, several studies have included microbial enzymes among the system-specific parameters. For example, Boajian Wu developed a PBPK model to evaluate the impact of GIT glucuronide hydrolysis of SN-38 Glucuronide, a key inactive metabolite of the chemotherapeutic irinotecan, on the pharmacokinetic profile of the active compound, SN-38. In this two-compartment model, encompassing the liver and gut, Wu found GIT microbial β-glucuronidase activity increased intestinal exposure to SN-38 but not systemic exposure [49]. Recently, Zimmermann et al., used gnotobiotic mouse studies involving a specific gut colonist that varied in its encoding of single enzymes to quantify brivudine metabolism *in vivo* and to construct a pharmacokinetic model to quantitatively predict microbiome contributions to systemic drug and metabolite exposure and to distinguish host and microbe contributions [50].

5.1.2. Constraint-based reconstruction and analysis (COBRA)

COBRA methods use formalized metabolic models to simulate, analyze and predict metabolic phenotypes including how microbes utilize various metabolic processes, host-microbe interactions and microbe-microbe interactions [51,52]. In the context of drug metabolism, Swagatika et al., employed COBRA methods to model the effects of commonly used drugs, including statins, anti-hypertensives, analgesics and immunosuppressants, on human metabolism [53]. They found that diet shapes human metabolism and elimination of acetaminophen and statins [53]. In particular, a low L-cysteine vegetarian diet resulted in a reduction in sulfation and excretion of acetaminophen metabolites. Reduced sulfation can be attributed to low levels of sulfur containing compounds such as L-cysteine, which contributes to the biosynthesis of a critical co-factor, phosphoadenylyl sulfate, for sulfation reactions [53].

There have been notable efforts to integrate the strengths of PBPK and COBRA methods [54,55]. Krauss et al., combined COBRA and PBPK methods to more accurately predict allopurinol pharmacokinetics and pharmacodynamics. Allopurinol is a preventative antigout medication that prevents increases in uric acid levels and alters gut microbiota composition [56]. The authors predicted the pharmacological effects of allopurinol on the biosynthesis of uric acid and reported a 69.3% decrease in uric acid concentrations which is supported by clinical data [55]. Future use of these approaches will enable both a systems level and targeted mechanistic understanding of host-microbiome metabolism.

6. Bringing insights from microbiome-drug interaction studies into the clinic

6.1. Microbiome metabolic phenotyping

The impact of the microbiome on the efficacy and toxicity of the chemotherapeutic irinotecan and the cardiac drug digoxin are relatively well characterized (Fig. 1b). In the case of metastatic colorectal cancer patients receiving irinotecan (CPT-11), microbial β-glucuronidases hydrolyze the glucuronide group from the major inactive metabolite of CPT-11, SN-38 glucuronide. A build-up of SN-38 in the colon causes epithelial cell damage that contributes to severe diarrhea in some patients [15,57]. Using a combined shotgun metagenomics and targeted metabolomics approach, a group previously identified a phylogenetically diverse set of bacterial β-glucuronidases and transporter proteins that are associated with high turnover of SN-38 glucuronide and a potentially elevated risk of irinotecan dependent toxicity [46]. Defining the metabolic and metagenomic basis of variability in drug metabolism using *ex vivo* incubations of drugs with human fecal samples may suggest putative biomarkers of a patient's risk of poor drug efficacy and safety.

6.2. Developing drug metabolism classifiers

To date metabolic phenotyping studies of microbe-drug interactions pairing DNA or RNA high throughput sequencing with metabolomics reveal that the level of gut microbiome complexity linked to drug metabolism varies between drugs [45,46,58]. A major hurdle is understanding what microbiome features identified through these preclinical studies, using model systems or human fecal samples as a proxy for the gut microbiome, will translate into accurate surrogate

endpoints for clinical studies. For example, the presence or absence of a particular microbe or enzyme in a sequenced fecal sample may not have the power to predict drug metabolism.

One approach to overcome this hurdle is to combine features using machine learning to identify the combinations of features most strongly predictive of drug metabolism. One such supervised learning approach is the "random forest" [59,60] method, which can be used to combine chemical, molecular, and clinical features. Initially one could define drug metabolism as a binary value where every sample is labeled as either "high" or "low" based on drug concentrations in a fecal sample from a patient. A receiver-operator curve plotting true-positive and false-positive rates can then be used to assess performance on different combinations of feature sets [61]. To target specific features that drive predictions one can calculate the importance of individual features to prediction accuracy by calculating the mean decrease in accuracy per feature [60]. This analysis outputs the highest performing feature set and classifier to be used with future patient data for a given drug (Fig. 2).

6.3. Clinical trials

6.3.1. CPT-11

There are a number of clinical trials investigating the efficacy of probiotics to modulate microbiome-dependent adverse drug responses. A randomized, double blind design was carried out to investigate the potential for probiotic use to minimize CPT-11 induced toxicity. Patients were randomized in to a probiotic group (PRO) and a placebo group (PLA). 39% of patients in the PRO group experienced grade 3–4 diarrhea while 61% of participants in the PLA group experienced diarrhea (Table 1) [62]. Future studies of a similar design may also address how diet influences microbiome β-glucuronidase activity and patient toxicity by including metagenomics or metatranscriptomic sequencing from fecal samples to assess microbiome function.

Recent efforts to reduce CPT-11 toxicity also include targeted inhibition of microbial enzymes that convert the inactive form of the drug to its active form. Wallace et al., 2010, identified potent *Escherichia coli* β-glucuronidase inhibitors which substantially reduce CPT-11 induced toxicity in mice while having no effect on the orthologous mammalian enzyme [15]. A clinical trial establishing the safety and efficacy of this approach in human population has the potential to yield valuable insight into the efficacy of targeted, small molecule modulators of specific microbiome functions.

6.3.2. Tacrolimus

Tacrolimus is an immunosuppressant commonly used for kidney transplant recipients. A narrow therapeutic range limits its efficacy: underexposure increases the risk of graft rejection and over-exposure increases the risk of drug-related toxicity [63]. An ongoing clinical trial focused on identifying biomarkers of successful discontinuation of immunosuppressants including tacrolimus for patients with liver disease, includes microbiome profiling as a secondary outcome measure for a trial (Table 1). However, preclinical research provides compelling evidence of a role of the gut microbiota in patient outcomes.

In a pilot study of kidney transplant recipients, patients who required a 50% increase in the standard dose of tacrolimus to maintain therapeutic levels had a greater abundance of *Faecalibacterium prausnitzii* [64]. Subsequently, Guo et al., reported that tacrolimus is

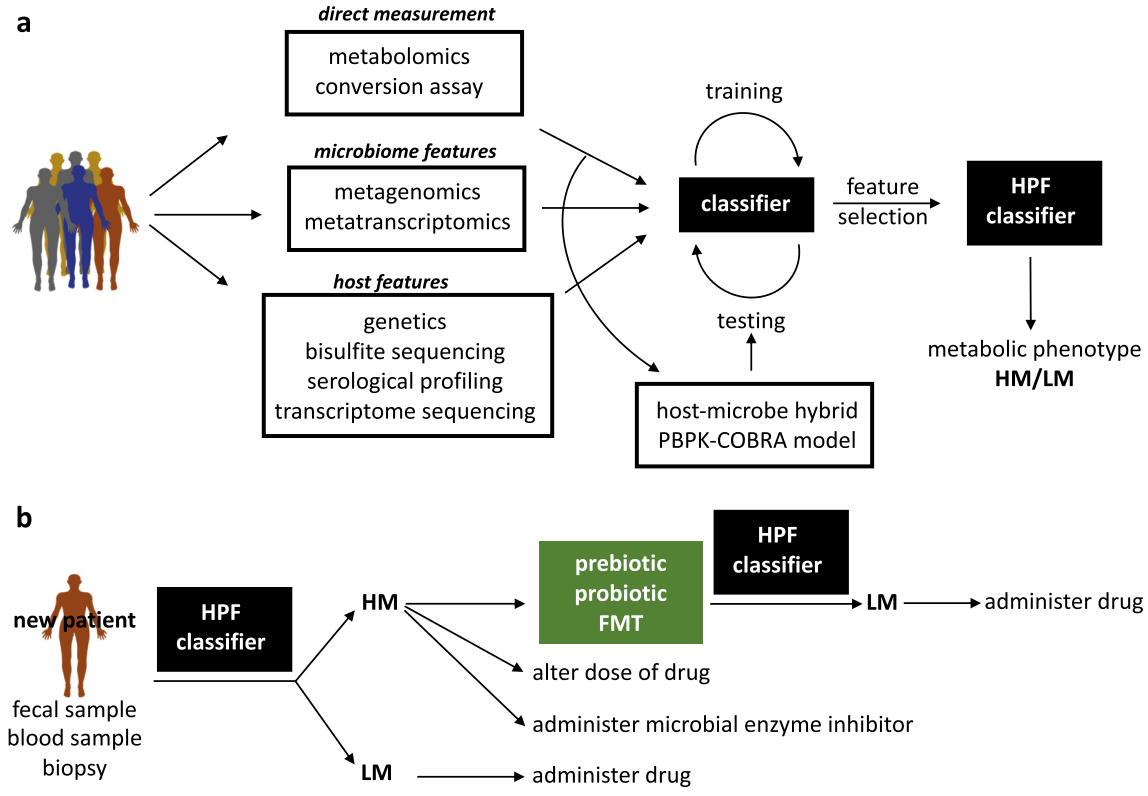

Fig. 2. Pipeline for metabolic phenotyping and modulation of microbiome driven adverse drug responses. a The construction of a high performance classier (HPC) to distinguish high drug metabolizers (HM) from low drug metabolizers (LM). For patients treated with therapeutic drugs that are susceptible to glucuronidation, such as irinotecan and NSAIDs, being a HM may reflect an elevated risk for drug-dependent toxicity. The main steps for metabolically phenotyping of HM and LM patients include data aggregation and preparation as input features for classifier training and testing, followed by the selection of key features that predict outcome and evaluation of classifier performance. The feature space for the classifier can be derived from preclinical and clinical studies and might include multi'omic data derived from both microbiome and host studies. This data can be integrated into hybrid COBRA-PBPK models to gain further predictive and mechanistic insight into drug pharmacokinetic profiles and aid in the identification of key host and microbiome parameters. b The HPC can be used to stratify new patients taking susceptible therapeutics into either HM or LM 'metabotypes' based on non-invasive fecal sampling alone or in addition to host biological samples. HM patients may undergo pre-treatment therapy, ranging from the use of probiotics and prebiotics to FMT, to modulate the microbiome towards a LM profile and improved treatment efficacy and safety.

Table 1
Clinical trials investigating microbiome intervention and profiling approaches to improve drug efficacy and safety.

Drug (s)	ATC Classification	Target Outcome	Microbiome Intervention	Phase	N	NCT	Status
Xanthohumol	Anti-cholesterol, Anti-inflammatory	Establish PK	Microbiome profiling	1	32	NCT03735420	Not yet recruiting
Metronidazole	Anti-infective	↑ Efficacy	Probiotic: Lactobacillus GG	4	0	NCT00304863	Withdrawn
Antibiotic-unspecified	Antibiotic	↓ Toxicity	Probiotic: BioGaia *Lactobacillus reuteri*	NA	73	NCT02127814	Completed
Irinotecan	Antineoplastic agents	↓ Toxicity	Probiotic: PROBIO-FIX INUM	3	100	NCT02819960	Recruiting
Irinotecan	Antineoplastic agents	↓ Toxicity	Probiotic: Colon DophilusTM	3	46	NCT01410955	Completed
Irinotecan	Antineoplastic agents	↓ Toxicity	Antibacterial: Cefpodoxime	1	20	NCT00143533	Completed
VEGF-TKI	Antineoplastic agents	↓ Toxicity	Probiotic: Activia yogurt containing Bifidobacterium lactis DN-173010)	NA	20	NCT02944617	Recuriting
Dacomitinib	Antineoplastic agents	↓ Toxicity	Probiotic: VSL 3	2	236	NCT01465802	Completed
Tenofovir	Antiviral	↑ Efficacy and Safety	Fecal Microbiota Transplant	NA	64	NCT02689245	Completed
Chemotherapy-unspecified	Chemotherapy-unspecified	↓ Toxicity	Probiotic: VSL 3	NA	20	NCT03704727	Recruiting
Tacrolimus	Immunosuppreseant	↑ Efficacy and Safety	Microbiome profiling	4	148	NCT02498977	Recruiting
Pembrolizumab	Monoclonal antibody	↑ Efficacy	Fecal Microbiota Transplant	2	20	NCT0334113	Recuriting
Aspirin	NSAID	↓ Toxicity	Probiotic:	2	109	NCT03228589	Completed
Aspirin	NSAID	↓ Toxicity	Microbiome profiling	NA	100	NCT03450317	Recruiting

converted into less potent metabolites by *Faecalibacterium prausnitzii* and other Clostridiales in monocultures as well as by the fecal microbiota from healthy individuals [65]. Given that the host physiological and pharmacokinetic parameters relating to tacrolimus are well defined, including the identification of host CYP3A4*22 and CYP3A5*3 polymorphisms linked to variable tacrolimus levels [66], there is an opportunity to integrate known data regarding host and microbe metabolism of the drug into an integrated PBPK and COBRA model.

6.3.3. Xanthohumol

Xanthohumol is a prenylated flavonoid and promising anti-cholesterol and anti-inflammatory candidate therapeutic. The mechanisms for its antiatherogenic properties are diverse and include the inhibition of triglyceride synthesis, prevention of low density lipoprotein oxidation and the promotion of reverse cholesterol transport in macrophages [67,68]. *In vitro*, xanthohumol has strong antimicrobial activity against *Bacteroides fragilis* and toxigenic, clinically relevant, strains of *C.difficile* [69]. Microbial metabolism has been linked to the bioactivity and toxicity of xanthohumol. For example, the gut microbiota converts xanthohumol into 8-prenylnaringenin, an estrogenic phytoestrogen, and then further metabolizes the compound into less potent end products [70]. *Eubacterium ramulus*, from the abundant human microbiome genus *Eubacterium*, metabolizes xanthohumol extensively *in vitro*[70]. How the microbiome contributes to xanthohumol efficacy and toxicity is the focus of an ongoing Phase I randomized, interventional clinical trial (Table 1).

7. Conclusions and future prospects

The extent to which the gut microbiome influences variability in population level therapeutic drug efficacy and toxicity is unknown. Furthermore, we have limited insight into the underlying mechanisms, enzymes, metabolites and species that play key roles in microbiome-drug interactions. A broader map of the metabolic potential of gut microbes will support the development of predictive models of how drugs and foods are modified by the host microbiome, enabling crucial insight into the microbial enzymes and pathways that are responsive to drugs.

Collectively, mechanistic animal model studies, high throughput sequencing and computational approaches used to investigate the microbiome-drug interactions, represent a pipeline for the prediction and modulation of gut microbiome driven adverse drug responses in the clinic (Fig. 2). A shift away from snapshot study designs towards longitudinal human studies that monitor microbiome function over time and at varying levels of granularity may accelerate our discovery of population-level variability in drug response. Longitudinal study designs, depending on their resolution, offer unique insights into how microbial communities respond to a particular perturbation [71].

8. Outstanding questions

Among the outstanding questions to address through preclinical studies and randomized clinical trials are: Is a patient's pre-treatment microbiome predictive of her drug response outcome? What microbiome features are most predictive? What is the temporal stability of patient microbiome phenotypes? How does diet and antibiotic use impact therapeutic drug treatment? What host factors are key modulators of microbiome activity that may shape drug response outcomes? Addressing these questions will enable us to reengineer microbial interactions to better promote drug safety and efficacy.

9. Search strategies and selection criteria

Clinical trial data for this review was identified by searches of ClinicalTrials.gov in addition to PubMed and references from relevant articles using the search terms “microbiome”, “microbiota”, “drug”, “metabolism”, “drug treatment”, “gene expression”, “metagenomics”, “prebiotic”, “probiotic”, “intervention”,“16 s” and “NOT ‘review’[Publication Type]”. Only articles published in English between 2010 and 2019 were included with an exception for those introducing key terms for the first time; preference was given to articles published between 2016 and 2019.

Conflict of interest statement

Dr. Guthrie has nothing to disclose.
Dr. Kelly has nothing to disclose.

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