SUPPLEMENTARY INFORMATION

The Microbiome and the Gut-Liver-Brain Axis for CNS Clinical Pharmacology: Challenges in Specifying and Integrating *In Vitro* and *In Silico* Models

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In a relatively new research field that is growing at the rate of 10,000 research articles a year and is accompanied by broad coverage in the popular press, it is almost impossible to provide a comprehensive review that spans both the historical record and the state-of-the-art. There is also a broad spectrum in the depth of coverage of the various topics connected by this review. We have identified a large number of articles that should inform the reader about the intersection of the human microbiome, clinical pharmacology, environmental toxicology, and the central nervous system. Given the reasonable restriction in the number of references allowed, we have chosen to present many points in the main article with minimal references, and provide, as appropriate, parallel text in this Supplementary Information with more complete references. Hence, this supplement is presented as a complementary guide to reading in greater depth and a "review of the reviews," mixed with selections from primary literature. We have attempted to minimize redundancy between the main article and this supplement, but some has been necessary to maintain the context of the citations in both places. We also apologize to the many researchers whose work we were unable to highlight.

INTRODUCTION

Several articles provide additional information about metabotypes[1, 2, 3], other metabolomic profiles[4, 5, 6] and the development of high-dimensional biomarkers.[4, 7, 8]

With regard to the microbiome and human disease,[9, 10] there are a rapidly growing number of reports of differences between the gut microbiota of healthy and diseased patients, where the primary method for studying the microbiome involves comparing microbiota composition between individuals or model animals with shotgun metagenomics and 16S rDNA sequencing.[11]

As another example of a disease-related change to the microbiome, fecal samples of colorectal cancer patients seemed to be enriched with *Bacteroidetes*, whereas in the fecal samples of healthy controls, *Firmicutes* were one of the major phyla detected.[12] Further, it has been shown that variances in the gut microbiota can account for differences in outcomes for patients who suffer from recurrent *Clostridium difficile* infection.[13, 14]

In addition to reports of the microbiome affecting behavior, mood, and decision-making,[15, 16, 17, 18], the gut microbiota's influence has been implicated in multiple central nervous system (CNS) diseases such as Parkinson's disease,[19, 20, 21, 22, 23, 24] depression and anxiety,[19, 25, 26, 27, 28] autism spectrum disorder (ASD),[19, 25, 29, 30, 31, 32] attention-deficit hyperactivity disorder (ADHD),[25] schizophrenia,[25, 33] bipolar disorder,[33] multiple sclerosis (MS),[19, 34] Alzheimer's Disease (AD),[24, 34] glioblastomas,[35] and various non-CNS diseases such as obesity,[36] type 2 diabetes,[36] glucose intolerance,[36] insulin resistance,[36] acne,[37] atopic dermatitis,[37] psoriasis,[37] colorectal cancer,[11] non-alcoholic fatty liver disease (NAFLD),[36, 38] irritable bowel syndrome (IBS),[25] Crohn's disease (CD),[39] ulcerative colitis (UC),[39] substance use disorders (including opioid use disorder (OUD)),[40] fibromyalgia,[41] chronic pain,[42, 43, 44] stroke,[45] lung disease,[46] celiac disease,[47] and metabolic syndrome.[48] It is also important to recognize that genotoxic bacterial proteins such as the typhoid toxin directly inflict damage to the host's DNA.[49]

SIGNIFICANT FACTORS IN THE HUMAN-MICROBIOME INTERACTION

Biochemical Signaling from Gut Microbiota to the Human

To provide support for the interactions shown in **Figure 1** (see main article), it is worthwhile to examine how the primary postbiotics can alter the host's physiological state by reviewing the roles of tryptophan metabolites, bile acids, short-chain fatty acids (SCFAs), and immune system signaling.

1. Tryptophan Metabolism

The essential amino acid tryptophan is primarily produced from dietary protein metabolism and enters the vascular system in the intestinal tract. Enterochromaffin cells in the digestive tract metabolize tryptophan to serotonin, which is stored systemically in platelets. Spore-forming bacteria in the gut modulate the induction of serotonin in the colon and blood, and in doing so regulate GI motility and clotting.[50] Free circulating tryptophan can cross the blood-brain barrier (BBB) to support serotonin synthesis within the brain. Additional tryptophan metabolism is provided by the kynurenine and aryl hydrocarbon receptor ligand pathways.[51] These tryptophan metabolites are associated with regulation of gut and brain immune homeostasis, gut and brain inflammatory response, inflammatory bowel disease, gut barrier function, and depression.[40, 52] Furthermore, mouse models of autism spectrum disorder are associated with impaired serotonin production, which is correlated with a decrease of *Blautia*,[30] and rat models with fecal transplantation from depressed patients had alterations in tryptophan metabolism.[25] Tryptophan can directly influence the brain, where tryptophan metabolites affect microglia activity, leading to a reduction of CNS inflammation.[34]

2. Bile Acids

Bile acids are metabolites of cholesterol catabolism, which affects nutrient absorption and gut immune homeostasis. Primary bile acids are transported from the liver to the gut, where they undergo biotransformation.[40] Bile acids digest dietary lipids, regulate lipid metabolism, and are important for small intestine epithelial barrier function. The primary bile acids include cholic acid, chenodeoxycholic acid, and muricholic acid. Using 7α-dehydroxylation, *Blautia* converts the primary bile acids into secondary bile acids, which include deoxycholic acid, lithocholic acid, and ursodeoxycholic acid. Further, there are taurine-conjugated and -deconjugated forms of primary and secondary bile acids. *Bifidobacterium* and *Lactobacillus* produce bile salt hydrolase that deconjugates primary and secondary bile acids from taurine and glycine. Mouse models of autism spectrum disorder have deficient bile conversion, suggesting a link between bile metabolism and ASD.[30]

3. Short-Chain Fatty Acids

The three most common SCFAs are acetate, propionate, and butyrate, with acetate being approximately three times more common than propionate and butyrate.[38] SCFAs are primarily generated by the gut microbiota-driven fermentation of dietary fibers in the colon and the metabolism of dietary fats or dietary proteins in the absence of dietary fibers. SCFA metabolites are used for energy in the colon, and some SCFAs are transported to the liver by the hepatic portal vein. SCFAs are released from the liver for circulation. Local SCFAs affect gut membrane permeability, and circulating SCFAs can cross the BBB to affect neural and glia cells.[40] SCFAs

also can affect BBB permeability and act as a histone deacetylase inhibitor.[38, 40] There is evidence of alterations in the amount of SCFAs in ASD children, but it is unclear whether there is an increase or decrease, as the evidence points both ways.[25]

4. Immune System

The immune system plays a central role in those interactions between the human and its gut microbiome that are associated with CNS diseases, including schizophrenia,[25] glioblastomas,[35] Parkinson's[35] and Alzheimer's diseases,[35] and multiple sclerosis,[35] all associated with changes in the immune system. There are three main ways the gut microbiota interacts with the immune system: inflammasomes, type-I interferon signaling (IFN-I), and NF-κB signaling. Inflammasomes are signaling complexes that activate in response to microbial and endogenous threats. Pattern-recognition receptors (PRRs) are involved with inflammasome activation. NLRC5 acts to negatively regulate NF-κB and IFN-I signaling to modulate innate immune system homeostasis. Gut microbiota are able to change inflammation through inflammasome signaling.[35]

IFN-I is a cytokine that influences innate immunity, adaptive immunity, and maintenance of host homeostasis. IFN-I is induced by pathogen-associated molecular patterns (PAMPs) and IFN-I secretion depends on the PRRs. IFN-I signaling can be utilized for protective effects for the host, such as inducing anti-viral responses. Host INF-I signaling influences microbiota composition. Glioblastomas are associated with altered IFN-I signaling.[35]

The NF-κB family of transcription factors influences innate immunity, adaptive immunity, and maintenance of the immune system. Alterations of gut microbiota composition influence different inflammatory diseases by regulating innate immunity and NF-κB signaling. Parkinson's disease and Alzheimer's disease are associated with a pro-inflammatory reaction partially through NF-κB signaling.[35]

Through signaling with the immune system, the gut microbiota can influence immune cells' development in the CNS, such as *Bacteroides fragilis* promoting Th1 cell development and *Clostridium* promoting Treg cell differentiation. Furthermore, microbe-produced SCFAs activate inflammasomes and influence the differentiation of suppressive Tregs, and long-chain fatty acids (LCFAs) promote differentiation and proliferation of Th1 and Th17 cells while simultaneously increasing mRNA expression of pro-inflammatory factors. Hyperactive Th1 and Th17 cells can lead to infiltration of immune cells in the CNS and progression of multiple sclerosis, and recruitment of immune cells can lead to the progression of glioblastomas.[35] The translocation of microorganisms from the gut to lymphatic tissues via immune cells will both challenge and train the mammalian immune system.[53]

Gut Microbiota and Host CNS Interactions

In addition to the hypothalamic-pituitary-adrenal (HPA) axis and microglia interactions discussed in the main article,[20, 26, 34, 35] there is also an intriguing conference report that bacteria from the gut may migrate from the GI system directly to astrocytes in the brain to form a brain microbiota.[54]

1. The Vagus and Enteric Nervous Systems

The enteric nervous system (ENS) provides local control of digestive functions and an intimate connection between the stromal cells of the GI tract and the autonomic nervous system (ANS).

The ENS within the gastrointestinal wall regulates enteric processes that include immune response, detecting nutrients, motility, microvascular circulation, intestinal barrier function, and epithelial secretion of fluids, ions, and bioactive peptides.[55, 56, 57] The gut microbiota regulates maturation of the adult enteric nervous system via enteric serotonin networks.[58] The vagus nerve, connected to all of the layers of the digestive wall, serves as the connection between the CNS and the ENS, but in such a manner that it is never in direct contact with the gut microbiota. Thus, only indirect signals interact with the microbiota through the transport of postbiotic diffusion or by cells in the epithelium relaying luminal signals. Afferent vagal and hormonal signals from the gut to the brain regarding the quality and quantity of food affect the sensation of satiety, energy balance, and glucose homeostasis.[59] Enteroendocrine cells, which control motility, secretion, and food intake in the presence of luminal carbohydrates, triglycerides, and proteins, interact with the vagus nerve directly through the vagus nerve's serotonin-activating 5-HT3 receptors, and gut hormones such as cholecystokinin, glucagon-like peptide-1, and peptide YY affect the brain through receptors in the vagus nerve. SCFAs, LCFAs, and TLR4 also affect the vagus nerve.[60] The Dresden model of Parkinson's disease proposes that the microbiota and the vagus nerve are tied to the disease etiology, and that environmental toxins such as rotenone cause α-synuclein production in the gut. Once produced, the α-synuclein is then transferred from the enteric system to the CNS via the vagus nerve.[20] Once in the brain, α-synuclein disrupts mitochondria function, leading to the production of reactive oxygen species and the progression of the disease.[61] Traumatic brain injury can trigger increases in intestinal permeability. Is this the result of the neuro-enteric axis,[62] or might it also be associated with shifts in the microbiome?

2. The Inflammasomes (Covered in the main article)
3. Microglia (Covered in the main article)
4. HPA Axis (Covered in the main article)
5. Development (Covered in the main article)
6. Gut Microbiota Spatial Heterogeneity

The heterogeneity of the gut microbiome, both in types and densities along the length of the GI tract and with depth into mucosal layers, is coupled with physiological gradients in pH and pressure.[63] At the simplest level, it has been shown that there is large variability between the compositions of the microbes in the mucosa and the stool.[64] Next-generation Illumina gene sequencing of the highly preserved 16S rRNA gene has shown that there are gradients of microbial community composition: the mouth has the most phylogenic diversity, the stomach the least, and there is increasing diversity down the GI tract from the stomach to the stool.[65] Due to the diversity of the gut microbiota throughout the GI tract, there are differences in the way the microbial communities respond to various inputs. For instance, pyrosequencing the 16S rRNA gene (V1–2) after 8 weeks of Vitamin D3 supplementation showed that only the composition of the upper GI tract (gastric corpus, antrum, and duodenum) changed.[66] While the upper GI tract saw decreased counts of *Gammaproteobacteria*, including *Pseudomonas spp*. and *Escherichia/Shigella spp*., there were no measurable differences in the microbial composition in the terminal ileum, appendiceal orifice, ascending colon, and sigmoid colon, or in stools.[66] This might help to explain why Vitamin D3 supplementation may ameliorate inflammatory bowel disease.[67] A similar distribution was seen in healthy subjects, where *Enterobacteriaceae* were shown to increase toward the distal end of the GI tract (the sigmoid colon and rectum), whereas

Streptococcus species, *Comamonadaceae*, *Enterococcus* species, and *Corynebacterium* species had increased abundance in the proximal end of the GI tract (the cecum and transverse colon).[68] There also has been new research into "smart pills" that are able to collect intestinal fluid samples autonomously to measure gut microbe diversity with higher resolution and lower costs.[69, 70, 71, 72]

EXTERNAL INFLUENCES ON THE MICROBIOME

Xenobiotics and the Exposome

Here we provide specific details that can add a useful perspective to the material presented in the main review article.

Emulsifiers, a food additive, can disrupt the mucus layer that protects the gut epithelium from bacteria. Examples of emulsifiers in processed food include carboxymethylcellulose and polysorbate-80, which have been shown to affect SCFA levels found in feces. Non-caloric artificial sweeteners such as saccharin, sucralose, aspartame, and acesulfame K are associated with inducing glucose intolerance in humans and a disruption of the gut microbiota through an increase of *Enterobacteriaceae*, *Deltaproteobacteria*, and *Actinobacteria* phyla, and a decrease of probiotics *Bifidobacterium*, *Lactobacillus*, and *Bacteroides*. Polyphenols, commonly found in red wine, are associated with the increase of probiotics and the decrease of pathogenic bacteria.[73]

The gut microbiota is both resilient and plastic. It maintains a relatively stable steady state despite changes in diet or antibiotics.[74] However, it can be formed and guided toward different paths through diet or directed therapies. With this in mind, some targeted approaches to alter gut microbiota composition have been attempted, such as using *Clostridium scindens* to increase resistance to *C. difficile* infection by targeting the pathway of secondary bile acid.[75] There have been forays into bioengineering probiotics for prevention of colonization, production of antimicrobial factors, immunomodulation and cytoprotection, and regulation of virulence gene expression.[76] Another example of microbial manipulation is the use of a tungstate treatment to selectively inhibit molybdenum cofactor-dependent microbial respiratory pathways, thereby reducing inflammation in colitis.[77] Thus it is important to account for the effects of the gut microbiota when considering drug efficacy, availability, and toxicity.[74]

Probiotics, Prebiotics, Synbiotics, and the FDA

Lactobacillus is an example of a beneficial bacteria thqt is used as a probiotic to prevent stress-induced synaptic dysfunction and thereby reduce the response of the HPA axis to chronic stress and relieve the symptoms of anxiety and major depression disorder.[26] Probiotics also have been used to reverse symptoms of diseases such as IBS.[25] Other beneficial effects include anti-pathogen and anti-inflammation activity and mitigating symptoms of CNS disorders.[78] Probiotics also can potentially control body weight, adipose tissue, and inflammation and prevent metabolic syndrome.[48] So-called psychobiotics refer to probiotics that produce neurochemicals such as GABA, serotonin, and dopamine. These compounds have the potential to directly influence ENS signaling and indirectly influence host brain function and behavior.[79]

While prebiotics are indigestible to the host, they serve as microbial nutrients and act to promote the growth of beneficial bacteria populations. Bifidogenic prebiotics include inulin, oligofructose, fructo-oligosaccharides, galactose-containing oligosaccharides, and xylose-containing oligosaccharides.[48]

An example of a symbiotic, which promotes the beneficial effect of probiotics, is the combination of the prebiotic inulin with the probiotics *Bifidobacterium animalis, Lactobacillus acidophilus,* and *Lactobacillus paracasei.*[78]

Diet

The microbiome changes associated with various diets have been examined in some detail. The Western diet and lifestyle are associated with a high-calorie, high-fat, low-fiber diet and a sedentary lifestyle and produce a less diverse microbial ecology when compared to other diets, such as those high in fiber.[80] *Bifidobacterium* and *Eubacterium* are reduced and there is an increase of cancer-promoting nitrosamines and inflammation.[73] In addition to the production of TMA by the gut microbiome, a high-fat diet can induce inflammasome activation and has been shown to worsen the effects of antibiotics in *Oreochromis niloticus*.[35, 81] Metformin and berberine are two drugs that treat type 2 diabetes by reversing the microbiota modulations of a high-fat diet. The drugs decrease microbiota diversity but increase the number of SCFA-producing bacteria *Allobaculum*, *Bacteroides*, *Blautia*, *Butyricicoccus,* and *Phascolarctobacterium.*[73]

The Mediterranean diet is high in fiber and antioxidants and low in red meat, and it is associated with increased levels of fecal SCFAs and probiotics *Bifidobacteria*, *Lactobacilli*, *Eubacteria*, *Bacteroides* and *Prevotella*.[73] In general, the amount of SCFAs produced is dependent not only on the amount of fiber but also the type of fiber eaten.[82] Furthermore, gluten is metabolized in the gut primarily by *Firmicutes, Actinobacteria,* and *Proteobacteria.*[47]

Intermittent fasting is a diet that alternates between periods of fasting and non-fasting. When *Drosophila melanogaster* were fed on a 2-day non-fasting and 5-day fasting diet, triacylglyceride levels increased and led to a decrease of bacterial load in the gut.[83, 84] Intermittent fasting has also been investigated in mice. After a diet of fasting every other day, mice gut microbiota had a large increase of *Firmicutes*, which was shown to induce beiging of white adipose tissue, weight loss, and attenuation of metabolic dysfunction.[85] How might such protocols become a part of clinical pharmacology?

The ketogenic diet is a high-fat, low-carbohydrate diet that has been well established as a treatment for refractory epilepsy but is also used for treating autism spectrum disorder, Alzheimer's disease, metabolic syndrome, and cancer. The ketogenic diet alters the microbiota by decreasing alpha diversity (within a sample) and increasing the taxa *Akkermansia muciniphila* and *Parabacteroides. Akkermansia muciniphila* and *Parabacteroides* are associated with decreased systemic gamma-glutamylated amino acids and elevated hippocampal GABA/glutamate levels, which provide a seizure protective effect.[86] Children on the ketogenic diet had a decrease of *Bifidobacterium* and *Eubacterium rectale,* which is associated with production of the SCFAs acetate and butyrate, respectively.[87]

Fecal transplants in mice enhanced healthspan and lifespan.[88] More active lifestyles, such as those of professional athletes (and the possible use of nutritional supplements), result in increased gut microbiota diversity,[89] which suggests the possibililty of legal questions and sporting regulations regarding the possible use of fecal transplants in increasing an athlete's competitive advantage in sports.

Drugs

It is known that there are different passive permeability characteristics among the different portions of the human intestines.[90] There are simple differences of the ion permeability as well; for instance, the jejunum is highly permeable to sodium and chloride ions, while the colon is more anion- than cation-selective.[90] Early work on drug permeability within rat intestines has shown that there is a decrease in permeability to hydrophilic drugs and a significant increase in permeability for hydrophobic drugs in the small intestine.[91] Overall, a recent review has concluded that the varying factors throughout the GI tract, such as osmolality, the unstirred water layer, mucosal differences, and the presence of other fluids, all must be taken into account when considering how a body will take in a drug.[92] Within the small intestine, there is a thin and discontinuous mucous layer, composed mostly of MUC2, that is able to effectively facilitate molecular transport.[93] However, within the colon there are two layers of mucous, a thin, sterile inner layer and a thicker, more porous outer layer, to allow a symbiotic relationship with gut microbiota.[94] The mucous layers can affect drug absorption due to size restriction through the mucous[95] or electrostatic interactions between the drug and the mucin when the pH is greater than 2.6.[92] In addition, the unstirred water layer, which is a stagnant boundary layer above the epithelium, can affect drug absorption.[92] It has been shown that between 10% and 36% of transport resistance is due to the unstirred water layer,[96] which varies in thickness along the GI tract depending on the amount of villi within the space.[92] Further research has shown that it is not epithelial surface area that is the main determinant of drug absorption, but rather the mucus layer at the surface of the epithelium and the fluidity of the epithelial cell membrane.[97] Diet can affect drug absorption by regulating enzymes involved in drug metabolism, limiting drug bioavailability.[98] Note that these layers could severely compromise the ability to obtain representative samples of the microbiota of each region.

In addition to variances in the way a drug is absorbed throughout the GI tract, it has been shown that the location within the GI tract changes the way a drug is metabolized. Phase I metabolism, which is the introduction of a reactive group by means of oxidation, reduction, and hydrolysis, predominantly occurs in precision-cut, illium intestinal slices, and phase II metabolism, which is the conjugation with polar moieties occurring by means of glucuronidation, sulfation, acetylation, and methylation, mostly takes place in precision-cut, colon intestinal slices.[99, 100] This is heavily due to the distribution of P450 enzymes, which account for about half of overall elimination of commonly used drugs.[99]

The gut microbiota affects drug efficiency both directly and indirectly through multiple mechanisms.[101] Some direct means of interaction include acetylation, deacetylation, and demethylation.[102] Such mechanisms can sometimes be helpful, as in treating inflammatory bowel disease with sulfasalazine, which is turned into the active drug 5-aminosalicylic acid by microbial enzymes.[102] However, the gut microbiota can indirectly reduce the efficacy of a drug, as is the case with acetaminophen (paracetamol), which must compete for sulfonation by the liver with the microbial metabolite p-cresol, ultimately leading to some drug toxicity.[103] Postbiotics also can affect liver metabolism, such as the expression of cytochrome P450 enzymes.[63] To take these factors into account, researchers have recently published a genome-scale metabolic reconstruction for 773 members of the gut microbiota so that physiologically based pharmacokinetic models are more accurate.[104]

Wilson and Nicholson[105] and Thiele *et al.*[101] provide a number of informative examples of how the gut microbiome interacts with drug metabolism, efficacy, and toxicity. Oral drugs

interact with the gut microbiota first, and intravenous (IV) drugs interact with the liver before the gut microbiota. Interactions of the gut microbiota with drugs include reduction, hydrolysis, dihydroxylation, dealkylation, demethylation, decarboxylation, acetylation, deamination, and deconjugation. Liver interactions are primarily oxidations, conjugations, reductions, and hydrolyses. Enterohepatic cycling involves the transfer of metabolites between the gut and liver and can result in drugs being reactivated by gut microbiota. The liver acts as the way to cause metabolites to enter circulation. Metabolites are eliminated in feces or by the kidneys. [105]

One of the pharmacological challenges is to identify whether human microbiota can serve as agonists to G-protein coupled receptors (GPCRs). In a recent demonstration, a panel of bacteria were selected to create a forward chemical screen that can be used to detect small molecules produced by gut microbiota that interact with host GPCRs.[106] It was shown that small molecules produced by the gut microbiota can activate multiple GPCRs, including orphan GPCRs, serving as an unanticipated source of GPCR ligands that might adversely affect drug action.

Opioids

As a final example of the emerging complexity of opioid clinical pharmacology and its implications for precision medicine, suppose that our Vitruvian human in **Figure 1** were to ingest 200 ml of grapefruit juice every day for five days, and be treated on day four with a 10 mg oral dose of oxycodone hydrochloride. The mean area under the oxycodone concentration-time curve, the peak plasma concentration, and oxycodone half-life would be increased by factors of 1.7, 1.5, and 1.2, respectively, all a result of the grapefruit juice inhibiting first-pass intestinal CYP3A4-mediated oxycodone metabolism and transporter proteins such as P-glycoprotein (P-gp) and organic anion transporter polypeptide.[107] In addition, grapefruit juice decreases the production of noroxycodone and noroxymorphone, and increases production of oxymorphone. One might reasonably assume that there are many more as yet unidentified interactions between these drugs, the microbiome, and the gut-liver-immune-brain axis GLIBA.[108] The roles of endogenous opioids that are a dopamine metabolite produced by mammalian cells[109, 110] are worthy of detailed study, could have significant biological, medical, and social implications, and may play a role in some of the interactions in **Figure 1**.

DECODING MICROBIOME-HUMAN INTERACTIONS

Pharmacogenomics/Microbiomics (Covered in the main article)

Mathematical Modeling of the Microbiome-Host Interaction

One method for *in silico* modeling of these interactions is constraint-based reconstruction and analysis (COBRA), in which a metabolic reconstruction of an organism is assembled in a bottom-up manner on the basis of reaction stoichiometry and physicochemical properties obtained from genome annotations and biochemical and physiological data.[111] This reduces the system to a set of linear equations that is able to provide physiologically relevant solutions.[101] This type of system has been used, for example, to investigate how the body interacts with levodopa, the most common drug administered for Parkinson's disease.[101] From this approach, it was determined that plasma-levels of levodopa were most sensitive to the gastric emptying rate and the loss of bioavailability due to microbial activity.[101] Further work to predict a drug's effects have come from research within the field of quantitative systems toxicology (QST),

which is an approach to quantitatively understand the toxic effects of a chemical on a living organism, from molecular alterations to phenotypical observations, through the integration of computational and experimental methods.[112] One of the fundamentals of QST is the use of molecular descriptors and physicochemical properties to make accurate *in silico* predictions in quantitative structure-activity relationships (QSARs).[112] In addition, pathway analysis tools, such as Ingenuity Pathway Analysis and DAVID/KEGG, are being used to create network-based models of biological systems.[113] Models such as these have been used to determine the molecular mechanisms responsible for sunitinib cardiotoxicity and identify a prophylactic intervention.[114] Multi-omic techniques under development for rapid determination of drug mechanism of action should be readily extensible to organ-chip systems with microbiomes.[115] The quantity of the data and the variety of interactions will exceed the capacities of standard relational databases, with graph databases offering the greatest potential, at least for describing the interaction network.[116, 117, 118]

Assays for Understanding Host-Microbiome Interactions (Covered in the main article)

Organs-on-Chips for *In Vitro* Studies of Microbiome-Organ-Organ Interactions

1. The Rationale for Microphysiological Systems (MPS) GLIBA Models

Shuler began pioneering work on organs-on-chips in the 1990s and remains active in the field, [119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135] with some of his work directly related to toxicology.[119, 120, 135, 136] A growing number of original papers and reviews are relevant to the MPS and the microbiome GLIBA (M-GLIBA),[137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147] with substantial progress in the implementation of the four organs of the GLIBA: the gut,[148,124, 129, 148, 149, 150, 151, 152] the liver,[153, 131, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163] and the neurovascular unit and/or the blood-brain barrier (BBB).[122, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178] Zhang and Radisic provide an excellent overview of the rapidly expanding commercial market.[179]

Organoids are often a useful intermediate step between a biopsy or stem cells and a tissue chip, and they can be used in MPS organoid chips,[151, 180, 181, 182, 183, 184, 185] but this discussion is beyond the scope of this review. Organoids are already being used to propagate cells from a patient's cancer biopsy to predict the response to different cancer therapies using a variety of live-cell assays, including patient-derived xenografts and organoids.[186, 187, 188, 189]

2. Single Organ Chips Needed for the M-GLIBA (Covered in the main article)

3. Multiple-Organ MPS Models

There is a small but expanding literature on multi-organ MPS.[128, 130, 132, 133, 135, 139, 145, 147, 161, 162, 163, 174, 185, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200] Boeri *et al.* provide a review that focuses on liver multi-organ communication with the gut, microbiota, and brain.[201] Logsdon *et al.* review how the BBB connects the microbiome to the brain via bacterial and host cytokines, peripheral immune cells, and other secreted factors.[202] The coupling between different organs can either be physical, as demonstrated by several groups using either integrated or connected chips,[132, 163, 193, 198] or functionally by transferring effluent media from the output of one chip into the input of another organ chip.[199, 200, 203] One advantage of the functional approach is that it is easy to adjust the fraction of the media in one organ that has been conditioned by another, whether done

manually[203] or by an automated pipetting robot.[200] Functional coupling does not even require that the different organs have to be co-localized, as long as the biochemical signals of interest can survive freezing and transport,[203] and offers the advantage of ready access to the input and output of the media for each organ. The need to examine the metabolic coupling between different cell types, particularly endothelial and stromal cells, can be addressed using connected organ chips for which the cell types that are normally cultured in close proximity or even in contact are instead spatially separated but joined fluidically.[204] The evaluation of juxtacrine signaling, for example between astrocyte end feet, pericytes, and endothelial cells, might benefit from the comparison of physically coupled co-cultures with separated ones.

In any multiple organ application, it is important to recognize that recirculation will increase the ability of a small population of cells in each organ to condition some volume of common media over time as compared to single-pass perfusion, that the common media will have to be periodically or continuously refreshed and a fraction removed, since the multiple organ chip will need to be fed and is not likely to have sufficient kidney or liver function to detoxify the recirculating media by excreting toxic metabolites, and that the total volume of media being recirculated must be scaled properly to the numbers of cells used, lest the circulating signals be diluted below the threshold of physiological effect.[205, 206]. There are also economical and practical considerations in systems that couple multiple organs. If the system is large, expensive, and difficult to maintain, it is less likely to be used in moderately parallel experiments than one that is compact, inexpensive, and easy to use and replace should a component fail. Given the need to culture tissue chips for extended periods of time, it is important to have a system that can be readily sterilized. Multiple organs integrated on a single larger chip have the possible advantage of operating with smaller fluid volumes than those that are connected by tubing, discrete reservoirs, and/or pipettes. Integrated systems will have a lower probability of being fully functional than separated systems for which the most highly functional organ chips can be preselected and sub-optimal organs can be readily replaced, rather than having to seed all cells on a single integrated chip with the expectation that some organs on the chip will prove better than others. If the integrated chips are small, inexpensive, and do not require a large number of cells, this may not be an issue.

From this analysis, we conclude that it is most appropriate to use simple, massively parallel well-plate cultures for high-throughput screening, for example, for the effects of a drug or toxin on a specific receptor or transporter. Single-organ or organoid MPS chips are already available commercially for medium-throughput, high-content screening of phenomena that can only be reproduced in multicellular co-cultures, particularly those that involve either a 2D barrier separating different cell types, or multicellular interactions with a non-trivial extracellular matrix. There is growing recognition that microphysiological systems with multiple organs are better suited for high-content, low-throughput identification and recapitulation of complex pharmacological and toxicological kinetics and dynamics and organ-organ and drug-organ-organ interactions than are isolated monocultures on plastic, albeit at a much greater investment in operator training and experimental complexity.[132, 198, 207] That investment, while at first glance significant, needs to be compared not to high-throughput screening but to a long-term, multiple animal study, which may not reflect human physiology, pathology, or toxicology, and is also expensive. Returning to the focus of this review, studies of the interaction between human multiple organs and their individual microbiota may well be an ideal application of multiple, coupled organ chips.

4. MPS for Drug Discovery

A number of papers address drug toxicity[135, 136, 143, 208, 209] and pharmacology.[134, 195, 210, 211, 212, 213, 214] Of the many groups working on organs-on-chips, only a few are developing the pharmacodynamic (PD) tools that will be needed by clinical pharmacologists and toxicologists to fully utilize tissue chips.[127, 132, 134, 198, 215, 216] The reader is urged to study a comprehensive review of the iterative measurement-modeling connection between MPS and quantitative systems pharmacology.[217]

Although beyond the scope of this review, it is worthwhile to note that studies of the microbiota of male and female genitourinary systems[218, 219, 220, 221, 222, 223, 224, 225, 226] could be conducted using organ-on-chip models,[192, 227, 228, 229, 230, 231, 232] which might provide insights into the interplay between the vagina microbiome, human papilloma virus, inflammation, and cancer,[6] and the effect of the vaginal and gut microbiota on drug metabolism and efficacy.[233]

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