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DRUG METABOLISM AND DISPOSITION
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Minireview

Gut Microbiome Integration in Drug Discovery and Development of Small Molecules[S]

Patrick Jimonet, Céline Druart, Stéphanie Blanquet-Diot, Lilia Boucinha, Stephanie Kourula, Françoise Le Vacon, Sylvie Maubant, Sylvie Rabot, Tom Van de Wiele, Frank Schuren, Vincent Thomas, Bernard Walther, and Michael Zimmermann, on behalf of Medicen Microbiome Drug Metabolism Working Group

Medicen Paris Région, Paris, France (P.J.); Pharmabiotic Research Institute, Narbonne, France (C.D.); UMR 454 MEDIS, Université Clermont Auvergne, Clermont-Ferrand, France (S.B.D.); Global Bioinformatics, Evotec ID, Lyon, France (L.B.); Preclinical Sciences & Translational Safety, JNJ Innovative Medicine, Beerse, Belgium (S.K.); Biofortis, Saint-Herblain, France (F.L.V.); Translational Pharmacology Department, Oncodesign Services, Dijon, France (S.M.); Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France (S.R.); Center of Microbial Ecology and Technology, Faculty of Bioscience Engineering, Ghent University, Gent, Belgium (T.V.W.); TNO, Leiden, The Netherlands (F.S.); Lallemand Health Solutions, Blagnac, France (V.T.); Servier, Saclay, France (B.W.); and Structural and Computational Biology Unit, European Molecular Biology Laboratory, Heidelberg, Germany (M.Z.)

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ABSTRACT

Human microbiomes, particularly in the gut, could have a major impact on the efficacy and toxicity of drugs. However, gut microbial metabolism is often neglected in the drug discovery and development process. Medicen, a Paris-based human health innovation cluster, has gathered more than 30 international leading experts from pharma, academia, biotech, clinical research organizations, and regulatory science to develop proposals to facilitate the integration of microbiome science into drug discovery and development. Seven subteams were formed to cover the complementary expertise areas of 1) pharma experience and case studies, 2) in silico microbiome–drug interaction, 3) in vitro microbial stability screening, 4) gut fermentation models, 5) animal models, 6) microbiome integration in clinical and regulatory aspects, and 7) microbiome ecosystems and models. Each expert team produced a state-of-the-art report of their respective field highlighting existing microbiome-related tools at every stage of drug discovery and development. The most critical limitations are the growing, but still limited, drug–microbiome interaction data to produce predictive models and the lack of agreed-upon standards despite recent progress. In this paper we will report on and share proposals covering 1) how microbiome tools can support moving a compound from drug discovery to clinical proof-of-concept studies and alert early on potential undesired properties stemming from microbiome-induced drug metabolism and 2) how microbiome data can be generated and integrated in pharmacokinetic models that are predictive of the human situation. Examples of drugs metabolized by the microbiome will be discussed in detail to support recommendations from the working group.

SIGNIFICANCE STATEMENT

Gut microbial metabolism is often neglected in the drug discovery and development process despite growing evidence of drugs' efficacy and safety impacted by their interaction with the microbiome. This paper will detail existing microbiome-related tools covering every stage of drug discovery and development, current progress, and limitations, as well as recommendations to integrate them into the drug discovery and development process.

Introduction

Microbiome research has evolved significantly during the past 20 years, thanks particularly to the development and increased accessibility to next-generation sequencing technologies. With several hundred bacterial species identified in each human individual's gut, each of them harboring 4000 to 6000 genes, the number of microbial genes outnumbers the human genome by more than 100-fold (Rajilić-Stojanović and de Vos, 2014; Gilbert et al., 2018). This estimate is aligned with about

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ABBREVIATIONS: 5-ASA, 5-aminosalicylic acid; ADME, absorption, distribution, metabolism, excretion; ADMET, absorption, distribution, metabolism, excretion, toxicity; BCS, Biopharmaceutical Classification System; DDI, drug–drug interaction; DME, drug-metabolizing enzyme; INTEDE, Interactome of Drug-Metabolizing Enzymes; PBPK, physiologically based pharmacokinetics; PK, pharmacokinetics; SHIME, Simulator of Human Intestinal Microbial Ecosystem; SPF, specific pathogen free; TIM, TNO Gastro-Intestinal Model; UC, ulcerative colitis.

230,000 nonredundant gut bacterial genomes recently catalogued (Almeida et al., 2021; Kim et al., 2021). Such a large number of genes encode an extensive enzymatic repository, capable of metabolizing a broad spectrum of chemical compounds of endogenic and exogenic origin. Therefore, microbiota have been identified as potential contributors to xenobiotic biotransformation. The impact of such microbial metabolism on drug efficacy and/or toxicity has been suggested for a growing number of drugs (Noh et al., 2017; Zhang et al., 2018; Weersma et al., 2020).

The gut microbiota can impact drug metabolism in multiple ways: it can metabolize drugs directly; it can influence drug metabolism by impacting the activity of human drug-metabolizing enzymes (DMEs) (CYP450s, transferases; Dempsey and Cui, 2019); it can hydrolyze the conjugated forms produced by DMEs; and all of these modalities can occur concurrently (Kang et al., 2013; Enright et al., 2016; Li et al., 2016; Spanogiannopoulos et al., 2016; Noh et al., 2017; Dhurjad et al., 2022; Dikeocha et al., 2022; Pant et al., 2023; Yang et al., 2023). In addition, effective drug concentration can also be decreased by intracellular accumulation of the unmodified drug in microorganisms thus altering drug disposition (Klünemann et al., 2021). Importantly, medical drugs have been shown to be among the strongest factors influencing the composition and functions of the gut microbial ecosystem (Forslund et al., 2015; Maier et al., 2018; Bruno et al., 2019), coining the term of reciprocal microbiota drug interactions (Zimmermann et al., 2021).

These bidirectional drug–microbiome interactions need further scrutiny by the pharmaceutical industry, health and research professionals, as well as regulatory agencies. To evaluate how approaches to study drug–microbiota interactions could be included in future drug discovery and development processes, a working group has been formed by Medicen. It includes more than 30 leading experts from pharma, biotech, clinical research organizations, regulatory science, and academia (https://medicen.org/a-propos/filieres-axes-strategiques/). In frequent meetings, the team collected materials that were used as a basis to discuss potential implementation of microbiota-mediated drug metabolism into the drug discovery and development pipeline.

Pharmaceutical companies involved in the consortium have been confronted with these drug–microbiome interactions at some point of the development of new chemical entities. They have, or have not, used existing in silico, in vitro, and in vivo tools to better understand the potential impact of the microbiota on the safety and efficacy of molecules. However, the assessment often remains inconclusive, occurs late in the development process, and lacks integration into the drug discovery and development workflow. Review of the existing tools by the working group has highlighted major progress made to support many stages of drug discovery and development. However, increasing findings of drug–microbiota interactions pose a challenge to the industry, if more systematic approaches to address those interactions are to be implemented. The implementation of standardized tools, processes, reference materials, demonstration of predictability in humans, and clinical expertise will facilitate their adoption.

To establish whether understanding of the microbiome and drug–microbiome interactions will accelerate the discovery and development process of safe and efficacious drugs, the working group has identified two main areas, which will be further elaborated in this paper. The first one is how microbiome tools could support the R&D process from drug discovery to clinical proof-of-concept studies by allowing the early identification (and removal) of some risks, improving the safety and efficacy of drug candidates, and, when overlooked in due time, retrospectively explain in vivo metabolism profiles. The second one is about the modelling aspect and the need to generate predictive data representative of the human situation. Integration of microbiome considerations in these modelling approaches could in the future improve in vitro to in vivo extrapolation and capture interindividual variability in drug response (safety and efficacy).

Examples of Microbial Drug Metabolism

Early risk assessment within chemical series of clinical candidates has been integrated progressively in drug discovery over the past few decades (Maurer et al., 2022). Besides the targeted biological properties of a clinical candidate, its physicochemical and pharmacokinetic properties, as well as biochemical off-target interactions, influence the medical efficacy and safety of the drug candidate.

Typically, a thorough characterization of the absorption, distribution, metabolism, excretion (ADME) and safety profile of a compound is performed to accurately predict doses, exposures, drug–drug interactions (DDI), and toxicity liabilities. Early identified undesired ADME properties and DDI liabilities can be addressed through structural modifications of the chemical entity by medicinal chemists. Based on a multivariate optimization process, drug design is adapted for such an evaluation following iterative use of in silico tools and high throughput in vitro assays. Metabolic stability of the compound, mainly dependent on hepatic biotransformation, is one of the most critical ADME parameters and is determined early in the drug discovery process via liver microsomal or hepatocyte stability assays. An improved and more comprehensive early metabolite profiling in drug design and discovery has been implemented by Pfizer with real success in improving clinical candidate quality (Cerny et al., 2020). Early identification of metabolic pathways and metabolites supports prioritization of chemical series to favor the best drug profile and potentially avoid undesired properties. Cerny et al. have listed four questions that often present themselves to medicinal chemists optimizing compounds for candidate selection: 1) What are the important clearance mechanisms that mediate the disposition of my molecule? 2) Can metabolic liabilities be modulated in a favorable way? 3) Does my compound undergo bioactivation to a reactive metabolite? 4) Do any of the metabolites possess activity, either on- or off-target? An additional question necessary to support compound development relates to metabolites in safety testing (Cerny et al., 2020). These questions are also applicable to the microbiome field, raising the question of how to best address them, which we further discuss in this review.

Metabolism of drugs occurs mainly through drug-metabolizing enzymes, the main group of proteins responsible for metabolizing a large and diverse range of chemicals (Penner et al., 2012). Microbiome DMEs have been well described in recent reviews (Spanogiannopoulos et al., 2016; Wilson and Nicholson, 2017); whereas hepatic DMEs focus mainly on reactions aiming at the production of more hydrophilic molecules, microbiome DMEs stimulate primarily reductive and hydrolytic transformations. Therefore, drugs exposed to microbiome DMEs could generate, at least locally, metabolites not observed by liver-mediated transformations and hydrolyse conjugated drugs/metabolites formed in the liver and excreted in the gut via the bile. Note that sometimes the gut microbiota can produce metabolites more hydrophilic than the parent compound (e.g. tacrolimus; Guo et al., 2019). Furthermore, these drug microbiome-derived metabolites (that could be similar to or different from host-mediated metabolites) could potentially be absorbed through the gut epithelium and could then reach the blood circulation. A few examples of drugs metabolized by the gut microbiota and leading to bioavailable metabolites reaching the blood circulation have been reported (see Table 2 in Cai et al., 2023). In addition, many transporters are playing an important role in modulating drug pharmacokinetics (PK) properties, DDIs, and related efficacy and toxicity. Direct or indirect influence of the gut microbiota on drug transport has been shown in several experiments but still requires more studies (see tacrolimus example later and Zhang et al., 2021).

More surprisingly, conjugation by human gut bacteria of bioactive molecules into more hydrophilic metabolites was recently reported. Sulfonation of steroidal metabolites, including cholesterol or analogs such as pregnenolone or lanosterol, was identified and a commensal biosynthetic gene cluster characterized in a biochemical assay and mouse germ-free model. This unexpected metabolism could result in potential modulation of host immune responses through its influence on the Th17/Treg cell balance (Yao et al., 2022).

Well-known examples of gut microbiota metabolism are reductive reactions of prodrugs such as the azo precursors of antibacterial sulphanilamides, the azo precursor of the prodrug sulfasalazine used in the management of ulcerative colitis (UC), or nitro-containing benzodiazepines that are reduced to active amino drugs by nitroreductases (Misal and Gawai, 2018). It is important to note that reduction could lead to unwanted and toxic compounds as has been shown for metronidazole (Dingsdag and Hunter, 2018). Molecules bearing chemical moieties susceptible to reductive reactions should therefore be among the first candidates for their evaluation of microbiome-mediated metabolism.

Another example of gut microbial metabolism is decarboxylation of prodrugs such as levodopa. Levodopa is used to increase dopamine levels for the treatment of Parkinson's disease. Dopamine cannot cross the blood-brain barrier contrary to levodopa. Once levodopa has reached the central nervous system, it is metabolized in the brain to produce dopamine, the active therapeutic agent. However, in the gut lumen, levodopa can be decarboxylated by microbial enzyme such as *Enterococcus faecalis* tyrosine decarboxylase, reducing the bioavailability and brain concentrations of levodopa and therefore limiting its medical efficacy to improve symptoms of Parkinson disease patients (Jameson and Hsiao, 2019; Maini Rekdal et al., 2019; Beckers et al., 2022).

Glucuronidases are other well-known enzymes with the potential to hydrolyze glucuronide metabolites, potentially originating from enterohepatic recirculation following parenteral administration of the parent drug. This metabolism pathway has been observed for the anticancer agent irinotecan, thus leading to gut toxicity of the deconjugated metabolite SN38 (Wallace et al., 2015). Indeed, when the parent drug irinotecan is administered, it is converted by host carboxyesterase into its active form SN38 in blood serum and tissues (Yue et al., 2021). SN-38 is extensively glucuronidated in the liver and eliminated through biliary secretion in the intestine. In the intestinal lumen, however, bacterial β-glucuronidase expressed by the host microbiota deconjugates the glucuronic acid and regenerates the active form (SN38), which causes gastrointestinal damage and diarrhea that is dose-limiting for irinotecan. In both cases, L-dopa and irinotecan, the use of inhibitors of the respective gut-metabolizing enzymes has been suggested to improve drug efficacy, reduce drug toxicity, and decrease patient response variability (Cheng et al., 2019; Maini Rekdal et al., 2019; Bhatt et al., 2020).

5-Aminosalicylic acid (5-ASA or mesalamine or mesalazine) is another striking example of a drug recently proved to be metabolized into its inactive acetylated form (N-acetyl 5-ASA) by unexpected gut microbial enzymes (Mehta et al., 2023). 5-ASA is a first line treatment for UC but with a reported lack of response to treatment for about half of the patients (Ford et al., 2011). Ultimately, identification of responders and nonresponders based on individual microbiome analysis could improve UC treatment using metabolizing enzyme inhibitors or direct a move to second-line drugs.

Similarly, the commonly used immunosuppressant tacrolimus was recently shown to be converted by *Faecalibacterium prausnitzii* and other gut bacteria to a therapeutically less potent metabolite, previously not identified using hepatic microsomes. The in vitro observation was confirmed in human fecal samples. This newly identified route of elimination for tacrolimus potentially contributes to the low and variable exposure after oral dosing; it also supports the observed positive correlation between *F. prausnitzii* abundance and oral tacrolimus dose (Guo et al., 2019). Contribution of the gut microbiota to the highly variable PK of tacrolimus was recently confirmed by a study showing that the gut microbiota is able to influence the expression of efflux transporter ABCB1, which limits tacrolimus intestinal absorption (Degraeve et al., 2023).

This diverse set of drugs illustrates the growing number of reported drugs metabolized by the gut microbiome with an impact on drug efficacy and safety. Therefore, the working group asked the questions of whether the potential interaction of a drug with the gut microbiome should be more regularly assessed and at what stage of the drug discovery and development process this should occur (Javdan et al., 2020).

A consideration to make when addressing this question is why the microbiome has been overlooked for so long if it does play such a substantial role in drug metabolism and clearance.

Firstly, drug candidates moving through preclinical and clinical development were initially low molecular weight and soluble compounds, often highly bioavailable after oral administration [Biopharmaceutical Classification System (BCS I) and II] and therefore poorly exposed to the microbiota of the large intestine after their efficient absorption in the small intestine. It could be a major component explaining why drug–microbiome interaction has been underestimated apart from a few remarkable examples (e.g. L-dopa, irinotecan, sorivudine, nitrazepam, sulfasalazine, omeprazole).

However, clinical trials over the past 40 years have handled an ever-increasing structural complexity of small molecules (Shultz, 2019). Progressively, a growing number of compounds reached the market that categorize beyond the boundaries of the famous Lipinski rule of 5 (particularly compounds with molecular weight above 500 daltons), resulting in higher risk of poor intestinal absorption upon oral administration (Lipinski et al., 2001; Hamley and Jimonet, 2015), hence yielding compounds that are more easily exposed to the gut microbiota. This evolution highlights the possibility to identify oral drugs beyond the initial limits but also represents a greater challenge for medicinal chemists to control compound lipophilicity (measured logD), aqueous solubility, and resulting permeation properties (Tinworth and Young, 2020). With the increase in low permeability drugs (BCS III and IV) and decreased small intestine absorption, the colonic drug concentrations are not negligible and the drug exposure to the microbiome gains significance (Maier et al., 2018).

In addition, new modalities have been explored more recently that are covering a large chemical space with limited knowledge about their behavior in the gut, e.g. protein degraders, macrocyclic peptides, and oligonucleotides (Valeur and Jimonet, 2018). To illustrate, we could highlight the outstanding discovery and optimization by Merck scientists of orally bioavailable macrocyclic peptides as PCSK9 inhibitors (Johns et al., 2023). Despite a low oral bioavailability of 2%, obtained using permeation enhancers, phase 2b of MK-0616 showed efficacy at reducing LDL cholesterol with an acceptable safety profile (Ballantyne et al., 2023). Potential interaction with the gut microbiota and influence on the intestinal permeability will need to be closely evaluated.

A second explanation lies in the tremendous analytical progress that has revolutionized the microbiome field over the past 20 years, not only from a phylogenetic point of view but also from a functional point of view. It has described the microbiome as intrinsically highly diverse and characterized by a huge variability in composition and function between human subjects. Before the possibility to sequence microbiome genomes on large scales and at an affordable price, it was not possible to identify the presence of given bacterial enzymes and potentially related drug metabolism. Therefore, the influence of the microbiome on drug PK and medical response variability could not be easily proven. However, many studies have been reported the past 10 years to exemplify the involvement of specific gut bacteria on PK parameters of drugs such as digoxin, lovastatin, amiodarone, tamoxifen, acetylsalicylic acid,

or acetaminophen. Unfortunately, studies have been conducted mainly in rodents, with limited confirmation in human up to now (Džidić-Krivić et al., 2023). Pharmacomicrobiomics is a new field exploring the variability in drug response due to human microbiomes (Doestzada et al., 2018). The combination of an individual's genetic profiles with personal microbiome information will be a key driver toward stratification into human subpopulations (e.g. according to responder status or bioavailability status) and eventually a more accurate prediction of drug efficacy at the individual level (Steiner et al., 2022).

Changes in compound properties, new formulations (e.g. controlled-release formulation), as well as progress in gut microbiome knowledge and -omics technologies should stimulate the assessment of drug–microbiome interactions with the objective to improve drug candidate quality and individual response to treatment.

How Microbiome Tools Can Help Bring a Compound from Drug Discovery to Clinical Proof of Concept Studies

Drug Design, In Silico Databases, Predictive Models

Although some microbiome-encoded DMEs are well documented, reporting of drug–bacteria and drug–enzyme interactions is quite limited, and only a few databases, mentioned next, report data of suitable quality for in silico hypotheses and modeling.

A recent initiative collected 448 human DMEs, 599 microbial DMEs, the respective drugs known to be metabolized by these enzymes, and the interactions of DMEs with the microbiome, the host proteins, and the xenobiotics in a database of the Interactome of Drug-Metabolizing Enzymes (INTEDE) (Yin et al., 2021). The list of DMEs and their interactome is mainly based on a literature review combined with human DME tissue and disease-specific expression patterns collected from public data. The INTEDE initiative sheds light on the importance and complexity of how joint contribution of human and microbial DMEs can modify overall drug metabolism, how the microbiome interacts with DMEs and can thereby alter DME functionality, and finally how xenobiotics may modulate DMEs. A possible enhancement of INTEDE content to parallel human DME expression profile for microbial DMEs would be adding their presence/absence information deduced from publicly available metagenomics data from healthy and disease cohorts. The functional potential from microbial DMEs could be extrapolated from online microbial genome databases as well as publicly available metagenomics data, the latter of which would also allow stratification across healthy and disease cohorts according to presence of DMEs (Qin et al., 2010).

The MASI database is another literature-based initiative structuring the current microbiota-active-substance-disease knowledge (Zeng et al., 2021) counting 1051 pharmaceuticals and 406 dietary, herbal, probiotic, or environmental substances with 806 microbial species linked to 56 diseases (13,191 interactions). Even if both INTEDE and MASI are only focused on the currently approved drugs or drugs under clinical evaluation, they point out the importance of systematically collecting and organizing this type of knowledge in an integrated and structured manner. With the advent of large language models we can anticipate that the development of more comprehensive databases will undergo significant advancements.

Computational modeling of microbial communities with genome-scale reconstruction of their metabolic networks is recognized as a complementary approach to both in vitro and in vivo experiments. AGORA2 (Assembly of Gut Organisms through Reconstruction and Analysis, version 2) represents one of the latest initiatives in the realm of consortium modeling applications, specifically focused on predicting the transformation of known microbial drugs (Heinken et al., 2023). AGORA2 is particularly impressive due to its extensive coverage of the human microbiome, encompassing 7302 strains, 1738 species, and 27 phyla. Moreover, AGORA2 reconstructions are fully compatible with the human (Brunk et al., 2018) and whole-body metabolic models (Thiele et al., 2020) therefore enabling investigating host–microbiome co-metabolism in silico. To include the microbiome in the very early phase of in silico drug discovery, it is essential to 1) build a repository of compounds, 2) identify chemical conversions that modulate bioactivity, 3) couple this to drug-metabolizing enzymes that could facilitate such conversions, and 4) eventually couple this to microbial genome databases where more adequate gene annotation may help to link DME-encoding genes with chemical structures and putative microbial metabolites. It would allow the generation of hypotheses about the potential behavior of chemical moieties when they are subjected to given bacteria and their metabolizing enzymes. The scientific community's proactive efforts in this field are demonstrated through the recent publication of the SIMMER platform, which combines chemoinformatics and metagenomics approaches to predict bacterial species and enzymes capable of known biotransformation in the human gut (Bustion et al., 2023).

Machine learning models could be developed as soon as sufficient high-quality data become available and assist in the effective establishment of drug–bacteria interactions databases. Training such machine learning models has been exemplified to enable the prediction of drug–microbiome interactions for new compounds (McCoubrey et al., 2021a; McCoubrey et al., 2021b). From a set of 455 drugs susceptible to metabolism or bioaccumulation, the authors developed a model for predicting whether or not a new drug would be substantially metabolized or accumulated by intestinal microbiota. Similarly, a new data-supported prediction tool, named GutBug and freely available on the web, has been recently reported (Malwe et al., 2023).

The new database under development within the Microbiome Project of the Pistoia Alliance will bring up a larger and data-rich set for more finely tuned models to be matured through experimental validation cycles (https://www.pistoiaalliance.org/projects/current-projects/microbiome/). Defining specific data formats, standards, and repositories for microbiome drug/compound interaction (or more specifically metabolism) information could significantly improve future models and therefore predictions.

While computational PK–pharmacodynamics models are crucial for guiding drug development across vast chemical libraries, either based on superior disposition properties (e.g. solubility, high intestinal absorption, low clearance) or biological efficacy (e.g. substance functionalization for enhanced tissue or receptor affinity), the impact of microbiome-based in silico prediction models in the drug discovery trajectory remains to be assessed. With the ongoing enhancement of data quality, it will certainly be possible to add microbiome metabolism hypotheses to the in silico repertoire of compound properties and predictive parameters. As for any physicochemical or absorption, distribution, metabolism, excretion, toxicity (ADMET) parameters, significant gut metabolism alert would trigger the need for experimental screening to validate or not the prediction. These in silico models could be highly instrumental in upfront stratification of individuals based on bioavailability or responder status. Moreover, these models could be a crucial aid in risk prediction, for instance for accumulation of toxic phase I drug metabolites due to microbiome interference with first pass drug clearance or to more accurately predict fatal health risks from drug interactions as illustrated in the Okuda et al. (1997) study.

In Vitro Screening Assays and the Need for "Standardized" Assays

Despite the state-of-the-art multiomics approach over the past decade to characterize the human microbiome, in particular the intestinal microbiome, it remains complicated to precisely define what a "healthy" microbiome is (Shanahan et al., 2021). The reason is well known: interindividual variability in microbiota composition and functionality is driven by a plethora of determinants such as age, sex, ethnicity, diet,

medication, numerous other environmental factors, and even factors throughout life such as birth mode and childhood infections. Hence, it is extremely challenging to predict drug–microbiome interactions as the prescription of a drug is generally used to correct a disease, which may itself be accompanied by an alteration of the microbiome associated with the host. It would therefore also be necessary (theoretically) to define the state of the microbiota of those patients who are to receive a drug, which, depending on the disease, can be just as complicated as defining a healthy microbiota. Additionally, almost all microbiota models are based on stool samples reflecting only partially the digestive tract (Shalon et al., 2023).

In this context, it is difficult to recommend the use of specific, standardized in vitro models for testing the metabolism of drugs by gut microbiota. Solutions do exist, however, which can help to de-risk the development of a drug whose activity could be modified by the microbiota.

The first and most intuitive solution is to test drug modification by isolated bacteria in high-throughput 96-well plate configurations, as presented in several recent studies (Maier et al., 2018; Zimmermann et al., 2019a). In the future, it could be envisaged to set up standardized panels of strains covering a maximum of metabolic activities, as predicted by full genome sequencing and/or simple enzymatic tests. The panels of strains to be tested as a priority could then be chosen according to the chemical structure of the molecules under development and their greater or lesser probability of susceptibility to certain types of enzymes [bacterial beta-glucuronidases, for example (Candeliere et al., 2022)]. The conditions under which the tests are conducted (growth conditions, growth phase of the bacteria, drug vs. bacteria concentration ranges, etc.) should be standardized, which may be difficult for strict anaerobic bacteria that are likely difficult to cultivate. Moreover, the growth medium to which microbial cultures are subjected may very much determine whether or not a chemical compound is converted: this may depend on the microbe's intrinsic metabolic or co-metabolic potency, on available macronutrients (C- or N-source), and on specific co-factors for DMEs. Despite the increasing availability of gut microbial strains from culture collections (Poyet et al., 2019; Liu et al., 2021), this approach will probably remain limited by the difficulty, or even impossibility, to cultivate certain microbial species, independent of their abundance in the intestinal ecosystem (Yang et al., 2021).

The use of defined intestinal bacterial communities is an interesting and promising alternative, taking into account the possible cooperation of different species/strains in the drug metabolism as such. Yet, it also allows to maintain drug-metabolizing species/strains that rely on cross-feeding with certain co-factors from other endogenous gut microbes before they can be effectively cultivated. Several approaches can be used to generate simplified gut bacterial communities to which drugs are exposed (for a recent review of synthetic microbiota generated by different teams; see van Leeuwen et al., 2023). This may involve mixing microbial strains, which are initially grown individually, and incubating them together for a specific time to assess drug metabolism (Cheng et al., 2022). One can also try to co-cultivate strains in the same fermenter over several generations, thus creating a more or less complex consortium to which the drug candidate will be exposed (Blaustein et al., 2021; El Houari et al., 2022).

An increased level of complexity in microbial populations can be obtained by in vitro incubation of human-derived fecal microbiota samples, thus leading to the creation of a diversified microbiota to which the drug can be exposed (Aranda-Díaz et al., 2022). These methods have the advantage of generating relatively complex and stable microbiota, which can eventually be supplemented with other strains of interest carrying specific enzymatic activities and which are therefore likely to be standardized for recurrent testing. Yet, the extent to which interindividual variability in microbiota composition needs to be captured with high-throughput methods needs to be further investigated.

While waiting for more standardized models, Van De Steeg and colleagues recently reported an ex vivo fermentation screening platform in which human fecal microbiota pooled from several individuals were subjected to simulated colon conditions (van de Steeg et al., 2018). A set of 12 drugs (omeprazole, simvastatin, metronidazole, risperidone, sulfinpyrazone, sulindac, levodopa, dapsone, nizatidine, sulfasalazine, zonisamide, and acetaminophen) was incubated with human-derived microbiota under strictly anaerobic conditions. Composition of the human microbiota in the assay was representative of the microbiota found in the colon; the diversity of the microbial composition was maintained during incubations and was reproducible between experiments. Five out of the 12 included drugs (sulfasalazine, sulfinpyrazone, sulindac, nizatidine, and risperidone) showed microbiota-based biotransformation after 24 hours of incubation in the ex vivo fermentation assay, thus demonstrating that drug metabolites formed by microbial metabolism can be detected in a qualitative manner. These data were in accordance with the previously reported in vivo metabolism of the five drugs (see van de Steeg et al., 2018, and Table 1). Development of this in vitro system is ongoing toward a more quantitative prediction of drug disposition in human. Furthermore, this approach allows the use of microbiota samples coming from a single donor, opening the opportunity to evaluate inter- and intrapersonal variability to drug metabolism and therefore a primary step toward personalized medicine. By selecting a set of, e.g., 100 donors based on either microbiome diversity or phenotypic diversity (age, geography, lifestyle, diet), a microbiome predisposition score comparable to genetic predisposition analysis could be developed as a novel standardized assay.

As mentioned, standardized assays are not yet available, but screening assays, microbiome models, and reference compounds are described and could already be used during the drug discovery and development process. Their adoption, in combination with available in silico data as described earlier, should be seen as an opportunity to alert as early as possible on potential microbiome involvement in drug metabolism and its related consequences on efficacy, safety, and response to treatment variability (Fig. 1).

TABLE 1

In vitro metabolism of drugs using human fecal samples; confirmation in in vivo experiments

Drug	Metabolic reaction observed in vitro	Metabolic reaction observed in vivo: species	Reference
Sulfasalazine	Azo reduction	Rat and human	Peppercorn and Goldman, 1972
Sulfinpyrazone	Sulfoxide reduction (deoxygenation)	Rabbit and human	Strong et al., 1987
Sulindac	Sulfoxide reduction (deoxygenation)	Rabbit and human	Strong et al., 1987
Nizatidine	Nitrogen oxide reduction (deoxygenation)	Human	Basit et al., 2002
Risperidone	Isoxazole scission due to N-O bond reduction and subsequent hydrolysis of imine intermediate	Rat, dog, and human	Mannens et al., 1993

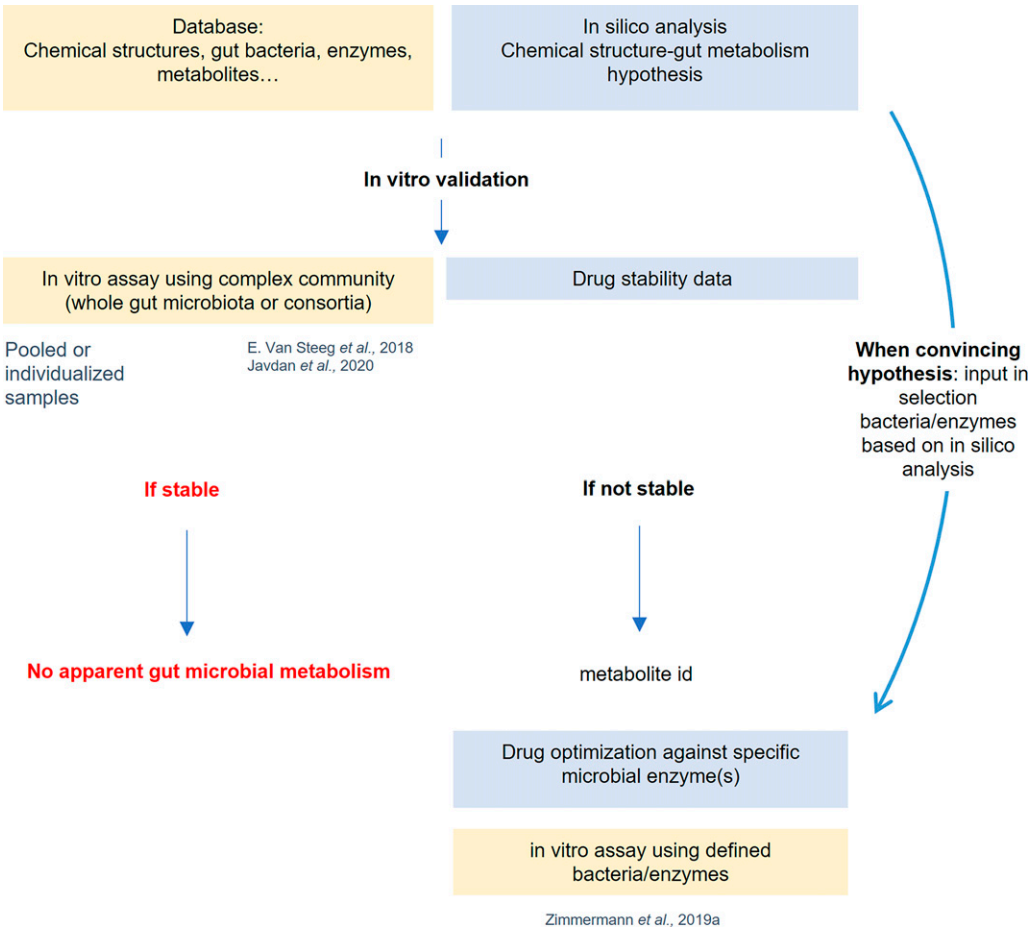

Fig. 1. Evaluation of gut microbiome metabolism in drug discovery.

In Vitro/Ex Vivo Models to Mimic the Human Gastrointestinal Tract

The ideal strategy for studying drug–microbiota interactions is to directly investigate these interactions inside the human body. However, clinical studies are hampered by ethical, regulatory, and budgetary constraints and are often limited to end-point measurements, making it more difficult to fully grasp the dynamics of drug metabolism and to identify the sites at which drug metabolism take place. After all, the composition of the fecal microbiota is fundamentally different from the other gastrointestinal compartments, both longitudinally from the oral cavity to the colon and cross-sectionally from the digestive lumen to the intestinal epithelium (Van den Abbeele et al., 2011). In vivo studies in mammals constitute a relevant alternative to human assays in the preclinical stage but with limitations mainly due to differences between human and animal diet and digestive physiology, obviously impacting gut microbiota composition and functionalities (Hugenholtz and de Vos, 2018). Furthermore, the 3R principle widely encourages the development of alternative in vitro approaches to reduce the number of animals used in scientific research. In vitro models of the digestive tract are therefore of utmost importance for human gut research.

Human in vitro gut models have been developed in a wide range going from simple static monocompartmental models to the most complex dynamic multicompartmental ones. Simple static models of the upper gastrointestinal tract reproduce one or several of the successive oral, gastric, and/or small intestinal phases of human digestion by changing pH conditions and adding appropriate digestive secretions in a single vessel maintained at body temperature (Minekus et al., 2014). Compared to static systems, dynamic models reproduce pH kinetics, variation in digestive secretions flow rate, and/or chyme transit in monocompartmental (Human Gastric Simulator, Dynamic Gastric Model) (Kong and Singh, 2010; Thuenemann et al., 2015) or multicompartmental model systems [Continuous Transfer Digestion Model, the Dynamic Digestion System, or the In Vitro Digestion System (Tompkins et al., 2011; Ménard et al., 2015), TNO Gastro-Intestinal Model (TIM)-1 (Minekus et al., 1995; Cordonnier et al., 2015; Denis et al., 2016)]. However, all these model systems only reproduce the physicochemical and enzymatic parameters of digestion and do not include microbiota.

The full implementation of gut microbiota is effectively integrated in several colon models, including the simplest ones, which are static batch culture systems (Roupar et al., 2021; Hernandez-Sanabria et al. 2020). Those models are inoculated with human-derived fecal microbiota and maintained under anaerobic conditions to simulate colonic fermentation but without any renewal of the nutritive medium. Such an approach is limited in time by substrate availability (24 to 72 h) and fluctuations in parameters like pH that are not regulated. In contrast, dynamic colonic models are based on the principle of semicontinuous or continuous fermentation and just like batch systems are inoculated with fecal samples. Such models are maintained under anaerobiosis and reproduce colonic temperature, pH, redox conditions, and transit time while providing the appropriate nutritional conditions mimicking human ileal effluents. This allows maintaining functional microbiota up to several weeks (or even several months with specific adaptations) without

microbial washout (Fehlbaum et al., 2015). The most characterized colonic in vitro models are probably the Reading Model (Gibson et al., 1988), the TIM-2 (Minekus et al., 1999), the Polyfermentor Intestinal Model (Zihler Berner et al., 2013), and the artificial colon (Cordonnier et al., 2015). Only a few models such as the well-known Simulator of Human Intestinal Microbial Ecosystem (SHIME) (Molly et al., 1993; Van de Wiele et al., 2015) or the SIMulator of the Gastro-Intestinal tract (Barroso et al., 2015) are including the entire gastrointestinal tract, from the stomach to the colon. Several configurations of the colonic compartments include the use of three-stage bioreactors in series to mimic the different sections of the human colon (Gibson et al., 1988; Cinquin et al., 2006; Van de Wiele et al., 2015) or the addition of mucin beads to distinguish luminal from mucosal colonic environments and their associated microbiota (Van den Abbeele et al., 2013; Deschamps et al., 2020; Roussel et al., 2020).

Up to now, most of the studies involving in vitro human gut models and pharma compounds have been performed in upper gastrointestinal systems devoid of resident microbiota. The main objectives were to assess the impact of oral formulation (Dickinson et al., 2012), digestive physicochemical parameters such as bile secretion (Pentafragka et al., 2022), or fasted versus fed status (Verwei et al., 2016) on the kinetics of release, dissolution, and bioaccessibility of drugs in the digestive lumen.

Concerning the integration of gut microbiota in human gut models for studying drug–microbiome interactions, only a handful of studies have been conducted, ranging from simple batch models to complex dynamic ones like the SHIME. The most frequently studied active substances are antibiotics (Verdier et al., 2021; Calatayud et al., 2022; Endika et al., 2023), followed by chemotherapeutics (Vanlancker et al., 2017), with the authors commonly investigating the effects by drugs on gut microbiota composition, diversity, and metabolic activities. After antibiotic disturbances of the microbiota, some strategies aiming to restore microbial equilibrium have also been tested such as a probiotic mixture of 8 bacterial strains upon clindamycin treatment in the TIM-2 (Rehman et al., 2012), a blend of 9 probiotic organisms plus 10 digestive enzymes after administration of 5-fluorouracil and vancomycin as microbiome-disrupting drugs in the SHIME (Ichim et al., 2018), or autologous fecal microbiota transplantation after ciprofloxacin treatment in the artificial colon (Verdier et al., 2021). Similar to the upper gut models, most of the studies in the colon models were performed under healthy conditions, i.e. using fecal microbiota from healthy volunteers. However, Maccaferri and colleagues (Maccaferri et al., 2010) evaluated the impact of rifaximin, a rifamycin derivative used in the treatment of Crohn's disease, on gut microbiota structure and microbial metabolic profiles using the MacFarlane and Gibson system inoculated with fecal samples from patients affected by colonic active disease. Interestingly, Van de Wiele's team further assessed whether colon microbiota modulated the anti-inflammatory efficacy of celecoxib, used in the treatment of chronic inflammatory disorders, through the coupling of a simplified epithelial inflammation model with in vitro batch incubation of fecal microbiota (Hernandez-Sanabria et al., 2020). Lastly, using a simplified but high-throughput 96-deep well plate culture of human gut microbiota, it was shown by metaproteomic analysis that gut microbiota growth curves, taxonomy, and functional responses were different depending on whether metformin was added during the lag, exponential, or stationary phase. These results suggest that the timing for drug stimulation should be considered when studying drug–microbiome interactions (Hao et al., 2020).

As described earlier, compared to their wide potential, the use of in vitro gut models to assess the bidirectional interactions between gut microbiome and pharma compounds still remains in its infancy. Such in vitro approaches need to enable a high level of experimental control and reproducibility, excluding confounding environmental or dietary factors, and therefore enable in-depth mechanistic studies on drug–microbiota interactions. The selection of an in vitro model primarily depends on the research question to be answered, the targeted gastrointestinal compartments, and the number of tested compounds. Dynamic and multicompartmental models enable a multiparametric control that allows for mechanistic investigations, with in vitro conditions closely related to the in vivo situation. However, these models are not always best suited for screening studies, since they are labor intensive and time consuming, hence allowing for only a limited number of independent repetitions. While most of current studies focus on the effect of active pharma substances on microbiota composition and function, inversely in vitro gut models can be relevant to investigate drug metabolism or degradation by gut microbes. Importantly, current in vitro studies only assessed drug interactions with fecal or colonic-oriented fecal microbes whereas most of available drugs are absorbed in the upper human gut (Murakami, 2017). It would therefore be highly interesting to further develop in vitro small intestinal models to assess drug–microbiome interactions with specific human small intestine microbes (Cieplak et al., 2018; Calatayud et al., 2019; Stolaki et al. 2019; Deyaert et al., 2023; Malik et al., 2023). Lastly, in vitro gut models can advantageously integrate many factors responsible for interindividual variabilities in drug absorption or metabolism, such as age, diet, variation in microbiome composition, and diversity between individuals.

In Vivo Models: How Do We Select the Most Relevant Animal Models—Translation Between Species?

The multimodality of drug–microbiome interactions makes it necessary, during the research and development process of a drug, to use animal models, which combine gut microbiota and DME in a comprehensive physiological environment. Indeed, to date, no alternative system has proven capable of recapitulating the complexity of a living organism.

The choice of animal models depends on the results of the previous stages—in silico, in vitro, and ex vivo—of the research and development process. Indeed, the objective of the in vivo stage is to validate the results of the previous stages, to provide additional information, and to detect unexpected deleterious effects that would lead to the reinitiation of in silico, in vitro, and ex vivo studies in order to reduce identified risks. A large panel of animal models does exist (Supplemental Table 1), and strengths and drawbacks of the main ones are highlighted in this section. A suitable combination of these different models should allow for successful coupling between the early R&D stages and the in vivo phase and increase the chances of translating the results into the clinical phase (Zimmermann-Kogadeeva et al., 2020; Dodd and Cann, 2022; Liu et al., 2022; Moossavi et al., 2022).

Academic studies on microbiota–drug interactions have mainly used rodents carrying their natural microbiota (conventional rodents), with a specific pathogen free (SPF) sanitary status. Their comparison with germ-free rodents (without microbiota) helps to highlight the possible role of the microbiota in the metabolic fate of the investigated drug (Peppercorn and Goldman, 1972; Brandi et al., 2006; Selwyn et al., 2015a; Selwyn, 2015b; Zimmermann et al., 2019b). These SPF models have several limitations: composition of the microbiota varies according to environmental factors, including the supplier and the animal facility where the studies are carried out, and the SPF condition, while preventing interference with pathogenic microorganisms, leads to a reduction of the microbiota diversity, which may compromise the maturation and physiological reactivity of the animal (Ericsson et al., 2015, 2018; Rausch et al., 2016; Wolff et al., 2020; Long et al., 2021). It may then be preferable to use rodents carrying a wild rodent microbiota (Rosshart et al., 2019).

Other studies use germ-free rodents that are colonized with one or several bacteria whose genome encodes enzymatic activities that may play a role in the metabolism of the investigated drug, based on preliminary in silico or in vitro studies. These models are a remarkable tool to verify and characterize this role in vivo (Humblot et al., 2007; Haiser et al., 2013, 2014; Zimmermann et al., 2019b). More specifically, they enable dissection of microbiota and host contributions to the drug metabolism and building of pharmacokinetic models to quantitatively predict the microbiota contribution to systemic drug and metabolite exposure (Zimmermann et al., 2019b). However, these models have the disadvantage of an extremely simplified microbiota and, hence, of an incomplete physiological maturation and reactivity. Therefore, the results need to be supplemented by experiments with animal models carrying a complete gut microbiota to quantitatively verify the contribution of the microbiota under actual physiological conditions.

In recent years, research on the gut microbiota has increasingly made use of animal species other than rodents. Indeed, they can sometimes be advantageously replaced by invertebrates (e.g. nematodes, insects) or non-mammalian vertebrates (e.g. fish), whose physiology and host–microbiota relationships are, in some aspects, common with mammals. As they can be used in large numbers and are less expensive than rodents, they are well suited for screening similar to those carried out in vitro (Erkosar et al., 2013; Dirksen et al., 2016; García-González et al., 2017; Scott et al., 2017; Catron et al., 2019; Douglas, 2019; Ericsson, 2019; Kumar et al., 2020; Ludington and Ja, 2020; Poupet et al., 2020; Lu et al., 2021; Radeke and Herman, 2021; Matthewman et al., 2023). As with any result obtained from a simple model, it must then be confirmed in a complex system with close similarity to humans to enable extrapolation. In this context, one should not forget the large animal models such as pigs, often dismissed for cost reasons yet interesting thanks to their physiological proximity to humans and the possibility of simulating human pathologies (Zhang et al., 2013; Clayton et al., 2018; Coelho et al., 2018; Vlasova et al., 2018; Ericsson, 2019; Matthewman et al., 2023).

Whatever the animal species used, the natural microbiota can be replaced by a microbiota of human origin. These "humanized" models have been widely used in academic gut microbiome research for several years (Arrieta et al., 2016). They have the great advantage of considering the specific composition and functions of the human microbiota, making the results more easily transferable to humans (Arrieta et al., 2016). They also have the advantage of allowing the study of unbalanced microbiota associated with pathological situations (Aguanno et al., 2022). However, in addition to the fact that they cannot reproduce the original ecosystem with a high fidelity due to the fact that the genetic, immune, physiological, and dietary environments of the recipient animals are different from that of humans (Arrieta et al., 2016), they suffer from the absence of a standardized and widely accepted fecal microbiota transfer protocol. Should we consider a single or multiple human donors? Under what conditions should human fecal samples be stored to optimize the engraftment of the microbiota in the recipient animals? Is it preferable to use germ-free or conventional recipient animals (after depletion of the original microbiota)? What should the practical modalities of inoculation of the recipient animals be, and how much time is necessary for the stabilization of the transferred microbiota? All these variations still make it challenging to integrate this type of model into the study and development of a drug (Wos-Oxley et al., 2012; Kennedy et al., 2018; Le Roy et al., 2019; Burz et al., 2019; Lundberg, 2019; Berland et al., 2021; Bokoliya et al., 2021; Gheorghe et al., 2021; Gopalakrishnan et al., 2021).

New models could overcome the drawbacks of this lack of standardization. These are models with simplified microbiota, whose composition is known, reproducible, and designed to simulate the functions of a complex microbiota and, consequently, host–microbiota interactions. The development of this type of model initially focused on the murine microbiota, leading for example to the "Schaedler flora," a consortium of eight bacterial strains that gives the animals physiological characteristics close to those of conventional animals (Wannemuehler et al., 2014; Wymore Brand et al., 2015). This microbiota can then be supplemented by one or several other strains with specific functions (Lobel et al., 2020). Since then, several groups have developed more complex consortia, of murine or human origin, with the objective of reconstituting as faithfully as possible the functions of a native microbiota, while guaranteeing reproducibility of the microbiota over time and between laboratories (Rezzonico et al., 2011; Slezak et al., 2013, 2014; Uchimura et al., 2016; Eberl et al., 2020; Darnaud et al., 2021). Active research in this field should allow a continuous improvement of these models.

Overall, many animal models are available to study microbiota–drug interactions. While academic research currently uses a wide range of animal models to study microbiota–host interactions, most of the few studies on microbiota–drug interactions focus on conventional and germ-free rodents. Extending the use of other models, such as human and/or simplified microbiota models, would broaden the knowledge of microbiota–drug interactions and refine the drug research and development process.

Specific Clinical Requirements If the Gut Microbiome Is Integrated in the Clinical Development of Small Molecules

Today, when performing traditional ADMET studies for drug candidates, it is not yet common to actively assess the impact of gut microbiome composition/functions on the PK of the drug candidate (Hitchings and Kelly, 2019). However, it is now clear that differences in microbiome composition and function between healthy individuals and patients or even interpatient can explain variability in a PK profile (Cai et al., 2023). To document how the microbiome is responsible or not for the variation in a PK profile, it is important to measure these differences in microbiome composition/function.

Integration of gut microbiota in ADMET studies and in a more general way in the clinical development of any drug candidate would require integrating the specificities linked to microbiome sampling and analysis in the design of these clinical studies. Generating reliable microbiome data in clinical studies depends on three key factors:

- Technical aspects should be considered as early as clinical trial design in order to ensure that appropriate sampling, sample preservation, and storage occurs prior to analysis.
- Microbiome composition/function variability at the intraindividual level as well as within a given population and between healthy volunteers and patients may impact the size or characteristics of the population (definition of inclusion/exclusion criteria), as well as the sampling strategy within a given study.
- How data from microbiome studies is reported with respect to interpretation and comparability must be considered.

Technological Considerations. Integration of microbiome composition and/or function analysis in the clinical development of drugs will impact sampling and analytical method selection.

The type of sample, collection timepoints, collection device, collection procedure, sample quantity and quality, and storage conditions will depend on the question to be answered, the final destination of the samples, and the analytical method to be applied (e.g. metagenomics, metabolomics) (Vandeputte et al., 2017; Zubeldia-Varela et al., 2020). For example, in the case of analysis of the gut microbiota composition via next-generation sequencing methods, particular attention should be paid to the preservation of the DNA of the samples by direct freezing of the samples or the use of DNA stabilizers. If the objective is to

analyze the metabolites produced by the microbiome (both metabolites produced during fermentation but also metabolites generated from drugs), consideration should be made for the preservation of volatile metabolites and the avoidance of certain stabilizers. The potential for interference between sampling conditions and stabilizers should be discussed with the analytical lab.

The storage and shipping conditions are also key factors to consider. Depending on the study protocol and health status of the subject, the sampling can be performed at home. In this case, a feces collection kit must be provided for the patient to take home, and the collection instructions must be clearly explained by the clinicians. A detailed and precise storage procedure must also be provided to the patient, and a request must be made to the patient to document any deviation from the procedure provided.

Sampling Strategy and Population. Microbiome composition varies between individuals within healthy or patient populations affected by different diseases. Understanding how these variations in gut microbiome composition/functions may affect the PK of a given drug candidate could provide a first set of important data. However, it is important to understand that the suitability and predictability of data obtained in healthy individuals must be carefully analyzed before extrapolating it into the targeted population of the drug candidate. Disease and medical conditions are associated with modifications of the microbiome composition and/or function, which can lead to different levels of absorption, distribution, metabolism, or toxicity within a patient population when compared to healthy individuals. In addition, microbiome characteristics may also evolve along the course of a pathology. As such, collection timepoint(s) have to be adapted to the status of the patients, their transit time, any co-medication (e.g. acute or chronic treatment), and the particular phase of the pathology as well as the PK of the drug candidate. The definition of the collection timepoint(s) could be supported by preclinical data such as that obtained in artificial gastro-intestinal models (e.g. TIM or the SHIME) (see In Vitro/Ex Vivo Models to Mimic the Human Gastrointestinal Tract section) that can reproduce a patient's microbiome composition.

It is also important that the "metadata" for each sample (i.e. sampling conditions and subject characteristics) are accurately recorded. Given that the gut microbiota composition/function can be modified by numerous factors, it is important to thoroughly characterize the patient population included in the study and to document patient level metadata/potential confounding factors in order to avoid bias in data interpretation. These metadata can be, for example, diet, disease stage, comorbidities, concomitant drugs (before/during the study), physical activity, living place, genetic background, and others.

As there is an intraindividual variation of the microbiota, serial sample collection is recommended for each sampling timepoint (Johnson et al., 2020; Vandeputte et al., 2021).

- Baseline: at least three serial sample collections
- First week of treatment: sampling every day
- Second and third week of treatment: sampling every 2 to 3 days
- Then, depending on the duration of the treatment and duration of the study, sampling once a week
- End of the treatment: at least three serial sample collections

Clinical Study Reporting. The final point to consider is the reporting of clinical studies analyzing the microbiome. Too often the reporting is poor and limited, such that data are not reproducible and thereby prevent any further use in metadata or other types of reanalysis. A consortium has recently published the STORMS checklist: Strengthening The Organization and Reporting of Microbiome Studies (Mirzayi et al., 2021). This checklist for reporting on human microbiome studies, organized into six sections covering all sections of a scientific publication, propose the minimum information to report in order to facilitate manuscript preparation, peer review, and reader comprehension of publications but also comparative analysis of published results. A clear and complete description of the analytical procedure and bioinformatics pipelines used to generate the data should be included in the publication.

How Can Integration of the Microbiome Improve In Vitro to In Vivo Extrapolation and Capture the Interindividual Variability in Response and Toxicity?

Despite the extensive occurrence of microbiome-derived metabolism for many drugs in in vitro conditions, a lingering question remains regarding its significance and predictability in representing a crucial aspect of drug clearance in vivo. It is essential to acknowledge that the development and standardization of in vitro liver tools, along with their integration into predictive physiologically based pharmacokinetic (PBPK) models, have been time-consuming processes spanning many years. While the results are deemed acceptable for interspecies comparisons and drug–drug interactions, there is a recurring issue of underpredicting in vivo clearance when using liver tools alone, requiring the incorporation of intestinal metabolism and transporter-mediated uptake for specific drugs.

This paper endeavors to demonstrate that tools and models capable of predicting microbiome-derived metabolism are reaching a level of maturity that allow them to complement the existing framework. More significantly, these advancements aim to draw attention to specific cases where the microbiome plays a pivotal role in the efficacy and toxicity of drugs.

PBPK models are PK models based on physiological processes. Today, in silico predictions and PBPK modeling are used as a translational approach throughout drug discovery, hit-to-lead generation, preclinical development, and in clinical studies. These models integrate in silico, in vitro, and in vivo animal data to predict the PK parameters of a chemical (and its metabolites) within the body over time for animals and humans (Marshall et al., 2016).

PBPK models can therefore be a key element in the integration of microbiome data in the drug development process. They could be used to organize current knowledge on how the microbiome impacts the disposition of certain "model" drugs, to predict the effect of the the microbiome on PK parameters of drug candidates.

PBPK models have a particular value when animal models are less robust and help to bridge from in silico/in vitro human data to the potential impacts of drug microbiome interactions in humans. Once these models are built, they allow for introduction of interindividual variabilities and prediction of the behavior of a drug in infants and the elderly but also model disease situations.

Specific PBPK drug–microbiome models are available for key gut metabolic reactions with a good prediction of the in vivo pharmacokinetic behavior of the drug tested. It has been done, in a very pragmatic way, in the case of the azo bond reduction of sulfasalazine, with a simple inclusion of a poor absorption scalar of 0.001 to represent the extensive metabolism of the drug by the anaerobic bacteria present in the colon (De Sousa Mendes et al., 2018). For other reductases involved in the gut metabolism of levodopa, budesomide, nitrendipine, nimodipine, rivaroxaban, and sulfasalazine, a PK model using in vitro human microbiome data has been used to predict if activity in the proximal colon or in the distal ileum is likely to be clinically important for an oral form of these drugs (Vertzoni et al., 2018).

In the case of the sulfoxide reduction of sulindac and sulfinpyrazone, a PBPK model including degradation half-life obtained in fecal incubations was used to calculate an intrinsic colon clearance, which was used

as a model input parameter to facilitate its conversion to sulfide metabolite (Kostantini et al., 2022). For clonazepam, more complex reactions have been included covering nitro reduction followed by oxidation, glucuronidation, and enterohepatic cycling. This is true also for hydrolytic reactions in the case of the two antiviral drugs sorivudine and brivudine. Prediction of the microbiome contribution versus the liver in the mouse (conventional and germ free) has been realized using both in vitro incubations and in vivo systemic exposure data (Zimmermann-Kogadeeva et al., 2020).

Hydrolytic, glucurono-conjugations, enterohepatic circulation, and β-glucuronidase reactions have been modeled in the case of irinotecan with a two-compartment PBPK model, encompassing the liver and the gut. Gastrointestinal microbial β-glucuronidase activity has been included to predict the increased intestinal exposure to the active/cytotoxic metabolite SN-38 but not its systemic exposure (Tao et al., 2022).

Finally, in the case of daidzein, small and large intestine compartments, scaling of the kinetic Vmax parameters obtained in vitro with human fecal incubations, and liver S9 were included to predict the in vivo behavior of the drug, covering a range of metabolic reactions such as β-glucosidase, reductase, and racemase (Wang et al., 2022).

These examples show that the link between in silico/in vitro microbiome metabolization data and the in vivo profile of a drug can potentially be made early, to understand how the gut microbiome might impact the interindividual variability. Employing in silico/in vitro microbiome tools during the early phase of drug discovery can be important for colon targeting programs or BCS class III and IV drugs for which colonic absorption and therefore exposure to the microbiome will be relevant. Additionally, considering that animal models are only partially predictive of humans, PBPK modeling approaches are key tools to reduce the use of animals in drug development (Wadman, 2023).

Beyond these more specific drug microbiome models, "classical" whole-body PBPK models have been published for at least 50 drugs, including a number with an advanced compartment absorption and transit model (Thiele et al., 2017). The advanced compartment absorption and transit model considers nine gastrointestinal compartments: the stomach, seven small intestinal segments, and the large intestine. It represents pH-dependent drug solubility, controlled release, drug absorption by the stomach and colon, metabolism in the gut or liver, degradation in the lumen, changes in absorption surface area, changes in drug transporter densities, and changes in efflux transporter densities. All of these can be used to better predict PK behaviors of drugs in humans and even lead to generic PBPK drug–microbiome approaches if the in vitro part can be standardized and included on a systematic basis.

Quantitative Systems Pharmacology

When PBPK models are combined with a compound's effects (pharmacodynamics), one can evaluate the impact of the variability of the PK on drug response. It is possible also to include cellular PBPK in these models, but it is more difficult to account for microbial metabolism and dietary information. Quantitative systems pharmacology, an emerging field of modeling technologies that describes the dynamic interaction between biological systems and drugs, is supposed to encompass all these aspects (Helmlinger et al., 2019). A combined model of small intestinal metabolism and levodopa PK has been realized and can predict the influence of dietary amino acids on the bioavailability of levodopa in Parkinson's disease patients (Guebila and Thiele, 2016).

First Human Data to Validate a Drug–Microbiome PBPK Model

Once drug–microbiome interactions have been identified in drug discovery and/or preclinical studies and a PBPK approach helps to predict the potential impact in humans, it will require human data to validate the hypothesis before entering into further development phases.

Classical human data phase 1 dose escalation studies could be used to generate the first microbiome data in humans. However, the most appropriate study would be the ^{14}C radiolabeled drug metabolism study. As a recent example, a radiolabeled human ADME study of ozanimod, a new treatment for relapsing forms of multiple sclerosis, allowed the extensive characterization of the metabolism of this drug, with the identification of three main metabolic pathways, including a reductive anaerobic metabolism mediated by the gut microbiota and responsible for a predominant portion of the excreted dose via urine and feces (Surapaneni et al., 2021). This radiolabeled study highlighted the importance of the gut microbiota in the metabolic profile of a recently approved drug.

^{14}C radiolabeled studies can combine identification of metabolites in plasma, urine, and feces; microbiome profiling of the subject and the data can then be used to validate a PBPK model based on in vitro and/or in vivo animal data.

Historically these studies were performed during phase 1 clinical trials and are now progressively conducted later in the development after the single ascending dose and multiple ascending dose studies. However, due to advancements in detection methods' sensitivity,[14] C-microtracer studies [dose of ≤ 1 μCi (37 kBq) of radioactivity] have the potential to shift the human ADME study to earlier in clinical development. This can facilitate the generation of crucial initial human balance and metabolism data, a recommendation made by pharmaceutical companies (Young et al., 2023). Such studies would be particularly valuable for validating the impact of microbiome transformation on the drug of interest (see Fig. 2).

Conclusion, Perspective, and Working Group Recommendations

This perspective paper prepared by the Medicen working group highlights that the human microbiome can have major impacts on the safety, efficacy, and response variability of a large panel of drugs. This becomes particularly relevant for BCS class III and IV compounds with low permeability and/or solubility and beyond the rule of 5 compounds, all demonstrating increased exposure to the colonic microbiome due to limited absorption in the upper gastointestinal tract. Over recent years, tools have been developed to answer microbiome-related questions throughout the drug discovery and development process. Whether these tools are mature and robust enough to be fully integrated in the drug discovery and development process of a new chemical entity remains to be further elucidated. Today gut microbial metabolism is often neglected in the analysis of human drug metabolism. However, the recent example of ozanimod highlights the importance of understanding drug metabolism early in the drug development process to avoid delays in registration. In this case, microbial reductive pathways followed by anaerobic decarboxylation accounted for a significant part of ozanimod's metabolism and disposition, which could have been identified potentially through in vitro microbiome stability studies earlier during the drug discovery or development process.

To support drug discovery, the working group recommends the use of one of the available databases integrating gut bacteria, bacterial enzymes, and substrates/products chemical structures that have been recently published or are under development. They can help already to identify chemical moieties susceptible to microbial metabolism by gut commensals and therefore to foster an adapted development plan through available in vitro and in vivo assays. This in silico evaluation could be done as early as the hit-to-lead stage and integrated in the derisking strategy of lead compound selection. As examples of the highest priority, we could nominate compounds bearing similar chemical moiety to known drugs metabolized by gut bacterial enzymes, compounds susceptible to reductive or hydrolytic reaction, compounds with predicted poor oral bioavailability, or compounds designed to target the large intestine. They would all be highly exposed to the distal gut microbiota.

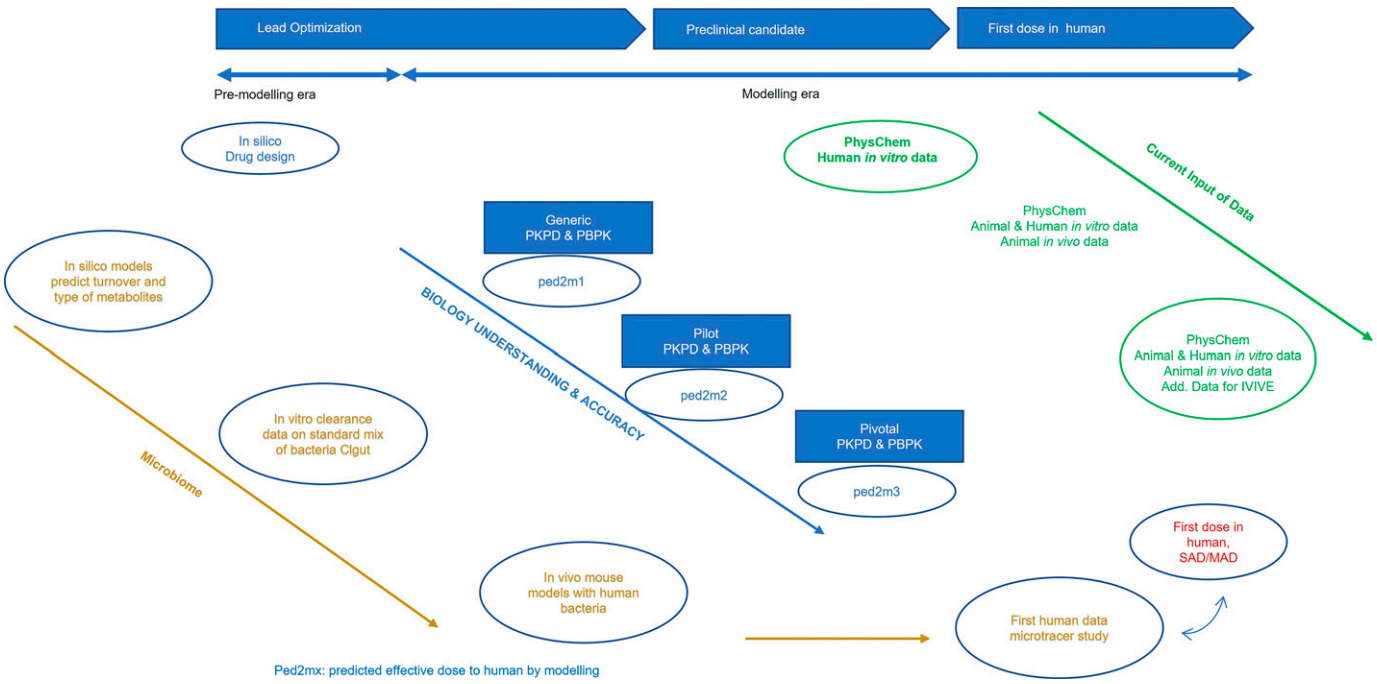

Fig. 2. Microbiome integration in preclinical and clinical modeling listing the type of approaches that could be used to evaluate the impact of the microbiome on a drug product; integration in the classical in vitro, physico-chemical, and modelling strategies that are in place in the pharmaceutical industries.

As discussed in this review, in vitro standardized assays are not yet available, but screening assays using either a specific strain, a defined consortium of bacteria, or a stable complex microbiome are described and could already be used to alert early on the potential involvement of the gut microbiome to metabolize a given drug (candidate). Particularly, experimental in vitro validation of in silico gut metabolism hypothesis would be a necessary step before moving to complex assays. Negative outcomes in such quite straightforward assays would minimize the risk of a costly late identification of an unforeseen metabolic pathway.

Successful integration of in vitro microbiome data into PBPK models to predict the in vivo behavior of a drug candidate has been realized for various reductive, hydrolytic, and oxidative pathways, including compounds undergoing enterohepatic circulation. This opens the way for generic models adaptable to drugs with similar pathways and potential reference substrates to calibrate in silico, in vitro, and in vivo animal models.

In case of a positive outcome in in vitro screening assays, or to evaluate compounds showing peculiar PK profiles, selected advanced drug candidates could be evaluated in human in vitro gut models and in animal in vivo models, humanized or not, to assess in a more physiological context the fate of the compound and its interactions with the gut microbiome and the host. As for in vitro screening assays, a whole microbiome or a model microbiome could be used.

Finally, the possibility to use fecal samples for microbiome assessment, either from healthy volunteers or from patients, would support patient stratification and sampling strategy in clinical studies. The recent 5-ASA example, showing a link between the presence of metabolizing enzymes that inactivate the drug and failure to treatment for UC patients, illustrates a critical role played by the microbiome and its metabolizing activity and suggests possible ways to a more efficient and personalized medicine (Mehta et al., 2023).

Ultimately, a full integration of studies evaluating the gut-mediated effects in drug discovery and development will require access to high-quality databases and related prediction tools, as well as the implementation of microbiome standards and reference compounds to develop standardized reproducible assays. Without waiting for them, we encourage researchers to use available tools and experiments to evaluate potential gut microbiota involvement in drug metabolism and safety as a real opportunity for better drug identification and individual treatment.

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Data Availability

The authors declare that all the data supporting the findings of this study are contained within the paper.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Jimonet, Druart, Blanquet-Diot, Boucinha, Kourula, Le Vacon, Maubant, Rabot, Van de Wiele, Schuren, Thomas, Walther, Zimmermann.

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Address correspondence to: Patrick Jimonet, Medicen Paris Région, 130 rue de Lourmel, 75015 Paris, France. E-mail: pjimonet@medicen.org