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Novel Approaches to Characterize Individual Drug Metabolism and Advance Precision Medicine

Klarissa D. Jackson, Brahim Achour, Jonghwa Lee, Raeanne M. Geffert, Jessica L. Beers, and Bethany D. Latham

Division of Pharmacotherapy and Experimental Therapeutics, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina (K.D.J., J.L., R.M.G., J.L.B., B.D.L.); and Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, Rhode Island (B.A.)

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ABSTRACT

Interindividual variability in drug metabolism can significantly affect drug concentrations in the body and subsequent drug response. Understanding an individual's drug metabolism capacity is important for predicting drug exposure and developing precision medicine strategies. The goal of precision medicine is to individualize drug treatment for patients to maximize efficacy and minimize drug toxicity. While advances in pharmacogenomics have improved our understanding of how genetic variations in drug-metabolizing enzymes (DMEs) affect drug response, nongenetic factors are also known to influence drug metabolism phenotypes. This minireview discusses approaches beyond pharmacogenetic testing to phenotype DMEs—particularly the cytochrome P450 enzymes—in clinical settings. Several phenotyping approaches have been proposed: traditional approaches include phenotyping with exogenous probe substrates and the use of endogenous biomarkers; newer approaches include evaluating circulating noncoding RNAs and liquid biopsy-derived markers relevant to DME expression and function. The goals of this minireview are to 1) provide a high-level overview of traditional and novel approaches to phenotype individual drug metabolism capacity, 2) describe how these approaches are being applied or can be applied to pharmacokinetic studies, and 3) discuss perspectives on future opportunities to advance precision medicine in diverse populations.

SIGNIFICANCE STATEMENT

This minireview provides an overview of recent advances in approaches to characterize individual drug metabolism phenotypes in clinical settings. It highlights the integration of existing pharmacokinetic biomarkers with novel approaches; also discussed are current challenges and existing knowledge gaps. The article concludes with perspectives on the future deployment of a liquid biopsy-informed physiologically based pharmacokinetic strategy for patient characterization and precision dosing.

Introduction

Interindividual variability in drug metabolism can significantly affect drug exposure and response (Zanger and Schwab, 2013). Over the past two decades, advances in pharmacogenomics have improved our understanding of how variations in genes that encode drug-metabolizing enzymes (DMEs), particularly the cytochrome P450 (CYP) enzymes, affect drug response (Tomalik-Scharte et al., 2008; Zanger et al., 2008; Zanger and Schwab, 2013). However, pharmacogenetics does not explain all the interindividual variation in drug metabolism phenotypes. For example, variation in CYP3A4 expression and activity is poorly described by germline pharmacogenetic testing (Klein and Zanger, 2013). In addition to genetic factors, nongenetic factors (e.g., age, ethnicity, epigenetics, drug interactions, lifestyle, diet, disease, etc.) can also impact P450-mediated drug metabolism (Klein and Zanger, 2013; Zanger and Schwab, 2013). Approaches to predict an individual's drug metabolism phenotype have been recognized as key components of precision medicine—the goal of which is to individualize drug treatment for patients to maximize efficacy and minimize drug toxicity (Gonzalez et al., 2017).

Several nongenetic factors are known to affect DME activity (Fig. 1) (Huang and Temple, 2008; Klein and Zanger, 2013; Zanger and Schwab, 2013). Variations in transcriptional regulation (by xenobiotic-sensing nuclear receptors) and epigenetic post-transcriptional regulation [by noncoding RNAs (ncRNAs) and DNA methylation] can influence DME expression (Ivanov et al., 2012; Kacevska et al., 2012; Klein and Zanger, 2013; Zhong and Leeder, 2013; Zanger et al., 2014). Coadministered drugs and dietary supplements can also increase or decrease DME activity (via induction or inhibition, respectively), which may enhance the risk for serious adverse drug reactions or treatment failure (Wilkinson, 2005). Moreover, disease states (e.g., nonalcoholic fatty liver disease and obesity)

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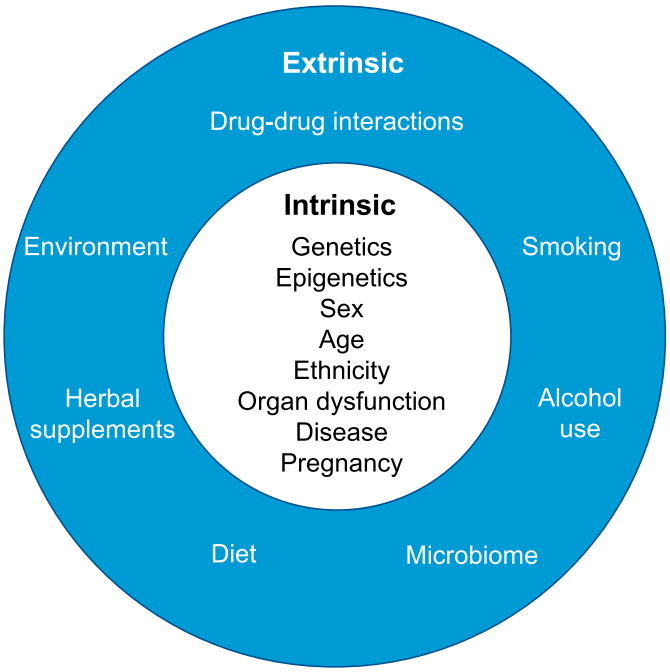

Fig. 1. Intrinsic and extrinsic factors that affect drug metabolism and pharmacokinetics. Figure adapted with permission from Huang and Temple (2008). ©2008 American Society for Clinical Pharmacology and Therapeutics.

can also alter DME activity (Woolsey et al., 2015). Given the myriad of intrinsic and extrinsic factors that can affect drug metabolism (Fig. 1) (Huang and Temple, 2008), clinically implementable phenotypic biomarkers are needed to accurately assess individual drug metabolism capacity across ethnically diverse populations (Tracy et al., 2016). This minireview focuses on approaches beyond pharmacogenetic testing that aim to determine drug metabolism phenotype in clinical settings.

To predict drug exposure and optimize drug dosing, it is important to understand an individual's drug metabolism capacity. Several approaches have been proposed to characterize individual drug metabolism, including phenotyping with exogenous probe substrates and endogenous biomarkers as well as more recent approaches evaluating circulating ncRNAs and liquid biopsy-derived markers relevant to DME expression and function. This minireview 1) provides a high-level overview of traditional and novel approaches to phenotype individual drug metabolism capacity, 2) describes how these approaches are being applied or can be applied to pharmacokinetic (PK) studies, and 3) discusses perspectives on future opportunities to advance precision medicine in diverse populations. Traditional and novel approaches to phenotype DMEs in clinical settings are discussed with a particular focus on cytochromes P450 (see Fig. 2). The major areas discussed include:

1. Traditional approaches (exogenous probe substrates, therapeutic drug monitoring)
2. Endogenous biomarkers
3. Circulating ncRNAs
4. Liquid biopsy

To provide a historical perspective, each section begins with a background of the field, starting with traditional approaches.

Traditional Approaches: Exogenous Probe Substrates and Therapeutic Drug-Monitoring

Clinical Phenotyping Drug-Metabolizing Enzymes. Pharmacogenetic testing has been used clinically for a few DMEs to predict a patient's drug-metabolizing phenotype prior to prescribing a medication (Pinto and Dolan, 2011). Information regarding a patient's drug-metabolizing phenotype is relevant when a medication has a narrow therapeutic index and is metabolized by an enzyme that is highly polymorphic and/or displays high variability in activity. Interindividual variability of cytochrome P450 expression and activity is very common. For example, while CYP3A4 is the most abundant P450 in adult human liver and intestine (Guengerich, 1999), greater than 30- to 100-fold variation has been observed in hepatic and intestinal CYP3A protein expression (Shimada et al., 1994; Paine et al., 2002; Zanger and Schwab, 2013). As noted earlier, genetic and nongenetic factors contribute to interindividual variability in DME expression and activity. Therefore, approaches beyond pharmacogenetic testing are needed to better understand a patient's drug-metabolizing phenotype.

Historical Perspective on P450 Phenotyping Approaches. Exogenous probe substrates have been used historically in clinical settings for phenotyping to determine an individual's drug-metabolizing capacity (Fuhr et al., 2007). An index substrate allows researchers to understand the activity of a DME (Fuhr et al., 2007). By definition, "index substrates have defined changes in systemic exposure when administered with strong inhibitors for a specific drug elimination pathway" (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/clinical-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions). Notable examples of early P450 phenotyping approaches include the erythromycin breath test (EMBT) for CYP3A activity and the dextromethorphan urine and saliva test for CYP2D6 activity.

Erythromycin is a macrolide used to treat a number of bacterial infections (Alvarez-Elcoro and Enzler, 1999). The EMBT was developed by Watkins et al. based on the observations that: *N*-demethylation of erythromycin was exclusively mediated by CYP3A4 in liver microsomes, and the carbon atom in the cleaved methyl group should be exhaled through the lungs as CO_2 (Baker et al., 1983; Watkins et al., 1989). When trace amount of ^{14}C-labeld *N*-methylerythromycin was injected intravenously into subjects, CYP3A4 activity was estimated by the amount of radioactivity recovered in the breath. Administration of CYP3A inducers or inhibitors increased or decreased breath $^{14}CO_2$ production, respectively (Watkins et al., 1989; Paine et al., 2002). Lown et al. showed that EMBT was correlated with CYP3A levels but not with CYP1A2, CYP2C8, CYP2C9, or CYP2E1 in microsomes prepared from patients with severe liver disease (Lown et al., 1992). Despite its responsiveness to changes in CYP3A activity, there are discrepancies in the literature regarding the performance of EMBT in clinics. As shown in previous studies, the lack of correlation between EMBT and CYP3A substrates could be due to erythromycin uptake and efflux by transporters (Schuetz et al., 1998; Kurnik et al., 2006; Frassetto et al., 2007). Thus, interactions with relevant transporters must be considered. CYP3A phenotyping with the benzodiazepine midazolam replaced EMBT for measuring CYP3A activity in vivo (Thummel et al., 1994). Midazolam is extensively

ABBREVIATIONS: AUC, area under the plasma concentration-time curve; cfRNA, cell-free RNA; CYP, cytochrome P450; DDI, drug-drug interaction; DM, dextromethorphan; DME, drug-metabolizing enzyme; DX, dextrorphan; EMBT, erythromycin breath test; EV, extracellular vesicle; HCV, hepatitis C; lncRNA, long non-coding RNA; miRNA, microRNA; ncRNA, noncoding RNA; PD, pharmacodynamics; PK, pharmacokinetics; PBPK, physiologically based pharmacokinetic modeling; PXR, pregnane X receptor; sEV, small extracellular vesicle; SU12662, *N*-desethylsunitinib; SULT, sulfotransferase; TDM, therapeutic drug monitoring; UGT, UDP-glucuronosyltransferase.

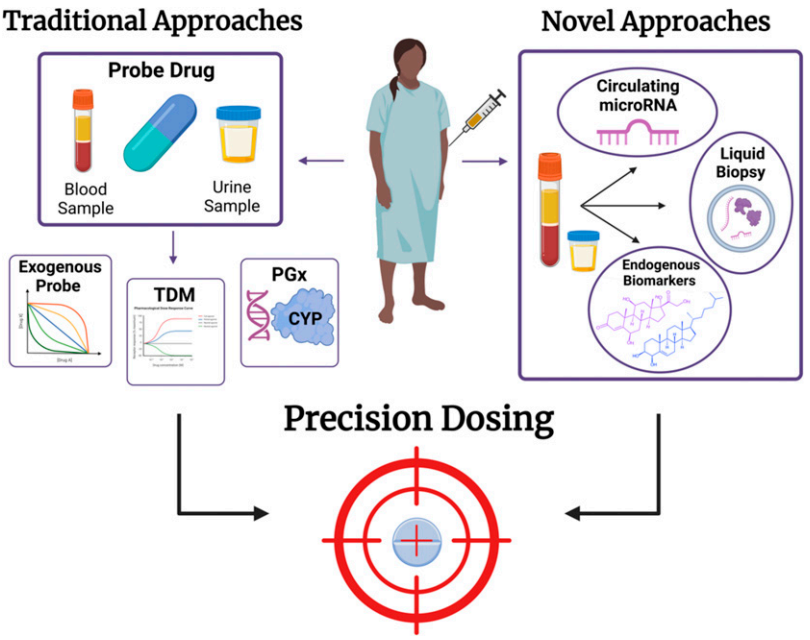

Fig. 2. Biomarkers of drug metabolism phenotype to facilitate precision dosing.

metabolized by intestinal and hepatic CYP3A; midazolam first-pass elimination following oral administration reflects the combined contributions of intestinal and hepatic CYP3A activity (Paine et al., 1996; Thummel et al., 1996).

Dextromethorphan (DM) has been used as a probe substrate to determine CYP2D6 activity (Frank et al., 2007; Wojtczak et al., 2007). *CYP2D6* is highly polymorphic, with significant variation across populations; an average of 5.4% of Caucasians and 2.38% of African Americans were identified as CYP2D6 poor metabolizers (Gaedigk et al., 2017). DM is an oral antitussive drug found commonly in over-the-counter cough medicines; it was chosen as a probe substrate because it has a good safety profile at low doses (Wojtczak et al., 2007). DM is *O*-demethylated by CYP2D6 to form dextrorphan (DX), the metabolite of interest for CYP2D6 activity (Chládek et al., 2000). DM is also metabolized by CYP3A via *N*-demethylation to form 3-methoxymorphinan (Chládek et al., 2000). Although DM has been examined as a probe substrate for both CYP2D6 and CYP3A, it is more commonly used for CYP2D6 activity (Jones et al., 1996). CYP2D6 activity is determined by measuring the metabolic ratio of DM/DX (Hu et al., 1998). DM and its metabolites are excreted primarily in urine (Schadel et al., 1995); in addition to urine, saliva and plasma samples can be used to measure the DM/DX metabolic ratio. As an example of its application, human subjects with a urinary DM/DX metabolic ratio of > 0.3 were assigned as CYP2D6 poor metabolizers (Chládek et al., 2000). Debrisoquine hydroxylation is another classic example used to demonstrate genotype-phenotype relationships with CYP2D6 activity (debrisoquine/4-hydroxydebrisoquine metabolic ratio) (Weinshilboum, 2003).

Current Approaches to P450 Phenotyping With Probe Substrates. Midazolam and caffeine have become the industry standards for phenotyping CYP3A activity and CYP1A2 activity in vivo, respectively (Thummel et al., 1994; Carrillo et al., 2000). As listed by the Food and Drug Administration guidance for drug development (https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers), other sensitive index substrates include tizanidine for CYP1A2, repaglinide for CYP2C8; tolbutamide and *S*-warfarin for CYP2C9; lansoprazole and omeprazole for CYP2C19; desipramine, dextromethorphan, and nebivolol for CYP2D6; and triazolam for CYP3A. An ideal probe substrate is highly selective for the enzyme being studied and has high turnover to form the metabolites of interest (Wu et al., 2021). To accurately quantify metabolites, key criteria are predictable Michaelis-Menten kinetics and lack of downstream metabolism or additional transporter activity (Foti et al., 2010). As many investigators prefer to use the same probe substrate in both in vitro and in vivo settings, probe substrates are typically considered in vitro prior to in vivo use (Yuan et al., 2002). Many P450 probe substrates have been validated and are used in vitro and in vivo (Walsky and Obach, 2004).

Drug cocktails containing multiple probe substrates are used to determine an individual's phenotype for multiple P450s simultaneously. Examples of drug cocktails are the "Geneva cocktail" and the "Cooperstown cocktail"; these can include as many as seven probe substrates at low doses (Streetman et al., 2000; Bosilkovska et al., 2014). Compared with individually dosing each probe substrate, cocktails can be used to determine an individual's phenotype more efficiently. Importantly, this approach only works if there is a high degree of specificity to each enzyme for the drugs being administered and if the drugs are all tolerated by the patient/volunteer (Fuhr et al., 2007). A review by Fuhr et al. (2007) provides a list of desirable properties and validation criteria for probe substrate phenotyping.

Therapeutic Drug Monitoring. Therapeutic drug monitoring (TDM) is used to measure the concentration of narrow therapeutic index drugs at specific intervals. When applied to an appropriate PK model, TDM can be used to make informed decisions about dose adjustments. In TDM, PK measures of drug exposure are monitored, including C_{max} or C_{min} plasma concentration or the area under the plasma concentration-time curve (AUC); in some cases, pharmacodynamic (PD) endpoints are monitored (Hiemke et al., 2018). The goal is to optimize the dose for a patient to facilitate individualized therapy. TDM is not necessary for all drugs; as noted earlier, it is especially important for drugs with a narrow therapeutic index, significant interindividual PK variability, well-established exposure-response relationships, and a defined target concentration range (Kang and Lee, 2009).

Digoxin and lithium were some of the first therapeutic agents to be prescribed and dose adjusted via TDM. Both drugs have very narrow therapeutic ranges: 0.8–2.0 ng/ml for digoxin and 0.6–0.8 mmol/L for lithium (Goldberger and Goldberger, 2012; Nolen et al., 2019). TDM benefits patients by providing an understanding of their response to the

drug and ensuring they remain in the therapeutic window to maximize efficacy and avoid toxicity. Clinical guidelines have been established for TDM with specific drugs in various therapeutic areas (Ashbee et al., 2014; Hiemke et al., 2018; Burns and Goldman, 2020).

Comparing Phenotyping With Probe Substrates and TDM. Despite their advantages, several disadvantages exist for both probe substate phenotyping and TDM. The major disadvantages of probe substrates are the lack of specific probes for most pathways and the invasiveness (administration of exogenous compounds) of the approach (Fuhr et al., 2007). Tolerability is also a concern for probe substates administering low doses of the substrate can address this concern (Fuhr et al., 2007). Sensitive and accurate analytical methods are needed to quantify the probe substate and metabolite(s) relevant to the phenotyping metric, and multiple samples may be required. For TDM, robust and accurate analytical methods are also needed. Specific timing of the PK sample collection is critical. For example, a retrospective analysis of randomly selected patients showed that, of 210 patients, the timing of serum digoxin monitoring was classified as "inappropriate" for 32% of the measurements (Mordasini et al., 2002). Additionally, clinician response to TDM results is important. A retrospective electronic medical record review of 90 patients showed that 21.1% of patients had serum digoxin levels outside of the therapeutic range. Of these patients, only one patient received a change in dose in response to their serum level results (Orrico et al., 2011). Care must be taken to ensure the appropriate sample timing and data interpretation for TDM.

Phenotyping and TDM are not often used simultaneously in the clinic; however, genotyping and TDM are frequently used (Ensom et al., 2001; Baumann et al., 2004). While genotyping provides information regarding variant alleles to predict PK/PD endpoints, phenotyping and TDM provide quantitative PK measures, such as enzyme activity or drug concentrations, which are influenced by both genetic and nongenetic factors: diet, smoking status, age, disease state, and so on. (Jang et al., 2016). Although fundamentally different, phenotyping and TDM may be used as complementary approaches to achieve the goal of precision medicine for patients.

Examples of P450 Phenotyping and TDM Clinical Applications. P450 phenotyping and TDM have been investigated for some tyrosine kinase inhibitors (e.g., imatinib, sunitinib, erlotinib) to facilitate dose individualization (de Wit et al., 2014, 2015a, 2015b; Parra-Guillen et al., 2017; Westerdijk et al., 2020; Clarke et al., 2021). Small molecule tyrosine kinase inhibitors used in cancer therapy are orally administered at fixed does; however, these drugs demonstrate high interindividual variability in PK (de Wit et al., 2015a). For example, sunitinib is metabolized primarily by CYP3A4 to the active metabolite *N*-desethylsunitinib (SU12662); sunitinib and SU12662 display a 30% to 50% interindividual variability in oral clearance (CL/F) (Faivre et al., 2006; Goodman et al., 2007; Houk et al., 2009; Yu et al., 2015c). A previous study showed that steady-state sunitinib and sunitinib + SU12662 AUC were significantly associated with midazolam AUC, a measure of CYP3A activity (de Wit et al., 2014). Midazolam AUC explained 51% of the variability in sunitinib AUC, suggesting that phenotyping CYP3A activity with midazolam may be a useful approach to guide sunitinib initial dosing (de Wit et al., 2014). Erlotinib is metabolized by CYP1A and CYP3A enzymes (Ling et al., 2006; Li et al., 2007); erlotinib PK is significantly influenced by smoking status (Hamilton et al., 2006). In 36 patients treated with erlotinib for non–small cell lung cancer, plasma clearance of erlotinib and its primary active *O*-demethylated metabolite OSI-420 were weakly correlated with caffeine clearance but not midazolam clearance (Parra-Guillen et al., 2017). Compared with P450 phenotyping, the authors suggested that erlotinib TDM may be a more suitable approach to optimize erlotinib dose; however, additional studies are warranted to define the target therapeutic range for erlotinib, and further, prospective studies are needed to validate the clinical benefit of erlotinib TDM (Parra-Guillen et al., 2017).

Endogenous Biomarkers

In addition to their role in drug metabolism, P450 enzymes metabolize many endogenous substrates, suggesting that endogenous substances have the potential to serve as biomarkers to study P450 phenotypes. Compared with exogenous probes, advantages of using endogenous biomarkers are: minimal blood sampling, which is more efficient for time and cost savings and more convenient for patients, and little risk for adverse events since no drug administration is required. Two recent reviews have summarized these points well (Mao et al., 2017; Magliocco et al., 2019). Briefly highlighted next are examples of endogenous biomarkers with a focus on CYP3A.

Of the multiple endogenous markers that have been reported to represent CYP3A activity, the metabolic ratios of 6β-hydroxycortisol/cortisol and 4β-hydroxycholesterol/cholesterol are the most well-studied markers of CYP3A activity (Lee et al., 2019).

6β-Hydroxycortisol/Cortisol. Cortisol is a steroid hormone produced by adrenal glands and released into blood to regulate blood sugar levels (Sherry, 2013). Ged et al. were the first to demonstrate the biotransformation of cortisol to 6β-hydroxycortisol by CYP3A activity (Ged et al., 1989). They showed that 6β-hydroxylase activity was 5.8-fold higher in liver microsomes from patients treated with the CYP3A inducer rifampicin compared with control donors and correlated with hepatic CYP3A protein content (Ged et al., 1989). In contrast, formation of 6β-hydroxycortisol was reduced when cortisol was incubated in human liver microsomes in the presence of CYP3A inhibitors (Abel and Back, 1993).

As demonstrated in a number of PK studies, both urinary 6β-hydroxycortisol and 6β-hydroxycortisol/cortisol ratio have been used clinically as noninvasive measures of CYP3A4 activity (Björkhem-Bergman et al., 2013; Shin et al., 2013; Lee et al., 2021). However, the level of circulating cortisol is known to be dependent on circadian rhythm (Saenger, 1983; Baker and Driver, 2007; Dvorak and Pavek, 2010). Cortisol excretion is the highest in the early morning and lowest around midnight (Saenger, 1983). Due to circadian rhythm variations in secretions, a 24-hour urine collection seems optimal to measure 6β-hydroxycortisol/cortisol ratio and has been used by many investigators (Ged et al., 1989; Bienvenu et al., 1991; Joellenbeck et al., 1992; Tran et al., 1999; Ohno et al., 2000; Peng et al., 2011).

Despite these efforts, several studies suggest that urinary 6β-hydroxycortisol/cortisol ratio is not an ideal biomarker of CYP3A activity. Chen et al. reported that midazolam clearance was poorly correlated with 6β-hydroxycortisol/cortisol ratio at baseline and following fluvoxamine-mediated CYP3A inhibition (Chen et al., 2006). Furthermore, 6β-hydroxycortisol/cortisol ratio did not predict midazolam clearance, EMBT results (Watkins et al., 1992; Kinirons et al., 1999), or CYP3A activity after inhibition with ritonavir or amprenavir (Gass et al., 1998). This is possibly due to the large interindividual and intraindividual variability of the urinary 6β-hydroxycortisol/cortisol ratio compared with midazolam clearance (mean coefficient of variation was 68.4% and 22.5% for urinary 6β-hydroxycortisol/cortisol ratio and midazolam clearance, respectively, at baseline) (Chen et al., 2006). Interestingly, Peng et al. suggested that formation clearance of the sum of 6β-hydroxycortisol and 6β-hydroxycortisone is a useful marker of CYP3A activity, possibly due to lower variation than the urinary 6β-hydroxycortisol/cortisol ratio (Hu et al., 2009; Peng et al., 2011; Shibasaki et al., 2013).

4β-Hydroxycholesterol. Cholesterol is an essential component of cell membranes and serves as a precursor for the biosynthesis of various

steroid hormones, bile acids, and vitamin D (Russell, 1992; Kliewer, 2005). Cholesterol forms numerous circulating oxysterols by both enzymatic and nonenzymatic reactions (Bodin et al., 2001). In vitro studies using recombinant enzymes showed that CYP3A4 catalyzed the formation of 4β-hydroxycholesterol (Bodin et al., 2001, 2002). A follow-up study demonstrated that the relative rates of 4β-hydroxycholesterol formation by CYP3A5 and CYP3A7 were 5.6% and 2.8%, respectively, compared with that catalyzed by CYP3A4 (Bodin et al., 2002). Interestingly, in vivo studies showed that plasma concentrations of 4β-hydroxycholesterol were associated with *CYP3A5* genotype (Diczfalusy et al., 2008; Suzuki et al., 2014), suggesting that CYP3A5 may be a contributor to 4β-hydroxycholesterol formation. The *CYP3A5*1* active allele results in high expression of functional CYP3A5 protein (Kuehl et al., 2001); Tomalik-Scharte et al. (2009) reported that the mean plasma 4β-hydroxycholesterol concentration and 4β-hydroxycholesterol/cholesterol ratio were higher with an increasing number of *CYP3A5*1* alleles. However, studies by Hole et al. and Lee et al. demonstrated that the *CYP3A5*3* inactive variant had no significant effect on plasma 4β-hydroxycholesterol concentrations in European and Korean populations, respectively (Hole et al., 2017; Lee et al., 2017). The reason for this discrepancy is unknown but may be due to population differences in expression of *CYP3A5* alleles and/or other factors.

Following a two-week induction period with rifampicin, the elimination half-life of 4β-hydroxycholesterol was reported as approximately 17 days (Diczfalusy et al., 2009). The long half-life of 4β-hydroxycholesterol results in stable plasma concentrations over time, which may be the reason for low intraindividual variability in untreated subjects. The current literature suggests that circulating 4β-hydroxycholesterol concentrations and the 4β-hydroxycholesterol/cholesterol ratio reflect interindividual variability in CYP3A activity; this variability can arise from factors including genetics, disease, and sex (Diczfalusy et al., 2008; Gebeyehu et al., 2011; Iwamoto et al., 2013; Gjestad et al., 2016; Hirayama et al., 2018; Gravel et al., 2019). For example, serum 4β-hydroxycholesterol levels were significantly higher in females compared with males (Hirayama et al., 2018). Lower plasma 4β-hydroxycholesterol concentrations have been reported in patients with inflammatory disease states, such as rheumatoid arthritis (Wollmann et al., 2017), nonalcoholic steatohepatitis (Woolsey et al., 2015), and type 2 diabetes (Gravel et al., 2019), compared with healthy controls. However, serum 4β-hydroxycholesterol levels were significantly higher in patients with hepatitis C (HCV) compared with healthy controls (Hirayama et al., 2018). These data suggest that 4β-hydroxycholesterol is of interest in monitoring changes in CYP3A activity during disease progression.

As summarized by Mao et al., several drug-drug interaction (DDI) studies have demonstrated changes in plasma 4β-hydroxycholesterol concentration in response to CYP3A inducers and inhibitors in healthy volunteers and patients with HIV, gallstones, and epilepsy (Mao et al., 2017). Mean 4β-hydroxycholesterol levels were also increased in Caucasian healthy volunteers treated with the CYP3A inducer rifampin (600 mg daily for one week) (Hautajärvi et al., 2018). In 10 HCV infected patients treated with ombitasvir/paritaprevir/ritonavir for 12 weeks, serum 4β-hydroxycholesterol levels decreased gradually during the first 4 weeks (36.3% at 2 weeks, 46.1% at 4 weeks), plateaued or slightly increased, and then returned to 10% less than pre-treatment baseline at 8 weeks after the treatment (Hirayama et al., 2018). These findings in HCV patients warrant further investigation.

Overall, the literature suggests that 4β-hydroxycholesterol or the 4β-hydroxycholesterol/cholesterol ratio is a reliable biomarker for phenotyping CYP3A induction. Whether 4β-hydroxycholesterol is a sensitive biomarker for CYP3A inhibition is not clear; studies have shown small or moderate changes in 4β-hydroxycholesterol plasma concentrations or 4β-hydroxycholesterol/cholesterol ratio with CYP3A inhibition (Tomalik-Scharte et al., 2009; Goodenough et al., 2011; Kasichayanula et al., 2014). Because treatment with an inhibitor should probably be long to detect reduced CYP3A activity, study design could be critical (Hole et al., 2017; Mao et al., 2017).

Comparison of Plasma 4β-Hydroxycholesterol With Midazolam. To examine the use of plasma 4β-hydroxycholesterol as a marker for CYP3A activity, studies have demonstrated significant, but weak to moderate, association between the plasma 4β-hydroxycholesterol concentration or normalized 4β-hydroxycholesterol/cholesterol ratio and oral or intravenous midazolam clearance (Tomalik-Scharte et al., 2009; Bjorkhem-Bergman et al., 2013; Shin et al., 2013; Gravel et al., 2019; Eide Kvitne et al., 2022). However, Lee et al. and Woolsey et al. found no relationship between the cholesterol metric and the midazolam metric (Woolsey et al., 2016; Lee et al., 2017). The reason for the discrepancies between the two metrics is not clear. As noted by others, the discrepancies may be due to the different half-lives of 4β-hydroxycholesterol and midazolam (Tomalik-Scharte et al., 2009; Mao et al., 2017). Following CYP3A inhibition, intravenous midazolam clearance was reduced by 286%, but normalized 4β-hydroxycholesterol did not change (Shin et al., 2013). Similarly, inhibition by ritonavir resulted in a more pronounced decrease in the midazolam metric than in the cholesterol metric (Tomalik-Scharte et al., 2009). Following CYP3A induction by rifampicin, the magnitude of the induction was more pronounced for oral midazolam clearance than the normalized 4β-hydroxycholesterol/cholesterol ratio (Bjorkhem-Bergman et al., 2013). However, other studies reported similar increases in the cholesterol metric and intravenous midazolam clearance (Shin et al., 2013; Kasichayanula et al., 2014). Oral midazolam clearance measures both hepatic and intestinal CYP3A metabolism while 4β-hydroxycholesterol reflects only hepatic CYP3A metabolism (Mao et al., 2017; Gjestad et al., 2019; Eide Kvitne et al., 2022), which may result in discrepant correlation between studies. The discrepancies in the literature regarding the association between cholesterol and midazolam metrics indicate that these metrics are not equivalent in their measurement of CYP3A-mediated metabolism. In addition, factors that affect not only the formation pathway but also the clearance pathways of 4β-hydroxycholesterol should be considered when evaluating the cholesterol metric (Neuhoff and Tucker, 2018). Early studies demonstrated the lack of correlation between CYP3A metrics (Kinirons et al., 1999; Masica et al., 2004). Studies have also reported discrepancies in the association of plasma 4β-hydroxycholesterol concentration with steady-state concentrations or oral clearance (CL/F) of other CYP3A substrates, such as quetiapine and tacrolimus (Vanhove et al., 2016; Gjestad et al., 2017; Vanhove et al., 2017; Neuhoff and Tucker, 2018). Identification and quantification of potential covariates may provide a better understanding of the relationship between the cholesterol and midazolam metrics and other CYP3A substrates (Tomalik-Scharte et al., 2009; Woolsey et al., 2016).

Plasma 4β-Hydroxycholesterol and PK Modeling. Although 4β-hydroxycholesterol has been used as a biomarker of CYP3A induction for many drugs or drug candidates, this biomarker has not been successful for precision dosing of some medications. Researchers found that pretransplant 4β-hydroxycholesterol did not improve dose predictions for kidney transplant patients taking tacrolimus (Størset et al., 2017; Vanhove et al., 2017). This could be due to the high levels of interindividual and intraindividual variability in tacrolimus exposure in the first days after transplant, which researchers believe could be independent of CYP3A activity (Vanhove et al., 2017). However, other groups have found evidence that a new drug candidate can be classified for CYP3A4 induction based on 4β-hydroxycholesterol level increases from baseline (Jiang et al., 2017). For example, 4β-hydroxycholesterol was used as a marker of CYP3A induction during treatment with enasidenib based on a PK/PD model for CYP3A induction. They found

that 4β-hydroxycholesterol gave an accurate measure of CYP3A induction, and induction was high with enasidenib use (Li et al., 2019b). Another group demonstrated CYP3A4 induction using 4β-hydroxycholesterol levels and created a physiologically based pharmacokinetic (PBPK) model to quantify CYP3A4 induction by ivosidenib (Bolleddula et al., 2021). There have been many successful and validated PK models using 4β-hydroxycholesterol as a marker of CYP3A induction. The literature shows that this biomarker may have utility with some drugs metabolized primarily by CYP3A4, but additional studies are warranted.

Circulating ncRNAs

As noted earlier, epigenetic regulation can influence DME expression (Ivanov et al., 2012; Kacevska et al., 2012; Klein and Zanger, 2013; Zhong and Leeder, 2013; Zanger et al., 2014). Epigenetic regulation refers to heritable genomic modifications that do not involve altering the DNA sequence; rather, genes may be regulated through DNA methylation, histone modification, chromatin remodeling, and ncRNA interference in response to environmental triggers such as diet, drugs, or stress (Peng and Zhong, 2015; Maldonato et al., 2022). Micro-RNAs (miRNAs) and long noncoding RNAs (lncRNAs) are two forms of regulatory ncRNAs that have been shown to alter drug metabolism. miRNAs are short, single strands of ncRNA approximately 22 nucleotides long that are involved in epigenetic regulation (Pogribny and Beland, 2013; Mori et al., 2019). miRNAs commonly participate in post-transcriptomic gene silencing by binding to a target strand of mRNA and either preventing its translation to protein or promoting its decay through deadenylation (Krol et al., 2010). lncRNAs are defined as ncRNAs that are greater than 200 nucleotides long (Dahariya et al., 2019). lncRNAs are a broad class of ncRNAs and may be classified based on their length, location, and intracellular target(s) (Chen et al., 2021). lncRNAs serve multiple functions: they regulate genomic modification, transcription, and nuclear transport through multiple actions, including direct DNA binding, post-transcriptional targeting of miRNAs and proteins, and histone modification (Fernandes et al., 2019; Chen et al., 2021;). They may also act as precursors to miRNA or inhibit the activity of other miRNAs by acting as an miRNA sponge (Ebert and Sharp, 2010; Kallen et al., 2013; Pan et al., 2015).

ncRNA Expression. While many miRNAs are expressed throughout the body, some predominately originate from specific tissues (Ludwig et al., 2016). miR-122, for example, is a liver-specific miRNA with a higher circulating concentration in the blood of patients with nonalcoholic fatty liver disease, hepatocellular carcinoma, and drug-induced liver injury; miR-122 has been studied as a biomarker for liver injury (Xu et al., 2011; Krauskopf et al., 2015; Pirola et al., 2015; Ludwig et al., 2016). miRNAs avoid degradation in circulation by binding to protein, high density lipoprotein, and/or as cargo within lipid exosomes excreted by cells (Vickers et al., 2011; Hannafon and Ding, 2013; Hammond, 2015). lncRNA may similarly be packaged into exosomes, allowing modulation of gene expression in neighboring cells (Hewson et al., 2016). Cell-free RNA (cfRNA) contained within exosomes is protected from the degradation that commonly occurs with cellular RNA, thus enabling a longer half-life; therefore, cfRNA in exosomes may capture RNA expression and shedding from tissuesover longer periods (Achour et al., 2021; Achour and Rostami-Hodjegan, 2022). Exosomal ncRNAs are present not only in plasma but also in saliva, urine, and feces, making them a potential noninvasive biomarker that may be measured through liquid biopsy (Weber et al., 2010).

Brief Historical Perspective and Key Recent Advances. Much of the study surrounding clinical applications of ncRNAs has been focused on its potential as a diagnostic marker for disease, particularly related to cancer and chemotherapy response (Sun et al., 2018; Guo et al., 2020). Given the role of miRNA in disease progression, molecular mimics and antisense inhibitors of miRNA have also been investigated as potential therapeutic targets (Hammond, 2015). The first evidence of miRNA regulation of DMEs was reported in 2006 by Tsuchiya et al., who found that miR-27b was involved in post-transcriptional regulation of CYP1B1; CYP1B1 is a P450 enzyme that metabolizes estradiol and is often overexpressed in certain types of cancer (Tsuchiya et al., 2006). Since this discovery, many associations have been identified between miRNAs and DME expression (see Table 1).

miR-27b has been shown to downregulate CYP3A4 expression (Pan et al., 2009). A recent study by Zastrozhin et al. found a significant negative correlation between CYP3A activity measured by urinary 6β-hydroxycortisol/cortisol ratio and the plasma concentration of miR-27b in patients taking the CYP3A substrate alprazolam (Zastrozhin et al., 2020). Another study developed a linear regression model to predict CYP2B6 activity, as measured by metabolism of the probe substrate efavirenz, in 72 healthy volunteers (Ipe et al., 2021). Researchers found that the addition of seven circulating miRNAs (miR-204–5p, miR-212–3p, miR-3649, miR-3941, miR-4254, miR-4442, and miR-6867– 5p) to a linear regression model accounting for CYP2B6 genotype-determined metabolizer status, age, sex, and race increased the predictive power of their model from approximately 8% to 45% (Ipe et al., 2021). Expression of phase II enzymes, including sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), are also modulated by ncRNAs (Yu et al., 2010; Li et al., 2020; Hu et al., 2022). For example, recent in vitro work found that both miR-196a-5p and miR-196b-5p downregulate *UGT2A1* expression in lung cell lines and human lung tissue (Sutliff et al., 2019). Similarly, expression of *UGT1A* mRNA has been negatively correlated with expression of miR-491-3p in human liver tissue (Dluzen et al., 2014). In 2015, Pan et al. proposed that epigenetic alteration of membrane transporters, DMEs, and cell cycle regulators by lncRNAs may contribute to drug resistance in cancer (Pan et al., 2015; Smutny et al., 2021). Though less is known about the relationship between lncRNAs and DME expression, recent studies have shown that lncRNAs may act in tandem with miRNAs and nuclear receptors to alter drug metabolism. A report by Li et al. demonstrated that the hepatically enriched lncRNA LINC00844 acts as a sponge of miR-486-5p, thereby increasing mRNA expression of pregnane X receptor (PXR, encoded by the *NR1I2* gene), hepatocyte nuclear factor 4-alpha (*HNF4A*), *CYP3A4*, *CYP2E1*, and *SULT2A1* in response to acetaminophen-induced toxicity in HepaRG cells and primary human hepatocytes (Li et al., 2020). Two lncRNAs, HNF1α-AS1 and HNF4α-AS1, have also been identified as modulators of hepatocyte nuclear factors 1-alpha (*HNF1A*) and *HNF4A*, which in turn regulate expression of multiple P450 enzymes (Chen et al., 2018).

Current Challenges and Knowledge Gaps. Further study is needed to understand the interplay between multiple ncRNAs, nuclear receptor expression, and DME expression and activity (Fig. 3). Multiple in silico databases have been developed that combine in vitro data to predict relationships between ncRNA levels, gene expression, and drug response (Rukov et al., 2014); however, these have typically been used to identify relationships between a single miRNA-gene-drug response relationship. These associations, while useful, may fail to capture the many complex relationships between multiple ncRNAs exerting target-specific effects on multiple genes simultaneously; these associations may also not capture the ability of a single miRNA to impact gene expression through interaction with multiple targets (Rukov et al., 2014). Of note, identification and examination of a single ncRNA biomarker from a large pool of candidates may result in low statistical power due to the many comparisons performed and can limit reproducibility of

TABLE 1

Drug-metabolizing enzymes and transporters regulated by miRNAs

Enzyme	miRNA(s)	Model System(s) and Identification Method(s)	Reference(s)
P450 enzymes			
CYP1A1	miR-18b, miR20b	Correlation, mRNA expression (human immortalized lymphoblastoid cells)	Wang et al., 2009
	miR-892a	Reporter assay, mRNA/protein expression, functional assay (MCF7 human breast adenocarcinoma cells)	Choi et al., 2012
CYP1A2	miR-122-5p	mRNA/protein expression, reporter assay (HepaRG cells)	Gill et al., 2017
	miR-320	Reporter assay, mRNA/protein expression (rats)	Wei et al., 2018
	miR-132-5p	Correlation, reporter assay, EMSA (HepG2, Huh-7, and HepaRG cells)	Chen et al., 2017
CYP1B1	miR-27b	Correlation, reporter assay, protein expression, functional assay (MCF7 cells)	Tsuchiya et al., 2006
	miR-187-5p	mRNA/protein expression, reporter assay, functional assays (human NSCLC cell lines)	Mao et al., 2016
	miR-200c	mRNA/protein expression, reporter assay, functional assays (RCC cells)	Chang et al., 2015
CYP2A3	miR-126	mRNA/protein expression (rats)	Kalscheuer et al., 2008
CYP2A6	miR-126-5p	mRNA/protein expression, functional assay, reporter assay (transfected HEK293 cells)	Nakano et al., 2015a
CYP2B6	miR-204-5p, miR-212-3p, miR-3649, miR-3941, miR-4254, miR-4442, miR-6867-5p	Correlation with 8-OH efavirenz metabolite ratio (72 healthy human subjects)	Ipe et al., 2021
	miR-25-3p	mRNA/protein expression, correlation, reporter assay, EMSA (human hepatocytes, transfected HepaRG cells)	Jin et al., 2016
	miR-1275	correlation with 8-OH efavirenz metabolite ratio (200 human subjects), mRNA expression (200 human subjects and human liver tissue), reporter assay (HepG2 cells)	Burgess et al., 2018
CYP2C8	miR-103, miR-107	Reporter assay, mRNA/protein expression, correlation (human hepatocytes)	Goldstein, 2001; Zhang et al., 2012
CYP2C9	miR-128-3p	EMSA, reporter assay, mRNA/protein expression, correlation (HepG2, HepaRG, and HEK293 cells)	Yu et al., 2015a
	miR-130b	mRNA/protein expression, reporter assay (HepaRG cells)	Rieger et al., 2015
	miR-155-5p	mRNA expression, reporter assay, correlation (human liver tissue and HepaRG cells)	Kugler et al., 2020
CYP2C19	miR-103, miR-107	Protein expression (human hepatocytes)	Goldstein, 2001; Zhang et al., 2012
	miR-29a-3p, miR-23a-3p	EMSA, mRNA/protein expression, correlation (HEK293 and HepaRG cells)	Yu et al., 2015b
	miR-155-5p	mRNA expression, reporter assay, correlation (human liver tissue and HepaRG cells)	Kugler et al., 2020
CYP2D6	miR-101, miR-128-2	mRNA/protein expression, reporter assay, correlation to tramadol metabolism (SH-SY5Y, U251, and HepG2 cells; orchiectomized growth hormone receptor knockout mice and rats)	Li et al., 2015
	miR-370-3p	Correlation, EMSA, mRNA/protein expression (HepaRG, HepG2, and HuH7 cells)	Bock et al., 1994; Zeng et al., 2017
CYP2E1	miR-214-3p	Correlation (human liver tissue), EMSA, mRNA/protein expression, activity assay (HepG2 cells)	Wang et al., 2017
	miR-552	EMSA, mRNA/protein expression, reporter assay (PLC/PRF/5, HEK293, and HepG2 cells)	Miao et al., 2016
	miR-570	Reporter assay, mRNA/protein expression, correlation (human liver tissue, HEK293 and HepaRG cells)	Nakano et al., 2015b
	miR-378	Reporter assay, mRNA/protein expression, correlation, functional assay (human liver tissue, HEK293 cells)	Mohri et al., 2010
	miR-132, miR-212	Reporter assay, mRNA expression (primary rat hepatocytes)	Shukla et al., 2013
CYP2J2	let-7b	Reporter assay, protein expression, functional assay (mice, human lung tissue, multiple recombinant cell lines)	Chen et al., 2012
CYP3A	miR-1, miR-532-3p, miR-577, miR-627	Reporter assay, protein expression, correlation (HEK293 cells, human liver tissue)	Wei et al., 2014
	miR-27b	Reporter assay, mRNA/protein expression, functional assay (PANC1, LS-180, HEK293 cells)	Pan et al., 2009
	miR-27a	Reporter assay, mRNA/protein expression, correlation (Hep3B and HEK293 cells)	Shi et al., 2015
	miR-628-3p, miR-641	Reporter assay, mRNA expression (HepaRG cells)	Yan et al., 2017
	miR-206	mRNA expression, reporter assay (human liver microsomes, HEK293 cells, HepG2 cells)	Liu et al., 2016
	miR-122-5p	mRNA/protein expression, reporter assay (HepaRG cells)	Gill et al., 2017
	miR-224-5p	mRNA expression, reporter assay, correlation (human liver tissue and HepaRG cells)	Kugler et al., 2020
CYP7A1	miR-122a, miR-422a	Reporter assay, mRNA expression (primary human hepatocytes, HepG2, Huh7 cells)	Song et al., 2010
CYP24A1	miR-125b	Reporter assay, mRNA/protein expression, functional assay (KGN and MCF-7 cells)	Komagata et al., 2009
Cytochrome b_5	miR-223	Reporter assay, mRNA/protein expression, correlation (human liver microsomes, human liver tissue, HepG2 cells)	Takahashi et al., 2014

TABLE 1 *continued*

Enzyme	miRNA(s)	Model System(s) and Identification Method(s)	Reference(s)
UGT enzymes			
UGT1A	miR-21-3p, miR-103b, miR-200a-3p, miR-376b-3p, miR-1286	Reporter assay, mRNA expression, correlation (HEK293 cells, primary human hepatocytes)	Papageorgiou and Court, 2017a
	miR-141-3p	Reporter assay, mRNA/protein expression (HEK293, Huh7, Caco-2 cells)	Papageorgiou and Court, 2017a; Tatsumi et al., 2018
	miR-491-3p	Reporter assay, mRNA/protein expression, correlation (Hep3B, HEK293, A-549, HuH-7, HepG2, Caco-2 cells)	Dluzen et al., 2014
UGT2A1	miR-196a-5p, miR-196b-5p	Reporter assay, mRNA expression, correlation (HEK293, H146, H1944 cells)	Sutliff et al., 2019
UGT2B4	miR-135a-5p, miR-410-3p	Reporter assay, mRNA/protein expression, correlation (HepG2, Huh7 cells)	Wijayakumara et al., 2017
	miR-216b-5p	Reporter assay, mRNA/protein expression, correlation (HEK293, SK-HEP-1, HepG2, Hep3B, Caco-2, MCF-7, A-549, Huh7 cells)	Dluzen et al., 2016
UGT2B7	miR-142-3p, miR-513c-3p, miR-1293, miR-4317, miR-4483	Reporter assay, mRNA expression, correlation (HEK293 cells, human liver tissue)	Papageorgiou and Court, 2017b
	miR-216b-5p	Reporter assay, mRNA/protein expression, correlation (HEK293, SK-HEP-1, HepG2, Hep3B, Caco-2, MCF-7, A-549, Huh7 cells)	Dluzen et al., 2016
	miR-3664-3p	Reporter assay, mRNA/protein expression, correlation (HepG2 and Huh7 cells); Reporter assay, mRNA expression, correlation (HEK293 cells, human liver tissue)	Papageorgiou and Court, 2017b; Wijayakumara et al., 2017
UGT2B10	miR-216b-5p	Reporter assay, mRNA/protein expression, correlation (HEK293, SK-HEP-1, HepG2, Hep3B, Caco-2, MCF-7, A-549, Huh7 cells)	Dluzen et al., 2016
UGT2B15	miR-103b, miR-376b-3p, miR-455-5p, miR548as-3p, miR-605, miR-624-3p, miR-770-5p, miR-3675-3p, miR-3294, miR4292, miR4712-5p, miR-6500-5p	Reporter assay, mRNA expression, correlation (HEK293 cells, human liver tissue)	Papageorgiou and Court, 2017b
	miR376c	Reporter assay, mRNA/protein expression, correlation (LNCaP cells); reporter assay, mRNA/protein expression, functional assay, correlation (LNCaP, DU145, PC3M, PC3M-LN4 and MDA PCa2b human PCa and HEK293 cells)	Wijayakumara et al., 2015; Margaillan et al., 2016
SULT enzymes			
SULT1A1	miR-631	Reporter assay, mRNA/protein expression, correlation (ZR75-1, MCF7, and MCF10A cells; human liver and platelet cytosol)	Yu et al., 2010

EMSA, electrophoresis mobility shift assay; Reporter assay, luciferase-based assay.
Table adapted from data found from Nakano and Nakajima (2018); Li et al. (2016); Li et al. (2019a).

results. Care should be taken to ensure that studies are adequately powered and multiplicity concerns have been addressed (Otani et al., 2019).

As demonstrated by Li et al., different ncRNAs may act together to influence nuclear receptor and subsequent DME gene expression in response to stimuli (Li et al., 2020). These relationships may also act in a reciprocal fashion, wherein nuclear receptor activation regulates expression of ncRNAs (Smutny et al., 2021). A recent study by Dempsey and Cui identified multiple lncRNAs that are regulated by the nuclear receptors PXR and constitutive androstane receptor (Dempsey and Cui, 2019). The interrelated roles of miRNA, lncRNA, and genetic polymorphisms in regulating PXR expression are reviewed extensively by Smutny et al. (2021). A recent ex vivo study by Tantawy et al. found significant associations between miR-107 and nine different transcription factors that regulate expression of CYP3A enzymes; the transcription factors include estrogen receptor alpha (*ESR1*), PXR, hepatocyte nuclear factor 3-beta (*FOXA2*), *HNF4A*, and peroxisome proliferator activated receptor alpha (*PPARA*) (Tantawy et al., 2022). These results suggest that miR-107 does not directly regulate CYP3A expression by binding to mRNA for CYP3As; rather, miR-107 may modulate the expression of multiple key transcription factors affecting P450 expression (Tantawy et al., 2022). As liquid biopsy analysis of cfRNA in exosomes continues to gain interest, it is important to note that a mechanism for the association between circulating exosomal miRNA and the metabolic clearance rates of DME substrates remains largely unknown (Pridgeon et al., 2022).

Comparison of ncRNA Analysis With Other Phenotyping Approaches. Given the role of ncRNA in regulating DME expression, it is possible that changes in circulating ncRNA may be reflected in

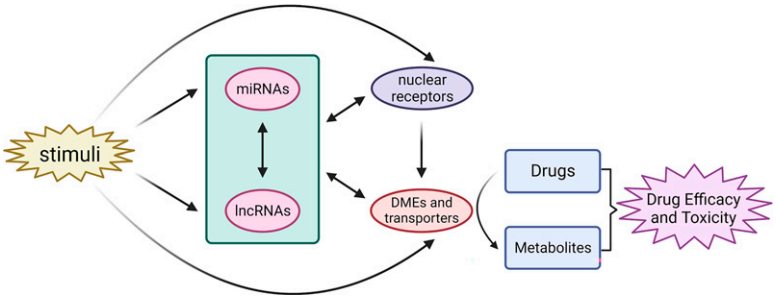

Fig. 3. Epigenetic regulation of drug metabolism. Both miRNAs and lncRNAs modulate the expression of nuclear receptors, drug-metabolizing enzymes, and transporters involved in drug metabolism and disposition in response to stimuli, leading to downstream effects on drug efficacy and toxicity. Figure adapted with permission from Maldonato et al. (2022) (Taylor & Francis Ltd, http://www.tandfonline.com).

changes to PK parameters that affect drug and metabolite exposure (Fig. 3) (Ingelman-Sundberg et al., 2013; Li et al., 2016; Maldonato et al., 2022). While many studies have demonstrated associations between miRNA levels and DME expression (see Table 1), very few have directly examined the relationship between miRNA and circulating drug or metabolite concentrations in vivo; even fewer studies have been devoted to the influence of lncRNA. As the reader is no doubt aware, some known and many unknown factors influence the relationships between genotype, mRNA expression, protein abundance, and drug metabolism, making observation of these associations difficult. While miRNA and lncRNA have been shown to influence DME expression and subsequent metabolism, this is only one of many potential causes for missing heritability in drug metabolism phenotype (Jukic et al., 2022). Rather than viewing ncRNAs as a replacement for TDM or other absorption, distribution, metabolism, and excretion phenotyping approaches, Rowland et al. contend that circulating ncRNA could instead be used as an additional piece of information alongside a patient's DME and transporter genotype and clinical characteristics in precision medicine-informed care (Rowland et al., 2022).

While there is increasing in vitro and ex vivo evidence of the role of ncRNAs in DME expression, more clinical studies are needed to assess the external validity and relevance of circulating ncRNA as a marker for drug PK/PD (Maldonato et al., 2022). For these reasons, more traditional approaches (i.e., use of clinical index enzyme substrates) remain the gold standard for drug metabolism phenotyping.

Perspective on Future Directions. As mentioned previously, an improved understanding of the mechanisms and pathways involved in epigenetic regulation of DMEs is needed to develop effective predictive models for PK parameters based on ncRNA (Stern et al., 2016; Maldonato et al., 2022). Current research opportunities in this area include developing quantitative systems pharmacology-based models that integrate RNA interference data with patient specific clinical and "omics" data to implement model-informed precision dosing and improve current approaches to drug discovery (Achour and Rostami-Hodjegan, 2022; Darwich et al., 2021; Stern et al., 2016). For example, Li et al. developed a systems-based model that integrates 1241 reactions between genes, RNA, protein, and epigenetic regulation mechanisms, including 241 miRNAs, to describe factors influencing drug action in the epidermal growth factor receptor signaling pathway (Li et al., 2012). With continued research examining the epigenetic pathways associated with DME expression and activity, similar models may be developed for predicting absorption, distribution, metabolism, and excretion characteristics.

Liquid Biopsy

Plasma- and serum-derived extracellular vesicles (EVs) have recently been proposed as a minimally invasive "liquid biopsy" for characterizing individual DME and transporter phenotypes ex vivo (Rodrigues and Rowland, 2019). Increasing interest has developed in the scientific community related to EVs because they have the potential to serve as biomarkers of human health and disease, drug delivery tools, and diagnostic markers (Kim et al., 2018; Shah et al., 2018; Sahoo et al., 2021; Newman et al., 2022a, 2022b). The use of EVs for companion diagnostic and drug delivery applications is beyond the scope of this review. Methodology and application of EVs to phenotype PK characteristics have been reviewed by Rodrigues and Rowland (2019) and Useckaite et al. (2021). The following sections provide a high-level overview of this topic and briefly describe recent advances in the application of EVs to evaluate individual patient phenotypes in PK/PD research.

EVs and the Nature of Their Cargo. EVs are lipid bilayer membrane-encapsulated particles that are released by different tissues throughout the body into clinically sampled biofluids, such as blood, cerebrospinal fluid, and urine (Useckaite et al., 2021). EVs are a heterogenous mixture of particles broadly classified based on their biogenesis pathway and size (reviewed by Yáñez-Mó et al., 2015; van Niel et al., 2018; Jeppesen et al., 2023). EVs include exosomes (50–150 nm in diameter), microvesicles (100–1000 nm in diameter), and apoptotic bodies (50–5000 nm in diameter) (Useckaite et al., 2021). Small EVs (sEVs) are less than 200 nm in size (Théry et al., 2018). Exosomes are formed from endosomal maturation and fusion of multivesicular endosomes with the plasma membrane; microvesicles are formed by direct budding of the cell membrane (Yáñez-Mó et al., 2015; Useckaite et al., 2021). The lipid membrane composition of exosomes differs from that of the originating cell membrane; membrane proteins, such as tetraspanins [e.g., cluster of differentiation (CD) 9, CD63, CD81], are enriched in exosomes (Yáñez-Mó et al., 2015; Useckaite et al., 2021; Jeppesen et al., 2023).

Knowledge in the EV field is rapidly evolving: a recent review outlined emerging advances in the understanding of EVs as well as nonvesicular extracellular nanoparticles (Jeppesen et al., 2023). EVs play a role in cell-cell communication in normal physiology and pathophysiology (Useckaite et al., 2021; Jeppesen et al., 2023). EV cargo is rich in molecular biomarkers, which include proteins, nucleic acids (miRNA, tRNA, rRNA, mRNA, DNA), metabolites, and lipids from the cells of origin (Useckaite et al., 2021). The composition of EV cargo differs based on the biologic fluid (serum versus urine) (Useckaite et al., 2021); EV shedding by cells may be affected by various factors, including disease and age (Achour et al., 2021, 2022).

EVs in PK/PD Research. Plasma and serum-derived EVs have attracted significant attention in recent years as a less invasive approach (compared with tissue biopsy) to characterize interindividual variability in DMEs and transporters (Rodrigues and Rowland, 2019). Proteins and mRNA from > 500 DMEs and transporters, as well as > 80 Food and Drug Administration-approved drug targets, have been detected in EVs isolated from plasma and/or serum (Kumar et al., 2017; Rowland et al., 2019; Achour et al., 2021, 2022; Rodrigues et al., 2021). Of interest to drug PK, the following DMEs and transporters have been identified in plasma- and serum-derived EVs: P450 1A1, 1A2, 2A6, 2B1, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5; UGT 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15; OATP 1B1, 1B3, 2B1; ABC B1, G2, C2, C4, C6, C9 (Kumar et al., 2017; Rowland et al., 2019; Achour et al., 2021; Useckaite et al., 2021; Achour et al., 2022). Table 2 shows examples of studies evaluating EV-derived PK biomarkers in specific populations.

Kumar et al., (2017) first described isolation and quantification of P450 enzymes (2E1 and 3A) in plasma exosomes and demonstrated ex vivo activity (Kumar et al., 2017), suggesting EV biomarkers may be applied to study drug-induced toxicity. Rowland et al. (2019) demonstrated that CYP3A4 mRNA, protein, and activity can be quantified from plasma-derived EVs as an approach to phenotype CYP3A4 in healthy volunteers (Rowland et al., 2019). Specifically, EV-derived CYP3A4 mRNA, protein, and ex vivo activity (midazolam 1'-hydroxylation) significantly correlated with in vivo CYP3A activity, as measured by midazolam apparent oral clearance (CL/F) (Rowland et al., 2019). The EV-derived CYP3A4 biomarkers were also correlated with within-subject changes in CYP3A activity (midazolam CL/F) following CYP3A induction by rifampicin (pre- and post-rifampicin treatment) (Rowland et al., 2019).

Achour et al. (2021) reported a quantitative link (correlation) between the plasma-derived EV RNA expression of 12 DMEs, particularly P450 enzymes, and 4 drug transporters with liver protein expression using plasma and matched liver tissue samples after normalizing for liver-to-plasma shedding (Achour et al., 2021). Although not very well understood, exosome shedding is, in essence, a physiologic process that is altered under pathologic conditions; exosome shedding adds another

TABLE 2

Recent studies evaluating EV-derived biomarkers for PK applications

Liquid Biopsy	DMD Biomarker(s) EV Cargo Assayed	Study Population (ethnicity)	Number of Participants and Sex	Application	Reference
Plasma EVs	CYP and UGT protein (proteomics, ELISA) CYP and UGT mRNA (qRT-PCR) CYP3A activity assay: midazolam 1'-hydroxylation; UGT activity assay: 4-methylumbeliferone glucuronidation	Caucasian/White (genotyped as *CYP3A4 *1/*1* and *CYP3A5 *3/*3*)	n = 6 males	Induction DDI for CYP3A	Rowland et al., 2019
Plasma EVs	cfRNA (RNAseq): 171 enzymes and 362 transporters, including CYPs, UGTs, ABCB1, ABCG2, ABCC2, SLCO1B1/1B3/2B1	Caucasian/White; liver cancer patients	n = 16 males; n = 13 females	Patient stratification and PBPK modeling of CYP3A substrates	Achour et al., 2021
Serum EVs	CYP2D6, CYP3A4, CYP3A5; OATP1B1, OATP1B3 protein (proteomics) CYP2D6 activity (dextromethorphan O-demethylation to dextrorphan)	Caucasian/White (genotyped for *CYP2D6*, *CYP3A4*, *CYP3A5*)	n = 10 males; n = 3 females (nonpregnant); n = 3 (pregnant)	Pregnancy and drug induction studies	Rodrigues et al., 2021
Plasma EVs	CYP3A4 (proteomics)	Caucasian/White; healthy volunteers (genotyped for *CYP3A5*)	n =10 males	Drug induction of CYP3A4	Rodrigues et al., 2022
Plasma EVs	cfRNA (RNAseq): 159 enzymes, 336 transporters, including CYPs, UGTs, ABCB1, ABCG2, ABCC2, SLCO1B1/1B3/2B1	Caucasian/White; patients with cardiovascular disease (genotyped for *CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5*)	n = 23 males; n = 7 females	Patient phenotyping	Achour et al., 2022
Plasma EVs	CYP2E1 (Western blot, activity assay)	Alcoholic and healthy cohorts	n = 17 males; n = 6 females	Alcohol induction of CYP2E1	Cho et al., 2017
Plasma EVs	cfRNA (RNAseq): neonatal Fc receptor (FcRn)	Caucasian/White; liver cancer patients	n = 16 males; n = 13 females	PBPK of mAbs disposition	Barber et al., 2023

FcRn, neonatal Fc receptor; mAbs, monoclonal antibodies; RNAseq, RNA sequencing.

variable with which to contend when interpreting variability in PK data. Determination of such variability becomes critical when the patient cohort includes a heterogeneous mix of diseases (Achour et al., 2021). In the study by Achour et al. (2021), PBPK simulations with three CYP3A substrates (alprazolam, midazolam, and ibrutinib) indicated that using liquid biopsy-determined hepatic CYP3A4 content for dose stratification and individualization significantly reduced interindividual variability in drug exposure (AUC) compared with uniform oral dosing (Achour et al., 2021). These findings suggest that the liquid biopsy may be a useful strategy to characterize individual drug metabolism capacity and inform individualized drug dosing. The authors extended their findings to the disposition of therapeutic antibodies by linking neonatal Fc receptor expression (*FCGRT*) in plasma exosomes to abundance in liver tissue (Barber et al., 2023). In another study of cardiovascular disease patients, cfRNA expression of P450s 1A2, 2B6, 2C9, 3A4, and ABCB1 from plasma-derived EVs was significantly correlated with P450 and P-glycoprotein activities, as measured by Geneva cocktail probe substrates in dried blood spots (Achour et al., 2022). The study also showed that genotype data (CYP1A2, 2C9, 2C19, 2D6, 3A5) had limited capacity to capture phenotype variability independently. The quantitative correlation explored between the EV cargo and abundance/activity in their tissue of origin is an essential requisite to facilitate implementation of precision dosing strategies by providing patient characterization data compatible with PBPK modeling platforms, such as Virtual Twins (Fig. 4) (Polasek and Rostami-Hodjegan, 2020; Darwich et al., 2021).

Because cell types from all organs can produce EVs, isolation of tissue-specific EVs (e.g., liver-specific EVs) has been suggested to be important for the use of EVs to characterize the contribution of hepatic versus extrahepatic DMEs and transporters in PK studies (Useckaite et al., 2021). Rodrigues et al. (2021) recently reported the isolation of liver-specific EVs from serum using an immunoprecipitation approach; this approach selectively captures EVs expressing asiaglycoprotein receptor 1, a cell-surface protein enriched in hepatocytes (Rodrigues et al., 2021). Using proteomics, the study measured CYP2D6, CYP3A4, CYP3A5, OATP1B1, and OATP1B3 protein concentrations from serum-derived sEVs (Rodrigues et al., 2021). The results showed that liver-specific sEV CYP2D6 ex vivo activity (as measured by DM *O*-demethylation to DX) was associated with sEV CYP2D6 protein concentration and plasma DM/DX concentration ratio (Rodrigues et al., 2021). Moreover, the strong CYP3A4 inducer rifampicin (300 mg × 7 days and 600 mg × 14 days) increased serum-derived liver-specific sEV CYP3A4 protein by greater than threefold compared with baseline (Rodrigues et al., 2021). As expected, CYP3A5 protein was detected in liver-specific sEV in two subjects genotyped as *CYP3A5*1/*3* (CYP3A5 expressors) but not in CYP3A5 nonexpressers (*CYP3A5*3/*3*) (Rodrigues et al., 2021). In addition, liver sEV CYP3A4 protein concentration increased in serum from pregnant females by trimester (T1–T3), consistent with previous observations that CYP3A4 expression is induced during pregnancy due to pregnancy-related hormones (Rodrigues et al., 2021). Serum-derived liver sEV CYP2D6 protein concentration was also higher in T3 pregnant females compared with nonpregnant (T0) females (Rodrigues et al., 2021).

A more recent study used plasma-derived global and liver-specific sEVs to evaluate CYP3A4 induction by modafinil in healthy volunteers (Rodrigues et al., 2022). Subjects were genotyped for *CYP3A5*; the plasma 4β-hydroxycholesterol/cholesterol ratio was used as an endogenous biomarker of CYP3A activity (Rodrigues et al., 2022). The study demonstrated that liver-specific sEV CYP3A4 protein was significantly correlated with baseline plasma 4β-hydroxycholesterol/cholesterol ratio, particularly in CYP3A5 nonexpressers (*CYP3A5*3/*3*). This was not the case with non-liver EVs, indicating a major contribution of intestinal CYP3A (Rodrigues et al., 2022). Modafinil (400 mg) administration once daily for 14 days resulted in increased plasma 4β-hydroxycholesterol/

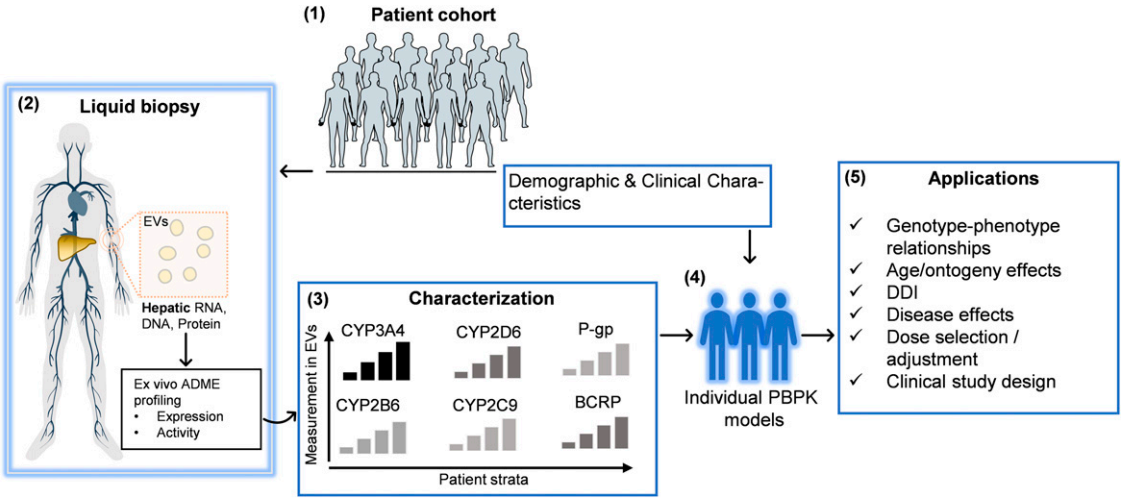

Fig. 4. Deployment of a liquid biopsy-informed PBPK strategy for patient characterization and precision dosing. Plasma is sampled from a patient cohort (1), followed by isolation of hepatic EVs and ex vivo ADME profiling of RNA and protein content using omics techniques (2). Characterization of PK pathways (enzymes, transporters) in EVs allows systems data to be collected (3), which together with patients' demographic and clinical characteristics are used to individualize PBPK models to generate virtual or digital twins (4). These models can be used for a range of applications in clinical study design, precision dosing, and disease effect modeling (5). ADME, absorption, distribution, metabolism, excretion.

cholesterol ratio and modest increases in liver-specific and non-liver sEV CYP3A4 protein, as measured by proteomics (Rodrigues et al., 2022). Although the changes in sEV CYP3A4 protein concentration induced by modafinil were not correlated with the modafinil-induced changes in plasma 4β-hydroxycholesterol/cholesterol ratio, the liver-specific sEV CYP3A4 protein concentration was significantly correlated with baseline plasma 4β-hydroxycholesterol/cholesterol ratio, as noted earlier (Rodrigues et al., 2022). Moreover, the increase in sEV CYP3A4 protein observed with modafinil treatment was used to successfully predict the plasma AUC ratios of CYP3A4 victim drugs following modafinil. These studies demonstrate the utility of liquid biopsy in studying induction DDI potential.

Challenges for PK Applications of Liquid Biopsy. Useckaite et al. (2021) described logistic and technical challenges of liquid biopsy applications. Matching the EV isolation approach to the downstream analytic approach was identified as a major challenge for the use of EV-derived cargo (mRNA, protein) (Useckaite et al., 2021). For example, resin precipitation methods for EV isolation (e.g., Exo-Quick) tend to precipitate highly abundant plasma proteins, such as albumin, with EVs, which may render them less suitable for downstream proteomic analysis (Useckaite et al., 2021). Precipitation methods, however, have the advantage of high yield and high throughput, allowing characterization of low abundance proteins and RNA transcripts (Achour et al., 2021). Density gradient ultracentrifugation is labor intensive and low throughput and therefore is not useful for routine analysis or clinical applications (Useckaite et al., 2021). Choosing the appropriate sample type (serum versus urine) is important to consider based on the research question (Useckaite et al., 2021); volume and quality of the biofluid are key determinants of the yield and quality of the macromolecular cargo (Achour et al., 2022). Quality control steps are therefore necessary to ascertain the suitability of samples. For DMEs, such as CYP3A4, which is expressed in the liver and intestine, hepatic and intestinal-derived EVs may contribute differentially to the CYP3A4 detected in EVs (Useckaite et al., 2021). Considering that active enzyme is predominantly expressed in enterocytes at the tip of intestinal villi, assessment of differences in shedding biology between intestine (into the feces and blood) and liver (into the blood) may allow better understanding of the contributions of these tissues to serum EVs. From a technical perspective, Rodrigues et al. (2021) suggest that immunocapture of liver-specific EVs is relevant to determine the hepatic contributions to CYP3A4 phenotype (Rodrigues et al., 2021). Connecting EV data to tissue abundance and further extrapolation to whole-organ PK has been identified as a challenge that is relevant to understanding the absolute abundance of the target protein (e.g., for PBPK modeling of hepatic clearance) (Useckaite et al., 2021). In addition, diurnal variation was shown to contribute to intrasubject variability in the liver-specific EV marker asiaglycoprotein receptor 1 in a small cohort of male and female healthy volunteers ($n = 10$) (Newman et al., 2021). EV concentration was 10-fold higher in males compared with females during the morning sample collection (Newman et al., 2021). These findings have implications for EV-based PK study design (e.g., time of day for sample collection), data analysis, and interpretation.

Knowledge Gaps for PK Applications of Liquid Biopsy. One of the main areas in which liquid biopsy research is expected to have an impact is noninvasive characterization of changes in PK pathways due to disease; very little evidence has been published in this area so far, except from opportunistic studies of surgical surplus or retrospective analyses of samples from previous clinical studies (Achour et al., 2021, 2022). As shown in Table 2, most of the studies investigating EV-derived PK biomarkers have been in predominately European ancestry populations. A knowledge gap exists in the characterization of EV-derived PK biomarkers in people from understudied populations, such as African ancestry and Indigenous American populations. Underrepresentation of non-European populations in drug metabolism and PK studies of EVs is a barrier to evaluating and implementing liquid biopsy-informed precision medicine strategies in these populations. Moreover, additional studies are needed to further evaluate EV-derived PK/PD biomarkers in special populations, such as pregnancy and pediatrics. Liquid biopsy remains a specialist area, with several challenging steps, ranging from isolation and purification of EVs to multiomics analyses of the enclosed RNA and protein content. These challenges led to the bulk of recent work being focused on characterization of enzymes and transporters in readily accessible biofluids, such as plasma, compared with more challenging systems, such as urine (Console et al., 2018) and cerebrospinal fluid, where evidence of utility is still lacking.

Current and Future Perspectives. While the application of EVs in PK research is a relatively new field with many unknowns, growing

evidence in the literature supports the use of the approach to characterize DME and transporter phenotypes in individual subjects. Recent articles have discussed the strengths and limitations of RNA-based approaches to profile DME expression using EVs (Achour and Rostami-Hodjegan, 2022; Pridgeon et al., 2022). Achour et al. (2022) noted that measurement of DME mRNA expression in tissue is not equivalent to expression of cfRNA in EVs due to the longer half-life of EVs and the observation that RNA in EVs has greater protection from degradation compared with cellular mRNA (Achour and Rostami-Hodjegan, 2022). Comparisons were made between the different methods to characterize individual P450 phenotypes using EV-derived markers, including CYP mRNA, protein, and enzyme activity (Rowland et al., 2019; Rowland et al., 2022). Importantly, the authors acknowledge that liquid biopsy methods complement other approaches, such as pharmacogenetics and TDM, to inform precision dosing (Achour and Rostami-Hodjegan, 2022; Rowland et al., 2022). While TDM measures drug concentrations in a patient already on treatment to guide dose adjustments to achieve the target therapeutic concentrations, pharmacogenetics and liquid biopsy provide information on the patient's genotype and phenotype, respectively, to guide initial dose selection and identify patients who may require close monitoring (Achour and Rostami-Hodjegan, 2022; Rowland et al., 2022). Determining P450 phenotypes through liquid biopsy measures also complements pharmacogenetic analyses by permitting evaluation of genotype-phenotype relationships (Rodrigues and Rowland, 2019).

The endgame of liquid biopsy technology is to be deployed in conjunction with model projections to allow improved patient characterization and better informed dosing (Fig. 4). The requisites for an effective framework for model-informed precision therapeutics have been discussed elsewhere (Darwich et al., 2021), with the centerpiece being a validated patient characterization approach. The approach starts with the collection of "systems" data required to build a Virtual Twin, including demographic and clinical characteristics available in electronic health records of the patients (e.g., age, sex, ethnicity, body mass index, estimated glomerular filtration rate, etc.), with additional characterization of the metabolic and transport pathways relevant to the elimination of the drug substrates in a routinely available liquid biopsy (e.g., plasma). Such data are incorporated in a generic/base PBPK model that best matches the disease and/or the condition of the patients (e.g., obesity, pediatrics, pregnancy, renal/hepatic impairment), resulting in individual models representing each patient in the cohort (Polasek and Rostami-Hodjegan, 2020). The individualized models can subsequently be used in virtual clinical studies designed for different PK applications, such as DDI studies, dose selection/adjustment, disease effects, and ontogeny effects (Fig. 4). Validation of EVs for such applications will most likely require a concerted effort from industrial and academic research groups. Once validated, liquid biopsy measures can then be integrated with other PK tools, such as genotyping and endogenous biomarkers, in a modeling and simulation framework for individual phenotyping (Rodrigues and Rowland, 2019; Useckaite et al., 2021). Evidence of this integrated approach is emerging in the literature, which provides support for liquid biopsy as a promising tool in the PK tool kit (Rodrigues et al., 2021, 2022).

TABLE 3

Summary of approaches for DME phenotyping

Approach	Description and Current Applications	Challenges, Knowledge Gaps, and Future Directions
Exogenous probe substrates	• Clinical index substrates are well established for some DMEs • Commonly used to measure changes in DME activity in DDI studies	• Procedure is invasive; involves administering exogenous probe substrate(s) • Requires a validated analytical method • Limited to a small number of DMEs because of lack of specific substrates
Therapeutic drug monitoring	• Involves measuring plasma or serum drug concentrations in patients already taking the drug • Used clinically for some narrow therapeutic index drugs to adjust dose based on target PK and/or PD endpoints	• Requires a validated analytical method • PK sample collection must be timed appropriately • Drugs monitored must have well-established exposure-response relationships • Requires dedicated equipment in a hospital setting
Endogenous biomarkers	• Approach is less invasive compared with exogenous probe substrates • May be measured in plasma or urine (e.g., plasma 4β-hydroxycholesterol/cholesterol, urine 6β-hydroxycortisol/cortisol) • Used in some clinical DDI studies to evaluate enzyme induction (e.g., CYP3A) • Baseline levels may be used to compare individual DME activity	• Mixed results have been reported for the correlation of endogenous biomarkers with exogenous probe substrate PK metrics • Factors besides DME activity may contribute to interindividual and intraindividual variability in endogenous biomarker measurements • Lack of specific endogenous biomarkers makes this approach challenging for clinical applications
Circulating ncRNA	• Includes microRNA and lncRNA involved in epigenetic regulation of gene expression • ncRNA are present in circulation bound to proteins, lipoproteins, and in sEVs (e.g., exosomes) • May be used as diagnostic markers for disease	• More studies are needed to understand the interplay between multiple ncRNAs, nuclear receptor expression, and DME expression and activity • More clinical studies are needed to assess the external validity and relevance of circulating ncRNA as markers for drug PK/PD • Quantitative application of the approach has not been demonstrated
Liquid biopsy	• Involves characterizing DMEs and transporters in plasma- and serum-derived sEVs • sEVs, such as exosomes, contain proteins, DNA, cell-free RNA, metabolites, and lipids • If sensitive assays are available, DME mRNA, protein, and ex vivo activity can be measured • Has been evaluated for clinical DDI studies or individual DME phenotyping	• Methods for isolating and characterizing sEVs and sEV-derived cargo are technically challenging • Must account for interindividual and intraindividual variability in exosome shedding • Requires correlation between sEV cargo and DME abundance/activity in the tissue of origin • More studies are needed for clinical validation • Additional studies are needed to evaluate sEV-derived PK/PD biomarkers in understudied ethnic populations and special populations

Conclusion

In conclusion, several approaches have been proposed to characterize individual drug metabolism phenotypes; each approach has advantages and disadvantages. Table 3 provides a summary of the approaches discussed and highlights some key concepts. Ongoing studies are required to address the current challenges and knowledge gaps related to novel phenotyping approaches: circulating ncRNAs and liquid biopsy. Concerted efforts between multidisciplinary teams are needed to validate novel approaches and implement best practices in clinical settings. The integration of novel approaches with well-established methods to characterize individual PK and drug metabolism phenotype will likely have the highest potential for success in precision medicine. Moreover, increasing the inclusion of understudied ethnic populations in future studies will be critical to advance the science and application of PK biomarkers in diverse populations.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Jackson, Achour, Lee, Geffert, Beers, Latham.

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Address correspondence to: Dr. Klarissa D. Jackson, UNC Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, 3320 Kerr Hall, CB# 7569, Chapel Hill, North Carolina 27599-7569. E-mail: klarissa.jackson@unc.edu