



# The Use of Microdosing for In vivo Phenotyping of Cytochrome P450 Enzymes: Where Do We Stand? A Narrative Review

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## Abstract

Cytochrome P450 (CYP) enzymes play a central role in the elimination of approximately 80% of all clinically used drugs. Differences in CYP enzyme activity between individuals can contribute to interindividual variability in exposure and, therefore, treatment outcome. In vivo CYP enzyme activity could be determined with phenotyping. Currently, (sub)therapeutic doses are used for in vivo phenotyping, which can lead to side effects. The use of microdoses (100 µg) for in vivo phenotyping for CYP enzymes could overcome the limitations associated with the use of (sub)therapeutic doses of substrates. The aim of this review is to provide a critical overview of the application of microdosing for in vivo phenotyping of CYP enzymes. A literature search was performed to find drug–drug interaction studies of CYP enzyme substrates that used microdoses of the respective substrates. A substrate was deemed sensitive to changes in CYP enzyme activity when the pharmacokinetics of the substrate significantly changed during inhibition and induction of the enzyme. On the basis of the currently available evidence, the use of microdosing for in vivo phenotyping for subtypes CYP1A2, CYP2C9, CYP2D6, and CYP2E1 is not recommended. Microdosing can be used for the in vivo phenotyping of CYP2C19 and CYP3A. The recommended microdose phenotyping test for CYP2C19 is measuring the omeprazole area-under-the-concentration-time curve over 24 h ( $AUC_{0-24}$ ) after administration of a single 100 µg dose. CYP3A activity could be best determined with a 0.1–75 µg dose of midazolam, and subsequently measuring AUC extrapolated to infinity ( $AUC_{\infty}$ ) or clearance. Moreover, there are two metrics available for midazolam using a limited sampling strategy: AUC over 10 h ( $AUC_{0-10}$ ) and AUC from 2 to 4 h ( $AUC_{2-4}$ ).

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## Key Points

Information about sensitivity to changes in enzyme activity at the microdose level was available for five different probes at five cytochrome P450 enzymes.

The use of microdosing for in vivo phenotyping of CYP1A2, CYP2C9, CYP2D6, and CYP2E1 is not recommended on the basis of the current available evidence.

Microdoses of omeprazole (100 µg) and midazolam (0.1–75 µg) can be used for the determination of in vivo CYP2C19 and CYP3A activity.

## 1 Introduction

Cytochrome P450 (CYP) enzymes are a large superfamily of heme-containing enzymes that are involved in the metabolism of both endogenous and exogenous compounds [1, 2]. CYP enzymes play a role in the metabolism of approximately 80% of all clinically used drugs [3]. The activity of CYP enzymes is influenced by many factors, such as genetics, age, co-administration of exogenous compounds (e.g., drugs or food), and disease state [4–6]. Owing to these factors there is a large variability in CYP enzyme activity between individuals, which might contribute to heterogeneous therapy outcomes [7]. Dosing of drugs on the basis of CYP enzyme activity can result in more predictable therapy outcome [8–10].

The actual enzyme activity of an individual person could be obtained through *in vivo* phenotyping. *In vivo* phenotyping would consist of two steps: (1) the administration of a selective substrate of the enzyme (the probe), and (2) the quantification of the metric representing enzyme activity. This metric is often a pharmacokinetic metric representing clearance (Cl) or a metabolic pathway [11, 12]. The value of the metric would give an indication of the activity of the enzyme. For example, high enzyme activity would correspond in a high clearance value, while low enzyme activity would be associated with a low clearance value. There are currently no guidelines regarding the application of *in vivo* phenotyping, but recommendations regarding probe and metric selection could be acquired from the drug–drug interaction guidelines of the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) [13, 14]. The major disadvantage of the currently used phenotyping tests is the use of therapeutic or sub-therapeutic doses. Fuhr et al. formulated a list of validated phenotyping procedures of which all are used in the milligram range [11]. These (sub)therapeutic doses can result in side effects or therapeutic effects, such as (mild) sedation for low dose midazolam (a sedative drug and a selective substrate of CYP3A) or hypoglycaemic effect for low dose tolbutamide (an antihyperglycemic drug and a selective substrate of CYP2C9) [11, 15–17].

The use of microdosing within the field of *in vivo* phenotyping could overcome the above described limitations. A microdose has been defined as 1/100th of the anticipated therapeutic dose with a maximum of 100 µg [14]. Owing to their low dose, microdoses are assumed to be nontoxic and nonpharmacologically active [18]. Microdoses differ from subtherapeutic doses in the amount of drug administered. While microdoses are dosed at a maximum of 100 µg, subtherapeutic doses refers to any dose below the registered therapeutic dose. Originally microdosing has been used to quickly assess the pharmacokinetics of a new

drug prior to phase I clinical trials [19]. In recent years, the application of microdosing has been extended toward drug–drug interaction studies, site of action studies [20], and recently the application in *in vivo* phenotyping studies has been proposed [20, 21]. However, there is still much unknown about the application of microdosing for *in vivo* phenotyping.

The aim of this narrative review is to provide a comprehensive overview of the use of microdosing for *in vivo* phenotyping of CYP enzymes. Two aspects of *in vivo* microdose phenotyping tests will be discussed. Firstly, the sensitivity of the test to detected changes in the activity of the CYP enzyme will be reviewed. Before a phenotyping test can be used for individualized dosing, the test needs to be validated at the microdose level to ensure that it reflects the enzyme activity in many different settings [11]. Second, the linearity of the phenotyping metric over the dosing range from a microdose to the currently used (sub-)therapeutic dose (µg to mg) is discussed. Scalability over this dosing regimen would allow the extrapolation of the currently established knowledge on *in vivo* phenotyping at the (sub)therapeutic level to the microdose level. This could enable the application of microdosing for *in vivo* phenotyping since the current knowledge at the (sub)therapeutic level could be extrapolated to microdose level and only the necessary clinical studies need to be conducted to complete the necessary evidence for clinical application. Lastly, recommendations regarding the use of microdosing for the *in vivo* phenotyping of CYP enzymes will be formulated.

## 2 Methods and Literature Search

### 2.1 Literature Search

The literature search was performed in PubMed and Embase using the following terms: microdose\*, phase 0, microgram dose, phenotyp\*, drug–drug interactions, drug interactions, human microdosing trials, and cytochrome P-450 enzyme. The search was performed on 23 June 2023. Results were restricted to the English language and studies in humans. Additional studies were selected from review articles and reviewing the reference section of each article (citation snowballing). Initial selection was based on title and abstract, while inclusion was determined by full-text assessment. Continuing, studies were included when pharmacokinetic outcomes of phenotyping and/or drug interactions were available. Microtracer trials were excluded from this review as the total administered dose exceeds the maximum dose definition of a microdose (> 100 µg) [22].

## 2.2 Sensitivity of In vivo Phenotyping Methods

A frequently used method to determine the sensitivity of a phenotyping method is to measure the phenotyping metric under three conditions: (1) baseline (or control) where the metric is quantified in the absence of any factors influencing the enzyme, (2) inhibition where an inhibitor of the enzyme is co-administered with the probe, and (3) induction where an inducer of the enzyme is co-administered with the probe. The phenotyping method is considered sensitive to the respective enzyme activity when the metric significantly changes during inhibition and induction enzyme compared with baseline.

Three different levels of evidence level were defined: (A) significant difference in the metric at the microdose level between baseline and inhibition/induction, (B) difference in metric at the microdose level between baseline and inhibition/induction but no statistics were performed, (C) no significant difference in metric at the microdose level between baseline and inhibition/induction. An evidence level of A for probe sensitivity indicates that the phenotyping method can be used for in vivo phenotyping at the microdose level, level B indicates that there is a potential use for the phenotyping method at the microdose level, and level C indicates that the probe-metric combination is not suitable for in vivo phenotyping at the microdose level.

## 2.3 Pharmacokinetic Scalability

The scalability of microdose pharmacokinetics were determined by comparing pharmacokinetic parameters of the microdose with the pharmacokinetic parameters of the therapeutic dose. For the area-under-the-concentration-time-curve (AUC) the value extrapolated to infinity in  $\text{ng} \times \text{h/mL}$  was used, unless otherwise denoted, and presented dose-adjusted to 100  $\mu\text{g}$ . The maximum observed concentration ( $C_{\text{max}}$ ) is also presented as dose-adjusted to 100  $\mu\text{g}$ . Furthermore, the clearance (Cl) was reported in L/h (apparent Cl for oral administration), volume of distribution ( $V_d$ ) in L (apparent  $V_d$  for oral administration), and half-life ( $t_{1/2}$ ) in h. Pharmacokinetic data from single microdose studies were completed with literature data from therapeutic pharmacokinetic studies. Microdose pharmacokinetics were considered predictive if the mean observed values of the microdose and of the therapeutic dose were within a twofold of each other, which is the generally accepted criterion for the predictive value of microdose pharmacokinetics [22–25].

In this review, four evidence levels of scalability were defined. (A) High scalability: all pharmacokinetic parameters of parent and metabolite fell within the twofold criterion; (B) good scalability: all pharmacokinetic parameters of either parent or metabolite fell within the twofold criterion; (C) moderate scalability: the metric used for phenotyping

fell within the twofold criterion; (D) poor scalability: none of the pharmacokinetic parameters fell within the twofold criterion or the metric used for phenotyping falls outside the twofold criterion. Evidence level A and B indicate that results of in vivo phenotyping at the microdose level could be extrapolated to therapeutic dose for data interpretation, level C indicates that the results of in vivo phenotyping might be extrapolated from the microdose level to the therapeutic dose level if the respective metric is sensitive to changes in CYP enzyme activity, and D indicates that the results of in vivo phenotyping at the microdose level could not be extrapolated to therapeutic dose level.

## 3 Results of Literature Search

### 3.1 Included Literature

A total of 23 out of 827 screened articles were included in the current review. The selection process is depicted in Fig. 1. Of the 23 articles, 13 articles reported the sensitivity of microdose probes [21, 26–30]. These articles are summarized in Supplementary Table 1. Continuing, 17 articles were included for the evaluation of scalability of microdose pharmacokinetics to the therapeutic level [28, 29, 31–39]. Taken together, this review describes the application of in vivo phenotyping with microdoses of CYP1A2 with caffeine, CYP2C9 with glibenclamide, losartan, tolbutamide, and warfarin, CYP2C19 with lansoprazole and omeprazole, CYP2D6 with yohimbine, CYP2E1 with chlorzoxazone, and CYP3A4 with midazolam, quinidine, and verapamil. Details of the comparison between microdose and pharmacokinetics of therapeutic doses are described in Supplementary Table 2 for studies containing both microdose and therapeutic data and Supplementary Table 3 for studies describing only microdose pharmacokinetics. The latter studies were completed with studies reporting pharmacokinetics of therapeutic doses. The evidence levels for sensitivity and scalability for each microdose phenotyping test are reported in Table 1.

### 3.2 Cytochrome P450 Enzymes

#### 3.2.1 CYP1A2

**3.2.1.1 Caffeine** Caffeine is recommended as a probe for CYP1A2 activity by the EMA owing to its predominant metabolism by CYP1A2 to paraxanthine at therapeutic levels [13, 40]. At a microdose of 25  $\mu\text{g}$ , the AUC extrapolated to infinity ( $\text{AUC}_{\infty}$ ) of caffeine was sensitive to CYP1A2 inhibition, but this effect was smaller compared with the effect at a therapeutic dose of 250 mg (8.1-fold versus 13.7-fold, respectively) [21, 41]. The authors did not report any hypothesis regarding the observed difference, but it could

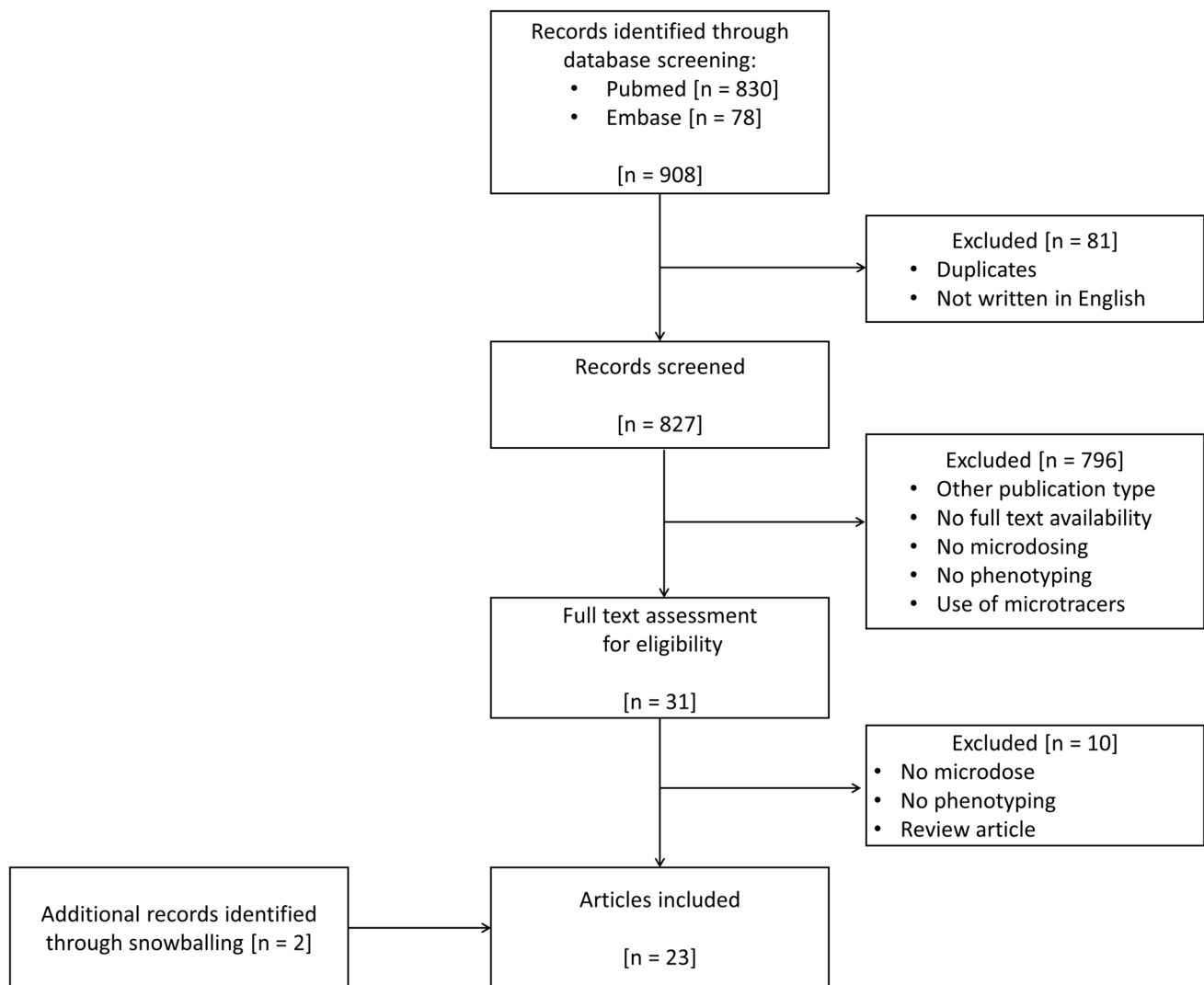


Fig. 1 Schematic overview of the selection process.

possibly be caused by difference in study design, participant populations, or a concentration-dependent contribution of CYP1A2 to the metabolism of caffeine. Currently, there is no information available about the sensitivity of CYP1A2 induction. The extrapolation of microdose metrics to therapeutic level is complicated owing to the difference in the above described effect size of CYP1A2 inhibition between the two dosing levels despite the dose-proportional pharmacokinetics of caffeine (0.25–250 mg) [21, 41–43]. Therefore, sensitivity data of the  $AUC_{\infty}$  of a caffeine microdose to induction needs to be obtained before clinical application could be considered (Table 1).

### 3.2.2 CYP2C9

**3.2.2.1 Glibenclamide** Glibenclamide is predominantly metabolized by CYP2C9 into its two main metabolites:

4'-hydroxy glibenclamide and 3'-hydroxy glibenclamide [44]. Therapeutic pharmacokinetics of glibenclamide (0.5–3.5 mg) are reported to be significantly different between CYP2C9 genotypes [45, 46], indicating that glibenclamide could be used as a probe for CYP2C9 activity. There is no information available about the sensitivity of phenotyping metrics to inhibition or induction of CYP2C9. However, a genotyping study reported the difference in  $AUC_{\infty}$  and  $t_{1/2}$  of a glibenclamide microdose (10  $\mu$ g) between genotypes, indicating a possible use as a microdose phenotyping probe [33]. However, no statistical analyses were performed. The pharmacokinetics of glibenclamide were moderate to poorly scalable from 10  $\mu$ g to 2.5 mg [33, 44, 47]. Taken together, glibenclamide shows potential as a microdose probe for CYP2C9 activity, but the sensitivity of  $AUC_{\infty}$  at the microdose level during enzyme inhibition and induction needs to be established before clinical application (Table 1).

**Table 1** Overview of probes and metrics available for in vivo phenotyping of Cytochrome P450 enzymes and the evidence level for their application

Enzyme	Probe	Dose (µg)	Route of administration	Metric	Evidence level		Comment	Recommendation	References
					Sens.	PK			
CYP1A2	Caffeine	25	PO	AUC <sub>∞</sub>	A	B	Only evidence for inhibition	Data is needed about performance of AUC <sub>∞</sub> during enzyme induction	[21, 41-43]
CYP2C9	Glibenclamide	10	PO	AUC <sub>∞</sub>	NA	C/D	No information about sensitivity	Sensitivity studies at microdose level need to be performed	[33, 44, 47]
	Losartan	100	PO	AUC <sub>∞</sub>	NA	B	No information about sensitivity	Sensitivity studies at microdose level need to be performed	[49]
	Tolbutamide	25	PO	AUC <sub>∞</sub>	C	B	Only evidence for inhibition	Sensitivity studies at microdose level need to be performed	[21, 51]
	Warfarin	10-100	PO	AUC <sub>∞</sub>	NA	C	No information about sensitivity	Sensitivity studies at microdose level need to be performed	[31, 33, 52, 53]
CYP2C19	Lansoprazole	50-70	PO	AUC <sub>∞</sub> Cl/F AUC 5-OH/LSP	NA NA NA	D D D	Difference in enzyme activity between genotypes	Sensitivity studies at microdose level need to be performed	[33, 55]
	Omeprazole	100	PO	AUC <sub>0-24</sub> AUC <sub>∞</sub> Cl/F	A/B	C/D			
CYP2D6	Yohimbine	50	PO	AUC <sub>∞</sub>	B	A/B		Sensitivity studies at microdose level need to be performed	[56, 57]
				AUC YH/11-OH YH	B	A/B			
				Cl/F	B	A/B			
CYP2E1	Chlorzoxazone	2.5-50	PO	AUC <sub>∞</sub> Cl/F	NA NA	A/B A/B		Sensitivity studies at microdose level need to be performed	[60, 61]
CYP3A4	Midazolam	75	PO	AUC 1-OH/MDZ	B/C	A/B	Only evidence for inhibition	Not suitable for <i>in vivo</i> phenotyping	[15, 20, 27-32, 34-39, 66, 68, 78-85]
			PO	AUC <sub>0-10</sub>	A	A/B	Only evidence for inhibition	Suitable for <i>in vivo</i> phenotyping at microdose level	
		PO	AUC <sub>2-4</sub>	A	A/B	Only evidence for inhibition	Suitable for <i>in vivo</i> phenotyping at microdose level		
		PO	AUC <sub>∞</sub>	A	A/B	Dependent on perpetrator dose and rout of administration	Suitable for <i>in vivo</i> phenotyping at microdose level		
		PO	Cl/F	A	A/B		Suitable for <i>in vivo</i> phenotyping at microdose level		
		PO	Cl <sub>met</sub>	A	A/B		Suitable for <i>in vivo</i> phenotyping at microdose level		
							No effect with cyclosporine with or without fluconazole		
Quinidine	100	PO	AUC <sub>∞</sub>	NA	B		Sensitivity studies at microdose level need to be performed	[66]	
			AUC 3-OH/QND	NA	B		Sensitivity studies at microdose level need to be performed		
			AUC N-OX/QND	NA	B		Sensitivity studies at microdose level need to be performed		
Verapamil	100	PO	AUC <sub>∞</sub>	NA	A/B		Sensitivity studies at microdose level need to be performed	[66]	
			AUC NVP/VPN	NA	A/B		Sensitivity studies at microdose level need to be performed		

AUC<sub>∞</sub> area-under-the-concentration-time-curve extrapolated to infinity, AUC<sub>0-24</sub> AUC from 0 to 24 h, AUC<sub>0-10</sub> AUC from 0 to 10 h, AUC<sub>2-4</sub> AUC from 2 to 4 h, AUC N-OX/QND AUC of N-oxide quinidine divided by quinidine, AUC NVP/VPN AUC of norverapamil divided by verapamil, AUC YH/11-OH YH AUC of yohimbine divided by 11-hydroxy yohimbine, AUC 1-OH/MDZ AUC of 1'-hydroxy midazolam divided by midazolam, AUC 3-OH/QND AUC of 3-hydroxy quinidine divided by quinidine, AUC 5-OH/LSP AUC of 5-hydroxy lansoprazole divided by lansoprazole, Cl/F apparent clearance, PK pharmacokinetics, PO oral administration, Sens. sensitivity

**3.2.2.2 Losartan** The major metabolic route for losartan is its metabolism by CYP2C9 into the E3174 metabolite [48]. There is no information available about the sensitivity of phenotyping metrics (AUC, Cl, or metabolic ratio) at the microdose level. The scalability of losartan pharmacokinetics over a dosing range of 100 µg to 50 mg was considered good in both healthy volunteers and hypertensive patients (See Supplementary Table 2) [49]. Before losartan can be used for in vivo phenotyping at the microdose level the sensitivity of the phenotyping metric to changes in CYP2C9 needs to be investigated at the microdose level.

**3.2.2.3 Tolbutamide** Tolbutamide is a recommended probe by the EMA owing to its predominant metabolism by CYP2C9 to 4'-hydroxy tolbutamide [13, 50]. The AUC<sub>∞</sub> of tolbutamide appears to be sensitive to CYP2C9 inhibition at the microdose level (25 µg) since inhibition resulted in an increase in AUC<sub>∞</sub> of 1.8-fold, but the change from baseline was not significant, possibly owing to the large variability in tolbutamide pharmacokinetics following the administration of the perpetrators compared to baseline [21]. This was attributed to use of moderate CYP2C9 inhibitors (ketoconazole and fluvoxamine) [21]. There is no information avail-



able about the sensitivity of  $AUC_{\infty}$  to CYP2C9 induction at the microdose level. Continuing, the pharmacokinetics were good scalable over a dosing range of 25  $\mu\text{g}$  to 125 mg [51]. Summarizing, there is currently insufficient evidence to recommend a microdose tolbutamide as a phenotyping probe for CYP2C9.

**3.2.2.4 Warfarin** S-warfarin is recommended as a probe for CYP2C9 activity at therapeutic level by the EMA [13]. Currently, there is no evidence that the pharmacokinetics of S-warfarin are sensitive to changes in CYP2C9 activity at the microdose level. A microdose genotyping study did not find any significant differences in warfarin  $AUC_{0-2}$  of a 10  $\mu\text{g}$  microdose between different CYP2C9 genotypes, indicating that warfarin might not be a sensitive probe for CYP2C9 activity at the microdose level [33]. However, the authors reported the quantification of racemic warfarin instead of (S)-warfarin and an incomplete capture of the pharmacokinetics as possible causes of the nonsignificant findings [33]. Furthermore, the pharmacokinetics of warfarin were moderately scalable from the microdose level (10–100  $\mu\text{g}$ ) to therapeutic level (5–7.5 mg; Supplementary Table 3) [31, 33, 52, 53]. In conclusion, S-warfarin cannot be currently recommended as a microdose probe for CYP2C9 activity (Table 1).

### 3.2.3 CYP2C19

**3.2.3.1 Lansoprazole** The predominant metabolic pathway for lansoprazole is its 5-hydroxylation by CYP2C19 to 5'-hydroxy lansoprazole [54]. Currently, there is no sensitivity information available at the microdose level but results from a pharmacogenetic-pharmacokinetic study suggests the possible sensitivity of lansoprazole  $AUC_{\infty}$  (50–70  $\mu\text{g}$ ) to changes in CYP2C19 activity, but no statistical analyses were performed [33]. The pharmacokinetics of lansoprazole demonstrated nonlinearity over a dosing range of 50  $\mu\text{g}$  to 30 mg (evidence level D) [33, 55]. Concluding, sensitivity studies at microdose level are needed before the application of lansoprazole as a microdose probe (Table 1).

**3.2.3.2 Omeprazole** Owing to omeprazole's good selectivity for CYP2C19 and its tolerability, it is recommended as a CYP2C19 probe by the EMA at therapeutic level [13]. At the microdose level the  $AUC_{0-24}$  is sensitive to inhibition and induction of CYP2C19 [26]. The effect size at the microdose level on  $AUC_{0-24}$  during both inhibition and induction of the enzyme was similar to the therapeutic level [26]. Furthermore, the pharmacokinetics of omeprazole were nonlinear over a dosing range of 100  $\mu\text{g}$  to 20 mg (evidence level D) [26]. Pharmacokinetics of omeprazole have been reported to be nonlinear over a dose range of 20–40 mg, which has been mostly attributed to the metabolic saturation of the

S-enantiomer of omeprazole. Moreover, S-omeprazole also inhibits CYP2C19 after multiple dosing, complicating the exploration of phenotyping results at the therapeutic dose level [26]. Taken together, a microdose of 100  $\mu\text{g}$  omeprazole is suitable as an in vivo phenotyping trope of CYP2C19 (Table 1) and might even be more predictive for CYP2C9 activity compared with a therapeutic dose.

### 3.2.4 CYP2D6

**3.2.4.1 Yohimbine** Yohimbine has been investigated as a probe for CYP2D6. At microdose level yohimbine  $AUC_{\infty}$ . The Cl/F and metabolic ratio of yohimbine to 11-hydroxy yohimbine seemed to be sensitive to perpetrators of CYP2D6 (see Supplementary Table S1) [56, 57]. However, the fold-difference in pharmacokinetic parameters at microdose was smaller compared with the therapeutic dose and no statistical tests were conducted. The pharmacokinetic parameters of yohimbine were not well scalable from microdose to therapeutic dose, while the metabolic ratio of yohimbine to 11-hydroxy yohimbine was good scalable (Supplementary Table S2) [57]. This was credited to the high variability in yohimbine pharmacokinetics [57]. Concluding, yohimbine seems to have potential as a CYP2D6 probe at the microdose level, but sensitivity to changes in CYP2D6 activity needs to be established at the microdose level before yohimbine could be considered as a microdose probe.

### 3.2.5 CYP2E1

**3.2.5.1 Chlorzoxazone** The metabolic ratio of 6'-hydroxy chlorzoxazone and chlorzoxazone is a commonly used metric for CYP2E1 activity [58, 59]. At this moment, there is no data available regarding the sensitivity of the metabolic ratio to perpetrators of CYP2E1 at the microdose level. Chlorzoxazone pharmacokinetics were linear over a dose range from 25  $\mu\text{g}$  to 5 mg [60, 61]. Currently, chlorzoxazone cannot be recommended as a microdose probe for CYP2E1 activity.

### 3.2.6 CYP3A

**3.2.6.1 Midazolam** Midazolam is a highly selective probe for CYP3A activity owing to its selective metabolism by CYP3A to its main metabolite 1'-hydroxy midazolam [62]. Furthermore, midazolam is a recommended CYP3A probe by the EMA [13]. Of the available phenotyping metrics,  $AUC_{\infty}$  and Cl/F had the best evidence for sensitivity (Supplementary Table 1) [5, 7–9]. The metabolic ratio of 1'-hydroxy midazolam/midazolam is thought to give a more accurate estimation of CYP3A activity because it correlates well with hepatic CYP3A4 content [63, 64] and is, therefore, the metric of preference [13]. Surprisingly, the meta-

bolic ratio was not sensitive to CYP3A induction [28]. The authors did not comment on this result, but concluded that the metabolic ratio determined 30 min after administration correlated well with midazolam Cl (Spearman correlation;  $p < 0.005$ ) [13]. Moreover, two metrics that are determined with limited sampling strategies,  $AUC_{0-10}$  and  $AUC_{2-4}$  were investigated. Both of these metrics were found to be sensitive to changes in CYP3A activity at the microdose level. Overall the pharmacokinetics of midazolam can be considered as good scalable over a wide range of doses (0.003–7.5 mg). Concluding, midazolam  $AUC_{\infty}$  and  $Cl/F$  are both suitable for in vivo phenotyping of CYP3A at the microdose level (Table 1).

**3.2.6.2 Quinidine** Quinidine is predominantly metabolized by CYP3A4 and CYP2D6 [65]. There is currently no information about the sensitivity of microdose phenotyping metrics for quinidine ( $AUC_{\infty}$ ,  $Cl/F$ , or metabolic ratio). Quinidine pharmacokinetics were considered to have good scalability (0.1–10 mg), while its metabolite quinidine N-oxide was not well scalable (Supplementary Table 2) [66]. Before its application as an in vivo phenotyping probe, sensitivity to changes to CYP3A activity at the microdose level should be established (Table 1).

**3.2.6.3 Verapamil** Verapamil is a substrate for both CYP3A and CYP2C8 enzymes [67]. It is currently unclear if any of the phenotyping metrics of verapamil ( $AUC_{\infty}$ ,  $Cl/F$  or metabolic ratio) are sensitive to changes in CYP3A activity. Verapamil had highly scalable pharmacokinetics from a dose of 100  $\mu$ g to 16 mg [66]. Continuing, the metabolic ratio for the main metabolite norverapamil and verapamil was good scalable over the same dosing range [66]. Sensitivity studies at the microdose level are essential to establish whether verapamil should be used as a probe for CYP3A owing its multiple metabolic pathways (Table 1)

## 4 Discussion

The suitability of microdosing for in vivo phenotyping of CYP enzymes was reviewed. Several recommendations can be made regarding the above described literature regarding the use of microdosing for the in vivo phenotyping for CYP enzymes (Table 1). The use of microdosing for in vivo phenotyping of CYP1A2, CYP2C9, CYP2D6, and CYP2E1 cannot be currently recommended since there is lacking evidence of the sensitivity of the phenotyping metrics of the respective probes. However, there is potential for caffeine, glibenclamide, and yohimbine as microdose probes for CYP1A2, CYP2C9, and CYP2E1, respectively. The  $AUC_{\infty}$  of a 25  $\mu$ g dose of caffeine was sensitive to CYP1A2 inhibition, but no evidence has been reported for CYP1A2

induction yet [21]. Moreover, both glibenclamide and yohimbine might be sensitive probes at the microdose level since microdose pharmacokinetics differed between different CYP2C9 and CYP2E1 genotypes, respectively [33, 56]. Microdosing could be used for the in vivo phenotyping of CYP2C19 and CYP3A4. For CYP2C19, the recommended microdose phenotyping test is the omeprazole  $AUC_{0-24}$  after a 100  $\mu$ g. CYP3A activity could be best determined with an oral 0.1–75  $\mu$ g dose of midazolam and the midazolam  $AUC_{\infty}$  or Cl. Moreover, there are two metrics available that are determined with a limited sampling strategy:  $AUC_{0-10}$  and  $AUC_{2-4}$ .

Three conclusion could be drawn based on the currently presented literature. Firstly, dose-proportional pharmacokinetics from (sub)therapeutic to microdose is not necessarily predictive of the sensitivity of a probe at the microdose level. This is illustrated by omeprazole and tolbutamide. For omeprazole,  $AUC_{0-24}$  is sensitive to changes in CYP2C19 but its pharmacokinetics are not linear, while for tolbutamide the pharmacokinetics are linear but the phenotyping metric at a microdose is not sensitive to changes in enzyme activity. Dose-nonlinear pharmacokinetics might be irrelevant at the microdose level since dose-nonlinear pharmacokinetics are often caused by saturable processes, such as metabolism, and a microdose will result in exposure below the threshold of saturation. For omeprazole, specifically, dose-nonlinear pharmacokinetics over a dosing range of 20–40 mg is thought to be caused by the metabolic saturation of the S-enantiomer and its auto-inhibition of CYP2C19 [26]. Secondly, microdoses might be more predictive of the in vivo enzyme activity. Owing to the low administered dose of microdoses, microdose pharmacokinetics are not influenced by saturable processes or auto-inhibition/induction that can occur at (sub)therapeutic doses, such as for omeprazole. Lastly, there is a need for recommendations regarding the design of studies investigating sensitivity of potential probes. Some sensitivity studies had suboptimal results owing to the choice of perpetrator [21], the choice of probe [33], or high variability in pharmacokinetics [21]. There are currently no guidelines on in vivo phenotyping of CYP enzymes. However, recommended probes and metrics in the drug–drug interaction guidelines from the EMA and FDA could be used [13, 14]. Characteristics of a validated probe are listed in these guidelines, but the use of microdosing for in vivo phenotyping is not discussed. However, the following recommendations can be made. A probe should be a selective substrate of the respective enzyme and in the case of a racemic drug, the isomer with the highest selectivity should be chosen as the probe (e.g., S-warfarin [33]). Perpetrators should be chosen on the basis of their strength of inhibition or induction of the enzyme in question [13, 14]. Moreover, the dose [68], duration of exposure [14, 68, 69], and time of administration of the perpetrator [14, 68] should

be chosen in such a way that the inhibition or induction of the enzyme is maximized. Lastly, if the probe is meant to be used in a phenotyping cocktail, drug–drug interaction studies at the microdose level should be conducted making sure that the individual probes in the cocktail do not influence each others metrics.

The ultimate aim of in vivo phenotyping is contribution to individualized dosing by explaining (in part) the variability in drug clearance. There are indications of the clinical relevance of in vivo phenotyping. Simvastatin dosing could be improved when CYP3A activity was taken into account [8]; accounting for the CYP3A phenotype in the dose calculation of irinotecan improved the predictability of the pharmacokinetic and toxicity profile [9], and midazolam CI was found to be highly correlated with sunitinib exposure and explained a large proportion in the observed interpatient variability in pharmacokinetics [10]. A second study reported a significant correlation between the metabolic ratio of midazolam and

sunitinib, but concluded that it did not predict variability in sunitinib clearance sufficiently to be useful in clinical dosing strategy [70]. Of these four studies, only the first study used a microdose for phenotyping [8]. Another study used a microdose cocktail to investigate the influence of renal impairment on the pharmacokinetics of the probes [71]. Lastly, microdosing could also be used to investigate time course of indication or inhibition [72, 73]. Taken together, in vivo phenotyping has the potential to beneficially influence pharmacological treatment.

The clinical application of microdose in vivo phenotyping for clinical dosing strategies is enabled by recent advances within the field of bioanalysis. Innovations made it possible to use relatively simple equipment, such as liquid chromatography tandem mass spectrometry (LC–MS/MS), for the quantification of extreme low plasma concentrations (Table 2) instead of accelerated mass spectrometry (AMS; Table 2). The advantages of LC–MS/MS over AMS are

**Table 2** Overview of the bioanalytical methods used for the quantification of drug concentrations in the support of in vivo phenotyping of cytochrome P450 enzymes at the microdose level

Compound	Matrix	Sample volume (µL)	Internal standard	Sample preparation	Quantification method	Run time (min)	LLOQ (pg/mL)	EMA/FDA <sup>c</sup>	Refs.
<sup>14</sup> C-caffeine	Plasma	190	Caffeine	PP	HPLC-AMS	30	5.21	No	[21]
Chlorzoxazone <sup>a</sup>	Plasma	500	<sup>3</sup> H <sub>2</sub> -chlorzoxazone	LLE	LC-MS/MS	5.5	2.5	Yes	[60]
Dextromethorphan <sup>a</sup>	Urine	100	n.r.	LLE	LC-MS/MS	n.r.	10	No	[33]
Glibenclamide	Plasma	200	n.r.	SPE	LC-MS/MS	n.r.	1	No	[33]
Lansoprazole <sup>a</sup>	Plasma	100	n.r.	LLE	LC-MS/MS	n.r.	10	No	[33]
Losartan	Plasma	1000	Candesartan	LLE	LC-MS/MS	n.r.	50	No	[74]
<sup>14</sup> C-midazolam	Plasma	190	Midazolam	PP	HPLC-AMS	30	5.75	No	[21]
<sup>14</sup> C-midazolam	Plasma	500	Midazolam	LLE	HPLC-AMS	NA	0.1 <sup>b</sup>	No	[31]
Midazolam <sup>a</sup>	Plasma	250	<sup>2</sup> H <sub>5</sub> -midazolam	SPE	UHPLC-MS/MS	2.5	0.05	Yes	[82]
Midazolam <sup>a</sup>	Plasma	1000	N-ethyloxazepam	LLE	Gas chromatography	NA	10	No	[78]
Midazolam	Plasma	750	Rosuvastatin	SPE	LC-MS/MS	6.5	5–200	No	[29]
Omeprazole	Plasma	300	Lansoprazole	LLE	LC-MS/MS	2.5	34	No	[26, 86]
Omeprazole	Plasma	100	<sup>2</sup> H <sub>3</sub> -omeprazole	PPE	LC-MS/MS	NA	10	Yes	[87]
Quinidine <sup>a</sup>	Plasma	500	<sup>3</sup> H <sub>2</sub> -Quinidine	PP	LC-MS/MS	4.5	5	No	[66]
<sup>14</sup> C-Tolbutamide	Plasma	190	Tolbutamide	PP	HPLC-AMS	30	5.84	No	[21]
Yohimbine	Plasma	25	<sup>13</sup> C <sup>2</sup> H <sub>3</sub> -yohimbine	LLE	LC-MS/MS	3	5	Yes	[57]
<sup>14</sup> C-warfarin	Plasma	500	Warfarin	LLE	HPLC-AMS	20	10 <sup>b</sup>	No	[31]
Warfarin	Plasma	200	n.r.	SPE	LC-MS/MS	n.r.	50,000	No	[33]
Verapamil <sup>a</sup>	Plasma	500	<sup>2</sup> H <sub>6</sub> -verapamil hydroxide	PP	LC-MS/MS	4.5	1	No	[66]

LLE liquid–liquid extraction, LLOQ lower limit of quantification, n.r. not reported, PP protein precipitation, SPE solid phase extraction

<sup>a</sup>Simultaneous quantification of metabolite

<sup>b</sup>Lowest measured concentration

<sup>c</sup>Validated according the guidelines of the European Medicines Agency (EMA) and/or US Food and Drug Administration (FDA)



the fast data acquisition, the independency of radioactive isotopes, and the higher accessibility in laboratories [74]. Another crucial aspect for clinical application is a suitable pharmaceutical formulation for microdose phenotyping test. At present, therapeutic formulations are adjusted, such as the dilution of intravenous infusions or dissolving and dilution or oral formulations. However, commercially available microdose formulations are desired to increase the reliability of the administered dose and to increase the accessibility of microdose phenotyping. Furthermore, patient burden of in vivo microdose phenotyping tests should be minimized. Limited sampling strategies could reduce the number of blood samples necessary for phenotyping as well as reducing the time spent at the clinic. Single-time concentrations or parent over metabolite concentrations could be investigated [75], or maximum posteriori Bayesian estimation using population pharmacokinetic models could be investigated [76, 77]. Moreover, oral administration would be preferable as well as less invasive sample collection methods, such as volumetric absorptive microsampling (VAMS) from a finger prick instead of venepuncture.

The current analysis has several strengths and limitations. First, the current narrative review represents a broad and comprehensive overview of the currently published literature regarding the application of microdoses to in vivo phenotyping of CYP enzymes. Moreover, the predefined evidence levels of sensitivity and scalability creates an objective measurement for the comparison of different phenotyping tests. However, owing to the narrative nature of the current analysis relevant citations could have been missed. The risk of missing literature was minimized by the inclusion of the snowballing procedure during literature selection. Lastly, narrative reviews carry a relative higher risk of bias in the selection and interpretation of the data. The risk of bias was minimized by including all the data used for the interpretation of the results presented here in the supplementary tables (Supplementary Tables 1, 2, and 3).

## 5 Conclusions

In this review we questioned whether microdoses could be used for in vivo phenotyping of CYP enzymes. On the basis of literature, in vivo phenotyping with microdoses is in its infancy. For most CYP enzymes, the use of microdoses for in vivo phenotyping cannot be recommended yet. For most probes, information is lacking regarding its sensitivity to changes in enzyme activity at the microdose level. However, for phenotyping of CYP2C19, a microdose of 100 µg omeprazole can be administered orally and the AUC<sub>0-24</sub> determined, while for CYP3A4 an oral 0.1–75 µg can be administered and the AUC<sub>∞</sub> or Cl calculated. Furthermore, a midazolam AUC<sub>0-10</sub> or midazolam AUC<sub>2-4</sub> can be

considered as metric. For clinical application, more studies are needed regarding the sensitivity of probes at the microdose level, the use of in vivo phenotypes in dosing strategies, limited sampling strategies, and less invasive sampling methods.

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## Declarations

**Conflict of interest** The authors have no competing interests to declare that are relevant to the content of this article.

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**Consent for publication** Not applicable.

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