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Abstract: Due to high basal interindividual variation in cytochrome P450 3A (CYP3A) activity and susceptibility to drug interactions, there has been interest in the application of efficient probe drug phenotyping strategies, as well as endogenous biomarkers for assessment of *in vivo* CYP3A activity. The biomarkers 4β-hydroxycholesterol (4βHC) and 6β-hydroxycortisol (6βHCL) are sensitive to CYP3A induction and inhibition. However, their utility for the assessment of constitutive CYP3A activity remains uncertain. We investigated whether endogenous plasma biomarkers (4βHC and 6βHCL) are associated with basal CYP3A metabolic activity in healthy subjects assessed by a convenient single-time-point oral midazolam (MDZ) phenotyping strategy. Plasma 4βHC and 6βHCL metabolic ratios (MRs) were analysed in 51 healthy adult participants. CYP3A activity was determined after administration of an oral MDZ microdose (100 μg). Simple linear and multiple linear regression analyses were performed to assess relationships between MDZ oral clearance, biomarkers and subject covariates. Among study subjects, basal MDZ oral clearance, 4βHC and 6βHCL MRs ranged 6.5-, 10- and 13-fold, respectively. Participant age and alcohol consumption were negatively associated with MDZ oral clearance ($p = 0.03$ and $p = 0.045$, respectively), while weight and female sex were associated with lower plasma 4βHC MR ($p = 0.0003$ and $p = 0.032$, respectively). Neither 4βHC nor 6βHCL MRs were associated with MDZ oral clearance. Plasma 4βHC and 6βHCL MRs do not relate to MDZ single-time-point metabolic phenotype in the assessment of constitutive CYP3A activity among healthy individuals.

It is well recognized that cytochromes P450 3A4 (CYP3A4) and CYP3A5 are important human drug-metabolizing enzymes with high interindividual variability in hepatic and intestinal activities. This is due to environmental, genetic, developmental, disease and seasonal control, including significant susceptibility to drug interactions [1–6]. Indeed, active CYP3A5 is genetically determined [7] while reduced CYP3A activity is associated with *CYP3A4*22* [8], and peroxisome proliferator-activating receptor alpha (*PPARα* rs4253728) [9] while increased CYP3A activity is linked with CYP oxidoreductase *POR*28* [10] polymorphism. Importantly, drug interactions such as those caused by enzyme inhibition with itraconazole and enzyme induction after rifampin treatment can result in a dramatic 400-fold range in CYP3A activity in human beings [11]. Furthermore, conditions including cirrhosis [12], chronic hepatitis C infection [13], critical illness [14], cancer [15,16] and kidney disease [17–19] are associated with reduced CYP3A activity. Given such wide differences in enzyme activity among individuals, there has long been interest in various methods to quantify *in vivo* CYP3A function.

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The most widely used and accepted method to assess CYP3A activity is to examine midazolam (MDZ) pharmacokinetics [20,21]. CYP3A phenotyping with MDZ has several advantages including rapid and specific elimination of CYP3A enzymes, sensitivity to a wide range of enzyme activity and ability to be administered orally or intravenously in the assessment of metabolism by first-pass organs. Conventional MDZ metabolic phenotyping strategy involves administration of 1–4 mg oral doses with sequential blood sampling over 24 hr. Variations of this approach to improve safety and practicality are the use of microdoses [22] and single-time-point [23] or limited sampling strategies [24].

Urinary 6β-hydroxycortisol (6βHCL) to cortisol metabolic ratio (MR) has had most widespread use as a non-invasive measure of CYP3A activity. Urinary 6βHCL MR is sensitive to both CYP3A induction and inhibition by drugs [25–28]. However, urinary 6βHCL MR is not solely dependent on CYP3A activity but also urinary elimination of cortisol and 6βHCL. Therefore, a minimally invasive index termed cortisol 6β-hydroxylation clearance has been proposed which requires analysis of both 6βHCL in urine and cortisol in plasma [26,29,30]. To our interest, the plasma 6βHCL to plasma cortisol MR has not previously been described as an alternative CYP3A activity metric.

Plasma 4β-hydroxycholesterol (4βHC) is an observed endogenous metabolite of CYP3A4-mediated cholesterol metabolism [31]. Induction and inhibition of CYP3A by

administration of anticonvulsants/rifampin and itraconazole increases [31,32] and decreases [33] plasma 4βHC concentrations, respectively. The MR of plasma 4βHC to total plasma cholesterol concentrations serves commonly as a measure of *in vivo* CYP3A activity [33,34].

The validity of urinary 6βHCL and plasma 4βHC MRs as CYP3A activity biomarkers in comparison with conventional MDZ phenotyping has been examined in several studies [27,28,34–38]. On balance, these reports have demonstrated that changes in urinary 6βHCL MR/6β-hydroxylation clearance and plasma 4βHC MR are correlated with alterations in MDZ pharmacokinetics. These findings indicate that urinary 6βHCL and plasma 4βHC have some utility in assessing alterations in CYP3A activity resulting from drug interactions. However, it is less clear whether these biomarkers are sensitive and capable of measuring constitutive CYP3A activity, which is known to have significant interindividual variability when determined as MDZ phenotype [23,34,36].

In this study, we compared convenient methods for assessing basal CYP3A activity in healthy subjects using plasma 4βHC and 6βHCL MRs and single-time-point MDZ microdose phenotyping.

Materials and Methods

Clinical protocol. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. This study was approved by the Human Subjects Research Ethics Board at Western University (Approval Number 18139). Informed consent was obtained from all individual participants included in the study. The study was conducted at the Centre for Clinical Investigation and Therapeutics, London Health Sciences Centre, London, ON, Canada. Subjects were deemed healthy for study inclusion according to physical examination, medical history and laboratory analysis. Beginning 1 week prior to study day, participants were asked to refrain from taking grapefruit or herbal products. Twenty-four hours prior to the commencement of the study, participants were also asked to refrain from caffeine, medication and alcohol consumption. After overnight fast prior to study day, baseline blood was obtained (~08:00) for analysis of plasma 6βHCL, 4βHC, cortisol and total cholesterol. Subjects were then administered 100 μg MDZ (Sandoz, Boucherville, QC, Canada) orally in water, and 3 hr thereafter, blood was obtained for measurement of plasma MDZ.

Genotyping. Single nucleotide polymorphisms (SNPs) associated with altered CYP3A activity were genotyped by TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA) for *CYP3A4*22* (rs35599367) and *CYP3A5*3* (rs776746), *PPARα* (rs4253728), and *POR*28* (rs1057868).

MDZ Quantification. Plasma samples were analysed for MDZ by liquid chromatography–tandem mass spectrometry (LC-MS/MS). MDZ and alprazolam standards were obtained from ThermoFisher Diagnostix (Waltham, MA, USA) and Toronto Research Chemicals (Toronto, ON, Canada), respectively. Plasma (500 μl) was spiked with internal standard (10 μl, 5 ng/ml alprazolam) and extracted with isopropyl ether. The organic layer was dried under nitrogen gas at 50°C and reconstituted in mobile phase. Analytes were separated by reverse-phase chromatography (Hypersil Gold, 50 × 5 mm, 5 μm; ThermoFisher, ThermoFisher Scientific, San Jose, CA, USA) using gradient elution with 0.1% formic acid in water and acetonitrile (Agilent 1200; Agilent, Santa Clara, CA, USA). The mass spectrometer (Thermo TSQ Vantage, ThermoFisher Scientific, San Jose, CA, USA) with heated electrospray ionization source was set in positive mode for the detection of MDZ and alprazolam with mass transitions 326.1 → 291.2 m/z and 309.0 → 280.9 m/z, respectively. The lower limit of quantification (LLOQ) for MDZ in plasma was 0.01 ng/ml. Assay bias and precision (CV %) were 11.8% and 6.8%, respectively.

6βHCL Quantification. Plasma samples were measured for 6βHCL using LC-MS/MS. 6βHCL and 6βHCL-D4 standards were purchased from Toronto Research Chemicals. Plasma (400 μl) was spiked with internal standard (8 μl, 8 ng/ml 6βHCL-D4), diluted with water and extracted using Oasis HLB plates (Waters, Milford, MA, USA). Analytes were separated and quantified using similar instrumentation and chromatography as above. 6βHCL and 6βHCL-D4 were detected in negative mode as formate adducts with mass transitions 423.1 → 313.3 m/z and 427.2 → 351.3 m/z, respectively. Calibration curves and quality control samples were prepared in Krebs–Henseleit bicarbonate buffer. The LLOQ for 6βHCL in plasma was 0.0625 ng/ml. Interday assay precision, as determined on 3 separate days, was 7.4%.

4βHC Quantification. Plasma concentrations of 4βHC were determined by picolinic acid derivatization and ultra-high-pressure liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS, Agilent 1290 coupled with Thermo TSQ Quantum Ultra) as described by Honda *et al.*, with modifications [39]. Plasma samples (50 μl) were spiked with internal standard (4βHC-D7; Avanti Polar Lipids, Alabaster, AL, USA) followed by saponification in 0.5 ml 1 M ethanolic KOH at 37°C for 1 hr. After the addition of 0.15 ml of water, sterols were extracted twice with 1 ml of hexane and the collected organic layers were allowed to evaporate to dryness at 80°C. Subsequently, 170 μl of a reagent mixture composed of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 μl) and triethylamine (0.2 μl) was added to each dried extract and incubated at 80°C for 1 hr to derivatize the sterols. The resulting mixture was extracted with hexane (1.5 ml) and the organic layer allowed to evaporate to dryness. The residue was reconstituted in mobile phase, and 20 μl was injected into the UHPLC-MS/MS. Standard curve samples containing 4βHC were prepared in Krebs–Henseleit bicarbonate buffer rather than plasma. Quality control samples contained 4βHC 5 ng/ml in Krebs–Henseleit bicarbonate buffer without added 4αHC. Analytes were separated on an Agilent Zorbax Eclipse Plus C18 column (100 × 2.1 mm, 1.8 μm) and gradient elution with mobile phases of 0.1% formic acid in water and 1:1 v/v acetonitrile: methanol. Under these conditions, retention times for 4αHC, 4βHC and 4βHC-D7 were 7.80, 8.10 and 8.00 min, respectively. Baseline separation was achieved between 4αHC and 4βHC. Analytes were detected in positive mode with mass transitions 635.4 → 146.5 m/z and 642.4 → 146.5 m/z for 4βHC and 4βHC-D7, respectively. The LLOQ for 4βHC in plasma was 2.5 ng/ml. Interday assay precision, as determined on 8 separate days, was 16%.

Cholesterol and cortisol quantification. Total cholesterol in plasma was measured by an enzymatic colorimetric method (Cholesterol E kit; Wako, Richmond, VA, USA), while total plasma cortisol levels were measured by ELISA (Cat. No. EA65; Oxford Biomedical Research, Burlington, ON, Canada).

Estimation of MDZ oral clearance. It has been previously reported that a single-time-point plasma sampling approach between 3 and 4 hr post-MDZ dose can be used to phenotype constitutive CYP3A activity [23]. We used plasma MDZ concentration *versus* time data from our

previous study of a healthy volunteer cohort administered oral MDZ microdose (100 μg) [40]. Linear regression analysis of total MDZ area under the plasma concentration–time curve (AUC) with the plasma concentration 3 hr post-dose was performed (Figure S1). This exercise yielded the following equation for MDZ exposure: MDZ AUC (ng/ml × min) = 8.591 $[MDZ]_{t = 3\ hr}$ + 0.112 (r^2 = 0.86, $p < 0.0001$), which was used to estimate MDZ AUC in the present study. MDZ oral clearance (CL/F) was calculated as dose divided by estimated MDZ AUC.

Statistical analysis. Univariate associations between participant demographics, CYP3A biomarkers and MDZ oral clearance were performed to obtain Pearson's correlation coefficients (ρ). We used the log-transformed values for CYP3A biomarkers, MDZ oral clearance and age to better describe the linear relationship between variables. Multiple linear regression was used to determine the contributions of demographic variables to biomarker MRs and MDZ oral clearance. Analyses were performed using R software (The R Project for Statistical Computing, www.r-project.org) and GRAPHPAD PRISM 5 software (GraphPad, La Jolla, CA, USA).

Results

Study cohort.

Fifty-one healthy participants (average age 28, 61% female) completed the study (table 1). The majority of subjects were Caucasian (57%). Average number of standard alcoholic beverages consumed per week was self-reported by study participants during health assessments. Forty-six of the 51 subjects consented for genetic analysis. Genotype frequencies for *CYP3A4*22*, *CYP3A5*3*, *PPARα* (rs4253728) and *POR*28* were within expected frequencies. No subjects were taking medications known to inhibit or induce CYP3A activity.

Table 1.
Study participant demographics.

Age (range)	28.2 (19–58)
Sex	
Male (%)	20 (39)
Female (%)	31 (61)
Weight (kg) (range)	71.1 (49.1–114.5)
BMI (kg/m^2) (range)	24.6 (19–36.5)
Ethnicity (% total)	
Caucasian	29 (57)
Asian/Pacific Rim	8 (16)
Middle Eastern	8 (16)
African	4 (8)
South Asian	1 (2)
Hispanic	1 (2)
Drinks/week (range)	3.1 (0–15)
Medication use (% total)	
Oral Contraceptives	14 (27.5)
Vitamin D	3 (6)
Iron Supplements	3 (6)
Multivitamins	3 (6)
Acetylsalicylic acid	1 (2)
Genotype[1] (Allelic Frequency, %)	
*CYP3A4*22*	5
*CYP3A5*3*	84
PPARα (rs4253728)	15
*POR*28*	26

[1]Genotype available for 46 of 51 participants.

Determinants of MDZ oral clearance and biomarker variation.

Modest, non-Gaussian interindividual variability was observed for each measure of CYP3A activity. Estimated MDZ oral clearance ranged 6.5-fold (45–292 l/hr, mean 112 l/hr; fig. 1A). Among subjects, plasma 4βHC MR ranged 10-fold (5.6–56.8 ng/mg, mean 17.7 ng/mg; fig. 1B), while plasma 6βHCL MR had 13-fold range (0.0021–0.0283, mean 0.081; fig. 1C). We explored whether each CYP3A activity measure was associated with participant demographics (age, sex, weight, BMI and ethnicity), alcohol consumption, medication use and relevant genotypes. With univariate analysis, increased weekly alcohol consumption was associated with decreased MDZ oral clearance ($\rho = -0.366$, $p = 0.008$; fig. 1A). Plasma 4βHC MR was associated with age and weight ($\rho = -0.307$, $p = 0.029$; $\rho = -0.468$, $p = 0.0005$, respectively; fig. 1B). No subject variables were correlated with plasma 6βHCL MR (fig. 1C). We did not find any association between *CYP3A4*22*, *CYP3A5*3*, *PPARα* (rs4253728) and *POR*28* alleles and endogenous biomarker MRs or MDZ oral clearance (table 2). Other considered variables did not associate with MDZ oral clearance, plasma 4βHC MR or plasma 6βHCL MR in univariate analyses.

Multiple linear regression analysis was performed with demographic covariates (age, sex, weight and alcohol consumption) to better understand the determinants of biomarker MRs and MDZ oral clearance. In the analysis of MDZ oral clearance, increasing age ($p = 0.030$) and alcohol consumption ($p = 0.045$) were associated with reducing MDZ oral clearance when sex and weight were adjusted in the model; 23.4% of the total variation in log MDZ oral clearance was explained by the four variables. For 4βHC MR, female sex ($p = 0.032$) and increased weight ($p = 0.0003$) were associated with reduced 4βHC MR when adjusted by age and alcohol (multiple $R^2 = 0.355$; table 3). There remained a lack of association between demographic variables and plasma 6βHCL MR after multiple linear regression modelling (table 3).

Correlation between MDZ oral clearance and biomarkers.

We found no significant relationships between MDZ oral clearance and plasma biomarker MRs (fig. 2A,B). Moreover, when 4βHC MR or plasma 6βHCL MR values were added to multiple linear regression models that included participant demographic variables, neither biomarker meaningfully increased the explained variation in MDZ oral clearance (Table S1). There was no relationship between plasma 4βHC MR and plasma 6βHCL MR (fig. 2C).

Discussion

In this study, we compared plasma biomarkers to MDZ metabolic phenotype, the probe test that is considered the gold standard for assessment of CYP3A activity. For this purpose, we used an orally administered MDZ microdose with plasma

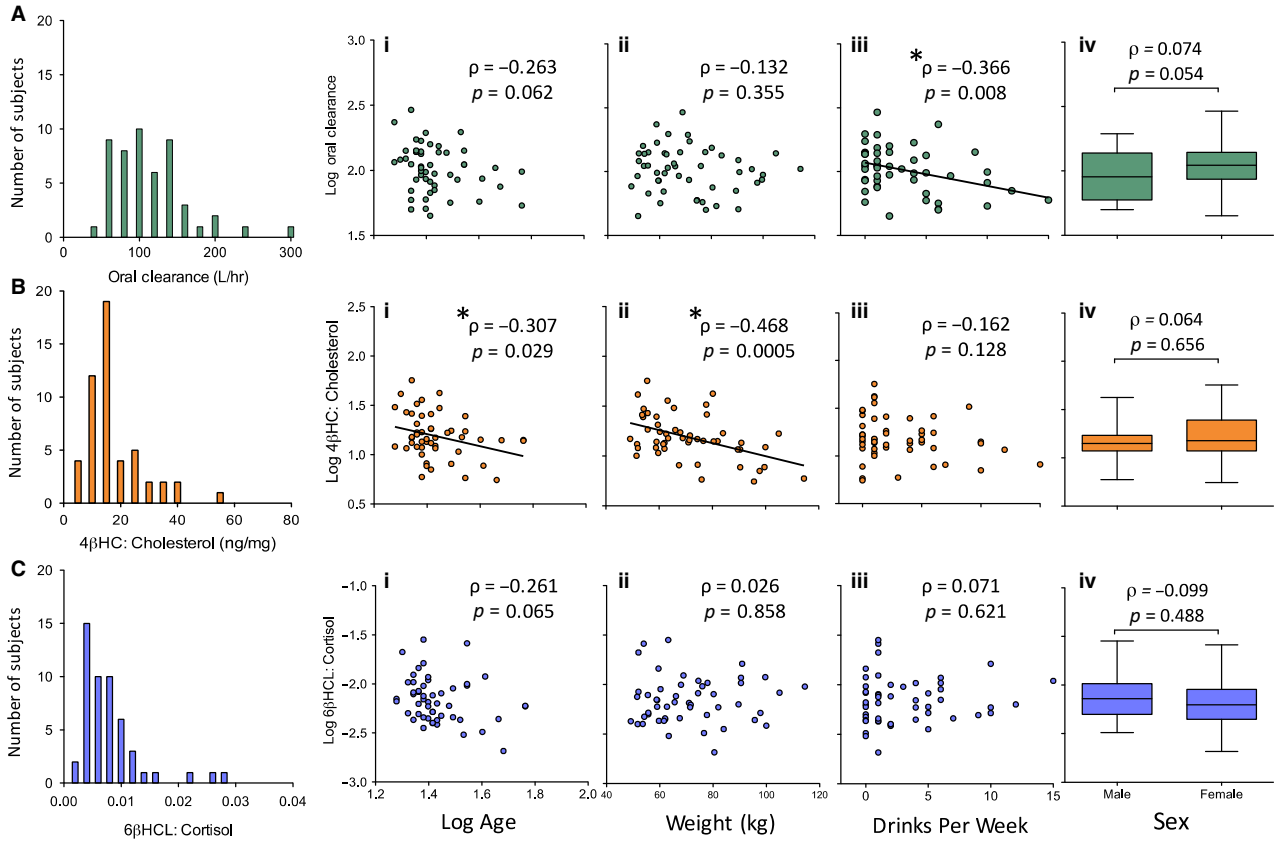

Fig. 1. Cohort distribution of CYP3A phenotype markers and association with demographic characteristics. Frequency distribution of (A) MDZ oral clearance, (B) 4βHC:cholesterol metabolic ratio and (C) 6βHCL:cortisol metabolic ratio in the study cohort (n = 51). (i–iv) Associations of log MDZ oral clearance, log 4βHC:cholesterol metabolic ratio and log 6βHCL:cortisol metabolic ratio with participant demographics including log age, weight, alcohol consumption and sex. (iv) Plots show median values (line), 25th to 75th percentile (box) and range (whiskers). CYP3A: cytochrome P450 3A; 4βHC, 4β-hydroxycholesterol; 6βHCL, 6β-hydroxycortisol; MDZ, midazolam; ρ: Pearson's correlation coefficient; *p*: *p*-value.

Table 2.
Relationships of gene variant carriers/non-carriers with corresponding MDZ oral CL or endogenous biomarkers.

	*CYP3A4*22*		*CYP3A5*[2]		*PPARα* rs4253728		*POR*28*	
Carrier status[1]	C	NC	Non-expresser	Expresser	C	NC	C	NC
n	5	41	32	14	14	32	22	24
MDZ Oral Clearance (l/hr) (±S.E.M.)	100 (±11)	116 (±8)	115 (±9)	110 (±12)	124 (±11)	110 (±9)	119 (±9)	110 (±11)
4βHC MR (ng/mg) (±S.E.M.)	11 (±2)	18 (±2)	16 (±1)	20 (±4)	16 (±2)	18 (±2)	18 (±2)	17 (±2)
6βHCL MR (±S.E.M.)	0.008 (±0.002)	0.008 (±0.0007)	0.008 (±0.0007)	0.008 (±0.002)	0.008 (±0.001)	0.008 (±0.001)	0.009 (±0.001)	0.006 (±0.0006)

[1]C, carriers (heterozygous + homozygous); NC, non-carriers.
[2]Non-expresser: *CYP3A5*3/*3*; expresser: *CYP3A5*3* heterozygous (n = 13) + *CYP3A5*3* non-carriers (n = 1).

exposure estimated using a single-time-point sampling strategy. The validity of this efficient approach is supported by the pharmacokinetic linearity of MDZ over a wide range of doses and the suitability of single-point sampling between 3 and 4 hr post-dose to predict MDZ AUC [23,41–43]. The lack of sedative effect also provides additional convenience and safety when compared to higher MDZ doses used in metabolic phenotyping. However, these advantages are counterbalanced by increased bias and reduced precision introduced by sparse sampling [24]. Our choice of oral *versus* intravenous MDZ phenotype should be considered in the interpretation of biomarker correlations. MDZ exposure after oral administration is

Table 3.
Multiple linear regression analysis of association of cytochrome P450 3A activity markers with subject demographics.

Model	Variable	Coefficient	*p*-Value
Log (MDZ Oral Clearance)	Log (age)	−0.476	0.030*
(R^2 = 0.234)	Sex	0.198	0.179
	Alcohol	−0.034	0.045*
	Weight	0.003	0.424
Log (4βHC MR)	Log (age)	−0.395	0.111
(R^2 = 0.355)	Sex	−0.369	0.032*
	Alcohol	−0.030	0.127
	Weight	−0.019	0.0003*
Log (6βHCL MR)	Log (age)	−0.538	0.081
(R^2 = 0.076)	Sex	−0.053	0.798
	Alcohol	0.006	0.787
	Weight	0.0008	0.900

*$p < 0.05$.

determined by both intestinal and hepatic CYP3A activity. It is generally considered that plasma 4βHC and 6βHCL levels result from metabolism in the liver. Significant endogenous 6βHCL formation may occur in the intestine because grapefruit juice, a well-known intestinal but not hepatic CYP3A inactivator, causes reduced urinary 6βHCL excretion [44]. At present, there is uncertainty regarding the contribution of intestinal CYP3A to plasma 4βHC concentrations. Nevertheless, oral MDZ phenotype was assessed in this study because we were motivated by the possibility that biomarkers could predict the pharmacokinetics of other orally administered, CYP3A substrate drugs.

For this study, we used plasma 6βHCL MR as an alternative to the traditional urinary biomarker analysis or 6β-hydroxylation clearance which requires both plasma and urine measurements. We anticipated that plasma 6βHCL MR would be less affected by factors influencing the excretion of 6βHCL or cortisol and potential intrarenal cortisol metabolism, which may alter assessment of cortisol 6β-hydroxylation activity. Given the known diurnal variation in plasma cortisol, plasma 6βHCL MR was assessed for all subjects in the morning (08:00). Imamura and colleagues recently reported a parallel diurnal variation in plasma 6βHCL levels [45], a finding that supports the validity of a simplified plasma 6βHCL MR analysis. Additional studies incorporating CYP3A induction and inhibition are required to fully assess the utility of plasma 6βHCL MR phenotyping approach.

The main findings of the present study are that plasma 4βHC and 6βHCL MRs are not associated with single-timepoint MDZ phenotype in healthy subjects at baseline. The current results with a cohort of moderate size (n = 51) confirm other studies reporting low correlations between plasma 4βHC MR and MDZ clearance after oral or intravenous administration in healthy subjects [34,38]. Moreover, the findings with plasma 6βHCL MR are consistent with the results of other reports that compared basal urinary 6βHCL MR with intravenous and MDZ oral clearance [36,46,47].

The lack of association between plasma biomarkers and MDZ oral clearance indicates potential differential contribution of factors regulating each biomarker level and MDZ pharmacokinetics. Indeed, we found that age, weight and alcohol consumption associate differently with the observed biomarker and MDZ exposures after univariate analyses (fig. 1). Moreover, results from multiple linear regression modelling indicate that age and alcohol were associated with MDZ oral clearance, while sex and weight related to 4βHC MR (table 3). Our findings are consistent with another report of reduced MDZ clearance in elderly compared to young men [48] while contrasting with another study that found no age-related effects [49]. The finding that alcohol intake has a negative association with MDZ oral clearance was surprising given that the amounts consumed were not expected to affect MDZ metabolism. In another study, decreased oral MDZ bioavailability together with a lack of alterations in systemic clearance was found in subjects who were moderate drinkers (14–21 drinks/week) [50]. The fact that plasma 4βHC MR is negatively correlated with weight (fig. 1C) is consistent with results from other studies [51,52]. Interestingly, there were no demographic variables that predicted 6βHCL MR albeit a previous report which showed that urinary 6βHCL MR decreases after the age of 50 [53].

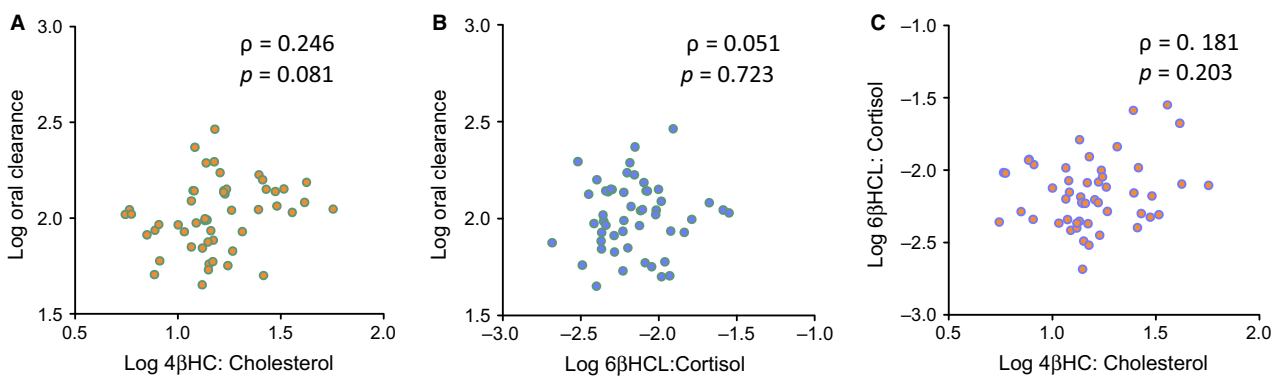

Fig. 2. CYP3A phenotype marker associations. Associations of (A) log 4βHC:cholesterol metabolic ratio and (B) log 6βHCL:cortisol metabolic ratio with log MDZ oral clearance. (C) Relationship between log 4βHC:cholesterol metabolic ratio and log 6βHCL:cortisol metabolic ratio. CYP3A: cytochrome P450 3A; 4βHC, 4β-hydroxycholesterol; 6βHCL, 6β-hydroxycortisol; MDZ, midazolam; ρ: Pearson's correlation coefficient; *p*: *p*-value.

In multivariable regression, but not in univariate analysis, we found that sex is a significant predictor of 4βHC MR, but unexpectedly females had lower values than males. This result differs from that of another study demonstrating higher plasma 4βHC levels in females [54]. Similarly, in multivariable regression analyses, we did not observe any association between sex and MDZ oral clearance or 6βHCL MR despite previous studies showing slightly increased MDZ clearance [55–57] and urinary 6βHCL MR [53] in women compared to men. While our ability to observe a sex difference in MDZ oral clearance may be attributed to the single-time-point microdose strategy, it is likely that our small sample size was a limiting factor. Indeed, it has been suggested that a sample size of 300 subjects would be necessary to adequately detect the subtle sex differences in oral MDZ pharmacokinetics [55]. It is interesting that with univariate analysis, we see a trend towards increased MDZ oral clearance in females ($p = 0.054$; fig. 1), indicating higher CYP3A activity.

With respect to genetic contributors to CYP3A phenotypes, we did not find any influence of *CYP3A4*22*, *CYP3A5*3*, *PPARα* (rs4253728) and *POR*28* alleles on endogenous biomarker MRs or MDZ oral clearance (table 2). It must be considered that the low numbers of subjects carrying less common variants (e.g. *CYP3A4*22*) and few with homozygous alleles (*CYP3A5*1/*1*) reduced the power to detect a genetic contribution for CYP3A activity measures. Indeed, the results differ from the observed impact of *CYP3A4*22* [58] and *POR*28* [59] on MDZ pharmacokinetics and CYP3A5 expresser status with plasma 4βHC MR [51,54]. However, the results are consistent with studies that, on balance, demonstrate a lack of effect of CYP3A5 expression with MDZ pharmacokinetics [60] and the absence of association of *PPARα* (rs4253728) and *CYP3A5*1* genotypes with urinary 6βHCL MR [61]. Two previous studies have shown that *CYP3A5* polymorphisms were not a relevant modulator of MDZ pharmacokinetics when studied at both microdoses and normal doses [43,62], and these findings are consistent with those that show a lack of *CYP3A5* genotype effects on MDZ pharmacokinetics at regular doses [60,62–65]. Therefore, we propose that our finding of a lack of association between *CYP3A5* genotype and MDZ oral clearance was not confounded by the low dose of drug administered. With respect to *CYP3A4*22*, its effects on MDZ pharmacokinetics have been described when the drug was administered at milligram doses [58,66]. It remains possible that the current MDZ microdose strategy may have affected the ability to detect an influence of *CYP3A4*22* on metabolic phenotype, but it is likely that the low number of subjects carrying this allele is the major contributing factor.

Participants in this study were racially diverse (table 1). We separately examined the relationships between 4βHC or 6βHCL MRs and MDZ oral clearance in the largest subgroup consisting of Caucasians (n = 29) and found a similar lack of correlations as we report in the analysis of the entire cohort. Furthermore, in examining only the Caucasian subgroup, similar relationships were found between demographic and genetic factors for each of the CYP3A activity measures after univariate and multivariable regression analyses. Therefore, the overall study findings did not differ after racial diversity factors were taken into consideration.

The relatively low interindividual range in basal CYP3A activity of the current subject cohort likely limited stronger correlations between the biomarkers and MDZ oral clearance. A 6.5-fold range in MDZ oral clearance was observed. In a previous study with a larger cohort of healthy participants, a 29-fold range in weight-normalized MDZ oral clearance was found [23]. Beyond constitutive CYP3A activity and especially towards the extremes of metabolism caused by potent enzyme induction and inhibition by drugs, both plasma 4βHC MR and urinary 6βHCL MR are functional metabolic metrics. However, their dynamic ranges are limited over the entire CYP3A activity spectrum, with 4βHC plasma level and urinary 6βHCL MR ranging approximately 20-fold [27,67,68] and 22-fold [28], respectively. This compares with 400-fold dynamic range seen with MDZ oral exposure [11]. Consequently, significantly larger sample sizes would be required to observe stronger correlations between biomarkers and MDZ metabolic phenotype when CYP3A variation is narrow [67]. From a practical perspective, the smaller dynamic range of the biomarkers has implications for their application in predicting therapeutic doses of CYP3A substrate drugs in patients who are not prescribed potent enzyme inducers or inhibitors.

In conclusion, given the observed lack of association of plasma 4βHC and 6βHCL MRs with single-time-point phenotyping with oral MDZ, the utility of these endogenous biomarkers for the assessment of constitutive CYP3A activity appears limited.

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Author contributions

Tirona and Woolsey participated in research design, performed data analysis and contributed to the writing of the manuscript; Woolsey, Beaton, Dresser, Gryn and Kim conducted the study; and Woolsey and Choi performed statistical analysis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Linear regression analysis of plasma MDZ concentration *versus* time in healthy volunteers (n = 19) administered an oral microdose of midazolam (100 μg).

Table S1. Multiple linear regression analysis of MDZ oral clearance with endogenous biomarkers and subject demographics.