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Original article

Estimation of cytochrome P-450 CYP1A2 activity in 863 healthy Caucasians using a saliva-based caffeine test

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A pronounced variability limits the usefulness of CYP1A2 phenotyping for drug therapy, for evaluating liver function, and for assessing the role of this enzyme in carcinogenesis. To identify and quantify sources of this variation, we estimated CYP1A2 activity in 863 healthy Caucasians using caffeine clearance derived from saliva concentrations before and 5–7 h after a caffeine test dose. Data from 786 individuals were eligible for evaluation (mean age 39 years, 415 women including 94 taking oral contraceptives, 401 non-smokers). Overall geometric mean (geometric SD) caffeine clearance was 1.34 ml min^{-1} kg b.w.$^{-1}$ (1.65). The effect of the following covariates was evaluated by analysis of covariance: age, sex, oral contraceptives, body height, body weight, body mass index, number of cigarettes smoked, tar exposure from smoking, several indices of dietary caffeine consumption, intake of sauerkraut, and country of residence (Germany, Bulgaria or Slovakia). Estimated changes relative to arbitrarily defined basal caffeine clearance (male, non-smoking, German resident) exerted by significant ($P < 0.05$) covariates were: coffee, 1.45-fold per litre of coffee drunk daily; body mass index, 0.99-fold per kg m^{-2}; smoking, 1.22-fold, 1.47-fold, 1.66-fold, and 1.72-fold for 1–5, 6–10, 11–20, and > 20 cigarettes smoked per day, respectively; oral contraceptives, 0.72-fold; country of residence, 0.81-fold and 0.74-fold for Bulgaria and Slovakia, respectively; female, 0.90-fold. These covariates explained 37% of overall variation. The 95% confidence interval of individual clearance was 0.46–2.20 times the predicted value. No relevant polymorphism was found for CYP1A2 activity when adjusted for covariate effects.

Keywords: cytochrome P-450 CYP1A2, phenotype, caffeine, reference values

Introduction

The rate at which xenobiotics including drugs are oxidized shows a wide interindividual variability. An approximately 10-fold activity range for cytochrome P450 enzymes is common in human liver microsomes from different donors (Böcker *et al.*, 1995, Rendic & Di Carlo, 1997). Clearance values of metabolically eliminated drugs differ accordingly among patients. Taking these pharmacokinetic characteristics into account by individual dosing is supposed to improve the risk-to-benefit profile in drug therapy. To this end, phenotyping for drug metabolizing enzyme activities by specific probe substrates gains increasing recognition. Phenotyping may be used in two ways: to determine the actual metabolic activity in an individual, and to identify and quantify sources of enzyme activity variation in large populations. Such data may in turn be used to predict biotransformation capacity and to individualize drug therapy without phenotyping each patient.

The human cytochrome P450 enzyme CYP1A2 is among those with pronounced inter-individual differences in activity (Balogh *et al.*, 1992; Butler *et al.*, 1992; Vistisen *et al.*, 1992; Sinha *et al.*, 1994; Schrenk *et al.*, 1998). This variability is reflected in the pharmacokinetics of drugs that are a substrate to CYP1A2,

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including theophylline, caffeine, acetaminophen, lignocaine, propranolol, propafenone, verapamil, imipramine, clozapine, tacrine and flutamide (Imaoka *et al.*, 1990; Woolf *et al.*, 1993; Fuhr *et al.*, 1996; Shet *et al.*, 1997). Additionally, human CYP1A2, together with CYP1A1, has the capability of activating most of the known procarcinogens (Rendic & Di Carlo, 1997). The clinical impact of this observation is supported by a correlation of CYP1A2 activity with human urinary bladder (Horn *et al.*, 1995) and colorectal (Lang *et al.*, 1994) cancer.

Tobacco smoke (Kalow & Tang, 1991a; Butler *et al.*, 1992; Vistisen *et al.*, 1992; Nakajima *et al.*, 1994; Schrenk *et al.*, 1998), grilled meat (Sinha *et al.*, 1994; Kall & Clausen, 1995), cruciferous vegetables (Vistisen *et al.*, 1992; Kall *et al.*, 1996) and drugs such as omeprazole (Rost *et al.*, 1994) and carbamazepine (Parker *et al.*, 1998) act as CYP1A2 inducers.

Many drugs, such as fluvoxamine (Brøsen *et al.*, 1993), quinolone antibacterials (Fuhr *et al.*, 1992), psoralens (Bendriss *et al.*, 1996), and oral contraceptives (Rietveld *et al.*, 1984; Abernethy *et al.*, 1985a; Vistisen *et al.*, 1992), to name a few, also ethanol (Rizzo *et al.*, 1997) and grapefruit juice and its components (Fuhr *et al.*, 1993), have an inhibitory effect on the enzyme. A lower CYP1A2 activity has been observed in women versus men at least in larger studies (Relling *et al.*, 1992; Vistisen *et al.*, 1992; Nakajima *et al.*, 1994; Carrillo & Benitez, 1996) and in patients with liver cirrhosis (Renner *et al.*, 1984; McDonagh *et al.*, 1991; Tanaka *et al.*, 1992; Denaro *et al.*, 1996). The liver is the major site for CYP1A2 expression in man and therefore, CYP1A2 activity might be a good test of functional liver mass (Cheng *et al.*, 1990; Fuhr, 1994; Jover *et al.*, 1997).

These characteristics of CYP1A2 and the availability of a non-toxic specific probe drug, i.e. caffeine (Kalow & Tang, 1993; Fuhr *et al.*, 1996; Rostami-Hodjegan *et al.*, 1996), make the enzyme a target of many phenotyping studies. In the apparent absence of a functionally relevant genetic polymorphism (Nakajima *et al.*, 1994; Sachse *et al.*, 1998), constitutional and environmental factors are the only assessed sources of CYP1A2 variability.

To date, the high variation in CYP1A2 activity and a lack of quantification for the effect of known contributors limit the usefulness of respective phenotyping procedures in epidemiological studies, in drug interaction trials and as a liver function tests. Thus, the objective of the present study conducted in 863 healthy Caucasian volunteers was to provide this quantitative estimation using a saliva-based caffeine test. We identified and quantified smoking habits, daily caffeine consumption, country of residence, intake of oral contraceptives by women, sex, and body mass index (BMI) as significant covariates of CYP1A2 activity. These findings may be used to predict individual CYP1A2 activity.

Materials and methods

The study was approved by the respective Medical Faculty Ethics Committees of the University of Frankfurt am Main and of the University of Cologne.

VOLUNTEERS

Participants were healthy as assessed on the basis of medical history and were aged ≥ 20 years. Defined exclusion criteria included a history of chronic or recent illness; intake of drugs (except oral contraceptives by women); intake of methylxanthines, grapefruit juice, broccoli or alcohol 12 h prior to and until the end of the investigation, regular consumption of more than 50 g of alcohol per day; vaccination, participation in another clinical trial or blood donation within the last 4 weeks; and pregnancy.

Trained staff interviewed the volunteers using a detailed questionnaire by which the following characteristics were documented additional to inclusion and exclusion criteria: sex, age, body height (as reported by the participant), body weight (measured), detailed smoking habits including tar content of cigarettes as labelled on the packages if available, average daily methylxanthine consumption, menstrual cycle history in women, exposure towards xenobiotics (e.g. occupational), diet on the previous day. Based on the reported daily intake of methylxanthine containing foods and beverages, daily caffeine intake was estimated according to its caffeine content reported in several internet websites which were mainly based on a United States Pharmacopeial Convention Inc. (1998). From this source, the following concentrations were applied: cola, 118 mg of caffeine per litre, coffee, 444 mg of caffeine per litre; cocoa, 42 mg of caffeine per litre; tea, 167 mg of caffeine per litre; and chocolate, 0.5 mg caffeine per gram of foodstuff.

STUDY PROCEDURE

The study was conducted in an ambulatory setting. If volunteers were eligible according to the inclusion and exclusion criteria, a pre-dose saliva sample of 2 ml was taken. Immediately thereafter, the caffeine test dose was administered as a cup of coffee prepared from 5 g of instant coffee (Nescafé Gold, Nestlé, Frankfurt, Germany). Actual caffeine doses varied between the product batches used and ranged from 129 to 154 mg (mean ± SD: 144 ± 7 mg). Within 5–7 h later (actual mean ± SD duration: 6.0 ± 0.3 h), the volunteers had to come back to the study site, and a second saliva sample was collected. Samples were stored at – 20 °C.

ANALYTICAL ASSAY

Caffeine and paraxanthine concentrations in saliva were estimated by high pressure liquid chromatography (HPLC) essentially as described (Fuhr & Rost, 1994). Briefly, a 200 µl aliquot was prepared by addition of 50 µl 20% trichloroacetic acid containing 100 mg/l of hydroxyethyltheophylline as the internal standard. After centrifugation, 25 µl of the supernatant were injected onto a Nucleosil 100 C18 reverse phase column (column dimensions 125 x 4 mm, 5 µm particle size, Macherey, Nagel, Düren, Germany) and eluted using a 4 mmol/l acetic buffer (pH 4.0) containing 1% of acetonitrile, 1% of methanol, and 1.6% of tetrahydrofurane (w/w). The initial flow was increased from 0.8 ml/min to 1.8 ml/min within 15 min. The samples were usually analysed in sets of 25 individuals each. At the beginning and end of each set seven calibration samples (void and 0.15–50 µmol/l of caffeine and paraxanthine) were placed. Unknowns were prepared and assayed in duplicate, with pairs of corresponding predose and postdose samples sorted once in ascending order according to the assigned volunteer number, and then again in reverse order. A quality control sample (0.5, 1.5, 5 or 15 µM of caffeine and paraxanthine) was measured every 10 unknowns. Work-up and/or measurement was repeated for the whole sample set if results of quality control samples were not appropriate, and for the unknown samples if duplicate measurements differed by more than 15%. Calibration was based on peak height ratios for ultraviolet absorption at 278 nm and data point weighting by the inverse of concentrations. Calibration and quality control samples were prepared from saliva of an individual with at least 3 days of methylxanthine abstinence which was checked for absence of caffeine and paraxanthine. Sample stability was confirmed with respect to freeze–thaw cycles and long-term storage at –20 °C for original samples, as well as to autosampler residence time for samples after work-up. Within-days precision (= coefficient of variation for repeated measurements) and accuracy (= mean difference between estimated and true concentration) was better than 10% in the concentration range of the calibration samples. Results of actual between-days precision and accuracy based on the quality control samples are shown in Table 1.

TREATMENT OF DATA

Calculations were made with the Microsoft Excel 7.0 spreadsheet software (Microsoft Corporation, Seattle, WA, USA), and statistical tests were carried out using SPSS 7.5 (SPSS Inc., Chicago, IL, USA). Caffeine clearance as an estimate of CYP1A2 activity *in vivo* was calculated using two different procedures:

The paraxanthine/caffeine ratio method

The molar concentration ratio of paraxanthine to caffeine measured in the saliva samples taken 5–7 h after a caffeine test dose was transformed to caffeine clearance values (Cl_{ratio}) essentially as described (Fuhr & Rost, 1994). We conducted a re-analysis of these data using cross-validation of different models. To this end, each clearance value was predicted separately based on the complete data set without this value. The assumption of a linear relationship between clearance and the saliva concentration ratio was confirmed. However, the intercept of the original equation describing the relationship between systemic caffeine clearance and the paraxanthine/caffeine ratio was recognized as negligible. Therefore, caffeine clearance was calculated using a simplified model according to equation 1:

$$Cl_{ratio} = C_{S,paraxanthine,\ postdose} \times C_{S,\ caffeine,\ postdose}^{-1} \times \Delta t_{postdose-predose}^{-1} \times 11.681 \quad (1)$$

where Cl = clearance (ml min^{-1} kg $b.w.^{-1}$); C = concentration (nmol/l); S, saliva; and t = sampling time (h).

The two-point method

The second calculation was based on caffeine concentrations only, determined in the predose ($C_{caffeine,\ predose}$) and the 5–7 h postdose ($C_{caffeine,\ postdose}$) saliva sample.

Table 1. Actual between-days performance of methylxanthine assay in saliva

Concentration in quality control samples	*15 µM*	*5 µM*	*1.5 µM*	*0.5 µM*
Paraxanthine				
Number of data points	88	89	82	86
Precision (%)	6.9	13.5	12.2	21.2
Accuracy (%)	5.4	–2.1	1.3	2.0
Caffeine				
Number of data points	89	90	79	84
Precision (%)	6.6	13.5	11.1	23.2
Accuracy (%)	2.2	–4.4	1.1	5.9

Precision, coefficient of variation for repeated measurements; accuracy, mean deviation of estimated from true concentration.

Measured saliva concentrations were converted to plasma caffeine concentrations by division by $f = 0.790$ (Fuhr *et al.*, 1993). Individual test doses were taken from the caffeine content in each batch of the coffee used as provided by the manufacturer and as confirmed by HPLC. These doses and the published volume of distribution for caffeine of 0.61 l kg b.w.$^{-1}$ (Benet *et al.*, 1996) were used to calculate fictional initial plasma caffeine concentrations $C_{\text{caffeine, 0}}$, assuming very rapid absorption of caffeine (Benet *et al.*, 1996; Liguori *et al.*, 1997) as in equation 2:

$$C_{\text{P, caffeine, 0}} = D \times \text{kg body weight}^{-1} \times Vd^{-1} + C_{\text{S, caffeine, predose}} \times f^{-1} \quad (2)$$

where C = concentration (nmol/l); P = plasma; D = dose (nmol); Vd = volume of distribution (l kg body weight^{-1}); S = saliva; f = saliva to plasma concentration ratio.

The change of logarithmic caffeine concentrations relative to the time difference between the predose and the postdose sample corresponds to the elimination constant, from which caffeine clearance was calculated as shown in equation 3, again taking the 0.61 l kg b.w.$^{-1}$ volume of distribution (Benet *et al.*, 1996) into account:

$$Cl_{\text{2-point}} = \ln(C_{\text{P, caffeine, 0}} \times C_{\text{S, caffeine, postdose}}^{-1} \times f) \Delta t_{\text{postdose–predose}}^{-1} \times Vd \quad (3)$$

where Cl = clearance (ml min^{-1} kg b.w.$^{-1}$); C = concentration (nmol/l); P = plasma; S, saliva; f, saliva to plasma concentration ratio; t = sampling time (h); and Vd = volume of distribution (l kg body weight^{-1}).

Non-compliance, i.e. intake of caffeine during the study period other than that provided by the investigator, was defined arbitrarily by two robust criteria: (i) a more than fivefold difference between clearance estimates obtained by the paraxanthine/caffeine ratio method and the two-point method; (ii) an increase rather than a decrease of fictional initial caffeine concentrations $C_{\text{caffeine, 0}}$ to $C_{\text{caffeine, postdose}}$ at the end of the sampling period

The data of individuals with suspected non-compliance were excluded from all further evaluations.

To compare the clearance estimates of both methods, a linear regression according to Pearson was calculated for logarithmic values.

Assuming a factorial model, logarithmically transformed clearance values from the two different methods were also used to identify sources of variation in CYP1A2 activity by separate analyses for the whole population, for Germans and for non-Germans. By analysis of covariance (ANCOVA) using the SPSS module 'general multivariate', we descriptively evaluated the effects of the following covariates on the dependent variables Cl_{ratio} and $Cl_{\text{2-point}}$.

Continuous covariates: body height (m); body weight (kg); body mass index (kg b.w. m^{-2}); daily tar exposure from smoking (mg); daily consumption of coffee (l), tea (l), cocoa (l), caffeinated soft drinks (l) or chocolate (g); daily caffeine consumption from coffee and cola only (mg); daily caffeine consumption from all sources (mg);

Nominal covariates: number of cigarettes smoked per day (for grouping see Table 2); age (grouped according to decades of age, see results section); intake of oral contraceptives (yes/no); sex; country of investigation; and intake of sauerkraut the day before the investigation (yes/no).

The evaluation was started with a complete model, taking all possible covariates and mutual interactions into account. Covariates without a significant contribution to overall variation ($P > 0.05$) were eliminated from the model. From partially overlapping covariates, e.g. dietary caffeine intake from all sources and coffee consumption, those with the more pronounced contribution on clearances variation were selected. The parameter estimates obtained for the remaining covariates were used to derive the following equation for prediction of individual clearance values (equation 4), corresponding to reference values for CYP1A2 activity:

$$\ln(Cl) = \text{intercept} + \text{slope}_1 \times F_1 + \ldots + \text{slope}_I \times F_I + V_1 + \ldots + V_j \quad (4)$$

where Cl = clearance (ml min^{-1} kg b.w.$^{-1}$); $F_1 \ldots F_I$ = significant continuous covariates; $V_1 \ldots V_j$ = indicator variables for significant nominal covariates).

The overall variance σ^2 corresponds to the mean sum of squares for the remaining error. Accordingly, the individual reference range for logarithmic caffeine clearance defined as its 95% confidence interval was approximated as the predicted logarithmic clearance $\pm$ ($\sigma\, t_{\text{n, 0.025}}$).

Possible differences of variance between subgroups were checked using Levene's test of equality of error variances.

For a descriptive approach to the distribution of logarithmic clearance data corrected for the influence of significant covariates, the following procedures were applied to ANCOVA residuals: normal distribution was first checked by the Kolmogorov–Smirnov test. The cumulative probability of residuals to be expected assuming normal distribution was then plotted versus its observed cumulative probability (P–P plot). Finally, a maximum likelihood ratio test was applied to examine

Table 2. Descriptive statistics for observed logarithmic clearance values (obs, mean ± SD if $n > 2$) as calculated from the paraxanthine/caffeine ratio in saliva (equation 1) compared to predicted individual logarithmic clearance values (pred) according to equation 5 and using parameter estimates shown in Table 4

Smoking class (cigarettes per day)	*1 (none)*			*2 (1–5)*			*3 (6–10)*			*4 (11–20)*			*5 (> 20)*			*All groups*		
	n	*obs*	*pred*	*n*	*obs*	*pred*	*n*	*obs*	*pred*	*n*	*obs*	*pred*	*n*	*obs*	*pred*	*n*	*obs*	*pred*
Germany																		
Men	208	0.29 ±0.39	0.30 ±0.16	16	0.59 ±0.34	0.53 ±0.14	22	0.87 ±0.29	0.74 ±0.19	32	0.92 ±0.33	0.84 ±0.16	10	0.95 ±0.55	1.04 ±0.20	288	0.44 ±0.46	0.43 ±0.28
Women without OCs	87	0.20 ±0.40	0.19 ±0.14	7	0.27 ±0.33	0.33 ±0.10	5	0.74 ±0.40	0.60 ±0.13	13	0.77 ±0.35	0.81 ±0.18	9	0.93 ±0.50	0.87 ±0.14	121	0.34 ±0.47	0.33 ±0.29
Women with OCs	49	–0.20 ±0.50	–0.18 ±0.09	7	–0.22 ±0.60	0.00 ±0.15	7	0.21 ±0.43	0.32 ±0.09	7	0.24 ±0.58	0.33 ±0.12	0	–		70	–0.12 ±0.53	–0.06 ±0.23
Bulgaria																		
Men	10	–0.07 ±0.38	–0.09 ±0.03	4	0.17 ±0.34	0.12 ±0.02	16	0.28 ±0.43	0.40 ±0.18	18	0.48 ±0.44	0.51 ±0.10	18	0.58 ±0.46	0.59 ±0.08	66	0.36 ±0.48	0.39 ±0.26
Women without OCs	34	–0.04 ±0.34	–0.13 ±0.07	36	0.02 ±0.53	0.08 ±0.07	35	0.26 ±0.40	0.28 ±0.06	60	0.38 ±0.42	0.43 ±0.08	21	0.58 ±0.34	0.51 ±0.09	186	0.24 ±0.47	0.24 ±0.24
Women with OCs	1	–0.88	–0.44	6	0.06 ±0.59	–0.25 ±0.05	5	–0.10 ±0.32	–0.03 ±0.05	7	0.31 ±0.80	0.14 ±0.04	2	0.55	0.12	21	0.11 ±0.63	–0.04 ±0.20
Slovakia																		
Men	4	–0.35 ±0.69	–0.13 ±0.07	5	0.42 ±0.23	0.13 ±0.11	0	–	–	0	–	–	8	0.21 ±0.35	0.41 ±0.09	17	0.14 ±0.49	0.20 ±0.24
Women without OCs	7	–0.22 ±0.37	–0.23 ±0.08	4	–0.02 ±0.32	0.03 ±0.07	0	–	–	1	0.71	0.47	2	0.30	0.40	14	–0.02 ±0.47	–0.01 ±0.27
Women with OCs	1	–0.18	–0.52	0	–		0	–	–	2	0.44	0.05	0	–	–	3	0.23 ±0.37	–0.14 ±0.34
All countries																		
Men	222	0.26 ±0.41	0.27 ±0.19	25	0.49 ±0.34	0.39 ±0.23	38	0.62 ±0.46	0.60 ±0.25	50	0.76 ±0.42	0.72 ±0.22	36	0.60 ±0.53	0.68 ±0.27	371	0.41 ±0.47	0.41 ±0.28
Women without OCs	128	0.11 ±0.40	0.08 ±0.20	47	0.05 ±0.50	0.12 ±0.12	40	0.32 ±0.43	0.32 ±0.13	74	0.45 ±0.43	0.50 ±0.18	32	0.66 ±0.44	0.60 ±0.20	321	0.26 ±0.47	0.26 ±0.27
Women with OCs	51	–0.22 ±0.50	–0.19 ±0.11	13	–0.08 ±0.60	–0.11 ±0.17	12	0.08 ±0.41	0.18 ±0.19	16	0.30 ±0.63	0.22 ±0.14	2	0.55	0.12	94	–0.06 ±0.56	–0.06 ±0.22
All countries/ individuals	401	0.15 ±0.45	0.15 ±0.24	85	0.15 ±0.51	0.16 ±0.23	90	0.42 ±0.48	0.42 ±0.25	140	0.55 ±0.48	0.55 ±0.24	70	0.63 ±0.48	0.63 ±0.25	786	0.30 ±0.50	0.30 ±0.31

OC, oral contraceptive.

whether the apparently normal distribution could better be explained by the sum of two or more normal distributions. The more complex model was favoured if the two-fold difference between the respective log-likelihood values exceeded $\chi^2_{3,0.05}$. These approaches were applied to the whole data set and also to separate strata grouped according to smoking habits (grouping as in Table 2, additional group: all smokers).

Results

Eight hundred and sixty-three healthy Caucasians were included in the study after they had given their written informed consent. In the specimens of 20 volunteers, concentrations of methylxanthines could not be measured reproducibly due to analytical interactions with other saliva components. Data from 24 individuals were not evaluated since five of them had incomplete data, nine retrospectively turned out not to have fulfilled the inclusion criteria and in 10 smoking habits could not be classified appropriately because they smoked tobacco other than cigarettes. Additionally, 35 volunteers showed evidence of non-compliance and were excluded from analysis. Thus, data from 786 individuals were eligible for evaluation. Among these, 479 lived in Germany, 273 in Bulgaria and 34 in Slovakia. Most of the German volunteers were well-trained participants in clinical studies from the volunteer panel of drug companies, whereas the remaining population investigated mainly consisted of medical personnel. Of eligible volunteers, 415 were female including 94 women taking oral contraceptives (see Table 2). With respect to smoking behaviour, the largest group was that of non-smokers comprising 401 individuals. The age distribution of the population was as follows: < 30 years, $n = 192$; 30–39 years, $n = 256$; 40–49 years, $n = 179$; 50–59 years, $n = 124$; > 59 years, $n = 35$. Mean ± SD coffee consumption was 0.46 ± 0.38 l per day, and the mean body mass index was 24.5 ± 3.7 kg m^{-2}.

Predose paraxanthine and caffeine concentrations of 2.52 ± 2.64 μmol/l and 2.87 ± 4.98 μmol/l (arithmetic mean ± SD), respectively, in eligible participants increased to 5.08 ± 2.06 μmol/l and 8.07 ± 4.73 μmol/l measured in the 5–7 h postdose sample. Overall geometric means (geometric SD) of caffeine clearance was 1.34 ml min^{-1} kg b.w.$^{-1}$ (1.65) for the paraxanthine/caffeine ratio method (Cl_{ratio}, equation 1, see Table 2) and 1.19 ml min^{-1} kg b.w.$^{-1}$ (1.89) for the two-point method ($Cl_{2\text{-point}}$, equation 3). The coefficient of correlation for the comparison between the two methods (Fig. 1) was 0.833.

Logarithmic clearance data were found to be a linear function of the significant covariates including BMI, number of cigarettes smoked per day, intake of oral contraceptives, daily coffee consumption, country of residence and sex for both of the two caffeine clearance estimates tested. For the paraxanthine/caffeine ratio method 37% of overall CYP1A2 variability was explained in the whole study population ($P < 0.0005$), whereas for the two-point method only 31% could be explained ($P < 0.0005$) by the two models, respectively. Covariate estimates (equation 4) were very similar for these two methods. Therefore, the paraxanthine/caffeine ratio method was used for all further considerations. Results of the ANCOVA are summarized in Table 3.

Height and body weight used as separate covariates, age and sauerkraut consumed 1 day prior to the investigation did not show significant effects. The combined estimate of daily caffeine consumption (coffee, cola, cocoa, tea and chocolate) correlated less to CYP1A2 activity than coffee intake alone. The effect of caffeine calculated from the daily use of coffee and cola together was not more pronounced than the one which coffee exerted alone. Since replacing number of cigarettes smoked per day by the daily estimated exposure to tar from these cigarettes (available for German and Slovakian smokers only) did not increase the fraction of CYP1A2 variation explained by the covariates, the

Table 3. Results of the analysis of covariance based on logarithmic clearance values calculated using the paraxanthine/caffeine ratio method (equation 1)

Source of variation	*Degrees of freedom*	*Mean sum of squares*	F	*Significance*
Intercept	1	1.198	7.465	0.006
Smoking habit classification	4	7.161	44.609	0.000
Country of residence	2	2.684	16.721	0.000
Gender	1	1.696	10.568	0.001
Intake of oral contraceptives	1	7.385	46.003	0.000
Body mass index	1	0.991	6.174	0.013
Daily litres of coffee drunk	1	12.825	79.891	0.000
Error	775	0.161	–	–

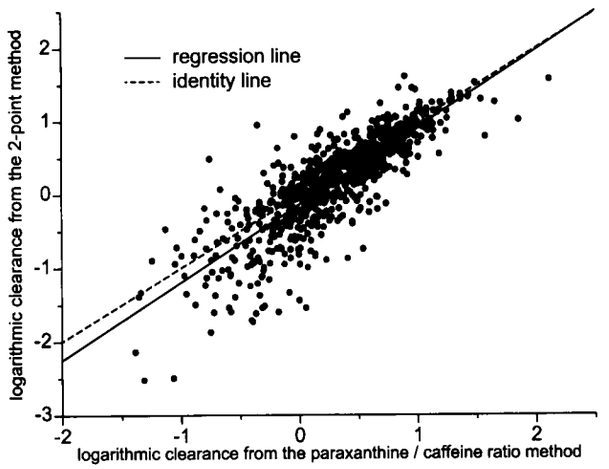

Fig. 1. Comparison of the two methods used for calculation of caffeine clearance. Linear correlation between the clearance estimates obtained by the paraxanthine/caffeine ratio method (Cl_{ratio}, equation 1) and the two-point method ($Cl_{2\text{-point}}$, equation 3).

former was used for the final evaluation. No interactions between the covariates were found. The remaining error not explained by the model was characterized by a variance of $\sigma^2 = 0.161$, from which the 95% confidence interval of 0.455–2.196 times the predicted value was calculated. This interval calculated from the individual covariate values corresponds to the individual reference range. The null hypothesis 'error variance of the logarithmic clearance is equal across groups' had to be rejected with $P = 0.004$ as the result of Levene's Test.

Applying equation 4 for the paraxanthine/caffeine ratio model, predictions for CYP1A2 activity could be made using the following equation:

$$\ln(Cl_{ratio}) = \text{intercept} + \text{slope}_{coffee} \times (\text{litres of coffee drunk daily}) + \text{slope}_{BMI} \times \text{BMI} + V_{\text{smoking habit index}} + V_{\text{country of residence index}} + V_{\text{oral contraceptive index}} + V_{\text{sex index}} \quad (5)$$

where Cl = clearance (ml min^{-1} kg b.w.$^{-1}$); $V_1 \ldots V_j$ = indicator variables.

Parameter estimates obtained in the ANCOVA for the whole population are shown in Table 4. Based on these parameter estimates, predicted individual data were compared to experimental data in Table 2.

When Germans and non-Germans were evaluated separately, 47% of the variation was explained in the German population and only 24% in the non-German group (using the paraxanthine/caffeine ratio model). However, the magnitude of the effects of covariates was similar in both populations.

One hundred and thirty-four of 786 volunteers (17.0%) were found to have a caffeine clearance lower than 0.86 ml/min/kg b.w., which was reported the

Table 4. Parameter estimates of covariates obtained for logarithmic clearance values using the paraxanthine/caffeine ratio method (equation 1)

Covariate	*Symbol used in equation 5*	*Estimate*	*95% Confidence interval*		*Mean resulting change of clearance (factor)*
			Lower bound	*Upper bound*	
–	Intercept	0.264	–0.015	0.542	–
Coffee intake (litre day^{-1})	Slope_{coffee}	0.368	0.287	0.449	1.445
Body mass index (kg m^{-2})	Slope_{BMI}	–0.010	–0.018	–0.002	0.990
Cigarettes/day					
Non-smokers	$V_{\text{smoking habit index}}$	0	–	–	Reference
1–5		0.195	0.065	0.324	1.215
6–10		0.383	0.253	0.509	1.467
11–20		0.504	0.386	0.621	1.655
>20		0.543	0.430	0.655	1.721
Oral contraceptives					
No	$V_{\text{oral contraceptive index}}$	0	–	–	Reference
Yes		–0.332	–0.236	–0.428	0.717
Country					
Germany	$V_{\text{country of residence index}}$	0	–	–	Reference
Bulgaria		–0.209	–0.356	–0.061	0.811
Slovakia		–0.303	–0.450	–0.156	0.739
Sex					
Male		0	–	–	Reference
Female	$V_{\text{sex index}}$	–0.111	–0.178	–0.044	0.895

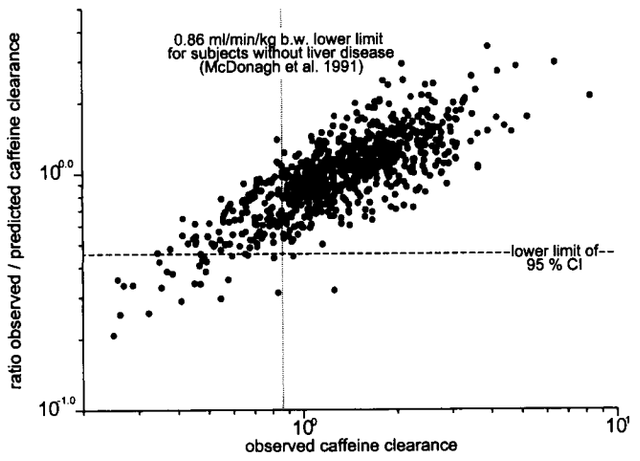

Fig. 2. Comparison of two methods to identify individuals with conspicuously low caffeine clearance. The antilog of residuals from the ANCOVA for logarithmic caffeine clearance values, corresponding to the ratio between observed and predicted clearance, was plotted against the clearance, both based on the paraxanthine/caffeine ratio method (equation 1). Dotted lines represent cut-off values to be used for the identification of individuals with conspicuously low CYP1A2 activity.

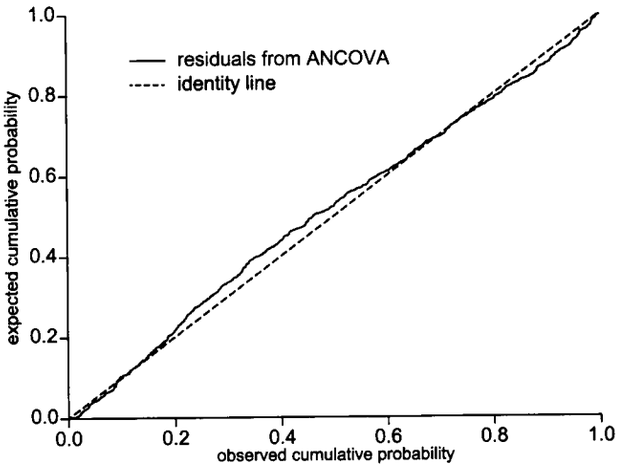

Fig. 3. *P–P* plot of ANCOVA residuals The distribution of residuals of logarithmic clearance values from ANCOVA based on the paraxanthine/caffeine ratio method (equation 1) in the whole data set (n = 786) is evaluated in this plot. Expected probability was calculated assuming normal distribution.

lower limit for healthy individuals without liver disease (McDonagh *et al.*, 1991). In contrast, 27 of the 786 volunteers (3.4%) were below the lower limit of the 95% confidence interval obtained in our model when the influence of the covariates was taken into account. The geometric mean for caffeine clearance in these 27 volunteers was 0.46 ml/min/kg b.w. and ranged from 0.25 ml/min/kg b.w. to 1.26 ml/min/kg b.w. Only two of these 27 indviduals had a caffeine clearance above 0.86 ml/min/kg b.w., whereas 109 of the 134 volunteers with clearance values below 0.86 ml/min/kg b.w. were above the lower limit of the adjusted 95% confidence interval (Fig. 2).

The assumed normal distribution of ANCOVA residuals from logarithmic caffeine clearance was not rejected by the Kolmogorov–Smirnov test, neither for the whole data set (P = 0.053) nor for the separate strata differing in smoking habits (P > 0.2 in all cases). Accordingly, the *P–P* plot which was almost identical for all groups did not support a clear-cut bi- or multimodal distribution of caffeine clearance, although histogram maxima were slightly decreased in favour of the extremes compared to normal distribution (Fig. 3). For the whole data set, but not for separate smoking strata, the maximum likelihood test showed that the overall distribution of residuals was best described by the sum of two separate normal distributions. These were characterized by means and SDs of $\mu_1 = 0.11$, $\sigma_1 = 0.28$ and $\mu_2 = -0.12$, $\sigma_2 = 0.47$, respectively, corresponding to a 1.26-fold difference between the groups for non-logarithmic clearance values. Some 52% of all observations were attributable to the first of the two normal distributions.

Discussion

The aim of the present study was to quantify the effect of known covariates and to assess possible additional covariates for CYP1A2 activity. To this end, we applied a saliva-based caffeine test in 863 healthy Caucasians from whom caffeine clearance could be evaluated in 786 volunteers. A linear regression model suggested that BMI, smoking, oral contraceptives, coffee consumption, country and sex are independently related to changes in CYP1A2 activity. Since effects of covariates on caffeine clearance have been repeatedly discussed (Grant *et al.*, 1983; Abernethy & Todd, 1985a; Kalow & Tang, 1991a; Kalow & Tang, 1993; Relling *et al.*, 1992; Vistisen *et al.*, 1992; Nakajima *et al.*, 1994; Horn *et al.*, 1995; Catteau *et al.*, 1995; LeMarchand *et al.*, 1997), we refer to these papers for further reading and limit our discussion to some new aspects.

COVARIATES

BMI

BMI was found as a new source of variability. We studied the effect of the latter since height and weight separately did not reach statistical significance, most probably due to the shared information with weight-normalized clearance (ml min^{-1} kg body $weight^{-1}$). For instance, a

lean individual with a BMI of 20 kg m^{-2} was estimated to have a 1.16-fold higher caffeine clearance than an obese individual with a BMI of 35 kg m^{-2}. This result is not supported by previous studies which found no difference in caffeine clearance between obese and lean individuals and thus indicated a negligible effect of obesity on caffeine metabolism (Abernethy *et al.*, 1985b; Caraco *et al.*, 1995b). No effect of obesity on drug clearance was also reported for theophylline (Gal *et al.*, 1978) and antipyrine (Abernethy *et al.*, 1981; Caraco *et al.*, 1995a). However, an increase in the volume of distribution for caffeine, theophylline and antipyrine, respectively, resulting in prolonged elimination half-lives in obese individuals was described by the same authors. This finding is difficult to explain in the light of the preferential distribution of these drugs in the extracellular space (Benet *et al.*, 1996) in accordance with their relatively low lipophilicity. From these considerations, it remains open to debate whether the small but significant effect of BMI in our study is caused by the simplified approach to estimate systemic caffeine clearance may also be sensitive to changes in V_d, or whether a large population as present in our study is required to detect a true BMI effect.

Smoking

The well-known dose-dependent inducing effect of smoking on CYP1A2 (Kalow & Tang, 1991a; Vistisen *et al.*, 1992; Kalow & Tang, 1993) was confirmed. We developed normal values for the following groups: non-smokers, 1–5, 6–10, 11–20, > 20 cigarettes per day. Polycyclic aromatic hydrocarbons are the major inducers of CYP1A2 activity found in cigarette smoke, but other components including nicotine, cadmium and certain pesticides may also contribute to this effect (Schein, 1995). Because tar and nicotine yields are highly intercorrelated with (Woodward & Tunstall-Pedoe, 1992) and tar is commonly accepted as an indicator of exposure towards toxic chemicals by smoking, we also investigated the effect of tar yields on caffeine clearance. The latter explained only a smaller proportion of CYP1A2 variability in comparison to the number of cigarettes smoked per day. This is in accordance with previous studies showing that machine-determined tar yields may be less representative indices of individual exposure to tobacco combustion products than the number of cigarettes smoked (Woodward & Tunstall-Pedoe, 1992; Coultas *et al.*, 1993). Poor self-reporting of brands (Coultas *et al.*, 1993) may also have contributed to this result.

Intake of oral contraceptives

The intake of oral contraceptives was related to a 1.39-fold lower caffeine clearance. Their inhibiting effect on caffeine metabolism is well documented (Patwardhan *et al.*, 1980; Rietveld *et al.*, 1984; Abernethy & Todd, 1985a). However, the magnitude of the effect differs from one study to another, most probably due to the lack of further specification concerning actual drug composition (depending on preparation and phase of menstrual cycle) and its pharmacokinetics, and duration of therapy. A correlation found between the content of oestrogens in oral contraceptives and inhibition of CYP1A2 supports the assumption that these are the main components affecting CYP1A2 activity (Balogh *et al.*, 1991; Meyer *et al.*, 1991). With respect to long-term effects of oral contraceptives on oxidative metabolism, it appears that after reaching a peak between the fourth and twelfth week, the changes diminish after 6 months (Meyer, 1990). For parameter estimation in the present study, we only took into account whether or not female participants were current users of oral contraceptives, because the number of women in possible subgroups was not sufficient for a more detailed analysis. However, the heterogeneity of exposure appears to be reflected by the highest variation in caffeine clearance in women with oral contraceptives (Table 2).

Daily coffee consumption

In accordance with Horn *et al.* (1995) and LeMarchand *et al.* (1997), daily coffee consumption (in litres) was related to a higher caffeine clearance; the size of the effect was similar in our and in these published studies. However, such an association was not found by Kalow and Tang (1991b) and Vistisen *et al.* (1992). All the previous studies were based on the consumption of coffee and/or tea expressed as daily number of cups. In a study conducted by Lelo *et al.* (1986), the number of drinks was a poor index of actual caffeine intake ($r^2 = 0.42$). Thus, we used a more detailed quantification of coffee intake and also tried to estimate caffeine intake from all relevant sources more precisely based on published food and beverage caffeine content (see method section). The exact mechanism why caffeine comsumption is correlated to caffeine clearance is still not clear. The existence of caffeine autoinduction of CYP1A2 was suggested from population studies (Horn *et al.*, 1995) and could be shown by increased liver microsome CYP1A2 activity and mRNA levels in rats on very high caffeine doses (Chen *et al.*, 1996; Goasduff *et al.*, 1996). Another hypothesis is that rapid oxidizers may consume more coffee because they metabolize it very quickly (Landi *et al.*, 1996). Surprisingly, in our study, caffeine intake calculated from daily intake of all methylxanthine containing foods and beverages explained a smaller fraction of in CYP1A2 variation than coffee consumption alone. This applied also to the effect of caffeine calculated from coffee and cola together. Recently, Liguori *et al.* (1997) found no difference in pharmacokinetics of caffeine from coffee or cola.

Thus, other substances in coffee besides caffeine may contribute to its inducing effect. Coffee beans are roasted at high temperatures and may therefore contain substances similar to the ones in grilled and pan-fried meat. The latter has a well-documented inducing effect on CYP1A2 (Sinha *et al.*, 1994; Kall & Clausen, 1995).

Country of residence

Country of residence was found as a significant covariate for caffeine clearance, with highest values in Germany. We did not expect this difference since all the participants were Caucasians and the study was conducted under the same conditions and by the same main investigators at all study sites. Race differences have been previously described (Grant *et al.*, 1983; Butler *et al.*, 1992; Relling *et al.*, 1992) but do not apply in our study. A possible explanation would be differences in dietary habits, including consumption of cruciferous and other vegetables, meat protein, alcohol and others that have been shown to influence CYP1A2 activity (Anderson *et al.*, 1979; Pantuck *et al.*, 1979; Vistisen *et al.*, 1992; Fuhr *et al.*, 1993; Kall & Clausen, 1995; Kall *et al.*, 1996; Landi *et al.*, 1996; Rizzo *et al.*, 1997). The low caffeine clearance found in Bulgaria and Slovakia also prompted us to investigate the effect of sauerkraut since this cruciferous vegetable is a major component of the Bulgarians' winter diet. In our study, sauerkraut had no effect. Regional differences in the tobacco type smoked may also contribute to country-specific findings (Butler *et al.*, 1992). Finally, the higher apparent dispersion of caffeine clearance data in non-Germans (Table 2) which was also found in the separate ANCOVA analyses for Germans and non-Germans may be attributed to a presumed poorer compliance, since most of the German participants were trained volunteers from clinical pharmacology units in pharmaceutical companies with experience in clinical studies. Clearly, further studies are needed to assess the background and the relevance of country-specific differences in apparent CYP1A2 activity.

Sex differences

Sex differences had a small effect in our study. This is in agreement with the results of other large studies (see Introduction) but was not found in small samples (Lang *et al.*, 1994; Schrenk *et al.*, 1998). Sex differences might not have reached statistical significance in these smaller populations because the interindividual variation from other sources was much larger than the sex related differences (Vistisen *et al.*, 1992).

Age

In adults, age had no significant effect on CYP1A2 activity in our study as in several other investigations with caffeine (Blanchard *et al.*, 1985; Relling *et al.*, 1992; Lang *et al.*, 1994; Catteau *et al.*, 1995; Landi *et al.*, 1996). In one study on clearance of the CYP1A2 substrate theophylline, age accounted for 31% of variability (Randolph *et al.*, 1986). However, this study was conducted in only 30 individuals compared with the 786 evaluated in this study. Therefore, it appears that there is no relevant influence of age on CYP1A2 activity in man.

Caffeine clearance and liver disease

Numerous studies have shown that caffeine clearance is decreased considerably in liver disease (e.g. Renner *et al.*, 1984; McDonagh *et al.*, 1991; Fuhr, 1994; Jover *et al.*, 1997). However, overlapping clearance ranges for healthy volunteers and patients were seen in most of these investigations. This is at least partly explainable by the covariates found in our study. From a statistical point of view, we were able to separate individuals with conspicuously low CYP1A2 activity, possibly related to liver disease, from those that have low CYP1A2 activity caused by the known effects of covariates (Fig. 2). Thus, it appears that adjusted 95% confidence intervals as reference ranges will have an increased specificity compared with the absolute cut-off value proposed by McDonagh *et al.* (1991). Clinical studies are required to evaluate this procedure.

Merits and limitations of this study

In the present study the paraxanthine/caffeine ratio in saliva (equation 1) proved to be fast, simple, relatively inexpensive and free of adverse effects. It had previously been shown to fulfil the recommended validation criteria (Kalow & Tang, 1993; Rostami-Hodjegan *et al.*, 1996), i.e. a close correlation with the in-vitro intrinsic caffeine clearance and the in-vivo systemic caffeine clearance (Fuhr & Rost, 1994; Fuhr *et al.*, 1996) as well as high specificity and sensitivity in computer simulations (Rostami-Hodjegan *et al.*, 1996). Additional support of the validity of the paraxanthine/caffeine ratio method (equation 1) comes from the close correlation of the results with those of the two-point method (equation 3) which in part are calculated from independent data. On the basis of previous studies (Kalow & Tang, 1991a; Balogh *et al.*, 1992; Butler *et al.*, 1992; Nakajima *et al.*, 1994; McQuilkin *et al.*, 1995; Carillo & Benitez, 1996) which did not show a pronounced intraindividual variability of caffeine clearance over periods of up to several weeks, we expect no relevant change of the phenotypic trait with time as long as covariates are unchanged, although long-term studies remain to be conducted.

However, different performances of the methylxanthine assay especially at low concentrations, caused by individual HPLC column characteristics and problems in pipetting of saliva for reasons of viscosity inconsistency

finally resulted in an unexpectedly poor assay precision (Table 1). With duplicate measurements of unknowns an improvement of precision by $2^{-0.5}$, corresponding to a 30% reduction in the coefficient of variation, is to be expected. As both paraxanthine and caffeine concentrations in saliva were higher than 1.5 µmol/L even in most predose samples, assay imprecision should account for approximately 10% of variation in caffeine clearance data.

The ANCOVA model used was based on the assumption that covariates exert a multiplicative effect on caffeine clearance and that variance is equal between groups. The latter was rejected by Levene's test. Inspection of variation of existing data (Table 2) however, suggests that this is no major limitation for the use of the model. Anyway, there is no alternative for quantification of covariate effects since nonparametric methods equivalent to ANCOVA do not exist. Thus, one has to bear in mind that the model used provides only an approximate description of the data.

The unknown degree of compliance is a further limitation of the study. It appeared as not feasible to conduct this large study under inpatient conditions for both practical and financial reasons. The ambulatory setting, however, does not preclude protocol violations. Clearance would be underestimated in cases with intermittent caffeine intake between the predose and the postdose sample. Vice versa, caffeine intake immediately before predose sampling would result in overestimated clearance because of saliva contamination with caffeine and resulting overestimation of $C_{caffeine,\,0}$. It is not known to which extent our arbitrary criteria for non-compliance indeed excluded non-compliant individuals. Additionally, incorrect self-reporting of coffee and alcohol consumption, brand and number of cigarettes smoked per day, regular use of medication, etc., cannot be excluded.

Finally, other environmental factors which we did not take into account such as the limitation of methylxanthine abstinence to 12 h predose only, dietary peculiarities, physical activity (Vistisen *et al.*, 1992), sleep deprivation (Kamimori *et al.*, 1992), and/or passive smoking (Horn *et al.*, 1995) may have influenced caffeine clearance additionally. We were aware of these limitations but decided to neglect them in the study design for feasibility reasons that apply to this study but also to the conduction of the phenotyping test for its anticipated applications in epidemiological studies or in estimation of liver function.

Despite these limitations, the remaining 63% of variability not explained by our model seems to be not fully attributable to its limitations and provides evidence for the existence of further unknown factors. These may also be genetic in nature although a genetic polymorphism with a pronounced effect on activity has not been found (Nakajima *et al.*, 1994), as identified intron mutations in the CYP1A2 gene might affect transcriptional efficacy or be linked to other CYP1A2 polymorphisms (Eaton *et al.*, 1995; Sachse *et al.*, 1998). A new cytochrome P450 enzyme of the CYP1 family, i.e. CYP1B1, was recently discovered, which has substrate specificity overlapping with CYP1A2 (Shimada *et al.*, 1997). Furthermore, a genotype combination for two other enzymes, i.e. CYP1A1 Ile/Val plus GSTM1*0, was associated with a more rapid apparent caffeine metabolism (McLeod *et al.*, 1997). Genetic differences of caffeine clearance may also be brought about by a different inducibility of CYP1 A enzymes (Landi *et al.*, 1996; Schrenk *et al.*, 1998). Finally, a gene–gene interaction with coordinate regulation of CYP1A2 and the UDP-glucoronyltransferase UGT1.6 (Bock *et al.*, 1994) has been reported.

Indeed, bi- or trimodal distributions of caffeine clearance, which have tentatively been related to genetic causes, have been reported and/or discussed by several authors (Butler *et al.*, 1992; Carrillo *et al.*, 1994; Rostami-Hodjegan *et al.*, 1996; Schrenk *et al.*, 1998). Although the polymorphisms observed may also be attributable to other confounders, mainly renal function polymorphisms when caffeine metabolism was estimated by urinary metabolite ratios (Tang *et al.*, 1994; Notarianni *et al.*, 1996; Rostami-Hodjegan *et al.*, 1996), we also found that the distribution of residuals from our model in the whole data set was best described by the sum of two normal distributions. This bimodality should apply to basal CYP1A2 activity and not to CYP1A2 inducibility since distributions were very similar in groups differing in smoking habits. The apparent polymorphism however, may as well be explained by non-specific deviations from normal distribution, e.g. by a minor skewness as seen in the *P–P* plot (Fig. 3) or by more general limitations of our model. Additionally, the two distributions identified in our sample have a substantial overlap, which would make it impossible to allocate individuals to possible underlying genotypes from caffeine clearance data only. Therefore, even if this bimodality is real, which would have to be confirmed by identification of its cause, it seems to be of minor importance for overall variation in caffeine clearance.

In summary, our results provide useful information for the application of CYP1A2 phenotyping. The quantitative estimation of the effect of covariates should enable a more precise prediction of the optimal individual dose of CYP1A2 substrates. It should allow a better comparability of CYP1A2 activity between different populations, thus reducing the number of individuals required in epidemiological studies. Our results are also expected to facilitate the identification of individuals with conspicuously low CYP1A2 activity related to liver disease.

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