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D. Schrenk · D. Brockmeier · K. Mörike
K. W. Bock · M. Eichelbaum

A distribution study of CYP1A2 phenotypes among smokers and non-smokers in a cohort of healthy Caucasian volunteers

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Abstract *Objective*: To analyse distributions of a urinary ratio of caffeine metabolites (MR_c) representative of cytochrome P450 (CYP) 1A2 activity in a cohort of Caucasian German healthy volunteers and to re-assess the effects of smoking and oral contraceptives on the range and type of MR_c distribution.
Methods: A cohort of volunteers comprising 192 individuals (96 males, 96 females) was divided into subgroups according to smoking and/or use of oral contraceptives. The CYP1A2 substrate caffeine was administered, and urine was collected for 6 h and analysed for representative caffeine metabolites. Distribution of a CYP1A2-dependent MR_c was analysed using cumulative distribution (probit) plots and Rosin-Rammler-Sperling-Weibull (RRSW) functions.
Results: Cumulative distribution curves for males, and females, without further subgrouping for smoking habits and/or oral contraceptive steroid (OCS) consumption, showed slightly higher MR_c values, i.e. slightly higher CYP1A2 activities, in males. Significantly higher MR_c values were found in smokers of both sexes than in non-smokers. The distributions among female non-smokers or smokers with and without OCS were nearly superimposible, however. For the two male subgroups, the sum of two RRSW functions resulted in a better adjustment to the data than a unimodal skewed distribution. A weak correlation between MR_c and the number of cigarettes smoked per day was found.
Conclusion: The inducing effect of smoking on CYP1A2 activity was confirmed, whereas no significant inhibitory effect of oral contraceptives was observed. The finding that the data are compatible with bimodal distributions in non-smokers suggests a significant impact of genetic factors on MR_c. Among smokers, data were also compatible with bimodal distributions, i.e. with the existence of a "non-responder" phenotype concerning CYP1A2 induction by compounds present in tobacco smoke.

Key words Caffeine metabolism, Cigarette smokers

D. Schrenk (✉) · K.W. Bock
Institute of Toxicology, University of Tübingen,
Wilhelmstrasse 56,
D-72074 Tübingen, Germany
Tel. +49 7071 2974945; Fax +49 7071 292273;
e-mail: dieter.schrenk@uni-tuebingen.de

K. Mörike · M. Eichelbaum
Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology,
Auerbachstrasse 112,
D-70341 Stuttgart, Germany

D. Brockmeier
Clinical Research Department H 840,
Section of Biometry, Hoechst AG,
D-65926 Frankfurt am Main, Germany

Introduction

Both genetic and environmental factors influence the activities of a number of drug metabolising enzyme activities in man. For the cytochrome P450 (CYP) family of mono-oxygenases, which play a predominant role in phase I of drug metabolism, these factors have been studied extensively. For example, genetic polymorphism has been shown to be the basis of dramatic differences in CYP2D6 expression [1]. Lack of CYP2D6 activity is correlated with the 'poor metaboliser' phenotype, showing a lower rate of metabolism of a number of clinically important drugs [2]. Much work has also been carried out with regard to inter-individual variations in expression of members of the CYP1A gene family. CYP1A2, the principal CYP1A isozyme in human liver [3, 4], catalyses the metabolism of a number of drugs such as the *O*-deethylation of phenacetin [5]. Furthermore, CYP1A2 is the major catalyst in the metabolic activation of carcinogenic aromatic amines, such as 2-naphthylamine or 4-aminobiphenyl [6], it specifically catalyses the *N*-3-demethylation of caffeine to paraxanthine [7–9], and it is involved, at least in part, in the *N*-1- and *N*-7-demethylations of caffeine [9].

The use of caffeine as a test drug for non-invasive assessment of CYP1A2 avtivity has also been established

[10]. For this purpose, urinary caffeine metabolites are determined by means of high-performance liquid chromatography (HPLC) some hours after ingestion of coffee or after administration of pure caffeine. Both the ratio of the sum of caffeine metabolites formed in a CYP1A2-dependent manner versus the caffeine metabolite 1,7-dimethyluric acid (17U) [10, 11], and the ratio of 1,7-dimethylxanthine + 17U versus unmetabolised caffeine [12] are in use.

A well-established factor inducing CYP1A2 activity is cigarette smoking [10–12]. Other factors which have been shown to induce caffeine metabolism in humans are extensive physical exercise [11], the use of the anti-ulcer drug omeprazole [13] and a diet rich in cruciferous vegetables [11]. The use of oral contraceptive steroids (OCS) was reported to decrease CYP1A2-dependent metabolic activities [14–15] in humans. A similar steroid action on CYP1A2 activity is thought to underlie the reduced caffeine clearance during pregnancy [16].

In a previous report [17], a urinary metabolic ratio for CYP1A2-catalysed caffeine metabolism was studied, in a cohort of 192 healthy Caucasian volunteers from Germany, showing only results for males. In the present study, the results for females including users of OCSs are also shown. Furthermore, distribution analysis was performed showing that bimodal distributions correlated well with the data obtained from smokers and non-smokers of both sexes, suggesting the existence of other than smoking-related, e.g. genetic and/or dietary, factors influencing CYP1A2 activity.

Materials and methods

Subjects

A random population sample of 192 Caucasian volunteers (96 males, 96 females), essentially identical to that described previously [17], were recruited for the study by public announcement. They were determined healthy by means of medical history, a physical examination and laboratory tests, paying particular regard to plasma creatinine levels. The mean age was 29 years (range 18–58 years) in all subjects: 30 years in males (range 18–58 years) and 29 years in females (range 18–55 years). The mean body weight was 67 kg (range 47–100 kg) in all subjects: 73 kg in males (range 60–100 kg) and 64 kg in females (range 47–82 kg). The volunteers were not taking any drugs, with the exception of OCS. Exclusion criteria were pregnancy, liver or kidney disease, or an allergy to caffeine. Smokers (37 males) specified their daily cigarette consumption. Of the female subjects, 43 were smokers and/or users of OCS. The protocol was approved by the ethics committee of the Robert Bosch Hospital, Stuttgart, and each subject gave written, informed consent.

Experimental protocol

After voiding their bladder at 15.00 hours, the subjects took 200 mg caffeine (one tablet Coffeinum 0.2 g, Comprette, Cascan) and collected their urine for 6 h in a bottle containing 50 ml 1 mol·l^{-1} sodium citrate-HCl buffer. The pH was adjusted to 3.0 to avoid conversion of 5-acetylamino-6-formylamino-3-methyluracil (AFMU) to 5-acetylamino-6-amino-3-methyluracil (AAMU). Furthermore, the participants were instructed to keep the urine cool until transfer to the laboratory, where aliquots were stored at −20 °C until analysis. The use of caffeine, caffeine-containing food or beverages or alcohol-containing beverages was not allowed from the evening prior to the test until the end of urine collection.

Analysis of urine metabolites

Caffeine metabolites were analysed as described by Campbell et al. [10]. The following caffeine metabolites were identified by spiking with the standard compounds: AFMU, 1U (1-methyluric acid), 1X (1-methylxanthine), and 17U. The ratio of [AFMU + 1X + 1U]/17U was used as an index for caffeine oxidation. Paracetamol was added as internal standard before the extraction step.

The excreted amount of each metabolite was calculated from the corrected area of the respective peak (using Waters baseline software), the amount of urine analysed and the total urine volume. Repeated analyses of a subgroup of 15 urine samples revealed an analytical accuracy of at least 96%. The intraindividual variation of the results was tested with four volunteers who repeated the test. The intraindividual difference did not exceed 12% for a single metabolite of interest and 18% for metabolic ratios for caffeine (MR_c).

Data analysis

MR_c values were sorted into those of females and those of males. Females were further subdivided into groups of subjects who neither smoked nor used OCS (non-smoker/non-OCS), those who did not smoke but used OCS (non-smoker/OCS), those who smoked but did not use OCS (smoker/non-OCS) and those who smoked and used OCS (smoker/OCS); males were subdivided into groups of smokers and non-smokers. Cumulative frequencies $F(MR_c) = n/N$ (with n = number of subjects with a MR_c equal to or smaller than a certain value, and N = total number of subjects) were plotted versus MR_c. Presenting the data as cumulative frequency was decided on purpose because it has many advantages over the simple histograms. Cumulative frequency curves, in terms of a step function, show all individual data clearly. Each increase of the step function by one probability unit ($1/N$) represents a single individual. If several individuals have the same MR_c, this is revealed by a step height of more than one probability unit which, in most cases, can be read easily from the graph. In contrast, histograms require pooling of many data into one class, with a resulting loss of individual data. Furthermore, histograms require subjective selection of the class width and the class boundaries, leading to pronounced variability.

Differences between groups were tested with the Kolmogorov-Smirnov test at $\alpha = 0.05$. Additionally, cumulative frequencies were depicted as probit plots for untransformed MR_c values. A sum of Rosin-Rammler-Sperling-Weibull (RRSW) functions [18, 19] was adjusted to the cumulative frequency data:

$$F(MR_c) = \sum_j a_j \cdot (1 - e^{-\{\lambda_j \cdot (MR - \tau_j)\}^{\beta_j}})$$

where a_j = fraction of the total number of subjects assigned to the j-th distribution; λ_j = a scaling factor; β_j = slope factor; and τ_j = dislocation of the RRSW function on the MR axis.

This adjustment was carried out for each subgroup separately. The HOEGIP-PC software [20], according to the least-square principle [21], was used for fitting. Additionally, each adjustment was performed with β_j fixed to 3.57, for which the RRSW function reached a good approximation to the normal distribution function [22]. To support the appropriateness of model selection, Akaike's information criterion (AIC) [23] was calculated for each adjustment. Furthermore, fitting results were compared using the F-test with the quotient of the mean sum of squares (MSQ) computed for the respective pairs.

From the adjustments, non-central moments m_1 and m_2 were calculated from these mean values (x_j) and standard deviations (s_j) as follows [24]:

$$m_1 = \Gamma(1+\frac{1}{\beta_j}) \cdot \frac{1}{(\lambda_j)} \qquad m_2 = \Gamma^2(1+\frac{1}{\beta_j}) \cdot \frac{1}{(\lambda_j)^2}$$

$$x_j = m_1 + \tau_j \qquad s_j = m_2 - (m_1)^2$$

where j, β_j and τ_j have the meaning as described above and $\Gamma(u)$ is the gamma function. MR_c values as a function of the number of cigarettes consumed per day were subjected to linear regression analysis for males and females separately.

Chemicals

AFMU was a generous gift from Dr. W. Kalow, Department of Pharmacology, University of Toronto, Canada. 1U, 17U, 1X, and 1,3,7-trimethylxanthine (caffeine), used as HPLC standards, were from Sigma (Taufkirchen, Germany). HPLC-grade chloroform and methanol were from Baker (Gross-Gerau, Germany).

Results

MR_c data of caffeine oxidation were available from 192 subjects: 96 males and 96 females. Among the males, 59 were non-smokers and, among the females, 54 were non-smokers/non-OCS users, 20 were non-smokers/OCS users, 12 were smokers/non-OCS users, and 10 were smokers/OCS users. Among the individuals tested, a broad range of MR_c values for caffeine oxidation was observed, namely between 2.3 and 14.0 in males, and between 2.2 and 11.5 in females. Figure 1 shows the probit plot of data for males and females separately. When comparing the two cumulative distribution curves for males and females, without further subgrouping for smoking habits and/or OCS consumption, the difference was statistically significant showing higher MR_c values in males. This was also true when male and female non-smokers were compared (not shown). Since the probit plot revealed curvilinearity with at least two linear phases, the data were analysed in detail taking into account the factors 'smoking' and 'use of OCSs'.

The two cumulative frequency distributions for male smokers and male non-smokers from this study have already been published [17]. The difference between the two curves was significant, showing higher ratios for smokers (Fig. 2A). In Fig. 2B, the cumulative frequency distributions for the four subgroups of females are shown. Significant differences between the cumulative distribution curves for female non-smokers/non-OCS versus smokers/non-OCS, as well as non-smokers/OCS versus smokers/OCS were obtained. In contrast, neither the difference between female non-smokers/non-OCS and non-smokers/OCS, nor the difference between female smokers/non-OCS and smokers/OCS was significant. A further detailed analysis (not shown) indicated that the type of OCS used might have an influence on the outcome, whether or not the use of OCS affect MR_c. It was found that among the 20 non-smokers/OCS-users, those ($n = 11$) using monophasic OCS had a MR_c of 3.8 (0.8) and those ($n = 9$) using either triphasic or cyproterone acetate-containing OCS had a MR_c of 4.7 (1.2).

A single RRSW function and the sum of two and three RRSW functions with the slope parameters β_j fixed to 3.57 (quasi-normal distribution) were adjusted to the cumulative frequency data. The result of adjustment

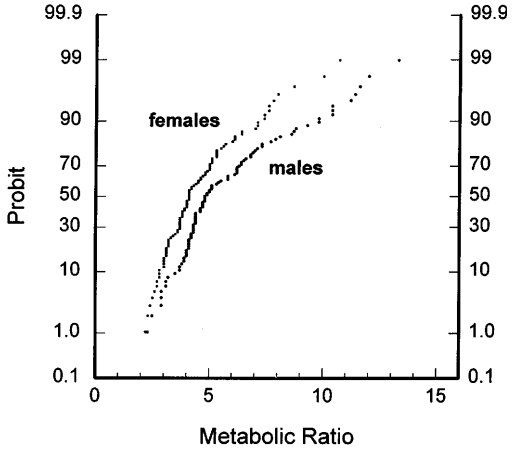

Fig. 1 Probit plots of the metabolic ratio of caffeine oxidation in healthy German female and male volunteers

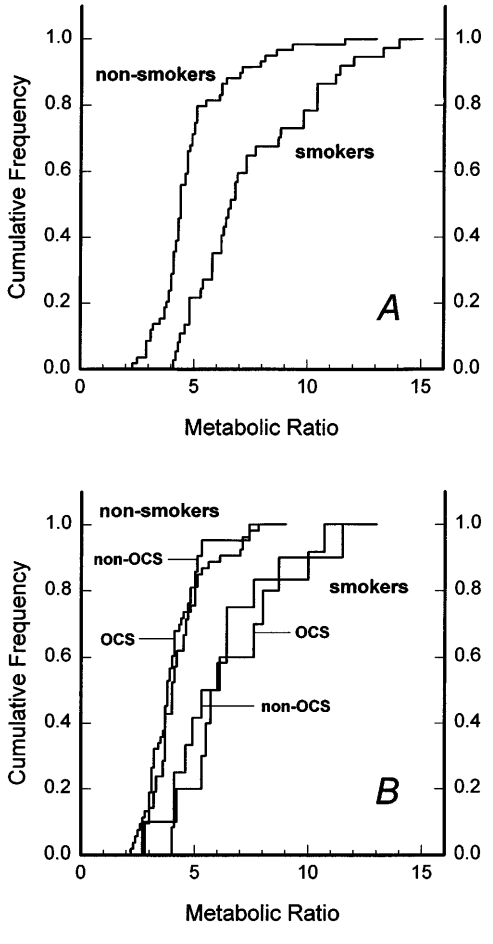

Fig. 2 Cumulative frequency distributions of the metabolic ratio of caffeine oxidation in male non-smokers and smokers (**A**), and in female non-smokers/non-OCS, non-smokers/OCS, smokers/non-OCS and smokers/OCS (**B**)

with a single RRSW function was unacceptable in each case. A reasonable adjustment was obtained when the sum of two RRSW functions was used as model function. No further significant decrease in the MSQ was obtained when the number of RRSW functions was increased to three, except in the case of the data for male smokers. For the sake of comparison, the adjustment using two RRSW functions is also presented in the latter case. When the slope parameters β_j were not fixed to 3.57, the MSQ decreased slightly but not significantly, indicating that the assumption of normally distributed populations is reasonable.

Adjustment of data for male non-smokers and smokers (Fig. 3A, B) using two RRSW functions with the slope parameters β_j fixed to 3.57 (quasi-normal distribution) revealed two distinct distributions. Additionally, the graphs show the according density functions for the two cumulative RRSW functions. In Fig. 3C and D, the results of adjustment of data for female non-smokers and smokers are shown, again with β_j fixed to 3.57. In both instances, two distinct distributions could be calculated. The similarity of the two density functions for male and female non-smokers with respect to their ratio and dispersion is apparent. The similarity is not as clear when the density functions for male and female smokers are compared. The results of adjustment are summarised in Table 1. Adjustments using logarithmic MR_c values also led to two distinct distributions in all four cases (not shown). Using uni-model skewed distribution as a model (not shown), the chosen model (two normal distributions) was significantly better for the two male subgroups, but not for the female subgroups.

Table 1 Adjustment of Rosin-Rammler-Sperling-Weibull functions to the metabolic ratios of caffeine oxidation (cumulative frequency data of Fig. 2) in non-smokers and smokers. *SD* standard deviation

	Males	Females
Non-smokers		
Fraction 1	0.74	0.80
Mean 1	4.23	3.67
SD 1	0.56	0.78
Fraction 2	0.26	0.20
Mean 2	6.39	6.16
SD 2	1.99	1.60
Smokers		
Fraction 1	0.73	0.59
Mean 1	5.91	5.05
SD 1	1.30	1.18
Fraction 2	0.27	0.41
Mean 2	10.75	7.88
SD 2	1.06	2.45

Figure 4A and B shows the results of linear regression analysis of MR_c versus the number of cigarettes consumed per day (according to questionnaire data) for both males and females. For both subgroups, coefficients of determination of $r^2 = 0.228$ for males and $r^2 = 0.309$ for females were obtained, suggesting a weak correlation of both parameters. Omitting the non-smokers from the analysis did not strengthen the correlation.

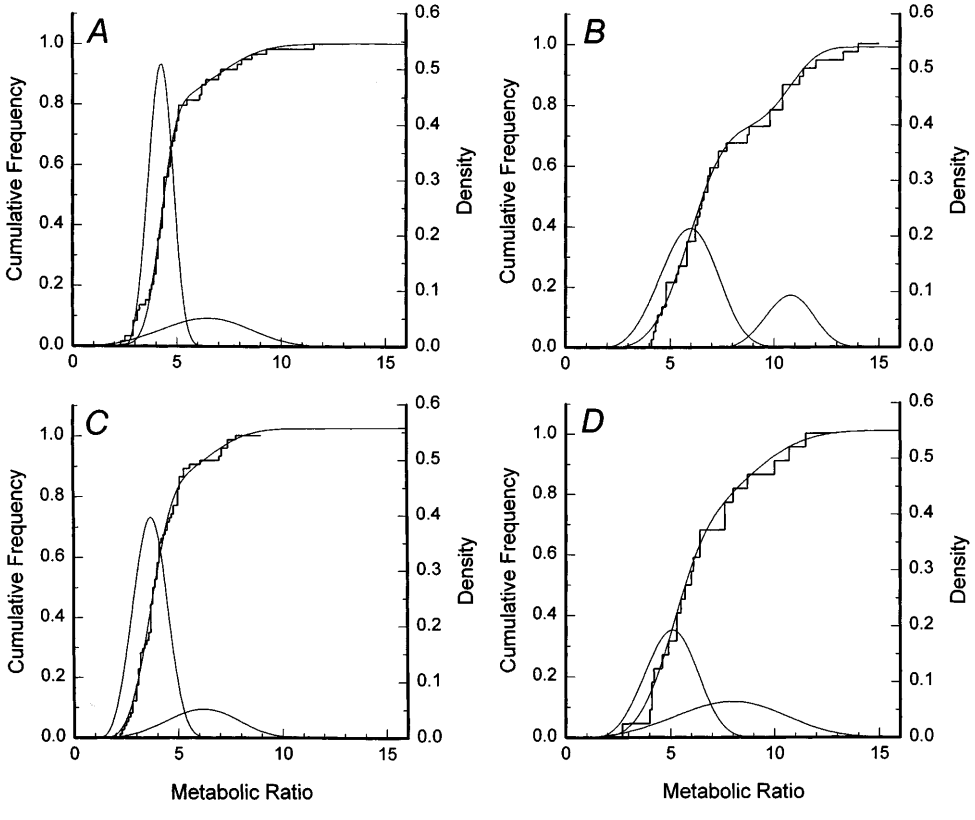

Fig. 3 Rosin-Rammler-Sperling-Weibull distribution analysis of metabolic ratio of caffeine oxidation in male non-smokers (**A**), male smokers (**B**), female non-smokers (**C**) and female smokers (**D**)

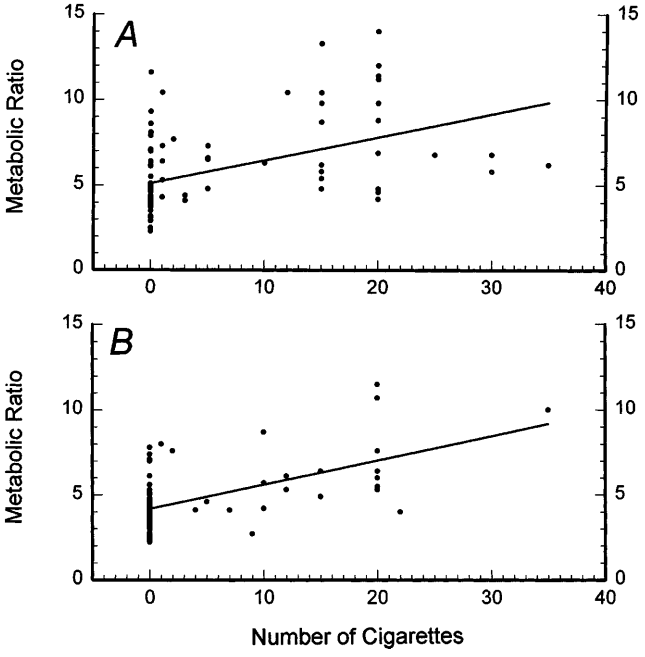

Fig. 4 Linear regression analysis of metabolic ratio of caffeine oxidation versus number of cigarettes consumed per day (according to questionnaire data) in male (**A**) and female (**B**) German volunteers

Discussion

In this report, data are presented from an assay of CYP1A2 activity in vivo in which caffeine was administered orally, urine was collected for 6 h after dosing and caffeine metabolites were analysed by HPLC. The urinary MR_c [AFMU + 1X + 1U]/17U, which mainly reflects CYP1A2 activity [10], varied sixfold among male subjects and fivefold among female subjects, and is similar to results previously reported by Kalow and Tang [25].

In agreement with other studies [11, 25], cigarette smoking significantly enhanced MR_c values, probably resulting from polycyclic aromatic hydrocarbons and related CYP1A2-inducing compounds present in tobacco smoke. Likewise, smoking has been demonstrated to stimulate phenacetin metabolism [26], is associated with increased amounts of immunoreactive CYP1A2 in human liver microsomes [5] and leads to decreased plasma half-lives of caffeine in vivo [27]. The number of cigarettes smoked per day was weakly correlated to the MR_c data as previously reported by others [11].

Distribution analysis was in agreement with the existence of slow and intermediate CYP1A2 phenotypes in a population of healthy Caucasians of both sexes. In previous reports [11, 25], distribution analysis was based on logarithmic MR_c values, whereas for the distribution analysis presented here, non-logarithmic MR_c values were used. The change in the slope of the cumulative frequency curve while MR_c values increase does not depend, however, on whether MR_c values are untransformed or log-transformed; these changes are evident in both presentations. The percentage of individuals with apparent intermediate/high MR_c values was 26% among male non-smokers and 20% among female non-smokers. The proportion of individuals with the apparent low MR_c phenotype ranged from 74–80% in both sexes.

Distribution analysis of MR_c values from smokers was also consistent with a bimodal distribution. As in non-smokers, bimodality was evident for both untransformed and log-transformed MR_c values. In addition to a large group of smokers with high MR_c values, a considerable number of smokers showed low to intermediate ratios, an observation also made previously by Vistisen et al. [11]. It remains to be elucidated whether this subgroup represents non-responders or is exposed to lower doses of inducing tobacco smoke constituents.

The bimodal normal-distribution model showed a better fitting to the data for the two male subgroups, but not for the female subgroups, compared with a unimodal skewed distribution. This result finally leaves the question open whether more than one subpopulation of the MR_c phenotype exists among male and/or female Caucasian non-smokers or smokers, respectively. The question of the validity of MR_c values has generated much debate; in particular, the issue of multimodal distribution has not been solved, since no convincing genetic basis for multimodality has been provided. In a recent paper, Rostami-Hodjegan et al. [28] pointed out that the sensitivity of the ratios to confounders is, in some cases, greater than their sensitivity to the activity of the enzyme that they are intended to mark. The MR_c values used in the present study were found not only to be closely related to CYP1A2, but also to vary directly with CYP2A6 activity. Furthermore, the authors found that this ratio, being insensitive to urine flow, was the most discriminant of bimodality [28].

The existence of a genetic polymorphism concerning inducibility of CYP1A2 has been suggested by Butler et al. [12]. The authors used the urinary molar ratio of [17X + 17U]/unmetabolised caffeine in the untransformed mode as a parameter for CYP1A2, obtaining apparent tri-linear probit plots for non-smoking populations from Arkansas, Italy and the People's Republic of China. The pros and cons of the use of the urinary amount of unmetabolised caffeine as part of the [17X + 17U]/unmetabolised caffeine ratio have been discussed in detail [12, 29]. The finding of a bimodal distribution of this ratio was confirmed for smokers and non-smokers in a Japanese cohort [30, 31]. However, the authors could not determine any differences in nucleotide sequence of the CYP1A2 gene clearly correlated with the bimodality of the phenotype including exons, exon–intron junctions or the 5′-flanking region. The ratio used in these papers was reported not to correlate with that used in the present study [32] ([AFMU + 1X + 1U]/17U).

A deficiency of CYP1A2-catalysed phenacetin *O*-deethylase was described by Devonshire et al. [33] to occur in about 10% of populations, and a genetic polymor-

phism for CYP1A2 has been suggested by a family study in which impaired phenacetin-*O*-deethylation ability was noted in two siblings [34]. However, the findings of this study were not confirmed by a larger, more recent study [35] using the same ratio ([AFMU + 1X + 1U]/17U) as in the present study. Other distribution patterns using this ratio have also failed, so far, to show clear multimodality, while data from smokers gave some hints that more than one subpopulation may exist [25, 36].

From animal and in vitro experiments, there is strong evidence that inducers present, for example, in cigarette smoke, bind to the aryl hydrocarbon (Ah) receptor which, upon ligand binding, forms a heterodimeric nuclear transcription factor with the Ah receptor nuclear translocator (ARNT) [37, 38]. Binding of the heterodimer to regulatory sequences in the 5′-flanking region of susceptible genes, such as the human CYP1A2, then results in their enhanced transcription [38]. In Ah receptor-deficient 'knockout' mice, both basal expression of CYP1A2 in liver and its inducibility by the potent inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) were diminished [39]. In human hepatocytes from six different donors, considerable differences in the efficacy of TCDD as an inducer of CYP1A activity were obtained [40], suggesting the possible existence of a polymorphism of TCDD inducibility in man. In a previous study [17] in the same population as that analysed here, a good correlation between MR_c and paracetamol glucuronidation as a functional parameter for a TCDD-inducible UDP-glucuronosyltransferase (UGT) was found. A number of heavy smokers exhibited both low MR_c values and low paracetamol glucuronidation, also indicating a lack of Ah receptor-mediated induction. It remains to be elucidated whether the different degrees of induction are related to polymorphic forms of the Ah receptor [41] and/or ARNT. The inhibiting effect of oral contraceptives on oxidative caffeine metabolism in non-smokers reported by various authors [11, 14, 15] was not significant in our study. It remains to be elucidated whether an impact of the type of contraceptive used, i.e. monophasic versus triphasic contraceptives, is valid for representative numbers of individuals and, if so, what the underlying mechanisms are. An apparent lack of inhibition was also found for a cyproterone acetate-containing contraceptive. Therefore, it appears reasonable in future studies to assess the influence of the type of contraceptive on MR_c values in a larger group of OCS users.

In conclusion, our findings show that the distribution of the untransformed [AFMU + 1X + 1U]/17U ratio, mainly reflecting CYP1A2 activity, is in accordance with a bimodal distribution model among both non-smokers and smokers in a representative Caucasian population from Germany. Since slightly 'better' fitting of a skewed unimodal model was found in females, a final decision on the distribution of MR_c values cannot be made based on our data only, but requires an additional hypothesis and additional data concerning the role of either genetic or epigenetic factors. The effects of oral contraceptives on oxidative caffeine metabolism may depend on the type of contraceptive used. Distribution of MR_c values is indicative of the existence of subgroups of responders and non-responders to inducers in cigarette smoke and/or in the diet. The most likely explanation for this finding, a genetic polymorphism of responsiveness of the Ah receptor signalling pathway, requires further investigation.

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