Heritability of Caffeine Metabolism: Environmental Effects Masking Genetic Effects on CYP1A2 Activity but Not on NAT2

J Matthaei[1], MV Tzvetkov[1], J Strube[1], D Sehrt[1], C Sachse-Seeboth[1], JB Hjelmborg[2], S Möller[2], U Halekoh[2], U Hofmann[3], M Schwab[3,4,5], R Kerb[3] and J Brockmöller[1]

Heritability of caffeine pharmacokinetics and cytochrome P450 1A2 (CYP1A2) activity is controversial. Here, we analyzed the pharmacokinetics of caffeine, an *in vivo* probe drug for CYP1A2 and arylamine N-acetyltransferase 2 (NAT2) activity, in monozygotic (MZ) and dizygotic (DZ) twins. In the entire group, common and unique environmental effects explained most variation in caffeine area under the curve (AUC). Apparently, smoking and hormonal contraceptives masked the genetic effects on CYP1A2 activity. However, when excluding smokers and users of hormonal contraceptives, 89% of caffeine AUC variation was due to genetic effects and, even in the entire group, 8% of caffeine AUC variation could be explained by a CYP1A1/1A2 promotor polymorphism (rs2470893). In contrast, nearly all of the variations (99%) of NAT2 activity were explained by genetic effects. This study illustrates two very different situations in pharmacogenetics from an almost exclusively genetic determination of NAT2 activity with no environmental modulation to only moderate genetic effects on CYP1A2 activity with strong environmental modulation.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ CYP1A2 activity can be measured *in vivo* as caffeine demethylation. CYP1A2 activity is relevant in drug treatment and toxicology but it is controversial how much of this activity is heritable.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ The heritability of human CYP1A2 activity is controversial. Specifically, it is unknown how much of the variation in CYP1A2 activity measured via caffeine pharmacokinetics is genetically or environmentally determined. In addition, the effects of single nucleotide polymorphisms discovered in genome-wide studies on caffeine pharmacokinetics are unknown.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

☑ Exposure to cigarette smoke and/or hormonal contraceptives can have a dominating effect on CYP1A2 activity. However, in the absence of such strong environmental modulators, CYP1A2 has a strong genetic component. NAT2 activity measured from caffeine metabolites showed extremely high heritability.

HOW THIS MIGHT CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS

☑ Estimates about heritability may strongly depend on environmental exposure and accordingly on sample selection. With all CYP1A2 metabolized drugs in individualized medicine, sex, smoking, hormonal contraceptives, and rs2470893 should be taken into consideration.

Caffeine pharmacokinetics and variation in the cytochrome P450 enzyme 1A2 (CYP1A2) have been extensively studied since the late 1970s.[1–3] It is a long-standing controversy, how much of the variation of human CYP1A2 activity is genetically determined.[4–9] This is medically relevant because activity of CYP1A2 determines the pharmacokinetics of theophylline, clozapine, and numerous other drugs.[10] In addition, CYP1A2 contributes to the detoxification or bioactivation of several (pro)carcinogens from tobacco smoke, several foods, and other endogenous or exogenous sources.

About 95% of oral caffeine clearance is mediated by CYP1A2 catalyzed demethylation.[11] Therefore, caffeine is frequently used as an *in vivo* probe drug for CYP1A2 activity.[12–14] Other enzymes also contribute to the further steps in caffeine biotransformation. Specifically, the minor caffeine metabolites (5-acetylamino-6-amino-3-methyluracil [AAMU] and 5-acetylamino-6-formylamino-3-methyluracil [AFMU]) are used to quantify *in vivo* the activity of arylamine N-acetyltransferase 2 (NAT2).[15–17] In addition, CYP2A6 and xanthine oxidase are involved in caffeine metabolism.[18–20]

Environmental factors are known to strongly affect caffeine biotransformation. The total caffeine clearance was about two-fold higher in persons smoking 30 cigarettes per day compared with nonsmokers[4] and was even about 10-fold higher after dioxin

[1]Institute for Clinical Pharmacology, University Medical Center, Göttingen, Georg-August University, Göttingen, Germany; [2]Department of Epidemiology, Biostatistics and Biodemography, University of Southern Denmark, Odense, Denmark; [3]Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology and University of Tübingen, Stuttgart, Germany; [4]Department of Clinical Pharmacology, University Hospital Tübingen, Tübingen, Germany; [5]Department of Pharmacy and Biochemistry, University of Tübingen, Tübingen, Germany. Correspondence: J Matthaei (johannes.matthaei@med.uni-goettingen.de)

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Table 1 Summary statistics of the demographic data

	Total	Sex		Age, y	Weight, kg	Height, m	BMI, kg/m^2
		Female	Male	Mean (range)			
	No.	No. (% of each group)	No.				
Entire sample							
MZ	86 (77)	52 (60)	34 (40)	25.8 (18–56)	67.1 (48.0–97.5)	1.72 (1.55–1.95)	22.6 (17.4–34.1)
DZ	26 (23)	18 (69)	8 (31)	22.3 (18–36)	67.7 (53.5–83.5)	1.71 (1.60–1.95)	23.1 (17.7–29.7)
13C caffeine sample							
MZ	58 (78)	40 (69)	18 (31)	25.3 (18–51)	64.6 (48.0–97.5)	1.70 (1.55–1.95)	22.4 (17.9–34.1)
DZ	16 (22)	14 (88)	2 (12)	22.1 (18–27)	66.1 (53.5–83.5)	1.69 (1.60–1.95)	23.2 (18.9–29.7)

BMI, body mass index; DZ, dizygotic; MZ, monozygotic.

intoxication.[21] These data show the significant effect of aryl hydrocarbon (Ah) receptor-mediated transcriptional regulation. However, the *in vivo* CYP1A2 activity also showed up to 20-fold variation among nonsmokers and this variation was assumed genetically determined.[5,7] On the other hand, thus far, only a minor fraction of the interindividual variation in CYP1A2 activity could be explained by defined variants in the genome.[22–24]

Recent large genomewide association studies identified genes possibly associated with habitual coffee consumption. Among these genes were *CYP1A2* and the Ah receptor, one of the transcriptional regulators of CYP1A2. Polymorphisms at both gene loci were associated with coffee consumption and consumption of caffeine-containing beverages.[25–27]

This twin study aimed to contribute to the question how much of the variation of caffeine pharmacokinetics as indicator of CYP1A2 activity is indeed genetically determined. Monozygotic (MZ) twins are 100% genetically identical and dizygotic (DZ) twins are, on average, 50% genetically identical. This provides the unique opportunity to differentiate between genetic and environmental effects using the design of a twin study.[28] The results of such a study may also be important in identifying priorities for future pharmacogenomic research because, only in cases of a high genetic influence, further studies to elucidate the underlying mechanisms might be promising. A secondary question of the present study was whether 13C caffeine may give data that are more reliable because with the extensive habitual use of caffeine, in some instances, the required strict dietary regulations may be difficult to be implemented or controlled.

The heritability in the variation of NAT2 as a minor pathway of caffeine biotransformation is well understood at the molecular level.[29,30] Therefore, the analysis of the acetylated caffeine metabolite may serve as a positive control of the power of the study design and data analysis approaches to estimate heritability.

RESULTS

Subjects

In total, 44 MZ and 14 same-sex DZ healthy twin pairs participated in the study. The subjects had a body mass index between 17 and 34 kg/m^2 and were between 18 and 56 years of age. All 58 pairs (116 subjects) received 50 mg caffeine orally. Per protocol, caffeine was administered on three independent occasions in a time interval of at least 7 days. However, for personal reasons, 5 subjects took only two caffeine doses and 10 subjects took only one. All subjects were asked to participate in an additional study period with 50 mg 13C caffeine orally. In total, 29 MZ and 8 DZ twin pairs participated in the additional study period.

There were no significant differences in age, body weight, height, and body mass index between the groups of MZ and DZ twin pairs (**Table 1**). There were no serious adverse events caused by the study drugs or study procedures.

Plasma caffeine and 13C caffeine pharmacokinetic data reflecting CYP1A2 activity

We observed strong variations in the concentration time curves after administration of caffeine and 13C caffeine (**Figure 1**). Fifty-six twin pairs were included in the calculations for area under the curve $(AUC)_{inf}$ as the primary endpoint and the additional pharmacokinetic parameters (**Tables 2 and 3**). Dietary habits were controlled in nutrition questionnaires. Intake of chargrilled meat had no statistically significant effects on the AUC_{inf} of caffeine. Interestingly, protein consumption was strongly associated with lower caffeine AUC ($P < 0.001$, Jonckheere-Terpstra Test). The median AUC_{inf} (95% confidence interval) ranged

Table 2 AUC_{inf} [mg*min/L] of caffeine

	Nonsmoking	Smoking
Male	506.5 (38.7%), $n = 28$	315.9 (50.1%), $n = 14$
Female (no HC)	768.3 (36.3%), $n = 18$	340.7 (43.3%), $n = 8$
Female (HC)	1,046 (32.0%), $n = 36$	787.3 (29.7%), $n = 8$

Data given as geometric mean (geometric coefficient of variation %). According to analysis of variance, sex ($P = 0.048$), smoking status ($P < 0.0001$), and hormonal contraceptives ($P < 0.0001$) were significant predictors of the AUC_{inf} without significant statistical interaction between contraceptives and smoking status. AUC_{inf} was significantly higher in nonsmoking women than in nonsmoking men and AUC_{inf} was significantly higher in women using hormonal contraceptives than in those without. AUC, area under the curve; HC, hormonal contraceptive.

Table 3 Caffeine pharmacokinetic parameters

	MZ		DZ	
	Nonsmoking $N = 64$	Smoking $N = 22$	Nonsmoking $N = 18$	Smoking $N = 8$
	Geometric mean (geometric coefficient of variation %) *Min–max*			
AUC_{inf}, mg*min/L	750.2 (51.6%) *224.8–2,368*	342.6 (55.0%) *130.5–975.1*	810.6 (41.0%) *401.2–1,549*	679.2 (40.0%) *409.8–1,295*
AUC_{300}, mg*min/L	259.1 (32.2%) *105.2–485.3*	176.4 (36.5%) *96.8–386.4*	254.4 (23.4%) *174.0–369.5*	244.8 (20.0%) *173.1–324.2*
Cl/F, L/min	0.068 (50.7%) *0.021–0.227*	0.147 (55.1%) *0.052–0.393*	0.064 (40.1%) *0.035–0.126*	0.077 (39.1%) *0.041–0.122*
$t_{1/2}$, min	385.2 (36.7%) *165.5–851.7*	233.1 (34.7%) *95.3–406.9*	436.2 (35.3%) *213.4–657.1*	402.5 (28.8%) *254.6–589.1*
C_{max}, mg/L	1.16 (28.0%) *0.55–2.10*	0.92 (36.4%) *0.59–1.92*	1.17 (20.1%) *0.80–1.63*	1.16 (16.9%) *0.95–1.45*
Ratio AUC_{300} caffeine/paraxanthine	3.55 (49.7%) *1.10–10.53*	1.94 (40.3%) *0.79–3.90*	3.37 (62.1%) *1.40–9.14*	2.68 (65.4%) *1.29–5.73*

AUC, area under the curve; Cl/F, total plasma clearance after oral administration; C_{max}, maximal plasma concentration; DZ, dizygotic twin pairs; MZ, monozygotic twin pairs; $t_{1/2}$, terminal half-life.

from 445.4 (394.4–466.1) mg*min/l in subjects with daily intake of protein to 1,029 (795.1–1,405) mg*min/l in subjects with intake of protein less than three times a month. Thus, high protein consumption may result in high caffeine clearance. According to the multiple linear regression analysis (**Table 4**), the AUC_{inf} of caffeine was significantly influenced by sex ($P = 0.014$), the number of cigarettes smoked per day ($P < 0.001$), hormonal contraceptive usage ($P < 0.001$), and by the *CYP1A1/1A2* promotor polymorphism rs2470893 ($P = 0.003$), which explained about 8% of the variation. For the entire study population, the median AUC_{inf} (95% confidence interval) in carriers of zero A alleles (rs2470893 G>A) was 864.1 (699.0–954.9) mg*min/l ($n = 60$) compared with 561.5 (458.9–620.3) mg*min/l ($n = 52$) in carriers of one or two A alleles ($P < 0.001$, Mann-Whitney U Test). When stratified for smoking, the effect of the A allele was particularly evident in the group of nonsmokers (**Figure 2f**). The *CYP1A2* (*CYP1A2*1F*, rs762551) genotype had no significant effect on AUC_{inf} (**Table 4**) in the entire study population, but in the subgroup of smokers the *CYP1A2*1F* genotype (rs762551) was associated with lower AUC_{inf} ($P = 0.04$). The *ABCG2* (rs2231142) and *AhR* (rs4410790) genotypes had no significant effect on AUC_{inf} (**Table 4**).

Table 4 Determinants of caffeine AUC_{inf} according to multiple linear regression analysis

	r (r^2)[a]	Coefficient	*P* value
All factors	0.75 (0.56)	–	<0.001
Sex	0.24 (0.06)	169.1	0.014
No. of cigarettes/d	–0.37 (0.14)	–24.2	<0.001
Use of hormonal contraceptive	0.48 (0.23)	377.8	<0.001
CYP1A1/1A2 (rs2470893)	–0.29 (0.08)	–144.2	0.003
CYP1A2 (rs762551; CYP1A2*1F)	–	–11.7	N.S.
ABCG2 (rs2231142)	–	–8.7	N.S.
AhR (rs4410790)	–	64.8	N.S.

AhR, aryl hydrocarbon receptor; AUC, area under the curve; CYP1A2, cytochrome P450 1A2.
[a]The correlation is given for the model with all factors and in the lines below the partial correlation coefficients are given for each significant factor alone. Coefficients indicate increase of AUC_{inf} in women, decrease per cigarette, increase with any current use of contraceptives, decrease per rs2470893 A allele, decrease per rs762551 A allele, decrease per rs2231142 A allele, and increase per rs4410790 A allele. The r^2 (coefficient of determination) may indicate the fraction of the total variation explained by all variables or by the respective factors given below. The four genotypes were selected because they were repeatedly and significantly associated with respective functions in earlier studies.[28–30]

We analyzed heritability in the entire study population, for the subgroups of nonsmoking men, nonsmoking women without hormonal contraceptives, all nonsmokers, and all without hormonal contraceptives. For the AUC_{inf} of the entire group, the Pearson's correlation coefficients were 0.84 for MZ and 0.68 for DZ twin pairs (**Figure 2a,b**). In the subgroup of nonsmoking twins who did not use hormonal contraceptives, the corresponding correlation coefficients were 0.95 for MZ and 0.63 for DZ twin pairs, respectively. Correlations of plasma concentrations at all times after caffeine administration were high for both MZ and DZ twin pairs, but rather consistently, correlations were higher between siblings of MZ twin pairs (**Figure 2e**).

In structural equation modeling, including the entire study sample, we found no heritability for AUC_{inf}. The model considering major influences of common environmental effects (72%) and unique environmental effects (28%) provided the best description of the data according to the Akaike criterion (common environmental effects [C] unique environmental effects [E] model; **Table 5**). The additive genetic effects (A) unique environmental effects (E) model revealed 74% of variation due to

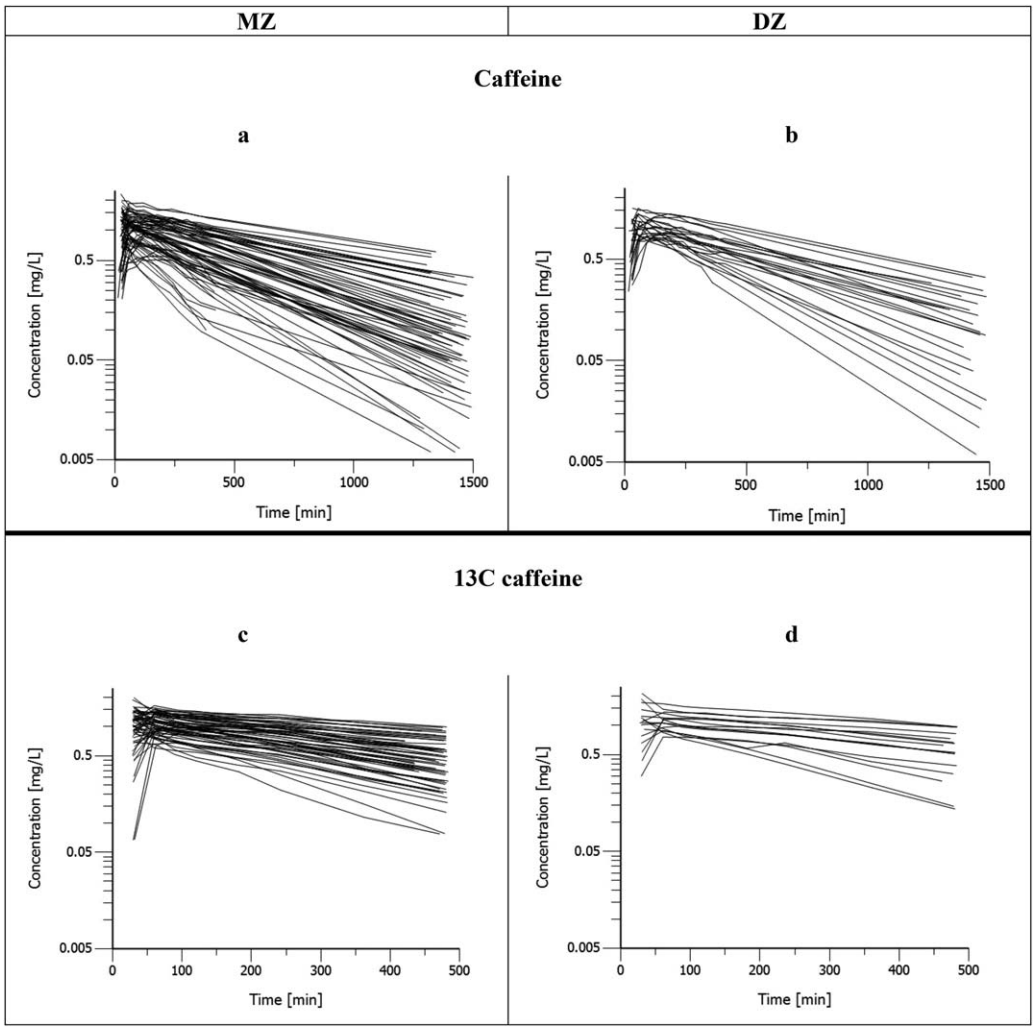

Figure 1 Concentration time curves after administration of caffeine and 13C caffeine. MZ, monozygotic twin pairs; DZ, dizygotic twin pairs. (**a** and **b**) Concentration time curves of one study day after administration of 50 mg caffeine. (**c** and **d**) Concentration time curves after administration of 50 mg 13C caffeine.

additive genetic effects and 26% due to unique environmental effects, but was not the most adequate model for the entire study population. However, the AE model gave the best description of the variation in the subgroup not affected by smoking and hormonal contraceptives with 89% of variation attributed to additive genetic effects. In line with these results, the estimations of heritability were similar when using the total oral clearance (Cl/F) as the parameter for CYP1A2 activity. For the entire study sample, common environmental effects explained 62% and unique environmental effects explained 38% of the variation of Cl/F (**Table 5**). In the subgroup of nonsmokers who had not used hormonal contraceptives, additive genetic effects explained 91% of the total oral clearance (**Table 5**).

Additional analyses were performed to evaluate the heritability of the variation of AUC_{inf} in all nonsmokers and in all subjects not using hormonal contraceptives. In these analyses, both for the subgroup of nonsmokers (29 MZ, 8 DZ) and for the subgroup of subjects who did not use hormonal contraceptives (26 MZ, 6 DZ), the AE model fitted best with about 71% and 91% of variation, respectively, explained by additive genetic effects.

Caffeine was administered on up to three different occasions, which allowed us to compare intraindividual vs. interindividual variation as a rough estimate for heritability.[31,32] In this calculation, the genetic component for the AUC_{inf}, the relative difference of intersubject variance minus intrasubject variance, was about 0.77 for the entire group and 0.86 for the group excluding smokers and users of hormonal contraceptives. Thus, up to 77% of the interindividual variation might be due to genetic factors, but, in this approach, this result reflects the sum of genetic factors and common environmental factors.

Results of the AUC_{300} were similar for both caffeine and 13C caffeine (**Table 3** and **Supplementary Table S1** online), which indicates a good fit of the pharmacokinetic parameters of unlabeled caffeine. Concerning the heritability estimated by structural equation modeling, our results were similar to estimations of heritability for the entire group of subjects with caffeine administration (**Table 5**).

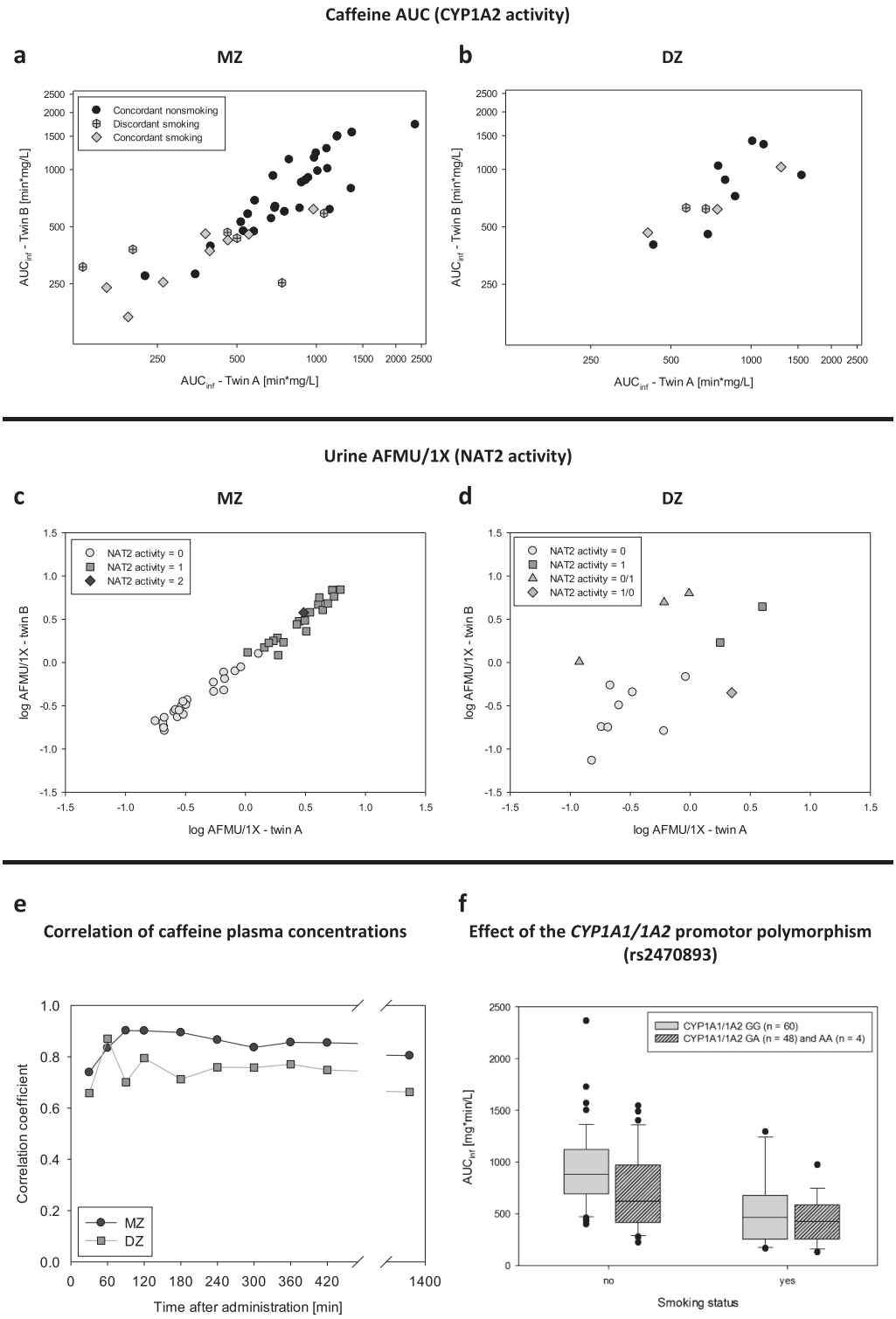

Figure 2 Heritability of cytochrome P450 1A2 (CYP1A2) and N-acetyltransferase 2 (NAT2) tested with caffeine. MZ, monozygotic twin pairs; DZ, dizygotic twin pairs; AUC_{inf}, area under the curve; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; 1X, 1-methylxanthine. (**a** and **b**) Correlations of the AUC_{inf} of the MZ and DZ twin pairs stratified to smoking status. (**c** and **d)** Correlations of the log ratio of AFMU over 1X for MZ and DZ twin pairs. The stratification to NAT2 activity groups is based on Cascorbi *et al.*[17] Subjects carrying homozygous the *4 allele are given an NAT2 activity of 2 indicating rapid enzyme activity. Subjects carrying homozygous or compound heterozygous **5A, *5B, *5C, *6A*, and **7B* were classified as having genetically predicted NAT2 activity of 0 indicating slow NAT2 enzyme activity. Heterozygous carriers of *4 were classified as having genetically predicted NAT2 activity of 1. For the DZ twins, NAT2 activities of 0/1 and 1/0 indicate the different NAT2 activities of the DZ siblings in certain twin pairs. (**e**) Correlation of the caffeine plasma concentrations to each timepoint after caffeine administration for MZ and DZ twin pairs. (**f**) Effect of the *CYP1A1/1A2* promotor polymorphism rs2470893 on the AUC_{inf} stratified by smoking status. In the group of nonsmokers, the AUC_{inf} was significantly different between carriers and noncarriers of the A allele ($P = 0.004$, Mann-Whitney *U* Test).

Table 5 Heritability of CYP1A2 activity as tested with caffeine and 13C caffeine

AUC							
Caffeine (n MZ = 43, n DZ = 13), AUC_{inf}							
Model	**A**	**D**	**C**	**E**	**χ^2**	***P* value**	**AIC**
Saturated	–	–	–	–	–	–	3,156
ACE	0.12	–	0.62	0.26	4.38	0.99	3,130
ADE	0.74	0.00	–	0.26	6.83	0.96	3,133
AE	0.74	–	–	0.26	6.83	0.98	3,131
CE	**–**	**–**	**0.72**	**0.28**	**4.60**	**0.99**	**3,129**
13C caffeine (n MZ = 29, n DZ = 8), AUC_{300}							
Saturated	–	–	–	–	–	–	1,808
ACE	0.03	–	0.77	0.2	29.29	0.015	1,807
ADE	0.80	0.00	–	0.20	33.32	0.004	1,811
AE	0.80	–	–	0.20	33.32	0.007	1,809
CE	**–**	**–**	**0.79**	**0.21**	**29.31**	**0.022**	**1,805**
Subgroup of nonsmoking subjects without hormonal contraceptives (n MZ = 15, n DZ = 4), Caffeine, AUC_{inf}							
Saturated	–	–	–	–	–	–	1,049
ACE	0.50	–	0.39	0.11	15.22	0.44	1,034.8
ADE	0.89	0.00	–	0.11	15.62	0.41	1,034.4
AE	**0.89**	**–**	**–**	**0.11**	**15.62**	**0.48**	**1,032.8**
CE	–	–	0.84	0.16	18.24	0.31	1,035.4
Cl/F							
Caffeine (n MZ = 43, n DZ = 13), clearance/bioavailability							
Saturated	–	–	–	–	–	–	–372
ACE	0	–	0.62	0.38	39.8	0.0004	–363
ADE	0.56	0	–	0.44	44.9	0.00007	–358
AE	0.56	–	–	0.44	44.9	0.0001	–360
CE			**0.62**	**0.38**	**39.8**	**0.0008**	**–365**
Subgroup of nonsmoking subjects without hormonal contraceptives (n MZ = 15, n DZ = 4) Caffeine, clearance/bioavailability							
Saturated	–	–	–	–	–	–	–148
ACE	0.26	–	0.65	0.09	16.7	0.33	–162.0
ADE	0.91	0	–	0.09	17.9	0.27	–160.0
AE	**0.91**	**–**	**–**	**0.09**	**17.9**	**0.33**	**–162.9**
CE	–	–	0.89	0.11	18.3	0.30	–162.5

The *P* values were calculated with respect to the saturated model. Best fitting model was chosen due to lowest AIC.
A, additive genetic effects; AIC, Akaike information criterion; AUC, area under the curve; C, common environmental effects; CYP1A2, cytochrome P450 1A2; D, dominant genetic effects; DZ, dizygotic twin pairs; E, unique environmental effects; MZ, monozygotic twins pairs.

Urine data of caffeine and 13C caffeine reflecting NAT2 activity

All 58 twin pairs collected urine for determination of ratio of AFMU over 1-methylxanthine (1X; see **Supplementary Table S2** online). There were no significant differences between the MZ and DZ twin pairs for the NAT2 activity groups with two slow NAT2 activity alleles and with one rapid and one slow NAT2 activity allele. In the linear regression analysis, about 73% of the variation of log AFMU/1X after caffeine intake was explained by NAT2 genotypes ($P < 0.001$). The Pearson correlation coefficients between the two siblings were 0.99 in the MZ group and 0.58 in the DZ group (**Figure 2c,d**).

Table 6 Heritability of NAT2 activity as tested with caffeine and 13C caffeine

Caffeine (n MZ = 44, n DZ = 14)							
Model	A	D	C	E	χ^2	*P* value	AIC
Saturated	–	–	–	–	–	–	27.24
ACE	0.87	–	0.12	0.01	15.53	0.41	12.77
ADE	0.99	0.00	–	0.01	15.65	0.41	12.89
AE	**0.99**	**–**	**–**	**0.01**	**15.65**	**0.48**	**10.89**
CE	–	–	0.88	0.12	108.24	0.000	103.48
13C caffeine (n MZ = 29, n DZ = 7)							
Saturated	–	–	–	–	–	–	6.63
ACE	0.90	–	0.09	0.01	31.76	0.007	8.38
ADE	0.99	0.00	–	0.01	31.80	0.007	8.42
AE	**0.99**	**–**	**–**	**0.01**	**31.80**	**0.01**	**6.42**
CE	–	–	0.90	0.10	104.31	0.000	78.94

The *P* values were calculated with respect to the saturated model. Best fitting model was chosen due to lowest AIC.
A, additive genetic effects; AIC, Akaike information criterion; C, common environmental effects; D, dominant genetic effects; DZ, dizygotic twin pairs; E, unique environmental effects; MZ, monozygotic twin pairs.

Structural equation modeling confirmed the high heritability of NAT2 activity. The model concerning additive genetic effects (A) and unique environmental effects (E) fitted best according to the Akaike information criterion. Ninety-nine percent of the variation of log AFMU/1X was assigned to additive genetic factors and just 1% to individual environmental effects (**Table 6**). With urinary (AAMU + AFMU)/1X as an alternative indicator of NAT2 reflecting possible decomposition of AFMU to AAMU, 98% of the variation was due to additive genetic effects. These results are in line with the calculations of the genetic component concerning the repeated application of caffeine and the corresponding repeated urine samples and resulted in similar heritability of about 0.98.

The results for the ratio of the urine metabolites AFMU over 1X after 13C caffeine administration (see **Supplementary Table S2** online) were calculated for 29 MZ twin pairs, 7 DZ twin pairs, and 1 additional DZ twin. For one dizygotic twin, the AFMU was below the limit of quantification. Within the linear regression analysis, 51% of the variation of AFMU/1X was determined by the NAT2 genotype. In conclusion, the results were similar as for unlabeled caffeine (**Table 6**).

DISCUSSION

We assessed the heritability of the variation of caffeine biotransformation in a study on monozygotic and dizygotic twin pairs. The variation of caffeine pharmacokinetics reflecting CYP1A2 activity was apparently determined by several environmental and genetic factors.

Twins may be handicapped *in utero* compared with single births. Therefore, one requirement in analyzing twin studies is to compare the results of the evaluated trait in twins to results from the general populations mostly not being twins.[33] In this respect, the pharmacokinetic parameters determined in our study were similar to those for the general population reported previously.[16,34]

Environmental factors, like smoking and usage of hormonal contraceptives, are known to affect CYP1A2 activity.[4,6,35,36] Accordingly, in our study, the AUC was significantly lower in smokers than in nonsmokers and significantly higher in women using hormonal contraceptives than in nonusers (**Table 2**). Extensive smoking is known to increase clearance of CYP1A2 metabolized drugs and, on the other hand, oral contraceptives with ethinylestradiol and gestodene are known to decrease clearance of CYP1A2 metabolized drugs.[36] A lower caffeine AUC, which was found with increasing amounts of protein consumption in food, may be interesting and medically relevant. However, our study was not specifically designed to address food effects and, therefore, replication studies are required for better understanding of the underlying mechanisms.

In the context of strong environmental effects and in the absence of strong genomic markers predicting CYP1A2 activity, it was of interest to estimate the relative contribution of genetic vs. environmental effects. We therefore estimated the heritability via structural equation modeling for the entire study group, as well as for the subgroups of twins, who are nonsmokers or do not use hormonal contraceptives. In the entire study group, common environmental effects seemed to influence most of the variation of caffeine demethylation as reflected by the AUC. However, in the subgroups of nonsmokers and subjects who did not use oral contraceptives, or both, additive genetic effects explained most of the variation in caffeine AUC. These results indicate that possible genetic effects are masked by smoking and the use of hormonal contraceptives. A twin study by Rasmussen *et al.*[7] investigated the role of genetic factors in CYP1A2 demethylation of caffeine by determination of urinary caffeine metabolites. In that study, a heritability of about 73% was estimated for nonsmoking subjects who did not use hormonal contraceptives. In another study, the heritability of coffee consumption ranged between 36 and 58%.[37] The repeated drug application of caffeine on up to three different occasions provided an additional approach to estimate heritability.[31,32] In these analyses, a genetic component of 77% was assessed for the entire study population. However, the 77% genetic component reflects genetic effects, environmental effects common to both siblings, and any environmental effects that are individually constant. For the subgroup of nonsmoking twins without use of hormonal contraceptives, the genetic component, as proposed by Kalow *et al.*[31] was 86%, which is in line with the result from the structural equation modeling.

One practical disadvantage of CYP1A2 testing with caffeine is the extremely widespread habitual use of coffee, making it often not easy to convince all study participants to abstain from all types of caffeine-containing beverages. One solution may be the administration of caffeine labeled with stable isotopes. As shown in **Figure 1**, **Table 3**, and **Supplementary Table S1** online, the results with both types of caffeine were similar. However, in other situations, like liver cirrhosis, with very long half-lives of caffeine the advantage of 13C labeled caffeine may be bigger.

Recent genomewide association studies identified several loci associated with coffee consumption.[25–27] Of these, we selected here only those polymorphisms that had significant and possibly pharmacokinetically relevant effects according to earlier studies. This included one functional polymorphism in ABCG2 (the putative efflux transporter of caffeine) as well as polymorphisms localized in or near the genes coding for CYP1A1 and CYP1A2 and the aromatic hydrocarbon receptor. Only one polymorphism (rs2470893), which is localized in the common promotor region between CYP1A1 and CYP1A2, had a significant effect on the variation of AUC_{inf} of caffeine in our study. We observed a lower AUC in carriers of the A allele. Therefore, the A allele may be associated with faster loss of caffeine efficacy and will be in line with the higher caffeine consumption associated with this variant.[26] The molecular mechanisms of this polymorphism may be due to differential SP1 transcription factor binding.[27] However, SP1 is a rather broad active transcription factor and other mechanisms should be considered. Other polymorphisms may be the truly functional ones and, as shown in **Supplementary Figure S1** online, the rs2470893 polymorphism is linked with multiple polymorphisms localized in or near *CYP1A2*. Other previously reported associations between genetic polymorphisms and caffeine pharmacokinetics or consumption[24,26,27] were not replicated in the present study. However, the sample size of our study was not powered to replicate these recent findings. On the other hand and in line with independent earlier data,[22,38] the subgroup of smokers with the *CYP1A2*1F* genotype (rs762551) had a lower caffeine AUC than carriers of the wild type *CYP1A2*1A*.

Our study showed that almost all (99%) of the variation of the *in vivo* NAT2 activity was inherited. Numerous polymorphisms predicting NAT2 activity have been identified in the last three decades,[39–41] but the nearly complete genetic control of NAT2 activity is only clear from the twin analyses presented in this study. In our study, the strong correlation among MZ twins contrasts with the much weaker correlation in DZ twins (**Figure 2c,d**). This not only directly illustrates the very high heritability, but also suggests the apparent lack of common environmental and epigenetic factors on NAT2 activity. Structural equation modeling showed that 99% of the variation of NAT2 activity in our study group was explained by additive genetic effects for both caffeine and 13C caffeine. These results confirm that the study design and the statistical method of structural equation modeling were appropriate to detect highly heritably traits, as there seems to be only very few environmental effects that modulate the *in vivo* NAT2 activity. In this sense, NAT2 activity is apparently an excellent control for the potential of a study design or data analysis method to detect a highly heritable trait.

In conclusion, the contribution of heritable effects to the variation of caffeine AUC and total oral clearance was only small compared with the strong environmental effects of smoking and the use of hormonal contraceptives. In subjects smoking and using hormonal contraceptives, genetic effects on CYP1A2 effects may be hidden by the strong environmental influences. Nevertheless, among nonsmokers not using hormonal contraceptives, up to about 90% of the variation in caffeine AUC may result from heritable effects. Thus, there will never be a simple answer as to how much of the variation in CYP1A2 activity is due to environmental vs. genetic effects because the answer strongly depends on the exposure of the selected study sample to the relevant environmental modulators. It was a very promising result that after almost two decades of searching for functional polymorphisms determining CYP1A2 activity, the impact of the rs2470893 polymorphism identified in genomewide association studies[25–27] was confirmed in a pharmacokinetic study, and this may stimulate further functional and clinical research on the questions of variation of *CYP1A1* and *CYP1A2* and its medical impact. At present, neither the rs2470893 nor the rs762551 (*CYP1A2*1F*) variant are promising biomarkers for individualized drug dosing. However, according to large epidemiologic studies, coffee consumption is inversely associated even with all-cause mortality[42–44] and, when viewed on an epidemiological basis, the moderate effects of the CYP1A2 genotypes may indeed modulate health effects of coffee in humans in an epidemiologically relevant manner.

METHODS

Subjects and study design

We studied caffeine metabolism in 44 MZ and 14 DZ twin pairs in Göttingen, Germany, in order to estimate the heritability of the variations of CYP1A2 and NAT2 activities. All volunteers gave their written informed consent before participation in the study. The study was approved by the ethics committee of the University of Göttingen and by the German Federal Drug Administration. It was registered at ClinicalTrials.gov (NCT01845194) and in the European clinical trials database (EUDRA-CT number: 2008-006223-31).

All healthy female and male twins aged between 18 and 65 years were eligible for inclusion. DZ twins were only included if they were the same sex. Health status was assessed by a detailed medical history, a medical examination, a normal electrocardiogram, and normal clinical laboratory results. The latter included sodium, potassium, calcium, aspartate aminotransferase, creatinine, total bilirubin, hemoglobin, erythrocyte, thrombocyte, and leucocyte counts. In addition, a normal urine status was required before inclusion. The smoking status and the use of hormonal contraceptives were assessed on the day of the screening procedures. Smoking of two cigarettes per week or less was classified as nonsmoking. Except for hormonal contraceptives, no other drugs were allowed for 1 week prior to probe drug application and for 48 hours thereafter. Caffeine intake was prohibited during 1 week before each study day and until the last blood sampling after administration of caffeine. Dietary habits were recorded in adapted standardized nutrition questionnaires[45] where total food protein intake was calculated as the mean of items "meat," "sausage," "fish," "dairy products," and "eggs" of that questionnaire.

Two formulations of caffeine were administered. First, the entire sample of 44 MZ and 14 DZ twin pairs received a tablet with 50 mg caffeine orally (Percoffedrinol; Lindopharm, Hilden, Germany) on up to three different occasions. Caffeine was administered in a modified cocktail approach together with 0.2 mg midazolam (intravenously), 2.5 mg torsemide (intravenously), 5 mg metoprolol (intravenously), and 50 mg talinolol (orally). Not all substances are known to interact with caffeine pharmacokinetics, particularly at the very low doses administered in this study. Caffeine was administered 10 minutes after the end of the 20-minute metoprolol infusion.[46] In the second study period, a subgroup of 29 MZ and 8 DZ twin pairs received a single dose of 50 mg (trimethyl-13C3) caffeine (99% 13C), obtained in powder form from Cambridge Isotope Laboratories (Cambridge, MA) and provided in a gelatin capsule. The 13C caffeine was administered orally in a cocktail together with 1 mg midazolam, 0.25 mg torsemide, 2.5 mg talinolol, 5 mg pravastatin, and 5 mg codeine. This add-on study utilizing 13C caffeine was

included to control for possible errors due to habitual caffeine consumption. Blood samples were drawn before caffeine intake and up to 23 hours after every dosage (0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 23 hours after intake of caffeine and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, and 8 hours after intake of 13C caffeine, respectively). All subjects stayed at the clinical research unit for the first 7 hours (8 hours) after caffeine (13C caffeine) administration and then returned for the last blood sampling at 23 hours after caffeine administration. One MZ and one DZ twin pair had to be excluded from the pharmacokinetic analyses of caffeine because of missing blood sampling on the last timepoint 23 hours after caffeine administration. Urine sampling was performed 4–7 hours and 5–8 hours after intake of caffeine and 13C caffeine, respectively. Blood samples were centrifuged (10 minutes, 4°C, 4000 rpm) and plasma and urine were stored at −20°C until the performance of the concentration analyses. Urine samples were not acidified to prevent AFMU from spontaneous deformylation to AAMU because there are indications that this process may begin already within the bladder and/or AAMU may be derived by another NAT2-dependent pathway.[47,48]

Drug concentration analysis

Quantification of caffeine and paraxanthine in plasma. For determination of caffeine and paraxanthine, plasma was spiked with internal standard solution (2H9-caffeine and 2H3-paraxanthine) before quantification by liquid chromatography tandem mass spectrometry.

For quantification of 13C3-caffeine and 13C2-paraxanthine, plasma was spiked with internal standard solution (2H9-caffeine and 2H6-paraxanthine) and diluted with water. Diluted plasma samples were prepared on a Strata X 30 mg (8E-S100-TGB) SPE plate (Phenomenex, Aschaffenburg, Germany), preconditioned with methanol and water. The SPE plate was washed with water, dried for 10 minutes at 50 psi, and eluted with methanol. The methanol eluate was evaporated to dryness and the residue dissolved in mobile phase. After centrifugation, the supernatant was used for liquid chromatography tandem mass spectrometry analysis.

Quantification of urinary metabolites. Urine samples were centrifuged before analysis. Urine and internal standard solution (2H3-AFMU, 2H3-AAMU, and [13C4,15N3]1-methylxanthine) were added to ammonium acetate buffer and, after thorough mixing and centrifugation, the supernatant was used for liquid chromatography tandem mass spectrometry analysis.

Liquid chromatography tandem mass spectrometry analysis

We used an Agilent 6460 triple quadrupole mass spectrometer (Agilent, Waldbronn, Germany) coupled to an Agilent 1200 high-performance liquid chromatography system. Ionization mode was electrospray. Electrospray jetstream conditions were as follows: capillary voltage 3500 V, drying gas flow 10 L/min nitrogen, drying gas temperature 325°C, sheath gas temperature 350°C, sheath gas flow 11 L/min, capillary voltage 3500 V, nozzle voltage 1000 V in positive, and 500 V in negative mode. High-performance liquid chromatography separations were performed with gradient runs using (A) 0.1 % formic acid in water, and (B) 0.1 % formic acid in acetonitrile as mobile phases at a flow rate of 0.4 mL/min. Caffeine and paraxanthine were analyzed on a Strategy 5 Pro column (100 × 2.1 mm; Interchim, France). For analysis of 13C3-caffeine and 13C2-paraxanthine, a Poroshell 120 EC-C18 column (100 × 2.1 mm; Agilent, Waldbronn, Germany) was used. Urinary metabolites of caffeine or 13C3-caffeine were separated on a Synergi Hydro RP column (150 × 2.1 mm I.D., 4 µm particle size; Phenomenex, Aschaffenburg, Germany). The mass spectrometer was operated in the multiple reaction monitoring mode. The multiple reaction monitoring transitions, fragmentor voltage, and collision energy for analytes and internal standards are summarized in **Supplementary Table S3** online. Standardization of the analytical assays was performed with calibration samples prepared in plasma in the concentration range from 0.005–2.913 µg/mL for caffeine and 0.005–2.702 µg/mL for paraxanthine, and 0.003–1.972 µg/mL for 13C3-caffeine. Standardization for 13C2-paraxanthine was carried out with paraxanthine as reference compound from 0.001–0.549 µg/mL. Calibration samples in urine were prepared in the concentration range from 0.099–99.09 µg/mL for AAMU, 0.057–28.27 µg/mL for AFMU, and 0.166–83.07 µg/mL for 1-methylxanthine and were used for both, the unlabeled and the 13C labeled metabolites. Calibration curves based on internal standard calibration were obtained by weighted (1/x) linear regression for the peak-area ratio of the analyte to the respective internal standard against the amount of the analyte. The concentration of the analytes in unknown samples was obtained by linear regression analysis. Assay accuracy and precision were determined by analyzing quality control samples that were prepared like the calibration samples. In plasma, assay accuracy ranged from 88.3–108.4% for caffeine and 13C3-caffeine and from 89.8–101.3% for paraxanthine over the whole concentration range. Assay precision, expressed by the coefficient of variation, was always better than 4.5% for both analytes. Urinary metabolites could be determined with accuracy between 87.2 and 110.5% for AAMU, 88.6 and 112.0% for AFMU, and between 88.3 and 108.4% for 1-methylxanthine and a coefficient of variation below 4.9%.

Genotyping

An automated solid phase extraction method was used to isolate DNA from venous blood samples, according to the manufacturer's instructions (EZ1 DNA Blood 350 µl kit used with the Bio-robot EZ1, both from Qiagen, Hilden, Germany). *CYP1A1/1A2* (rs2470893), *CYP1A2* (rs762551), *ABCG2* (rs2231142), *AhR* (rs4410790), and *RxR* (rs3818740) polymorphisms, as well as the *NAT2* alleles **4*, **5A*, **5B*, **5C*, **6A*, and **7B* were genotyped by DNA sequencing using fluorescence-labeled dideoxynucleotides and detection via Gene Mapper version 3.7 Software (Applied Biosystems, Foster City, CA). Zygosity was assessed by analyzing variants in 23 genes carrying frequent polymorphisms. Monozygosity was concluded when no differences in both siblings of each pair was detected regarding the genes.

Statistics

Noncompartmental methods and the WinNonlin software (Pharsight Corporation, Mountain View, CA) were used to estimate the pharmacokinetic parameters. If there was a residual caffeine concentration in plasma before dosage, the extrapolated concentrations were subtracted from the measured concentrations prior to the pharmacokinetic analyses. In these instances, extrapolations were made based on one-exponential decline with each individual's elimination rate constant. AUC_{inf} of caffeine was calculated from time of dose by the linear/log trapezoidal rule and extrapolated to infinity based on the last predicted concentration and using the terminal elimination rate constant (lambda z). To compare pharmacokinetics of caffeine and 13C caffeine, the AUC_{300} was calculated from time of dose until 300 minutes after application. Further parameters included the total plasma clearance after oral administration (Cl/F) and the terminal half-life ($t_{1/2}$), which were calculated as $Cl/F = dose/AUC_{inf}$ and $t_{1/2} = \ln(2)/\text{lambda z}$, respectively.

We used the primary endpoints AUC of caffeine and 13C caffeine as well as the urinary metabolic ratios of the secondary caffeine and 13C caffeine metabolites AFMU and 1X in MZ and DZ pairs to resolve variation into additive genetic effects (A), dominant genetic effects (D), common environmental effects (C), and individually unique environmental effects (E).[49] Additionally, estimations for heritability were determined for Cl/F of caffeine as another reliable parameter for CYP1A2 activity.[16] The fit of the submodels was assessed by the Akaike information criterion. The structural equation modeling was done by using the "mets"-package for programming environment R.[50,51]

The genetic component was calculated by "genetic component" = $[sd_b^2 - sd_w^2] / sd_b^2$, as described by Kalow *et al.*[31] and Ozdemir *et al.*[32] As the two siblings of one twin pair cannot be assumed as independent units, the given genetic components for the caffeine plasma concentrations and concentrations of urine metabolites reflect the mean of 50

repetitions in calculation of the genetic component in which each used one sibling of each pair by random selection.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

J.M. and J.B. wrote the manuscript. J.B., D.S., M.S., and R.K. designed the research. J.M., J.B., M.T., J.S., D.S., C.S.-S., and U.H. performed the research. J.M., J.B., J.H., S.M., and U.H. analyzed the data.

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

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