Phenotype–Genotype Correlation Applying a Cocktail Approach and an Exome Chip Analysis Reveals Further Variants Contributing to Variation of Drug Metabolism

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Although great progress has been made in the fine-tuning of diplotypes, there is still a need to further improve the predictability of individual phenotypes of pharmacogenetically relevant enzymes. The aim of this study was to analyze the additional contribution of sex and variants identified by exome chip analysis to the metabolic ratio of five probe drugs. A cocktail study applying dextromethorphan, losartan, omeprazole, midazolam, and caffeine was conducted on 200 healthy volunteers. CYP2D6, 2C9, 2C19, 3A4/5, and 1A2 genotypes were analyzed and correlated with metabolic ratios. In addition, an exome chip analysis was performed. These SNPs correlating with metabolic ratios were confirmed by individual genotyping. The contribution of various factors to metabolic ratios was assessed by multiple regression analysis. Genotypically predicted phenotypes defined by CPIC discriminated very well the log metabolic ratios with the exception of caffeine. There were minor sex differences in the activity of CYP2C9, 2C19, 1A2, and CYP3A4/5. For dextromethorphan (CYP2D6), *IP6K2* (rs61740999) and *TCF20* (rs5758651) affected metabolic ratios, but only *IP6K2* remained significant after multiple regression analysis. For losartan (CYP2C9), *FBXW12* (rs17080138), *ZNF703* (rs79707182), and *SLC17A4* (rs11754288) together with CYP diplotypes, and sex explained 50% of interindividual variability. For omeprazole (CYP2C19), no significant influence of *CYP2C:TG* haplotypes was observed, but *CYP2C19* rs12777823 improved the predictability. The comprehensive genetic analysis and inclusion of sex in a multiple regression model significantly improved the explanation of variability of metabolic ratios, resulting in further improvement of algorithms for the prediction of individual phenotypes of drug-metabolizing enzymes.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Genetic variants in drug-metabolizing enzymes, i.e., CYP2D6, CYP2C9, and CYP2C19 contribute significantly to interindividual variability of metabolic ratios of respective probe drugs; however, there is still a large overlap of metabolic ratios of genotypically predicted phenotypes. Moreover, there is only limited data on the confounding factors such as sex.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ A comprehensive cocktail study was performed aiming to figure out, whether the application of an exome chip analysis approach reveals further variants contributing to explanation of interindividual variation of metabolic ratios of five probe drugs.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ This study further contributed to the understanding of sex to drug metabolisms and in particular revealed further putative genes contributing to the explanation of variation of drug metabolism of losartan (CYP2C9) and dextromethorphan (CYP2D6).

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ Genotype-based determination of phenotypes requires inclusion of further genetic variants to improve the accuracy. Sex seems to play only a modest role in drug metabolism, partly being affected by the concomitant application of contraceptives.

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Implementation of pharmacogenetics is increasingly considered in clinical practice. Despite some HLA-marker-related hypersensitivity reactions toward drugs such as abacavir or carbamazepine,[1] most hereditary pharmacogenetic traits are related to the metabolism of drugs, affecting their pharmacokinetics. Accordingly, dose recommendations have been developed based on genotype–phenotype relationships aiming to optimize mean plasma drug concentrations based on the genotype of a drug-metabolizing enzyme.[2] With the exception of cases of complete lack of enzyme activity known for CYP2D6 or CYP2C19 poor metabolizers, the phenotype may be subject to broad variability. Although optimized coverage of genetic variants of P450 enzymes led to improved prediction of individual phenotypes, there is still variation due to so far unknown factors.[3]

Phenotypic testing based on probe drug cocktails has been established in some centers in order to optimize drug therapy.[4] The advantage of phenotyping is the concurrent measurement of the metabolites produced from each probe drug allowing determination of the metabolic capacity of specific CYP isoforms and providing valuable insights into interindividual variations in drug. A limitation, however, is the fact that a phenoconversion can occur due to drug–drug interactions as exemplified by the normalization of the nortriptyline metabolism phenotype in ultrarapid CYP2D6 metabolizers in the presence of the CYP2D6 inhibitor paroxetine.[5] Moreover, the choice of probe drug and methodological factors have to be considered.

In the present study, a five-drug cocktail was administered to healthy volunteers to determine the phenotypes of five CYP450 enzymes applying one-batch LC/MS–MS analytics of the probe drugs dextromethorphan (CYP2D6), losartan (CYP2C9), omeprazole (CYP2C19), midazolam (CYP3A4/5), and caffeine (CYP1A2). Considering also the impact of sex, phenotypes were first correlated to established diplotypes of the five cytochrome P450 enzymes. In addition, we investigated first, if consideration of recently reported genetic variants associated with CYP2D6 and CYP2C19 metabolism may improve prediction of phenotypes. Moreover, an exome chip analysis was performed in order to identify further variants that may contribute to the explanation of interindividual variation of metabolic phenotypes.

METHODS

Clinical study

A single-center, open-label, nonrandomized, parallel clinical trial was conducted at the clinical pharmacology unit of the Institute of Experimental and Clinical Pharmacology of the University Hospital Schleswig-Holstein (Kiel, Germany). The study was registered as Eudra-CT 2010-024302-35 and as DRKS00017626 in the German Clinical Trial Registry, and the protocol was reviewed and approved by the local ethics committee.

In total, 200 healthy volunteers (82 female and 118 male) were enrolled in the clinical trial. The inclusion criteria were an age between 18 and 55 years, absence of serious pathologic history of cardiovascular, renal, pulmonary, hematopoietic, hepatic, or neuronal function, and pathological findings at the initial examination. Exclusion criteria were known intolerance to probe drugs and pregnancy. The study participants underwent an interview and initial clinical examination by a physician including urine and blood tests (blood count, electrolytes, creatinine, albumin, ALT, AST, γ-GT, and coagulation status) as well as 12 channel ECG. Only volunteers were included in the study who had no ECG abnormalities and whose laboratory parameters were within the normal range.

The healthy volunteers were asked to stop intake of any medication, including oral contraceptives, herbal preparations like St. John's wort as well as consumption of caffeine-containing food or beverages, grapefruit, and other citrus 3 days before the study day and to fast on the morning of the study day. Furthermore, the volunteers were asked to stop smoking and alcohol consumption 2 days before the study day. After initial blood sampling for pharmacogenetic analysis on the study day, the probe drug cocktail composed of 30 mg dextromethorphan (Hustenstiller-ratiopharm Dextromethorphan, Ratiopharm), 25 mg losartan (Losartan-ratiopharm, Ratiopharm, Ulm, Germany), 20 mg omeprazole (Antra-Mups, Astra-Zeneca, Hamburg, Germany), 2 mg midazolam (Midazolam-ratiopharm, Ratiopharm, Ulm, Germany), and 100 mg caffeine (Percoffedrinol, Lindopharm, Hilden, Germany) was orally administered with 200 mL of water (time 0 hour). The here used probe drugs are widely used in CYP cocktail approaches in the same (Inje cocktail)[6] or in slightly varying combinations (e.g., Karolinska cocktail[7] or the Geneva cocktail[8]). The healthy volunteers received a caffeine-free meal 4 h after cocktail administration. For phenotyping, urine was collected by the volunteers over 8 h starting at the time of drug administration (time 0 h) and venous blood samples were drawn at a single timepoint 5 h after drug administration. Blood samples were centrifuged at 4°C, 1000 × *g*, and blood serum and collected urine were stored at −80°C.

Analytics

Parent drugs and metabolites (for CYP2D6: dextromethorphan/dextrorphan, CYP2C9: losartan/E3174, CYP2C19: omeprazole/5-hydroxy omeprazole, CYP3A: midazolam/1-hydroxy midazolam and CYP1A2: caffeine/paraxanthine) were extracted in a single step and simultaneously assayed by LC–MS/MS as described previously.[9,10] 4-Hydroxychalkone (Sigma-Aldrich, Darmstadt, Germany) was used as internal standard. Dextromethorphan, dextrorphan, Losartan, E-3174 (Synfine, Richmond Hill, Canada), omeprazole, 5-hydroxy omeprazole, midazolam, 1-hydroxy midazolam (Sigma-Aldrich), caffeine (Fluka, Buchs, Switzerland), and paraxanthine were diluted in water and LC–MS grade acetonitrile (Roth, Karlsruhe, Germany) and used as standards.

For validation, calibration, and quality control, blank human serum and urine were obtained from healthy volunteers (having no medication or caffeine consumption) from the Department of Transfusion Medicine (University Medicine Greifswald). To ensure the absence of the analytes of interest, each calibration curve consisted of the double blank sample, which represents the respective blank serum/urine sample without any internal standard.

Standard and QC samples were made from stock solutions containing 100 µg/mL of the respective standard in water/acetonitrile (50:50, v/v), which were mixed either with water only (caffeine and paraxanthine) or human serum diluted 1:10 in water (all other compounds). Calibration standards and QC samples contained mixtures of each analyte at concentrations of 0.1 (calibration only), 0.25, 0.5, 1, 2.5, 5, 10, 20, 50, and 100 ng/mL.

Serum samples were subjected to a protein precipitation by the addition of ice-cold acetonitrile and subsequent centrifugation, whereas urine samples underwent liquid–liquid extraction with diethyl ether. The supernatants of both procedures were evaporated and then reconstituted for subsequent chromatographic analysis.

Samples were analyzed by LC–MS/MS consisting of an Agilent 1,260 HPLC system (Agilent, Waldbronn, Germany) coupled to the QTRAP 5500 triple-quadruple mass spectrometer (Sciex, Darmstadt, Germany). Chromatographic separation was performed on a X-Terra® MS C18 3.5 µm 2.5 × 100 mm column (Waters, Germany). Samples were stored in a cooled autosampler at 4°C, while the temperature of the column was adjusted to 50°C. An isocratic elution was applied using a 50:50 (v/v) mixture of ammonium acetate buffer (pH 3) and LC–MS grade acetonitrile as mobile phase at a flow rate of 0.2 mL/minute. The MS/MS analysis was done in the positive multiple reaction monitoring mode by monitoring

the following mass transitions (m/z): 272.2/215.0 and 258.1/157.0 for dextromethorphan and dextrorphan, 423.2/207.0 and 437.3/235.1 for losartan and E3174; 346.0/198.0 and 362.0/214.0 for omeprazole and 5-hydroxy omeprazole; 326.0/291.0 and 342.0/324.0 for midazolam and 1-hydroxy midazolam; 195.1/138.0 and 181.1/124.0 for caffeine and paraxanthine and 224.8/147.0 for the internal standard 4-hydroxy chalkone.

The method was validated between 0.1 and 100 ng/mL for all analytes and was shown to be of adequate specificity, precision, and accuracy (±15% relative error of the nominal values).

Phenotype determination

Phenotype determination was based on the logarithmic metabolic ratio calculated as $\log MR = \log_{10}$ (parent compound/metabolite). While logMR for omeprazole/5-hydroxy omeprazole, midazolam/1-hydroxy midazolam, and caffeine/paraxanthine were determined in serum, logMR for dextromethorphan/dextrorphan and losartan/E3174 were analyzed in serum and urine, leading to comparable results.

Genotype assessment

Genomic DNA was extracted from whole blood samples using the QIAamp DNA Blood Midi Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Genotyping was performed preferentially using pyrosequencing on a HS96 system (Qiagen, Hilden, Germany) as described previously or with TaqMan allelic discrimination assays (Thermo Fisher, Waltham, MA, USA) on an ABI Prism 7900 HT device (Thermo Fisher) according to the manufacturer's protocols. The following known functional variants were genotyped: *CYP1A2* (c.-9-154C>A, rs762551) allocated to **1F; CYP2C9* (c.430C>T, p.R144C, rs1799853) allocated to **2*, (c.1075A>C, p.I359L, rs1057910) allocated to **3*; *CYP2C19* (c.681G>A, rs4244285) allocated to **2*, (c.431G>A, R144H, rs17884712) allocated to **9*, (c.-806C>T, rs12248560) allocated to **17; CYP2D6* (c.775del, p.R259fs, rs35742686) allocated to **3*, (c.506-1G>A, rs3892097) allocated to **4*, (c.454del, p.W152fs, rs5030655) allocated to **6*, (c.841_843del, p.K281del, rs5030656) allocated to **9*, (c.100C>T, p.P34S, rs1065852) allocated to **10* if rs3892097 was absent, (c.985+39G>A, rs28371725) allocated to **41*; *CYP3A4* (c.522-191C>T, rs35599367) allocated to **22* and *CYP3A5* (c.219-237A>G, rs776746) allocated to **3*. The *CYP2D6* gene copy number variation including the deletion (**5*) was analyzed using a TaqMan copy number assay (Hs00010001_cn, exon 9) with TERT as reference assay (4403316, Thermo Fisher) according to the manufacturer's protocol. Other tier 1 SNPs recommended in the CPIC guidelines were not considered due to low frequency (<1%) in European Whites or their normal function (CYP2D6*2). All diplotypes were assigned to predicted phenotypes based on the respective CPIC guidelines,[11–13] hereafter named as CPIC-predicted phenotypes (**Table 1**).

Additional genotyping was performed for rs28379954, a variant in *NFIB* (Nuclear Factor I B) reportedly affecting the expression of *CYP2D6*,[14] rs17884712 (c.431G>A, p.A144P) allocated to *CYP2C19*9*, as well as rs2860840 and rs11188059, both in *CYP2C18* and described to define a novel *CYP2C* haplotype associated with ultrarapid metabolism.[15] For genotyping methodology, see **Tables S1 and S2**.

Exome chip analyses

To identify potential new variants, which might contribute to interindividual variance in the metabolism of the probe drugs, an exome chip analysis was performed using an Illumina HumanExome-12v1_A Beadchip (Illumina, San Diego, CA) covering 247,870 genomic markers. To determine significant associations between markers and metabolic ratios, the Wald test was performed followed by Holm correction with a significance threshold for the adjusted P-value of 0.05.

Confirmation of exome chip results

For confirmation of significant exome chip signals, the following SNPs were verified by pyrosequencing or TaqMan allelic discrimination assays: *CYP2C9* c.961+2337G>A,T (rs10509680), *COL11A2* c.4844C>T (rs2229792), *FBXW12* c.17C>T (rs17080138), *IP6K2* c.-122C>T (rs61740999), *SLC17A4* c.1114G>A (rs11754288), *TCF20* c.2164A>G (rs5758651), *ZNF703* c.919C>T (rs79707182), g.94645745G>A,T rs12777823. See **Tables S1 and S2** for further information on genotyping assays.

Statistics

Statistical analyses were performed using SPSS version 29.0 (IBM, Ehningen, Germany). Pairwise linkage disequilibrium (LD) was calculated by applying Haploview, version 4.2.[16] All SNPs analyzed in the present study were tested for deviations from Hardy–Weinberg equilibrium (HWE) using a chi-squared test. The metabolic ratios of the respective probe drugs as outcome variables were normalized by logarithmic transformation. The impact of SNPs, diplotypes, or predicted phenotypes (**Tables 1 and 2**; **Table S3**) on the logMR was analyzed using Kruskal–Wallis and Mann–Whitney U-tests. Multiple regression analysis was applied to quantify the observed variability of logMR of respective probe drugs. First, the individual impact of individual SNPs, diplotypes, and sex was tested separately. Factors showing significance were included in a multiple linear regression model followed by backward selection with a P-value threshold of 0.05. P-values of 0.05 were considered as statistically significant.

RESULTS

A total of 200 healthy white European volunteers (118 males and 82 females; mean age 27 ± 6 years) were enrolled in the present study, and all underwent genotyping and phenotyping. Information regarding smoking habits and usage of oral contraceptives was recorded. No serious adverse events occurred in the study cohort, as all observed adverse events were considered as mild.

Association of *CYP2D6* and exome chip variants to the metabolism of dextromethorphan

In the present study, the significant association of the established CYP2D6 phenotype groups (namely ultrarapid (UM), normal (NM), intermediate (IM), and poor (PM) metabolizers),[13,17] with logMR dextromethorphan/dextrorphan was confirmed in serum and in urine (**Figure 1a**; **Figure S2A**). There was no difference in the metabolic ratio between male and female volunteers (**Figures S1A,B and S2B,C**). We also found no effects of the previously described genetic variant in *NFIB* (rs28379954)[14] (**Figures S1C** and **S2D**). The exome chip analysis identified three additional SNPs in the genes of collagen type XI alpha 2 chain *COL11A2* (rs2229792, c.4844C>T), inositol hexakisphosphate kinase 2 *IP6K2* (rs61740999, c.-122C>T) and transcription factor 20 *TCF20* (rs5758651, c.2164A>G) to be significantly associated with increased urine metabolic logMR dextromethorphan/dextrorphan (**Figure S2E–G**). However, only the variants in *IP6K2* c.-122C>T (rs61740999) and *TCF20* T>C (rs575865) showed a significant association with the metabolic logMR of dextromethorphan/dextrorphan in serum, indicating a reduced activity of CYP2D6 (**Figure 1b–d**).

Stepwise multiple regression analysis revealed a contribution of *CYP2D6* diplotypes to interindividual serum logMR variability of 83% ($P < 0.0001$). By comparison, the consideration of the phenotypes predicted by CPIC on the basis of diplotypes contributed only 72% ($P < 0.0001$, **Table 3**). The additional inclusion

Table 1 Association between pharmacogenetically relevant cytochrome P450 SNPs and their diplotypes to log metabolic ratios (logMR) of respective probe drugs

P450	Parent compound/ metabolite	CPIC-predicted phenotype	Detected genotypes or diplotypes	Number of cases	Mean logMR[a]	*P*-value[b]
CYP2D6	Dextromethorphan/ dextrorphan	UM	*1xn/*1; *1xn/*41	8	−1.81±0.86	<0.001
		NM	*1/*1; *1/*9, *1/*10; *1/*41; *1x2/*4, *1x2/*10	116	−1.63±0.49	
		IM	*1/*3; *1/*4; *1/*5; *1/*6; *4/*9, *5/*10; *41/*41	64	−1.24±0.47	
		PM	*3/*4; *4/*4; *4/*5; *5/*5	12	0.88±0.20	
CYP2C9	Losartan/E-3174	NM	*1/*1	137	−0.21±0.18	<0.001
		IM	*1/*2; *1/*3; *2/*2	59	−0.03±0.23	
		PM	*2/*3	4	0.40±0.17	
CYP2C19	Omeprazole/5-hydroxy omeprazole	UM	*17/*17	9	−0.83±0.47	<0.001
		RM	*1/*17	61	−0.70±0.40	
		NM	*1/*1	75	−0.53±0.40	
		IM	*1/*2; *2/*17	53	−0.16±0.35	
		PM	*2/*2	2	0.79±0.07	
CYP3A	Midazolam/1-hydroxy midazolam		CYP3A5 *1/*1; *1/*3; CYP3A4 *1/*1	33	0.81±0.22	<0.001
			CYP3A5*1/*1; *3/*3; CYP3A4 *1/*1	149	0.79±0.21	
			CYP3A5*1/*3; CYP3A4*1/*22	2	1.04±0.12	
			CYP3A5*3/*3; CYP3A4 *1/*22	16	1.03±0.23	
CYP1A2	Caffeine/paraxanthine		*1/*1	17	0.16±0.26	0.449
			*1/*1F	86	0.17±0.22	
			*1F/*1F	97	0.13±0.19	

CPIC phenotype assignments for CYP diplotypes observed in the present study sample: PM=poor metabolizer, IM=intermediate metabolizer, NM=normal metabolizer, RM=rapid metabolizer, UM=ultrarapid metabolizer.
[a]Data are presented as mean±SD. [b]*P*-values are derived from the Kruskal–Wallis test.

of *IP6K2* c.-122C>T (rs61740999) only slightly improved the model containing the phenotypes predicted by CPIC to 73% ($P < 0.0001$), but not the model containing the individual diplotypes. The inclusion of *TCF20* T>C (rs575865) did not lead to any improvement in the models (**Table 3**). The *IP6K2* C>T (rs61740999) variant was detected in eight study participants, who also covered all phenotype groups (1 of 8 UM, 2 of 115 NM, 2 of 65 IM, and 3 of 12 PM, **Figure S1D**). All subjects had a significantly higher mean logMR dextromethorphan/dextrorphan compared with noncarriers (**Figure 1d**; **Table 2**). The distribution of TCF20 variants among CYP2D6 phenotypes is depicted in **Figure S1E**.

Impact of *CYP2C9* variants on losartan metabolism

Mean metabolic ratios given as logMR losartan/E-3174 were similar in serum and urine (data are shown only for serum). Overall, the mean logMR losartan/E-3174 differed significantly between normal, intermediate, and poor genotypically predicted CYP2C9 phenotypes (**Table 1**). Female volunteers exhibited a slightly higher mean logMR than males (1.2-fold, $P = 0.031$, **Figure S3A,B**).

The exome chip analysis revealed four additional SNPs significantly associated with mean logMR of losartan/E-3174 (**Table 2**). One SNP (rs10509680, c.961+2337G>A,T) was located in the *CYP2C9* gene, and three SNPs were located outside the cytochrome P450 *2C8/9/18/19* cluster, namely in the genes of the F-box/WD repeat-containing protein 12 *FBXW12* (rs17080138, c.17C>T), zinc finger protein 703 *ZNF703* (rs79707182, c.919C>T), and solute carrier family 17 member 4 *SLC17A4* (rs11754288, c.1114G>A). The *CYP2C9* intronic variant c.961+2337G>A (rs10509680) was almost entirely linked to *CYP2C9*3*, as all four **3/*3* and 18 out of 19 intermediate metabolizers carrying **1/*3* presented this variant (**Figure 2a**). A total of 13 heterozygous *FBXW12* c.17C>T (rs17080138) variant carriers were observed among all three CYP2C9 phenotype groups (**Figure 2b**). Within the *SLC17A4* gene, 131 subjects carried the variant c.1114G>A (rs11754288) in all phenotypes (**Figure 2c**). In contrast, the *ZNF703*

Table 2 Impact of putative functional variants in *CYP2C19*, *NFIB*, and SNPs identified in the exome chip analysis on metabolic ratios of probe drugs

SNP	Genotype (*n*)	Parent compound/metabolite	LogMR[a]	*P*-value[b]
Putative functional variants reported before				
*CYP2C19*9* c.431G>A, p.A144P (rs17884712)	G/G (200)		−0.48±0.46	
NFIB c.-122C>T (rs28379954)	T/T (183)	Dextromethorphan/dextrorphan	−1.36±0.80	0.760
	T/C (12)		−1.40±0.47	
	C/C (5)		−1.37±0.35	
SNPs identified by exome chip analysis[c]				
IP6K2 c.-122C>T (rs61740999)	C/C (192)	Dextromethorphan/ dextrorphan	−1.42±0.71	<0.001
	C/T (8)		0.08±0.92	
COL11A2 c.4844C>T (rs2229792)	C/C (184)		−1.44±0.69	0.001
	C/T (15)		−0.55±1.09	
	T/T (1)		0.60	
TCF20 c.2164A>G (rs5758651)	A/A (128)		−1.59±0.60	<0.001
	A/G (63)		−1.18±0.63	
	G/G (9)		0.63±0.75	
CYP2C9 c.961+2337G>A,T (rs10509680)	G/G (178)	Losartan/E-3174	−0.18±0.19	<0.001
	G/T (22)		0.18±0.27	
FBXW12 c.17C>T (rs17080138)	C/C (187)		−0.16±0.21	0.002
	C/T (13)		0.11±0.35	
SLC17A4 c.1114G>A (rs11754288)	G/G (69)		−0.23±0.23	<0.001
	G/A (90)		−0.14±0.20	
	A/A (41)		−0.01±0.21	
ZNF703 c.919C>T (rs79707182)	C/C (196)		−0.15±0.21	<0.001
	C/T (4)		0.34±0.38	
g.94645745G>A,T (rs12777825)	G/G (138)	Omeprazole/5-hydroxy-omeprazole	−0.65±0.39	<0.001
	G/A (58)		−0.19±0.32	
	A/A (4)		0.84±0.08	

[a]Data are presented as mean±SD. [b]*P*-values are derived from the Kruskal–Wallis test or Mann–Whitney *U*-test for two group comparisons, ns=not significant.
[c]Genotype frequencies are derived from confirmatory individual genotyping experiments.

c.919C>T (rs79707182) variant was only found in CYP2C9 PMs (1 of 4) and IMs (3 of 59) (**Figure 2d**). As can be seen in **Figure 2**, one outlier (marked in red) assigned to CYP2C9 IM was a carrier of the intronic *CYP2C9* SNP, as well as of *ZNF703* and *FBXW12* variant alleles.

The impact of *FBXW12*, *SLC17A4*, and *ZNF703* SNPs on losartan metabolism is illustrated in **Figure 3**. All three variants were associated with a significantly increased metabolic ratio. The percentage contribution of diplotypes, sex, and additional genetic variants to logMR was further investigated using stepwise multiple regression analysis. While *CYP2C9* diplotypes contributed to explaining 31% of interindividual variability in the logMR of losartan/E3174 ($P < 0.0001$), the additional consideration of sex explained 34% ($P < 0.0001$) and the addition of the three newly discovered SNPs led to an explanation of 50% of the interindividual variability ($P < 0.0001$, **Table 3**). The inclusion of diplotypes in the model was slightly superior to the inclusion of CPIC-predictable phenotypes. Only 46% could be explained by taking into account CPIC-phenotypes, sex, and *FBXW12*, *SLC17A4*, and *ZNF703* SNPs ($P < 0.001$, **Table 3**).

Association of *CYP2C19* and exome chip variants to the metabolism of omeprazole

There was a significant association of CYP2C19 genotype-predicted phenotypes with logMR omeprazole/5-OH omeprazole (**Table 1**). Female volunteers exhibited a slightly higher logMR than males (1.1-fold, $P = 0.0273$), which was specifically observed in the RM group (**Figure S4A,B**), Next, the potential impact on omeprazole metabolism was analyzed for the recently described *CYP2C:TG* haplotype.[15] In our study, there was a lack of association between the *CYP2C:TG* haplotype status and logMR omeprazole/5-OH omeprazole (**Figure 4a**; **Table S4**). Interestingly, there was one case carrying homozygously a CA diplotype in the *CYP2C18* locus. *CYP2C19* rs17884712 (*CYP2C19*9*), reportedly associated with decreased CYP2C19 activity,[18] was not detected among our samples. However, a significant association with the metabolic ratio was observed for

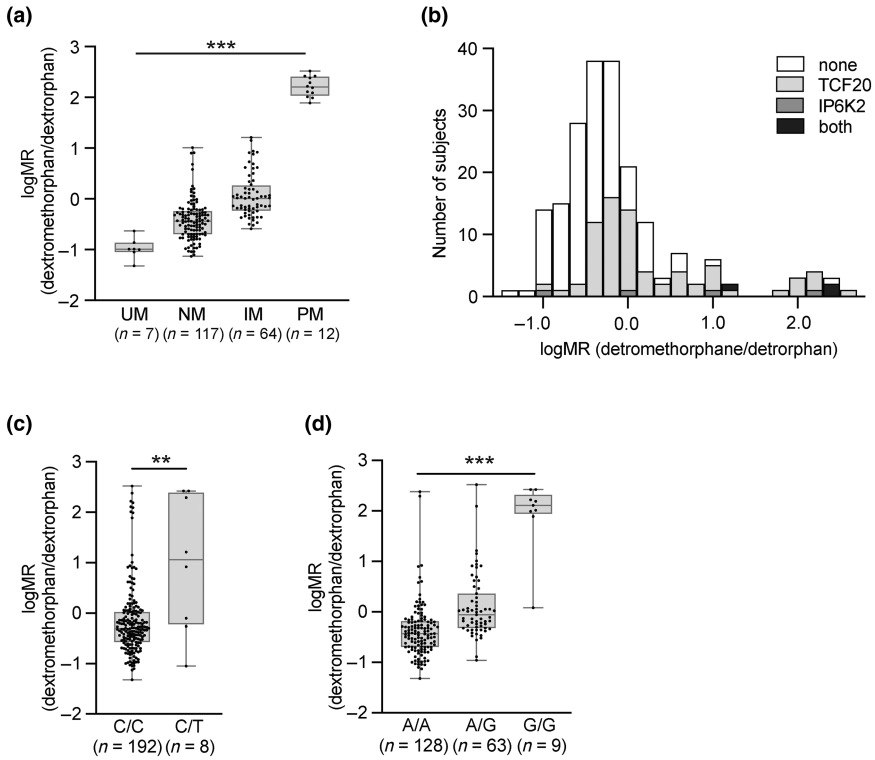

Figure 1 CYP2D6 phenotype–genotype correlation: (**a**) log metabolic ratio of dextromethorphan/dextrorphan in serum in relation to CYP2D6 established phenotype groups. (**b**) Histogram of log metabolic ratio. Subjects marked in violet were carriers of *IP6K2* and *TCF20* variants, while red marked subjects carried only the *IP6K2* and blue marked subjects only the TCF20 variant violet marked subject carry both variants. Impact of genotypes of (**c**) *IP6K2* (rs61740999), and of (**d**) *TCF20* (rs5758651) on metabolic ratios. The Mann–Whitney *U*-test, the Kruskal–Wallis test, ***$P < 0.001$.

rs12777823 (g.94645745G>T) located in the *CYP2C* cluster identified in the exome chip analysis (**Figure 4b**). This SNP was strongly linked to *CYP2C19*2* as reported previously.[19] Multiple regression analysis revealed that the *CYP2C19* genotype explained 30% of the interindividual variability of logMR omeprazole/5-OH omeprazole ($P < 0.0001$). The additional consideration of sex and rs12777823 increased the explanation of variability to 40%, while *CYP2C18* variants had no significant impact (**Table 3**).

Impact of *CYP3A* variants on midazolam metabolism

The metabolic ratios of midazolam/1-hydroxy midazolam did not differ significantly between *CYP3A5* expressors (**1/1* and **1/*3*) and nonexpressors (**3/*3*). Among the group of *CYP3A5* nonexpressors, however, *CYP3A4*22* allele carriers had a 1.7-fold increased mean logMR ($P < 0.001$) compared with *CYP3A4* wild-types. The difference was similar between the group being *CYP3A5* expressors and *CYP3A4*1/*1* carriers and the group of *CYP3A5* nonexpressors and *CYP3A4*1/*22* carriers indicating the major role of CYP3A4 for midazolam metabolism (**Figure 5a**).

On average, females had a slightly (1.2-fold) lower mean logMR compared with males ($P = 0.046$) (**Figure S5A**) that might be explained by the enhancing effect of ethinyl estradiol on CYP3A activity.[20] Since 93% of the female volunteers declared taking ethinyl estradiol-containing contraceptives until 3 days before the study, a separate analysis of female *CYP3A5* nonexpressors with the *CYP3A4*1/*1* genotype was performed. This group exhibited a significantly increased CYP3A activity (represented by lower logMR) compared with nonusers having the same genotypes. Female nonusers had similar activities as male volunteers (**Figure S5C,D**). Overall, multiple regression analysis revealed only a 10% contribution of the *CYP3A* diplotypes and sex to the interindividual variability of logMR midazolam/1-hydroxy midazolam ($P < 0.001$, **Table 3**). The exome chip analysis did not identify any other variants that contribute significantly to the metabolic ratio.

Impact of *CYP1A2* variants on caffeine metabolism

There was a lack of association of *CYP1A2*1F* on mean logMR caffeine/paraxanthine (**Figure 5b**). Females exhibited a significantly 1.3-fold higher mean logMR than men ($P < 0.001$). The same tendency was found in all genotype groups; however, the difference between females and males was only significant in *CYP1A2*1F* carriers (**Figure S6A,B**). Smokers ($n = 42$) showed a significantly higher CYP1A2 activity (represented by lower logMR) compared with nonsmokers ($n = 158$) (**Figure S6C**). By analyzing the interaction of sex and smoking, significant differences between females and males were found only in the group of nonsmoking *CYP1A2*1F* carriers (**Figure S6D–F**). Likewise, the multiple regression analysis revealed an 8% contribution of sex and smoking to interindividual variability of logMR caffeine/paraxanthine ($P < 0.001$; **Table 3**). There was no variant detected in

Table 3 Multiple regression analysis of factors, contributing to log metabolic ratios of probe drugs

	Consideration of genotypes or diplotypes[a]				
Dependent variable (logMR)	Independent variable included in analysis	Independent variable with significant impact after backward selection	Independent variable (*P*-value)	R^2	Model (*P*-value)
Dextromethorphan/dextrorphan	*CYP2D6*[a]	*CYP2D6*[a]	<0.001	0.83	<0.001
	CYP2D6[a]	*CYP2D6*[a]	<0.001	0.83	<0.001
	Sex				
	IP6K2 (rs61740999)				
	TCF20 (rs5758651)				
Losartan/E-3174	*CYP2C9*[a]	*CYP2C9*[a]	<0.001	0.31	<0.001
	CYP2C9[a]	*CYP2C9*[a]	<0.001	0.34	<0.001
	Sex	Sex	0.002		
	CYP2C9[a]	CYP2C9[a]	<0.001	0.50	<0.001
	Sex	Sex	0.003		
	FBXW12 (rs17080138)	*FBXW12* (rs17080138)	0.003		
	SLC17A4 (rs11754288)	*SLC17A4* (rs11754288)	<0.001		
	ZNF703 (rs79707182)	*ZNF703* (rs79707182)	0.004		
	CYP2C9 (rs10509680)				
Omeprazole/5-hydroxy omeprazole	*CYP2C19*[a]	*CYP2C19*[a]	<0.001	0.30	<0.001
	CYP2C19[a]	*CYP2C19*[a]	0.034	0.40	<0.001
	Sex	Sex	0.029		
	CYP2C18 (rs11188059)	rs12777823	<0.001		
	CYP2C18 (rs2860840)				
	rs12777823				
Midazolam/1-hydroxy midazolam	*CYP3A*[a]	*CYP3A*[a]	<0.001	0.10	<0.001
	Sex	Sex			
	Contraception		0.029		
Caffeine/paraxanthine	*CYP1A2*[a]	Sex	0.001	0.08	<0.001
	Sex	Smoking	0.020		
	Smoking				
	Consideration of CPIC-predicted phenotypes[b]				
Dependent variable (logMR)	**Independent variable tested**	**Independent variable with significant impact**	**Independent variable (*P*-value)**	**R^2**	**Model (*P*-value)**
Dextromethorphan/dextrorphan	*CYP2D6*[b]	*CYP2D6*[b]	<0.001	0.72	<0.001
	CYP2D6[b]	*CYP2D6*[b]	<0.001	0.73	<0.001
	Sex	*IP6K2* (rs61740999)	0.006		
	IP6K2 (rs61740999)				
	TCF20 (rs5758651)				

(Continued)

Table 3 (Continued)

Dependent variable (logMR)	Consideration of CPIC-predicted phenotypes[b]				
	Independent variable tested	Independent variable with significant impact	Independent variable (*P*-value)	R^2	Model (*P*-value)
Losartan/E-3174	*CYP2C9*[b]	*CYP2C9*[b]	<0.001	0.24	<0.001
	CYP2C9[b]	*CYP2C9*[b]	<0.001	0.27	<0.001
	Sex	Sex	0.002		
	CYP2C9[b]	*CYP2C9*[b]	<0.001	0.46	<0.001
	Sex	Sex	0.003		
	FBXW12 (rs17080138)	*FBXW12* (rs17080138)	<0.001		
	SLC17A4 (rs11754288)	*SLC17A4* (rs11754288)	<0.001		
	ZNF703 (rs79707182)	*ZNF703* (rs79707182)	0.001		
	CYP2C9[b]	*CYP2C9*[b]	<0.001	0.50	<0.001
	Sex	Sex	0.003		
	FBXW12 (rs17080138)	*FBXW12* (rs17080138)	<0.001		
	SLC17A4 (rs11754288)	*SLC17A4* (rs11754288)	<0.001		
	ZNF703 (rs79707182)	*ZNF703* (rs79707182)	0.001		
	CYP2C9 (rs10509680)	*CYP2C9* (rs10509680)	<0.001		
Omeprazole/5-hydroxy omeprazole	*CYP2C19*[b]	*CYP2C19*[b]	<0.001	0.29	<0.001
	CYP2C19[b]	Sex	0.035	0.37	<0.001
	Sex	rs12777823	<0.001		
	CYP2C18 (rs11188059)				
	CYP2C18 (rs2860840)				
	rs12777823				

[a]See **Table 1** for genotype or diplotype information. [b]See **Table 1** for CPIC-predicted phenotype information.

the exome chip analysis that contributed significantly to the metabolic ratio of caffeine.

DISCUSSION

In this study, we have applied a five-drug cocktail as a tool to phenotype the activity of specific CYP isoforms, relevant to drug metabolism. The single point measurement (5 hours in blood, 8 hours urine collection) of the metabolic ratio of the parent probe drug and its major metabolite applying a batch LC–MS/MS approach revealed reliable data and allowed a significant discrimination of diplotype-predicted phenotypes of CYP2D6, 2C9, 2C19, and 3A4/5, but not of CYP1A2. Numerous cocktail studies with partly different choices or dosages of probe drugs have been carried out so far. We selected probe drugs that have been applied successfully before.[6] The frequently applied Karolinska cocktail[7] used four of the same drugs but differed by applying 10 mg quinidine to phenotype CYP3A4. We used 2 mg of midazolam instead, since quinidine is known to be a CYP2D6 inhibitor affecting the dextromethorphan metabolism.[16] The Geneva cocktail[8] used flurbiprofen instead of losartan to phenotype CYP2C9 and further contained bupropion to phenotype CYP2B6 and fexofenadine for P-glycoprotein. Although we used higher doses in our approach, the five drugs were well tolerated by the volunteers.

The influence of sex on drug metabolism differed with respect to the various probe drugs. The strongest differences were observed for CYP1A2 and caffeine as female volunteers had a significantly lower activity than males. This finding is in line with earlier observations[21] and was not the result of a higher proportion of smoking in males,[22] as shown by a multivariate regression analysis considering the *CYP1A2* genotype, sex, and smoking habits. However, although participants were asked to stop smoking 2 days before the study, there was still a significant effect on CYP1A2 activity as indicated by reduced metabolic ratios of caffeine. This is possibly due to the remaining induction, since earlier studies demonstrated that smoking cessation

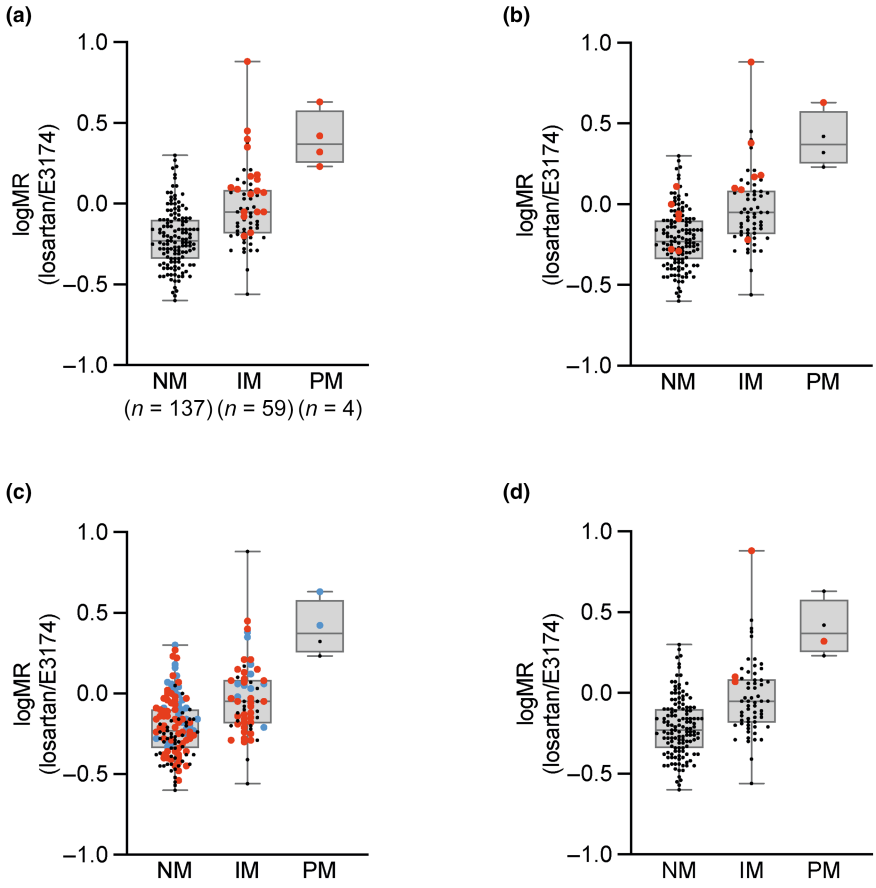

Figure 2 CYP2C9 phenotype–genotype correlation: log metabolic ratio of losartan/E-3174 in relation to *CYP2C9*1/*1* (NM), **1/*2*, **1/*3*, **2/*2*, (IM), and **2/*3* (PM). Cases carrying SNPs identified through the exome chip analyses are indicated as colored dots: heterozygous (red dots), homozygous (blue dots) (**a**) rs10509680 in *CYP2C9*, (**b**) rs17080138 in *FBXW12*, (**c**) rs11754288 in *SLC17A4*, (**d**) rs79707182 in *ZNF703*.

for 2 days led to a reduction in CYP1A2 activity by only 20%.[23] This shows the importance of considering smoking in clinical studies as carefully reviewed before.[24]

For CYP2C9, there is currently only sparse data on the effect of sex available.[25] In our samples, we could observe a significant tendency of lower activity in females.

Also, for CYP2C19, a tendency to lower activity in females was found. So far, sex differences in CYP2C19 were not observed in a Swedish,[26] but in a Pakistani omeprazole phenotyping study.[27] A small US-American study showed the same tendency, but did not reach significance.[28] In contrast, the activity of CYP3A4 was modestly but significantly higher in females, which is well established

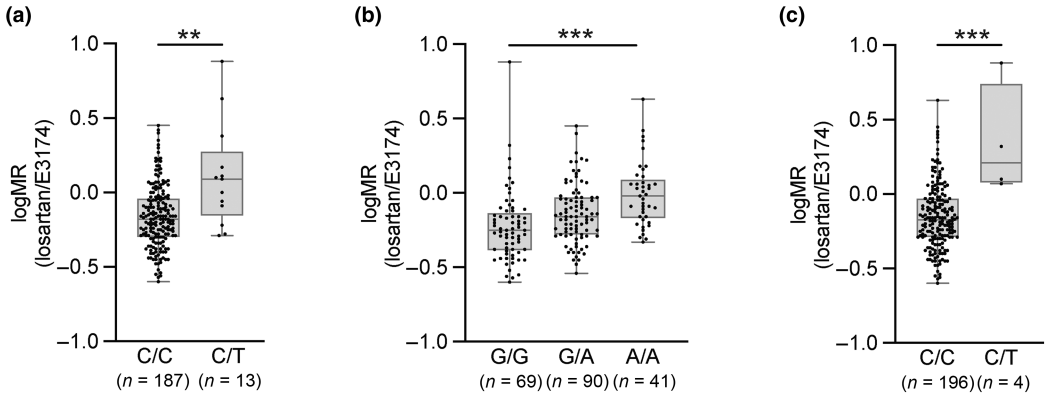

Figure 3 Impact of SNPs within the genes of (**a**) *FBXW12* (rs17080138), (**b**) *SLC17A4* (rs11754288), and (**c**) *ZNF703* (rs79707182) identified in the exome chip analysis to be associated with losartan/E-3174 metabolic ratio. The Mann–Whitney *U*-test, the Kruskal–Wallis test, ** $P<0.01$, *** $P<0.001$.

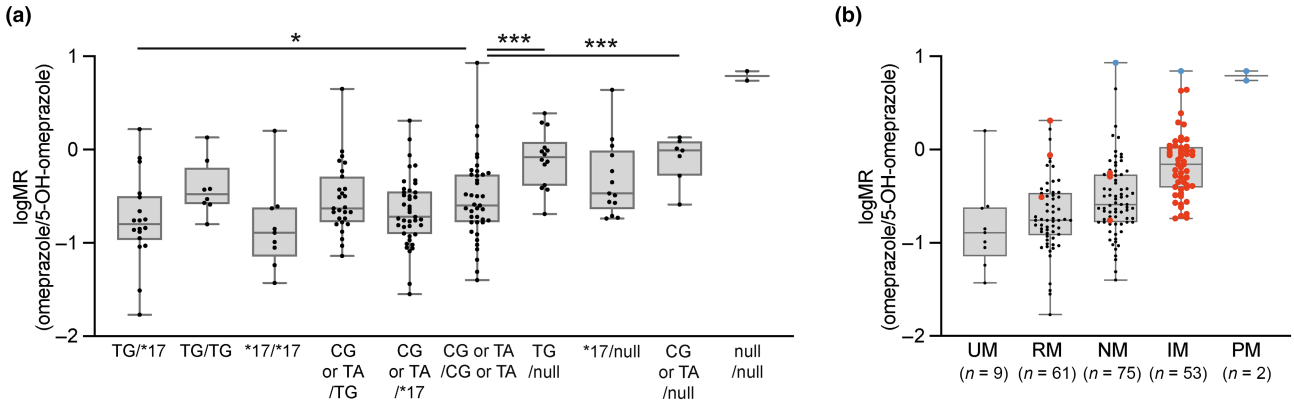

Figure 4 CYP2C19 phenotype–genotype correlation: log metabolic ratio of omeprazole/5-OH omeprazole in relation to *CYP2C9*2/*2* (PM), **1/*2* (IM), **1/*1* (NM), **1/*17* (RM) and **17/*17* (UM). (**a**) The *CYP2C:TG* had no significant impact on logMR. Cases are presented in the summarized *CYP2C* diplotype groups according to Bråten *et al*. For more details, see **Table S4**. Cases carrying CA containing diplotypes were excluded from this figure. Null = no function allele (*CYP2C19*2*). (**b**) Cases carrying *CYP2C19* rs12777823, which is strongly linked to *CYP1C19*2* are indicated as colored dots: heterozygous (red dots), homozygous (blue dots). Mann–Whitney *U*-test was performed for groups $n \geq 4$, ** $P < 0.01$, *** $P < 0.001$.

in vitro[29] and *in vivo*.[30] These results remained significant after including genotypes in a multivariate analysis. However, possibly this result is due to remaining the induction of CYP3A4 by ethinylestradiol taken as an oral contraceptive by 22 female volunteers before the study.

As expected, the allocation of dextromethorphan/dextrorphan metabolic ratios for CYP2D6 activity to CPIC phenotype groups showed highly significant differences. In particular, poor metabolizers were distinct from all other groups and metabolic ratios of CYP2D6 ultrarapid metabolizers could be better discriminated in serum (**Figure 1**) than in urine (**Figure S2**). Therefore, all further analyses were performed using data derived from serum dextromethorphan/dextrophan metabolic ratios. Although the medians of intermediates, normal and ultrarapid metabolizers were significantly different, there were large overlaps between the phenotypes, being in line with large past phenotyping studies[31,32] and the continuum of CYP2D6 activity described in the Consensus Recommendations from the Clinical Pharmacogenetics Implementation Consortium and Dutch Pharmacogenetics Working Group.[33]

Since studies in homo- and dizygote twins gave rise that further genetic factors are likely to contribute to interindividual variation of CYP2D6 metabolism,[34] it was therefore exciting to see that the exome chip analysis discovered three SNPs (*COLL11A2*, *IP6K2*, and *TCF20*) associated with dextromethorphan/dextrophan metabolic ratios in urine. However, only *IP6K2* and *TCF20* were confirmed in serum samples. All were related to elevated metabolic ratio (lower CYP2D6 activity). The findings were supported by the fact that there was a gene–dose effect for *TCF20* variants. rs575865 is located in the transcription factor 20 (*TCF20*) gene and was reported to be associated with an enhancer of CYP2D6 expression.[35]

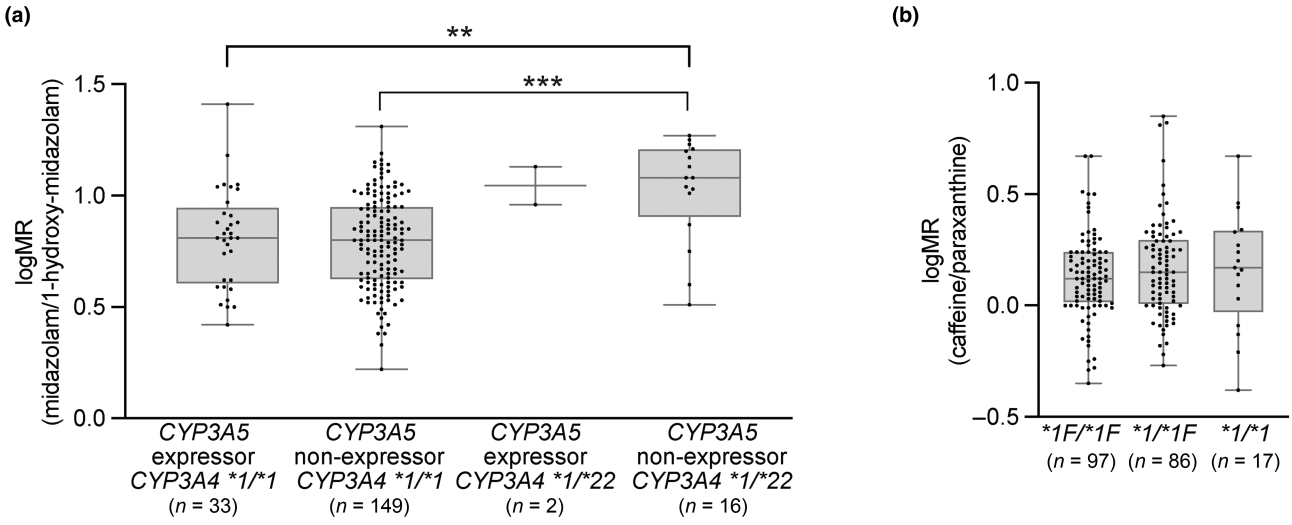

Figure 5 CYP3A4/CYP3A5 and CYP1A2 phenotype–genotype correlation. (**a**) CYP3A4/CYP3A5: log metabolic ratio of midazolam/1-hydroxy midazolam in relation to *CYP3A4*22* and *CYP3A5*3*. *CYP3A4*22* contributed significantly to decreased midazolam metabolism. (**b**) CYP1A2: log metabolic ratio of caffeine/paraxanthine in relation to *CYP1A2*1F* ($P = 0.760$.). Mann–Whitney *U*-test, **$P < 0.01$, ***$P < 0.001$.

Interestingly, the authors suggested in their paper to consider rs5758550 for *CYP2D6* genotyping panels to yield more accurate phenotype prediction. However, after inclusion into the multiple regression analysis of data from our study, *TCF20* was no longer significantly contributing to the explanation of variability of metabolic ratios in urine, but not in serum samples. The functional meaning of *IP6K2*, however, needs to be further elucidated and confirmed in independent cohorts.

For CYP2C9 significant differences in established genotype-predicted phenotypes of losartan/E3174 metabolic ratio considering *CYP2C9*2* and *2C9*3*[36] were confirmed. An additional SNP (rs10509680) located in the *CYP2C9* gene locus and almost completely linked to *CYP2C9*3* was identified in the exome chip analysis and proven by genotyping. This SNP, however, was previously described in two GWAS studies on warfarin responsiveness and maintenance doses.[37,38] Because of the almost complete linkage to the established low active allele *CYP2C9*3*, this SNP can be neglected in further genotyping approaches. Strikingly, the exome chip analysis revealed three variants outside the *CYP2C9* locus that were associated with diminished CYP2C9 activity as indicated by significantly increased losartan/E3174 metabolic ratios. In the multivariate model, these three variants together with sex improved the explanation of interindividual variability of losartan metabolism by 19%. So far, however, there is no rational for the functional influence of *FBXW12*, *SLC17A4*, and *ZNF703* on CYP2C9 activity or distribution of losartan. Recently, the *SLC17A4* locus was identified to be a novel triiodothyronine (T3) and T4 transporter.[39] Whereas no homozygous carriers of *FBXW12* and *ZNF703* could be found in our sample, there was a clear gene–dose effect for the variant in the *SLC17A4* gene, giving support that rs11754288 could have a functional impact. These are interesting findings, but confirmation in independent cohorts is required before further conclusions can be made.

Likewise, there was a distinct genotype–phenotype relationship of omeprazole/5-hydroxy omeprazole metabolic ratios to the established CPIC phenotype groups for CYP2C19. In addition, *CYP2C19* rs12777823 was identified in the exome chip analysis. This SNP located in the *CYP2C18/CYP2C19/CYP2C9/CYP2C8* gene cluster was described before by Shuldiner *et al.*[19] in relation to diminished response to clopidogrel. This polymorphism was found to be in strong linkage disequilibrium with *CYP2C19*2*. The multivariate analysis, however, revealed this SNP as an independent factor contributing to phenotypic variability. Therefore, it appears reasonable, to discuss rs12777823 as a further genetic marker in a clinical setting. Additional consideration of sex further slightly improved the model.

Of large interest was also the consideration of the recently described *CYP2C:TG* haplotype (rs2860840T/rs11188059G) in the *CYP2C18* locus reportedly being linked to ultrarapid metabolizers.[15] In accordance with previous studies, we observed no homozygous *CYP2C19*17* or *CYP2C19*2* carriers with the TG haplotype in our cohort, but identified some CA carriers. Furthermore, we were not able to confirm a contribution of the *CYP2C:TG* haplotype to the metabolic activity. This is in line with recent observations by Zubiaur *et al.*,[40] who determined the influence of *CYP2C:TG* on the pharmacokinetics of a number of substrates *in vivo*, the protein content, and CYP2C19 *in vitro* activity. These authors concluded that there is insufficient evidence supporting the clinical impact of *CYP2C:TG*.

Midazolam phenotyping confirmed that CYP4A4 is the major hepatic metabolizing enzyme, as there was no difference between CYP3A5 expressors and nonexpressors. The results confirmed the earlier described low CYP3A activity among *CYP3A4**22 allele carriers.[41] No further variant could be detected in the exome chip analysis.

Rs762551 present in *CYP1A2*1F* is considered to be associated with the inducibility of CYP1A2 in smokers[42] and often mentioned in relation to variability of CYP1A2 activity. In our sample, there was a lack of association with caffeine metabolism. However, based on our sex-specific analysis our results suggest that the observed differences in CYP1A2 activity are likely based on sex with only little effect of smoking. A limitation is the fact, however, that our study comprised only few smokers and all had to refrain from smoking before the beginning of the study. The exome chip analysis did not reveal any significant variant associated with caffeine/paraxanthine metabolic ratio.

Limitations of the study

This cocktail study determined only a single timepoint of the metabolic ratios of the probe drugs. Hence, intraindividual differences could be more pronounced compared with a full pharmacokinetic analysis obtaining the clearance and AUC. Moreover, deviations from the protocol, such as smoking or intake of caffeine prior to the study affecting CYP1A2 activity or any food-drug interactions, cannot be entirely excluded. We focused the genotyping panel on the most frequent variants in the Caucasian population. It can therefore not be excluded that consideration of less frequent genetic variants would have given a more precise figure of phenotype groups. However, the obtained genotype–phenotype relationships were in accordance with earlier studies.

With respect to exome chip analysis, the number of 200 volunteers is quite small and the functional impact on metabolic ratios needs to be confirmed in independent cohorts. However, all exome chip hits were further confirmed by other genotyping methods and the observed gene–dose effects give support for the findings. Last but not least, the functional consequences and pathways require further elucidation.

CONCLUSION

This genotype–phenotype study based on a trial of five-drug cocktails and metabolic ratio analysis at a single timepoint showed—with the exception of CYP1A2—a very well discrimination of genotype-predicted phenotypes defined by CPIC. The study found modest sex differences in the activity of CYP1A2, 2C9, 2C19, and CYP3A4, with the latter possibly due to contraceptive administration until 3 days before the study. In particular, the exome chip analysis revealed that additional variants in the *FBXW12*, *SLC17A4*, and *ZNF703* genes were significantly associated with the metabolic ratios of the CYP2C9 trial drug

losartan. Variants in *IP6K2* and *TCF20* were identified to contributing significantly to dextromethorphan metabolism, though only *IP6K2* remained significant after multiple regression analysis including CPIC-predicted phenotypes. *COL11A2* was found to affect only the metabolic ratios determined in urine samples. The inclusion of additional variants and sex in the multiple regression models improved partly significantly the explanation of variability in metabolic ratios, leading to further improvement of the algorithms for predicting individual phenotypes of drug-metabolizing enzymes for dose optimization.

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

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CONFLICT OF INTEREST

The authors declared no competing interests in this work. As an Associate Editor of *Clinical Pharmacology and Therapeutics*, Ingolf Cascorbi was not involved in the review or decision process for this paper.

AUTHOR CONTRIBUTIONS

R.B., H.B., and I.C. wrote the manuscript. R.B., S.O., W.S., A.F., and I.C. designed the research. R.B., H.B., S.O., M.K., L.E., and J.H. performed the research. R.B., H.B., S.O., M.H., and L.E. analyzed the data. S.O., W.S., A.F., and I.C. contributed new reagents/analytical tools.

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