Combinations of common SNPs of the transporter gene ABCB1 influence apparent bioavailability, but not renal elimination of oral digoxin

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Effects of different genotypes on the pharmacokinetics of probe substrates may support their use as phenotyping agents for the activity of the respective enzyme or transporter. Digoxin is recommended as a probe substrate to assess the activity of the transporter P-glycoprotein (P-gp) in humans. Current studies on the individual effects of three commonly investigated single nucleotide polymorphisms (SNPs) of the ABCB1 gene encoding P-gp (C1236T, G2677T/A, and C3435T) on digoxin pharmacokinetics are inconclusive. Since SNPs are in incomplete linkage disequilibrium, considering combinations of these SNPs might be necessary to assess the role of polymorphisms in digoxin pharmacokinetics accurately. In this study, the relationship between SNP combinations and digoxin pharmacokinetics was explored via a population pharmacokinetic approach in 40 volunteers who received oral doses of 0.5 mg digoxin. Concerning the SNPs 1236/2677/3435, the following combinations were evaluated: CGC, CGT, and TTT. Carriers of CGC/CGT and TTT/TTT had 35% higher apparent bioavailability compared to the reference group CGC/CGC, while no difference was seen in CGC/TTT carriers. No significant effect on renal clearance was observed. The population pharmacokinetic model supports the use of oral digoxin as a phenotyping substrate of intestinal P-gp, but not to assess renal P-gp activity.

During the last decades, membrane transporters demonstrated to play an essential role in the pharmacokinetics (PK) and -dynamics (PD) of many drugs, potentially explaining drug-drug interactions and pharmacogenetic sources of variability in drug effects1–3.

P-glycoprotein (P-gp), encoded by the gene ABCB1, is the first well-characterized membrane transporter4. It belongs to the ATP-Binding Cassette (ABC) family and acts as an efflux transporter located in the canalicular side of hepatocytes, the apical membrane of intestinal cells, the luminal side of the tubular cells in the kidney, as well as the apical membrane of brain microvessel endothelial cells, and placental syncytiotrophoblasts5,6. Effects of P-gp activity on plasma concentrations of a broad spectrum of endogenous and xenobiotic substances have been shown, including testosterone, aldosterone, antiviral drugs, anticancer drugs, immunosuppressant agents, antifungals and cardiovascular drugs7–13. Moreover, many in vivo studies showed that the induction or inhibition of P-gp could cause clinically relevant drug-drug interactions14.

To investigate transporter-based drug-drug interactions (TDDIs) of new compounds, digoxin is recommended as a P-gp phenotyping drug in humans by regulatory agencies15,16. Digoxin is a cardiac glycoside drug used in congestive heart failure and atrial fibrillation. The bioavailability of oral digoxin is approximately 60–80%,
and digoxin is mainly eliminated via glomerular filtration and active tubular secretion, with only minor contribution of non-cytochrome P450 enzymes and fecal excretion

In 1999, Greiner et al. demonstrated the importance of intestinal P-gp in its net transport of digoxin across the gut wall by administering digoxin orally and intravenously in combination with rifampin, a strong P-gp inducer

Furthermore, by showing that the co-administration of the P-gp inhibitor verapamil orally and digoxin intravenously resulted in a significant decrease in renal clearance, Pedersen et al. provided first evidence that renal P-gp might play an important role in digoxin elimination

However, recent evidence indicates that renal organic anion transporter polypeptide 4C1 (OATP4C1) might be rate-limiting for renal elimination of digoxin, although inhibition of OATP4C1 might be too weak to fully explain the reported effect of verapamil on digoxin clearance

Besides, Sato et al. showed that ritonavir strongly inhibits OATP4C1 in vitro with an IC50 of 8.5 µM, indicating a potential for clinically relevant drug-drug interactions. However, a recent clinical trial reported by Penzak et al. showed that the renal clearance of digoxin did not significantly change when administering digoxin orally before and after 14 days of oral ritonavir administration

Overall, it is not clear to which extent P-gp and OATP4C1 determine the renal elimination of digoxin.

Beyond TDDIs, ABCB1 polymorphisms might have an impact on P-gp activity and respective phenotyping results. Particularly, three commonly investigated single nucleotide polymorphisms (SNPs) in the protein-coding region, i.e., C1236T (rs1128503), G2677T/A (rs2032582) and C3435T (rs1045642), have been associated with changes in P-gp activity in vivo

Supplementary Table S1. The included publications were identified using PubMed by the search term "(ABCB1 OR MDR1 OR pgp) AND (variant OR polymorphism) AND pharmacokinetics AND digoxin". In total, 37 articles were identified. Of these, seven were review articles, five had no PK data evaluation, four were not related to digoxin, three were in vitro results, one was not in English, and in two cases the original articles could not be retrieved. Two additional relevant publications from the reference list of the review articles were additionally included. Two additional relevant publications from the reference list of the review articles were additionally included.

In addition, G2677T was highly correlated with C1236T (r2 = 0.76). Thus, any of the SNPs C1236T, G2677T and C3435T might be used as a reasonable albeit not fully reliable tagging SNP for the evaluation of a genotype effect.

Hoffmeyer et al. provided the first evidence on pharmacogenetic influences of P-gp activity in humans. Particularly, compared to wild-type, 3435TT carriers of P-gp were shown to have a 38% increase in peak steady-state concentrations (Cmax) after administration of 0.25 mg digoxin (p = 0.006) in Caucasians, suggesting a reduced intestinal secretion of digoxin and a reduced activity of P-gp related to the variant. However, in another early study, Gerloff et al. reported that the initial area under the curve (AUC0-4h) and Cmax values for 1 mg orally administered digoxin in Caucasians who carried 2677TT and 3435TT were not significantly higher than in subjects with 2677GG and 3435CC.

Until today, numerous evaluations on the geno/haplophenotype of ABCB1 on the pharmacokinetic parameters of digoxin in vivo have been published, and the results from 17 previous studies are summarized in Supplementary Table S1. The included publications were identified using PubMed by the search term "(ABCB1 OR MDR1 OR pgp) AND (variant OR polymorphism) AND pharmacokinetics AND digoxin". In total, 37 articles were identified. Of these, seven were review articles, five had no PK data evaluation, four were not related to digoxin, three were in vitro results, one was not in English, and in two cases the original articles could not be retrieved. Two additional relevant publications from the reference list of the review articles were additionally included.

Overall, the effects of the three ABCB1 SNPs, C1236T, G2677T/A, and C3435T, on digoxin pharmacokinetics are inconclusive, which might also be a consequence of considering only one of the 3 SNPs to define genotypes. Since the SNPs are in incomplete linkage disequilibrium, considering the SNP combination might be necessary to assess the role of common polymorphisms in digoxin pharmacokinetics accurately.

The SNP combinations CGC and TTT at positions 1236/2677/3435, respectively, are the most frequent combinations in the Caucasian population. In contrast to the assumption that the TTT combination might be too weak or too early, Schleyer et al. demonstrated that triple SNP variants (C1236T, G2677T, C3435T) expressed in Xenopus laevis oocytes had no effect on the efflux of digoxin. The efflux rate of digoxin after 45 min was 77.6 ± 11.9% in the triple SNP variant and 82.7 ± 13.6% for wild-type ABCB1.

To provide additional information on the effect of common ABCB1 SNP combinations on P-gp activity in vivo, we investigated the relationship between digoxin pharmacokinetics and SNP combinations in healthy Caucasian subjects using population pharmacokinetic modeling.
Results

Summary of subject demographics. Data from 40 healthy Caucasian subjects (22 female) with a mean age of 38 years (range 20–68), a mean height of 1.73 m (range 1.54–1.92 m), and mean body weight of 73.1 kg (range 54.4–97.0 kg) were available for the population pharmacokinetic analysis. All subjects were assigned to four SNP combination groups based on the genotype of P-gp. The SNP combinations were CG/C/G, CG/C/GT, CG/C/TTT, and TTT/TTT (as the respective combination of 1236/2677/3435 SNPs) with 9, 5, 15 and 9 subjects, respectively (see Table 1). Two subjects had different SNP combinations, CGT/TTT and CGT/CTT, and were excluded from the genotype covariate analysis due to the small group size. SNP frequencies of C1236T (0.425), G2677T (0.438) and C3435T (0.525) in the study population were similar to published data26, and did not show a significant deviation from Hardy–Weinberg equilibrium (χ² of 1.32, 0.745 and < 0.001, respectively).

For the SNP pairs C3435T/G2677T, C3435T/C1236T and G2677T/C1236T, a pronounced linkage disequilibrium was found (D’ of 0.78, 1.00, and 1.00; r² of 0.55, 0.67 and 0.95, respectively).

Digoxin empirical model. As a base model, a two compartment model with mixed first- and zero-order absorption and linear elimination described the data best. A summary of key model development steps is shown in Table 2. Visual predictive checks (VPCs) indicated a difference in apparent bioavailability and absorption shape between the two trials that was not captured well by the base model. Consequently, a significantly lower bioavailability was identified in trial I compared to trial II (-30.3%, drop in objective function value (OFV) by 72.6) and a significantly lower first-order absorption rate constant (Ka) (0.201 h⁻¹ vs. 0.636 h⁻¹, drop in OFV by 46.0) was identified in the test period of trial II compared to trial I and the reference period of trial II. When introducing additional estimates for bioavailability in trial I and for Ka in the test period of trial II, VPCs did not show any further misspecification.

Covariate model for effects of ABCB1 SNP combinations. Apparent bioavailability was estimated separately for each of the defined SNP combinations in the population pharmacokinetic model, resulting in a significant drop in OFV by 26.1 points. In addition, renal clearances (CLR), zero-order absorption durations (D2) and Ka were also computed separately for each SNP combination. However, the model did not improve significantly (drop in OFV by 1.27, 3.44 and 3.55 points, respectively). A non-parametric bootstrap with 1,000 samples was conducted for the model with separately estimated apparent bioavailabilities in each SNP combination group. Relative differences in apparent bioavailabilities and renal clearance between SNP combinations are summarized in Fig. 1. CG/C/GT and TTT/TTT carriers had an approximately 35% higher bioavailability com-

| Abbreviated description of SNP combination group | Number of subjects | Combinations of variants |
|-----------------------------------------------|-------------------|--------------------------|
| CG/C/GC                                      | 6                 | 1236 C/C + 2677 G/G + 3435 C/C |
| CG/C/GT                                      | 3                 | 1236 C/C + 2677 G/G + 3435 C/T |
| CG/C/TTT                                     | 7                 | 1236 C/T + 2677 G/T + 3435 C/T |
| TTT/TTT                                      | 6                 | 1236 T/T + 2677 T/T + 3435 T/T |
| CG/C/TT                                      | 1                 | 1236 C/T + 2677 G/T + 3435 T/T |
| CG/C/C                                       | 1                 | 1236 C/C + 2677 G/T + 3435 T/T |

Table 1. Observed distribution of genotypes for ABCB1. *38 out of forty subjects in the two trials were included in our evaluation of the effect of SNP combinations. P-gp, P-glycoprotein. **Contains one non-compliant subject.

| Model | Description | OFV     | AIC      |
|-------|-------------|---------|----------|
| Base model | 2-Compartment model with mixed-order absorption and linear elimination | -1,166.40 | -1,072.41 |
| Full model-selection of demographic and physiological covariates | Base model with separate estimates of bioavailability for different trials | -1,238.98 | -1,142.98 |
| | Model 2 with additional separate estimates for first-order absorption rate constants of test period of trial II | -1,284.96 | -1,183.58 |
| Final model with covariate | Final model with separate estimates of bioavailability according to CG/C/GC, CG/C/GT, CG/C/TTT, and TTT/TTT ABCB1 SNP combination groups | -1,311.09 | -1,206.58 |

Table 2. Model selection: Summary of covariate building steps for digoxin pharmacokinetics. Summary of population pharmacokinetic model selection. Starting from the base model with a 2-compartment model, separate estimates for bioavailability and first-order absorption rate constants were introduced into the model. Finally, covariates on the bioavailability of different SNP combination were computed. OFV, objective function value; AIC, Akaike information criterion.
pared to CGC/CGC, while CGC/TTT carriers had a similar bioavailability compared to CGC/CGC. However, no significant differences in renal clearance were observed among different SNP combination groups.

The final point estimates and bootstrap statistics of pharmacokinetic parameters are summarized in Table 3. VPC and Goodness of Fit (GOF) plots of plasma and urine data are shown in Figs. 2 and 3.

**Table 3.** Final model parameter estimates (model 4). IIV CV%, coefficient of variation on inter-individual variability; 95% CI, 95% confidence interval.

![Figure 1. Relative difference in (A) apparent bioavailabilities and (B) renal clearance comparing different SNP combination groups to the reference SNP combination CGC/CGC. Median and 95% confidence intervals (95% CI) of fixed effects parameter estimates obtained from a bootstrap. The vertical dashed line represents no difference compared to the reference SNP combination. Refer to Table 3 for further information.](image-url)
Figure 2. Visual predictive check of the final model (model 4) of (A) plasma, (B) urine. Solid (dashed) lines represent medians (5%, 95% percentiles) of observed concentrations; orange, blue and orange areas represent 95% confidence intervals of 5%, 50% and 95% percentiles predicted by the model. For a correctly specified compartmental model, observed medians should lie inside the middle blue boxes. Observed 95% percentiles should lie within the upper and 5% percentiles within the lower orange boxes.
studies included in this population pharmacokinetic evaluation, where C\text{max} and AUC_{0-24h} of digoxin were significantly higher in CGC/CGT and TTT/TTT, but not in CGC/TTT carriers, compared to CGC/CGC. In contrast, in a study by Xu et al. subjects carrying TTT/TTT showed a higher average C\text{max} and AUC_{0-4h} compared to CGC/CGC in a Chinese Han population, but the observed difference was not statistically significant. In previous studies, no systematic evaluation of the effect of CGT on the pharmacokinetics of digoxin has been conducted. Several previous studies showed the effect of 3435CT on the pharmacokinetics of digoxin. However,
we cannot identify the subjects as belonging to the CGT group since the SNPs at positions 1236 and 2677 were not considered. Therefore, there is currently not enough data to support a genotype effect of CGT. Kim et al. included CGC/CGT carriers in an ABCB1 genotype evaluation regarding the pharmacokinetics of fexofenadine. However, no statistical evaluation was conducted since there was only a single CGC/CGT carrier. We cannot explain why in our evaluation homozygous (but not heterozygous) carriers of TTT as well as the CGC/CGT genotype group had a different apparent bioavailability compared to homozygous wildtype carriers. Beyond the relatively small effects of the combined genetic variants and the pronounced inter-individual variability of apparent bioavailability, other possible explanations would include (1) the mechanisms by which each single SNP and/or the SNP combination may modify overall P-gp expression/activity; indeed, an effect of C3435T could be attenuated by the presence of the two further SNPs studied here; (2) whether a dominant model, a co-dominant model or a recessive model would be most appropriate to describe the relationship between genotype and P-gp expression/activity; for the presence of all three SNPs (TTT group), a recessive model would be in agreement with our findings; (3) effects of other covariates not assessed in the present analysis, such as further SNPs of ABCB1, SNPs in xenobiotic receptors involved in P-gp regulation etc.; (4) a chance finding based on the relatively low number of subjects in the CGC/CGT group (n = 5); this needs further investigation, and to this end, it would be interesting to assess individuals with two respective mutated alleles (CGT/CGT) but these were not present in our population. Although the employed model is of an empirical nature, net apparent bioavailability reflects the sum of intestinal absorption and intestinal as well as biliary secretion, which presumably results in enterohepatic circulation. The 35% higher bioavailability in TTT/TTT and in CGC/CGT carriers thus cannot be translated directly into a 35% lower activity of P-gp in these individuals. The results also do not allow to draw further conclusions on the underlying molecular mechanisms, such as reduction in ATP binding affinity, loss of ATP hydrolysis, modification of protein folding, or reduction in P-gp expression58. Published data on the effect of the TTT SNP combination on P-gp expression in the duodenum and intestine is equivocal56. Although there are contradictory data on whether ABCB1 genotype would influence P-gp activity in vitro or in vivo, the present study showed an effect of combined SNPs on digoxin apparent bioavailability along with the absence of a respective effect on renal clearance of digoxin. This casts further doubts on the role of P-gp as a rate-limiting transporter for digoxin elimination in the kidney. However, we cannot rule out that our limited sample size was insufficient to detect minor differences in renal clearance between groups. Also, in our previous NCA analysis, no significant difference in renal clearance was found between CGC/CGC and other SNP combination groups52.

When digoxin was given intravenously or orally with a strong P-gp inducer, renal elimination of digoxin was not relevantly affected, but the AUC0–144h for both administration routes and AUC0–3h, Cmax and bioavailability for oral administration were decreased. In addition, non-renal clearance (which might reflect intestinal and/or biliary clearance) for intravenous administration and tmax for oral administration were increased59. These findings also indicate that P-gp activity is not rate-limiting for the elimination of digoxin in the kidney. However, the impact of SLCO4C1 polymorphisms on the pharmacokinetics of substrates is unknown54. Whether SLCO4C1 (cytogenetic location: 5q21.1) is in linkage disequilibrium with ABCB1 (cytogenetic location: 7q21.12) has not been investigated yet. Therefore, we assume that polymorphisms of SLCO4C1 are randomly distributed in our study subjects, while we cannot exclude whether such polymorphisms may have an additional impact on the pharmacokinetics of digoxin.

In our empirical pharmacokinetic model, different values for apparent bioavailability were estimated for the two trials due to differences in study designs (see Table 3), probably attributable to the use of different concomitantly administered drugs. Thus, correcting for trial-specific differences was necessary to allow identification of two trials due to differences in study designs (see Table 3), probably attributable to the use of different concomitantly administered drugs. We cannot explain why in our evaluation homozygous (but not heterozygous) carriers of TTT as well as the CGC/CGT genotype group had a different apparent bioavailability compared to homozygous wildtype carriers. Beyond the relatively small effects of the combined genetic variants and the pronounced inter-individual variability of apparent bioavailability, other possible explanations would include (1) the mechanisms by which each single SNP and/or the SNP combination may modify overall P-gp expression/activity; indeed, an effect of C3435T could be attenuated by the presence of the two further SNPs studied here; (2) whether a dominant model, a co-dominant model or a recessive model would be most appropriate to describe the relationship between genotype and P-gp expression/activity; for the presence of all three SNPs (TTT group), a recessive model would be in agreement with our findings; (3) effects of other covariates not assessed in the present analysis, such as further SNPs of ABCB1, SNPs in xenobiotic receptors involved in P-gp regulation etc.; (4) a chance finding based on the relatively low number of subjects in the CGC/CGT group (n = 5); this needs further investigation, and to this end, it would be interesting to assess individuals with two respective mutated alleles (CGT/CGT) but these were not present in our population. Although the employed model is of an empirical nature, net apparent bioavailability reflects the sum of intestinal absorption and intestinal as well as biliary secretion, which presumably results in enterohepatic circulation. The 35% higher bioavailability in TTT/TTT and in CGC/CGT carriers thus cannot be translated directly into a 35% lower activity of P-gp in these individuals. The results also do not allow to draw further conclusions on the underlying molecular mechanisms, such as reduction in ATP binding affinity, loss of ATP hydrolysis, modification of protein folding, or reduction in P-gp expression58. Published data on the effect of the TTT SNP combination on P-gp expression in the duodenum and intestine is equivocal56. Although there are contradictory data on whether ABCB1 genotype would influence P-gp activity in vitro or in vivo, the present study showed an effect of combined SNPs on digoxin apparent bioavailability along with the absence of a respective effect on renal clearance of digoxin. This casts further doubts on the role of P-gp as a rate-limiting transporter for digoxin elimination in the kidney. However, we cannot rule out that our limited sample size was insufficient to detect minor differences in renal clearance between groups. Also, in our previous NCA analysis, no significant difference in renal clearance was found between CGC/CGC and other SNP combination groups52.

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In our empirical pharmacokinetic model, different values for apparent bioavailability were estimated for the two trials due to differences in study designs (see Table 3), probably attributable to the use of different concomitantly administered drugs. Thus, correcting for trial-specific differences was necessary to allow identification of trial-independent effects of ABCB1 SNP combinations. Furthermore, we introduced a mixed zero- and first-order absorption model since some plasma concentration profiles exhibited a double peak phenomenon, with a second peak occurring after 4 h to 8 h post-dose. The double peak phenomenon could not be attributed to a food effect, but is in line with previous evaluations of digoxin exhibiting a similar phenomenon55,56.

Another limitation of this study is that plasma and urine samples were only available up to 24 h after drug administration, which covers less than one elimination half-life of digoxin. However, the assessment of drug elimination should be reliable once absorption is completed. Indeed, the estimated renal clearance of digoxin was comparable with previously published data57. Despite the limitations, the chosen population pharmacokinetic approach allowed to correct for differences between trials and to describe the effect particularly attributable to SNP combinations based on a large and detailed dataset.

In the previous studies assessing the effect of ABCB1 genotypes on digoxin pharmacokinetics, noncompartmental methods were used, including Cmax, AUC0–t, time of maximum plasma concentration (tmax) or ClRg17–30. In general, a population pharmacokinetic evaluation might be advantageous to identify effects on parameters more closely related to physiological processes. Although our model described the data well, the complex interplay of drug absorption, intestinal secretion and biliary elimination could not be captured in detail by the empirical model solely based on oral administration of digoxin. The information on genotype effects that could be obtained by the present evaluation turned out to be supportive but not superior to the information obtained by noncompartmental analysis. Using semiphysiological models applied to datasets including both oral and intravenous administration, as has been used for drugs undergoing first pass metabolism such as midazolam58, might be a promising approach to learn more about the mechanism of ABCB1 genotype effects on digoxin pharmacokinetics.

Whether the observed relationship between the SNP combinations/haplotypes of ABCB1 and digoxin can be transferred to other P-gp substrates is currently not clear. For instance, in a previous study conducted in a Chinese population, 1236CC carriers had a lower Cmax (− 53%; p = 0.013), AUC0–∞ (− 40%; p = 0.04), and cumulative amount excreted in urine over 6 h (− 52%; p = 0.027) and a higher apparent oral clearance (+ 35%; p = 0.013) of cloxacinil, another P-gp substrate, as compared to carriers of 1236CC and 1236CT. Moreover, the homozygous CGC carriers also had a lower Cmax (p = 0.017), AUC0–∞ (p = 0.032), and cumulative amount excreted over 6 h in urine (p = 0.026) and a higher apparent oral clearance of cloxacinil (p = 0.002) compared to homozygous carriers
of TTT. Renal clearance of cloxacillin was not altered by the SNP combination TTT/TTT59. This result is very similar to the one obtained for digoxin. In contrast, in a study in Japanese subjects, homozygous carriers of TTT (defined as homozygous ABCB1*2 in the corresponding publication) compared to subjects who did not carry TTT had a lower renal clearance (p < 0.05) until urinary recovery (p < 0.01) of the P-gp substrate irinotecan and its metabolites, while there was no significant difference in the ratio AUC0–16h/Cre60.

Furthermore, Kim et al. showed that homozygosity for TTT (defined as homozygous ABCB1*2 in the corresponding publication) was related to a difference in AUC0–16h of orally administered fexofenadine, another P-gp probe substrate, in the opposite direction: The AUC was 40% lower compared to homozygous CGC (defined as homozygous ABCB1*1/*1)69, suggesting increased intestinal secretion for this SNP combination. Moreover, the effect of different ABCB1 SNP combinations on the pharmacokinetics of cyclosporine was also inconsistent in previous reports, which may be attributable to the high variability in the pharmacokinetics in the heart and renal transplantation patients61–64. As a potential explanation for discrepant findings, digoxin was suggested to bind to different sites of P-gp unlike other typical P-gp substrates65. Whether the SNP combination of P-gp alters the structure of the binding site for digoxin, but not for other substrates, is also unknown. Thus, the whole protein structure of different P-gp SNP combinations and related drug-protein binding needs further to be studied in the future.

Additionally, the DNA methylation level in the ABCB1 promoter may also influence the ABCB1 activity. Wu et al. evaluated both the effect of the SNP combination of ABCB1 and DNA methylation level in the ABCB1 promoter on digoxin pharmacokinetics. mRNA expression in intestinal epithelial cells showed no difference between homozygous CGC and homozygous TTT carriers (p = 0.087). However, mRNA expression of homozygous TTT-HM carriers, who had a higher degree of DNA methylation, was significantly decreased compared to homozygous TTT-LM carriers (lower degree of DNA methylation), homozygous CGC-LM and homozygous CGC-HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively). Subjects who carried homozygous TTT–HM had a significant higher AUC0–72h, AUC0–∞, Cmax and lower apparent oral clearance compared to homozygous TTT-LM carriers (lower degree of DNA methylation), homozygous CGC-LM and homozygous CGC-HM carriers, who had a higher degree of DNA methylation, was significantly decreased compared to homozygous TTT-LM carriers (lower degree of DNA methylation), homozygous CGC-LM and homozygous CGC-HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively). Subjects who carried homozygous TTT–HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively). Subjects who carried homozygous TTT–HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively). Subjects who carried homozygous TTT–HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively). Subjects who carried homozygous TTT–HM carriers by 31.1, 27.9 and 43.6% (p < 0.02, 0.013 and 0.008 respectively).

Conclusion

The empirical population pharmacokinetic evaluation showed that homozygous carriers of the TTT and CGC/CGT have a 35% higher apparent bioavailability of oral digoxin, while no effects of CGC/TTT on apparent bioavailability and of any ABCB1 variants on renal elimination were observed. These results support the use of digoxin as a phenotyping substrate of intestinal but not of renal P-gp activity. Our study suggests considering the effect of a combination of SNPs on the pharmacokinetics of digoxin, rather than focusing on single SNPs. This might play an important role in the design and refinement of transporter phenotyping studies, including the development of appropriate sampling schedules, and the mode of administration. Furthermore, protein expression, protein structure and/or P-gp affinity to digoxin or other substrates in different genotypes need to be further investigated.

Methods

Clinical trials. Data from 40 healthy Caucasian subjects receiving single oral doses of 0.5 mg digoxin in two clinical trials were analyzed57,58. In trial 1, a single dose of 0.5 mg digoxin was given concomitantly with 2 mg oral midazolam, 1 mg intravenous midazolam, 125 mg tolbutamide, 150 mg caffeine, 20 mg omeprazole and 30 mg dextromethorphan in the reference period. In the test period, the drugs of the reference period were combined with ethanol58. In trial 2, a single dose of 0.5 mg digoxin was given alone in the reference period and in combination with 10 mg adefovir, 500 mg metformin, 2 mg pitavastatin, and 100 mg simvastatin in the test period52. Blood and urine samples were collected up to 24 h after drug administration. The study design and the timing of blood and urine samples are summarized in Table 4.

Genotyping of ABCB1 was carried out using the DMET Plus Array (Affymetrix, Santa Clara, California, United States)59. To define SNP combination groups, the common ABCB1 SNPs C1236T, G2677T and C3435T were taken into account59. Deviation from Hardy–Weinberg equilibrium was assessed using a chi square test. Blood and urine samples of digoxin were processed with solid-phase extraction (Strata-X 30 mg/3 mL, product number: 8B-S100-TBJ, Phenomenex, Aschaffenburg, Germany). Digoxin-d3 was spiked in blood and urine sample as internal standard (product code: TRC-D446577-2.5MG, Toronto Research Chemicals, Toronto, Canada). Concentrations were quantified with a validated high-performance liquid chromatography-tandem mass spectrometry method (Agilent 1,260 Infinity, Agilent Technologies, Waldbronn, Germany/API 5,000, AB Sciex Germany GmbH, Darmstadt, Germany) as described previously50,58. The calibration range of digoxin in plasma and urine was 0.128 nmol/L to 38.4 nmol/L and 1.28 nmol/L to 384 nmol/L, respectively. All assays fulfilled the bioanalytical method validation criteria according to the FDA and the EMA guidelines56,57. Intra-day and inter-day inaccuracy and imprecision of all quality control samples were < 15%.

Data analysis. Digoxin empirical model. 2083 plasma and urine samples were included for the population pharmacokinetics analysis. All samples with concentrations below LLOQ were pre-dose samples and were...
Table 4. Summary of study designs used for pharmacokinetic analysis of digoxin. C\textsubscript{max}, maximal observed plasma concentration.

| Study   | Study design                                      | Number of subjects | Pharmacokinetic sampling | Dose regimen of digoxin | Identity and manufacturer of digoxin |
|---------|--------------------------------------------------|--------------------|--------------------------|-------------------------|-------------------------------------|
| Trial I | Reference period: digoxin 0.5 mg                 | 16 healthy Caucasian subjects (male = 8; female = 8) | Plasma (28 samples): -0.15 h pre-dose, and post-dose at 0:08, 0:20, 0:30, 0:45, 1:00, 1:15, 1:30, 1:45, 2:00, 2:05, 2:10, 2:20, 2:30, 2:45, 3:00, 3:30, 4:00, 4:30, 5:00, 6:00, 8:00, 10:00, 12:00, 14:00, 16:00, 18:00 and 24:00 h Urine (6 collection interval): Predose, 0-6 h, 6-10 h, 10-14 h, 14-18 h and 18-24 h | Single dose (2 tablets) | Digoxin 0.25 mg Mibe GmbH Arzneimittel, Brehna, Germany |

Effect of different ABCB1 SNP combinations. After identification of a reasonable base model, relationships between SNP combinations and pharmacokinetic parameters were evaluated by introducing different Ka, D2, apparent bioavailabilities and CLR in each SNP combination group in the population pharmacokinetic model. The changes in OFV were considered to identify significant relationships.

Compliance with ethical standards. Both clinical trials were approved by the Ethics Committee of the Faculty of Medicine, University of Cologne, Germany, and conducted in accordance with applicable regulations and the ethical principles described in the Declaration of Helsinki and the International Conference on Harmonization guidelines for Good Clinical Practice. The clinical trial I and clinical trial II are registered at clinicaltrials.gov with the IDs NCT02515526 and NCT02743260, respectively. Informed consent was obtained from all participants.

Data availability

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Received: 1 March 2020; Accepted: 10 July 2020
Published online: 27 July 2020

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Acknowledgements
We thank Samira Boussettaoui, Simone Kalis, and Ingrid Fehrenz for the technical support. Also, we would like to thank our colleagues at the Department I of Pharmacology, Clinical Pharmacology Unit, University Hospital Cologne, Germany, for their support during the conduct of the study, including Usman Arshad, Dominik Dahlinger, Monika Endres, Dirk Kroll, Kathi Krüsemann, Xia Li, Ahmed Abbas Suleiman, Doris Theisen, Yingying Tian, and Sami Ullah who were involved in the clinical trials. Moreover, we also appreciate the volunteers who joined in these two challenging clinical trials. These two investigator-initiated trials were essentially funded by the institutional budgets of the involved research organizations. In addition, C.-h.H. was partially financed by the graduate program in Pharmacology and Experimental Therapeutics at the University of Cologne which is financially and scientifically supported by Bayer HealthCare AG (Cologne/Wuppertal, Germany). M.G. was supported by a grant of the Government of Saudi Arabia, M. Schwab and E.S. were supported by the European Commission Horizon 2020 UPGs Grant 668353 and by the Robert Bosch Stiftung (Stuttgart, Germany).

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Participated in clinical study design: M.Stoffel, M.G. and U.F. Conducted experiments and clinical study: C.-h.H., M.Stoffel, M.G., E.S., M.Schwab., M.T. and U.F. Quantified the clinical trial sample: C.-h.H. Analyzed the data: C.-h.H., M.T., U.F. Wrote the manuscript: C.-h.H., M.T. and U.F. All authors reviewed the manuscript.
Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-69326-y.
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