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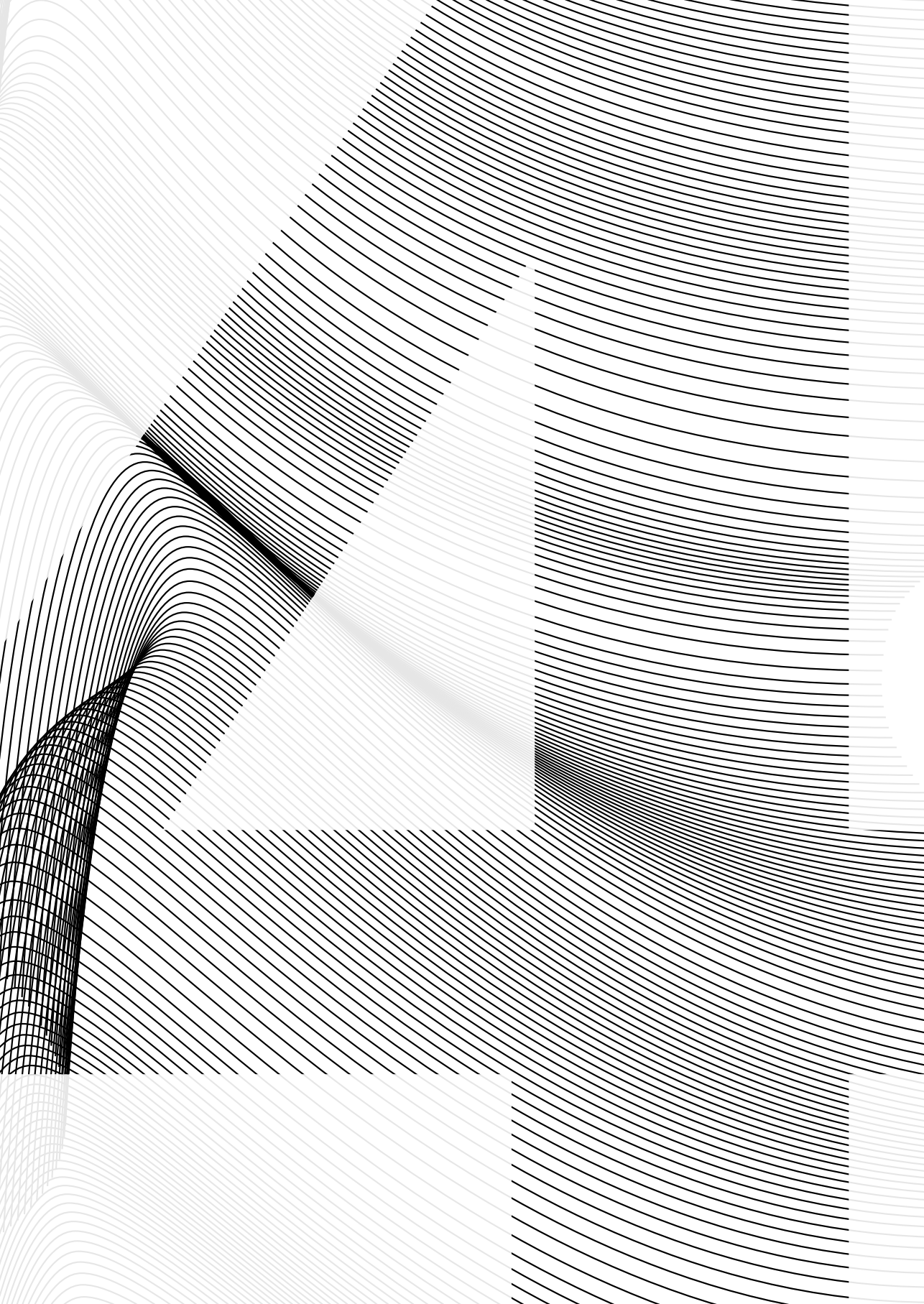
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# Short-Term Fasting alters Cytochrome P450-mediated Drug Metabolism in Humans

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## Abstract

Experimental studies indicate that short-term fasting alters drug metabolism. However, the effects of short-term fasting on drug metabolism in humans need further investigation. Therefore, the aim of this study was to evaluate the effects of short-term fasting (36h) on P450-mediated drug metabolism. In a randomized crossover study design, nine healthy subjects ingested a cocktail consisting of five P450-specific probe drugs (caffeine (CYP1A2), *S*-warfarin (CYP2C9), omeprazole (CYP2C19), metoprolol (CYP2D6) and midazolam (CYP3A4)) on two occasions (control study after an overnight fast and after 36h of fasting). Blood samples were drawn for pharmacokinetic analysis using nonlinear mixed effects modeling. In addition, we studied in Wistar rats the effects of short-term fasting on hepatic mRNA expression of P450-isoforms corresponding with the five studied P450 enzymes in humans. In the healthy subjects, short-term fasting increased oral caffeine clearance by 20% ( $p=0.03$ ) and decreased oral *S*-warfarin clearance by 25% ( $p<0.001$ ). In rats, short-term fasting increased mRNA expression of the orthologs of human CYP1A2, CYP2C19, CYP2D6, CYP3A4 ( $p<0.05$ ) and decreased the mRNA expression of the ortholog of CYP2C9 ( $p<0.001$ ) compared with the post-absorptive state. These results demonstrate that short-term fasting alters cytochrome P450-mediated drug metabolism in a nonuniform pattern. Therefore, short-term fasting is another factor affecting cytochrome P450-mediated drug metabolism in humans.

## Introduction

There is considerable variability in the rate of drug metabolism due to physiological, genetic, pharmacologic, environmental, and nutritional factors.<sup>1</sup> Nutritional factors modulate the activity of many enzyme systems in the liver which play an important role in the metabolism and elimination of drugs.<sup>2</sup> Interestingly, only a few studies are available in humans describing the role of short-term fasting on the activity of certain individual liver enzymes.<sup>2,3</sup> However, the effect of fasting on human drug metabolism in general, and on cytochrome P450 (P450) enzymes that are predominantly involved in human drug metabolism (CYP1A2, CYP2C9, CYP2C19, and CYP3A4) in particular, has not been studied in detail. Studies in animals indicate that short-term fasting alters drug metabolism.<sup>2-5</sup> Therefore, short-term fasting may contribute to both intra- and inter-individual variations in drug metabolism. Moreover, fasting related consequences such as malnutrition or cachexia are common in patients. For example, the prevalence of cachexia ranges from about 10% in patients with chronic heart failure or chronic obstructive pulmonary disease to about 70% in patients with advanced cancer.<sup>6</sup> Changes in drug metabolism due to alterations in nutritional conditions may potentially result in treatment failure, or, conversely, in unwanted side effects.

The effects of fasting on drug metabolism in experimental models can be explained by the altered activity of nuclear transcription factors. The nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR)<sup>7</sup> serve as xenosensors, which regulate the activity of many of the drug metabolizing P450 enzymes in animals<sup>8</sup>. Interestingly, short-term fasting increases the activity of both CAR and PXR.<sup>8,9</sup> However, the mechanisms regulating nuclear transcription factors are complex, and fasting can also attenuate PXR function, as studied by Buler *et al.*<sup>10</sup> Because the activities of CAR and PXR are altered by fasting and both transcription factors are involved in the regulation of drug metabolism in animals, we hypothesized that short-term fasting influences drug metabolism in humans as well.

Therefore, the aim of our study was to assess the effect of short-term fasting on the pharmacokinetics of five different drugs, metabolized via specific metabolic cytochrome P450-pathways by using a cocktail approach in healthy male subjects. To gain more insight in the effect of short-term fasting on P450-enzyme activity, the second aim of our study was to assess the effects of fasting on hepatic mRNA expression of PXR, CAR and P450 enzymes (CYP1a2, 2c11, 2d2 and 3a2) in rats, which correspond to the five studied CYP-enzymes in the healthy human subjects (CYP1A2, 2C9, 2C19, 2D6, and 3A4, respectively).<sup>11</sup>

# Materials and Methods

## Human study

### *Subjects*

Male subjects were eligible to participate in the study if they were  $\geq 18$  years old, healthy as determined by an experienced physician, and with renal and liver function tests without any clinical relevant abnormality. Only males were included because the hormonal status may affect hepatic enzyme function in women.<sup>12</sup> Subjects were excluded if they had major illness in the past 3 months. Other exclusion criteria were as follows: gastrointestinal disease that may influence drug absorption; abnormal values of aspartate aminotransferase<sup>13</sup>, alanine aminotransferase (ALT), bilirubin, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and creatinine; drugs of abuse or excessive alcohol intake ( $>3$  units of alcohol per day), use of alcohol for at least 2 days prior to each study day, strenuous exercise at least 3 days prior to each study day, defined as more than 1 hour of exercise per day, use of prescription or nonprescription drugs, consumption of caffeine-containing foods or beverages within 1 day prior to study, and consumption of grapefruit and grapefruit-containing products or starfruit for at least 2 days prior to each study day.

The study design was approved by the institutional ethics review board (ABRnr: NL40834.018.12). All subjects provided written informed consent before study entry and the study was conducted in accordance with the ethical standard of the responsible committee on human experimentation and with the Helsinki Declaration of 1975 (as revised in 2008).

### *Experimental design*

We designed an open-label, single-dose crossover intervention study. Subjects were randomly assigned for receiving a single oral administration of a drug cocktail on 2 occasions with a wash-out period of 4 weeks. In both study occasions, the drug cocktail was administered at 8:00 AM. In the control study, the subjects were fasting from 10:00 PM, the preceding evening to minimize the effect of food intake in the morning on the bioavailability of the drug cocktail. In the fasting study, the same subjects were fasting from 8:00 PM starting two evenings prior to the study, ensuring a duration of fasting of 36 hours at the moment the drug cocktail was administered. This period of fasting results in major changes in lipid metabolism.<sup>7,14</sup>

At noon, on both study occasions, the subjects were allowed to have a meal. To prevent differences in caloric intake between the two interventions to affect the pharmacokinetics of the drug cocktail, subjects consumed a standard fluid meal (Nutridrink Compact®, Nutricia, Zoetermeer, The Netherlands) containing 25% of their estimated daily energy

expenditure (25 kcal/kg/day). After another 4 hours, subjects were allowed to consume their habitual diet.

To standardize the 3 days preceding each study day, subjects were asked to keep a diary containing dietary instructions. Furthermore, adherence to the fasting period was checked by measuring the following biomarkers at baseline of both occasions: glucose, free fatty acids,  $\beta$ -hydroxybutyrate and acetoacetate.<sup>15</sup> Differences in biochemical parameters between both interventions were tested using a paired t test for normally distributed data and a Wilcoxon signed-ranks test for data that were not distributed normally. The normality of data distribution was assessed using the Shapiro-Wilk test.  $P < 0.05$  was considered significant. Statistical analysis was performed using IBM SPSS Statistics version 21.0.

A validated P450-probe drug cocktail was administered consisting of 100 mg caffeine (CYP1A2) (10 mg/ml ampoules, VUMC, Amsterdam, The Netherlands), 5 mg racemic warfarin (CYP2C9) (5 mg tablet, Crescent Pharma Ltd, Hampshire, United Kingdom), 20 mg omeprazole (CYP2C19) (20 mg capsule, Teva Pharmachemie, Haarlem, The Netherlands), 100 mg metoprolol (CYP2D6) (100 mg tablet, Teva Pharmachemie, Haarlem, The Netherlands) and 0.03 mg kg<sup>-1</sup> midazolam (CYP3A4) (1 mg ml<sup>-1</sup> oral solution, UMCG, Groningen, The Netherlands).<sup>16</sup>

Serial blood samples for calculation of pharmacokinetic (PK) parameters were collected after administration of the drug cocktail at t=0, t=1, t=2, t=3, t=4, t=5, t=6, t=7, t=8 and t=10 hours. Furthermore, PK samples at day 2, 3, 8 and 15 were obtained of which the latter two due to the long elimination half-life of warfarin. Plasma was separated by centrifugation and stored at -80°C until analysis.

### ***Bioanalysis of the CYP450-probe drugs***

A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the simultaneous determination of plasma concentrations of the five drugs used in the CYP450-probe cocktail was developed and validated. In brief, samples were deproteinized by a solution containing 420:80 acetonitril:methanol and spiked with internal standards (caffeine-D9, omeprazole-D3, warfarin-D5, metoprolol-D7 and midazolam-D5). Chromatography was performed on a Shimadzu LC-30 Nexera HPLC system (Shimadzu, Kyoto, Japan) using Thermo Scientific Hypersil Gold HPLC column (50 mm X 2.1 mm, 1.9  $\mu$ m) (Thermo Fisher Scientific, Waltham, MA, USA). This was coupled to a 5500 QTrap mass spectrometer (ABSciex, Framingham, MA, USA). The tandem mass spectrometry (MS/MS) system was operated using an electrospray in positive ionization mode. A three-component mobile phase contained the following: solvent A (ultra-pure water (Purelab option-Q)), solvent B (Acetonitrile (Biosolve)) and solvent C (2% formic acid/1% ammonium formate in water (Merck/Fluka). A gradient of solvent B from 2% to 90%

over 4 minutes was applied on the column and then cycled back to the initial condition. For the chiral compound *S*-warfarin an Astec Chirobiotic V column (150 mm X 2.1 mm, 5  $\mu\text{m}$ ) was used, and the MS/MS system was operated using an electrospray in negative ionization mode. The same three-component mobile phase was used, but with a gradient of solvent B on the column from 5% to 40% in 3.5 minutes, directly followed by 90% and then cycled back to 5%. For both columns, the temperature was maintained at 40°C and the autosampler temperature was kept at 10°C. The lower and upper limits of quantification (LLOQ and ULOQ) were 50-5000 ng ml<sup>-1</sup> for caffeine, 1-200 ng ml<sup>-1</sup> for metoprolol, 0.5-100 ng ml<sup>-1</sup> for midazolam, 2-500 ng ml<sup>-1</sup> for omeprazole and 4-1000 ng ml<sup>-1</sup> for *S*-warfarin. Linearity was  $R^2 \geq 0.996$  for all components except for *S*-warfarin ( $R^2 = 0.9954$ ). Accuracy was within the ranges of 80-120% for LLOQ; 85-115% for MLOQ and ULOQ. Also, the within-run and between-run precision fall within the predefined maximum criteria (relative standard deviation LLOQ +/- 20%, MLOQ and ULOQ +/- 15%).

### ***PK data analysis***

For each P450-probe drug plasma concentration time curves from all subjects were analyzed simultaneously using non-linear mixed-effects modeling software (NONMEM version 7.1.2., Globomax, Ellicott City, Maryland, USA). Nonlinear mixed effects compartmental modeling was preferred instead of non-compartmental data-analysis because of the ability to accurately study time dependent effects of fasting on the pharmacokinetics of the probe drugs.

### ***Structural model development***

Concentration data were log transformed for all compounds. The population models were built in a stepwise manner. Several compartment models with first order elimination were evaluated. Model parameters were quantified as oral clearance (CL/F), inter-compartment clearance (Q/F), volume of distribution of the central ( $V_1/F$ ) and peripheral compartment ( $V_2/F$ ). For all the compounds, with the exception of omeprazole, the absorption rate constant ( $K_a$ ) and lag-time ( $T_{lag}$ ) could not be estimated; respective values were fixed to 6 hours<sup>-1</sup> and 0 hour. For omeprazole transit compartments were incorporated in the model to account for the delay between drug administration and absorption from the gut.<sup>17</sup> The mean transit time (MTT) between the gut and systemic circulation was estimated by dividing the ratio of the number of transit compartments ( $n$ ) by the transition rate constant ( $K_{tr}$ ) between the compartments ( $MTT = n / K_{tr}$ ).

Inter- and intra-individual variability of the pharmacokinetic parameters was estimated using an exponential error model. For example clearance in the  $i$ th individual was estimated using

$$\frac{CL}{F} i, j = \frac{CL}{F} pop \times e^{(\eta_i + k_i)}$$



where  $CL/F_{ij}$  is the oral clearance for the  $i^{\text{th}}$  individual on the  $j^{\text{th}}$  occasion,  $CL/F_{\text{pop}}$  represents the population value, and  $\eta_i$  and  $\kappa_i$  represent the interindividual and intra-individual random effects with mean 0 and the respective variances  $\omega^2$  and  $\pi^2$ .

The difference between the  $k^{\text{th}}$  observed concentration of the  $i^{\text{th}}$  individual ( $Cobs_{i,k}$ ) and its corresponding model-predicted concentration ( $CPred_{i,k}$ ) was estimated with an additional error model

$$\ln(Cobs_{i,k}) = \ln(CLpred_{i,k}) + \varepsilon_{i,k}$$

where  $\varepsilon$  is the residual random error with mean 0 and variance  $\sigma^2$ .

The statistical package R, version 64 3.0.1 (The R Foundation for Statistical Computing) and Xpose version 4 were used for dataset checkout, exploration and model diagnostics.<sup>18</sup> Pirana software was used as an interface between NONMEM, R and Xpose.<sup>19</sup> Model diagnostics was based on the objective function values, precision of the parameter estimates, goodness-of-fit plots (plots of measured drug concentrations versus population (PRED) or individual (IPRED) predictions, and conditional weighted residuals (CWRES) versus time and PRED) and shrinkage, which was calculated for all random effects.<sup>20</sup>

### Covariate analysis

Correlations between fasting and pharmacokinetic parameters were evaluated by stepwise inclusion in the models.<sup>21</sup> For instance, the change of clearance due to fasting was evaluated using the equation

$$\frac{CL}{F} i = \frac{CL}{F} pop \times \theta_{fasting}$$

where  $\theta_{fasting}$  is the fractional change of clearance due to fasting.

To evaluate a possible time dependency of the effect of fasting on the pharmacokinetics of the studied compounds a time cut-point covariate model was used

$$\frac{CL}{F} i = \frac{CL}{F} pop \times \theta_{fasting} \quad \text{for } TIME \leq \theta_{cut}$$

$$\frac{CL}{F} i = \frac{CL}{F} pop \quad \text{for } TIME > \theta_{cut}$$

in which the pharmacokinetic parameter is increased or decreased before the time cut-point ( $\theta_{cut}$ ) and comparable with control after  $\theta_{cut}$ .

The selection of covariates was carried out using a forward and backward selection approach.

Statistical selection between models was performed using the log-likelihood ratio test for two hierarchical models, using the objective function value (OFV) produced by NON-MEM. During forward selection a covariate was selected if a decrease in OFV of 2.7 points ( $p=0.01$ , 1 degree of freedom) or 4.6 points ( $p = 0.01$ , 2 degrees of freedom) was observed. All selected covariates were added simultaneously in an intermediate “full” model. The importance of each covariate was re-evaluated by backward selection. Each covariate was independently removed from the intermediate model to confirm its relevance using  $p<0.05$  (delta OFV = 3.8 and 7.0 df=1 and 2) as requirement for confirmation.<sup>21,22</sup>

### ***Model validation***

The stability and performance of the final model were checked using simulation-based diagnostics (visual predictive checks) and bootstrap diagnostics.<sup>23,24</sup> Via the bootstrap re-sampling technique 1000 bootstrap datasets were generated by random sampling with replacement.<sup>23</sup> Bootstrapping was performed using the Perl modules Perl-speaks-NON-MEM.<sup>25</sup> Model validity was assessed by calculating median values and the 2.5<sup>th</sup> and 97.5<sup>th</sup> percentiles of parameter distribution generated by the bootstrap, and comparing them with the original estimates. Via the visual predictive checks, the 10<sup>th</sup> and 90<sup>th</sup> percentiles of the observed concentrations were compared with simulations of concentration-time profiles (1000 replicates) of the final model.

### **Animal study**

#### ***Rats***

Male Wistar rats weighing 250–280 grams (Charles River breeding Laboratories, Sulzfeld, Germany) were housed individually in a 12-hour light/dark cycle. On the day of the experiment, food was removed from the cages just before lights off for the 36-hour fasted rats ( $n=6$ ), and in the morning for the 24-hour fasted rats ( $n=6$ ). Control animals were fed ad libitum ( $n=6$ ). After 24 hours or 36 hours of fasting, rats were sacrificed with an overdose of nembutal (120 mg/kg BW). The liver was dissected, snap frozen in liquid nitrogen and stored in  $-80$  until further analysis. The study design was approved by the institutional animal welfare committee (DECnr: DIE102662).

#### ***Rat hepatic RNA isolation and qPCR***

Total RNA from rat liver was isolated using the Magna Pure apparatus (Roche Molecular biochemicals, Mannheim, Germany) and the Magna pure tissue III total RNA kit (Roche Molecular biochemicals, Mannheim, Germany). mRNA yield was determined using the Nanodrop (Nanodrop, Wilmington, Delaware USA) and cDNA was synthesized with equal mRNA input with the First strand cDNA synthesis kit for qPCR with oligo d(T) primers (Roche Molecular Biochemicals, Mannheim, Germany). Quantitative PCR was performed using the Lightcycler 480 and Lightcycler 480SybrGreen I Master mix (Roche

Molecular biochemicals, Mannheim, Germany). The primers used are displayed in Table 1. Quantification was performed using the LinReg software. Samples with a deviation of more than 5% of the mean efficiency value of the assay were excluded. Calculated values were normalized by the housekeeping gene values (Cyclophilin) which was selected to be the most stable among different groups.

### *Statistical analysis (animal study)*

Data are expressed as means  $\pm$  standard deviation (S.D.). Differences in means were tested by one-way analysis of variance.  $P < 0.05$  was considered significant. Statistical analysis was performed using IBM SPSS Statistics version 21.0.

**Table 1:** Primers for rat liver CYP450 mRNA isolation and quantitative polymerase chain reaction (qPCR)

Gene (Rat)	Human ortholog	Sequence	Annealing temperature (°C)
Rat PXR	Nr1i2	Forward: 5'-GGCCGATGTGTCAACCTACA-3' Reverse: 5'-AAGCCGCCATTAGGGTCTTC-3'	55
Rat CAR	Nr1i3	Forward: 5'-AGCAAGGCCAGAGACGCCA-3' Reverse: 5'-CCGGAGGCCTGAACTGCACAAA-3'	60
CYP1A2	CYP1A2	Forward: 5'-TACAACTCTGCCAGTCTCCAG-3' Reverse: 5'-TTGGGAACCTGGGTCCTTGT-3'	55
CYP2C11	CYP2C9	Forward: 5'-AAGCACAATCCGCAGTCTGA-3' Reverse: 5'-GGCATCTGGCTCCTGTCTTT-3'	55
CYP2D2	CYP2D6 CYP2C9	Forward: 5'-TATTGAGTGTGGCCAGTGGT-3' Reverse: 5'-GTGGACACAGAGAATCGCCT-3'	60
CYP3A2	CYP3A4	Forward: 5'-CTAAAGTTCTGCCACGGGA-3' Reverse: 5'-CCATGCATCAAGAGCAGTCA-3'	55
Cyclophilin	-	Forward 5'-ATGTGGTCTTTGGGAAGGTG-3' Reverse 5'-GAAGGAATGGTTTGTATGGGT-3'	55

## Results

### Healthy subjects

Nine healthy male subjects (mean age  $24.3 \pm 3.0$  years) received a single oral administration of the P450-probe cocktail after an overnight fast (control) and after 36 hours of fasting. There were no adverse events.

Table 2 demonstrates that the biomarkers for fasting (glucose, free fatty acids,  $\beta$ -hydroxybutyrate and acetoacetate) were all significantly altered in comparison with the control condition indicating compliance to the fasting protocol.

**Table 2:** Effect of 36 hours of fasting on biochemical parameters in healthy male subjects (n=9)

	Value Median (range)	Control Median (range)	Fasting (36h) Median (range)	p-value
Age (years)	24 (20-31)			
Height (cm)	187 (175-194)			
Body weight (kg)	79 (68-88)			
Glucose (mmol l <sup>-1</sup> )		5.1 (4.6-5.9)	4.4 (3.6-5.1)	0.018
$\beta$ -hydroxybutyrate (mmol l <sup>-1</sup> )		0.1 (0.0-0.4)	0.6 (0.3-1.6)	0.007
Acetoacetate (mmol l <sup>-1</sup> )		0.03 (0.01-0.15)	0.19 (0.08-0.33)	0.008
Free fatty acids (mmol l <sup>-1</sup> )		0.50 (0.15-0.91)	1.05 (0.76-1.55)	0.028

### Pharmacokinetics

The plasma concentrations versus time profiles in healthy subjects were described using a one-compartment model (caffeine, omeprazole), a two-compartment model (metoprolol, midazolam) and a three-compartment model (*S*-warfarine) (Table 3). Interpatient variability was estimated for clearance and central volume of distribution; inpatient variability was estimated for clearance.

Despite the study restrictions, preadministration plasma concentrations of caffeine ranging from 0 to 1010 mgL<sup>-1</sup> were observed, which impairs comparison of pharmacokinetic parameters between both interventions. To account for the variable pre-treatment intake of caffeine in the pharmacokinetic model, a fictive caffeine dose of 100 mg with variable bioavailability was administered 12 hours before administration of the probe cocktail. The fictive dose of caffeine was used to account for the unknown intake by some of the volunteers. The bioavailability and interindividual variability were parameters estimated in the NONMEM analysis with respective values of 6.7% and 370%. The value of 6.7% indicates that on average predose intake was low, whereas the high variability was caused by two volunteers with predose caffeine plasma concentrations. For omeprazole, nine transit compartments were incorporated in the model to account

for the delay between administration and absorption from the gut.<sup>17</sup> Mean transit time (MTT) was 1.4 hours and intraindividual variability in this parameter was 21% (Table 3).

The developed mixed-effects models described the observed data well as demonstrated by the goodness-of-fit plots shown in Figure 1. There were no trends in plots of conditional weighted residuals versus time and model predicted concentration (plots not shown). Shrinkage of intra- and inter-individual variability of the pharmacokinetic parameters and residual variability was less than 20%. Parameters were estimated with adequate precision; nonparametric bootstraps (n = 1000 replicates per model) showed that the final models were robust (Table 3). The validity of the models was further demonstrated by visual predictive checks (VPC) in which simulated data exhibited a central tendency and variability comparable to the observed data (Figure 2).

### Effect on pharmacokinetic parameters

**Caffeine (CYP1A2):** Fasting increased oral caffeine clearance (Cl/F) by 20% ( $\theta_{Cl/F, \text{fasting}} = 1.20$ , df=1, p=0.03, 95%CI: 1.03 – 1.37) (Table 3). After post hoc analysis, the median caffeine clearance (Cl/F<sub>posthoc</sub>) was 5.6 L/h (range: 3.9-10.7 L/h) in the control study and 7.5 L/h (range: 4.1-17.9 L/h) after 36 hours of fasting. This effect can also be appreciated in the visual predictive check plot (Figure 2a).

**S-Warfarin (CYP2C9):** Short-term fasting decreased oral S-warfarin clearance by 25% ( $\theta_{Cl/F, \text{fasting}} = 0.75$ , df=2, p<0.001, 95%CI: 0.67-0.83), until a time cut-point of 23 hours after administration of the cocktail. After post hoc analysis, the median S-warfarin clearance (Cl/F<sub>posthoc</sub>) in the control study was 0.18 L/h (range: 0.11-0.27 L/h) and decreased to 0.13 L/h (range: 0.01-0.20 L/h) after 36 hours of fasting.

Twenty-three hours after administration of the drug cocktail, there were no differences in oral S-warfarin clearance. Furthermore, fasting decreased the central volume of distribution by 15% ( $\theta_{V_{1/F}, \text{fasting}} = 0.85$ , df=2, p<0.001, 95%CI: 0.79-0.90); the corresponding time cut-point was 11 hours (Table 3). The effect of fasting on the pharmacokinetics of S-warfarin is demonstrated in the visual predictive check plot (Figure 2b).

**Omeprazole (CYP2C19):** Fasting did not affect oral omeprazole clearance (Cl/F) nor the volume of distribution (V<sub>1/F</sub>) in healthy subjects (Table 3, Figure 2c). Median post hoc estimates for oral omeprazole clearance after the control study and 36 hours of fasting were Cl/F<sub>posthoc</sub>=34 L/h (range: 18-67 L/h) and Cl/F<sub>posthoc</sub>=26 L/h (range: 16-81 L/h), respectively.

**Metoprolol (CYP2D6):** Metoprolol exposure of one subject clearly deviated from the other eight subjects (Figure 1d and 2d). Oral clearance (Cl/F) was 139 L/h for the typical subject and was 20% lower for the single subject characterized as a slow metabolizer. Fasting did not affect the pharmacokinetics of metoprolol (Table 3, Figure 2d). Median

post hoc estimates for clearance after the control study and 36h of fasting were  $Cl/F_{\text{post-hoc}} = 114$  L/h (range: 28-570 L/h) and  $Cl/F_{\text{posthoc}} = 137$  L/h (range: 28-545 L/h), respectively.

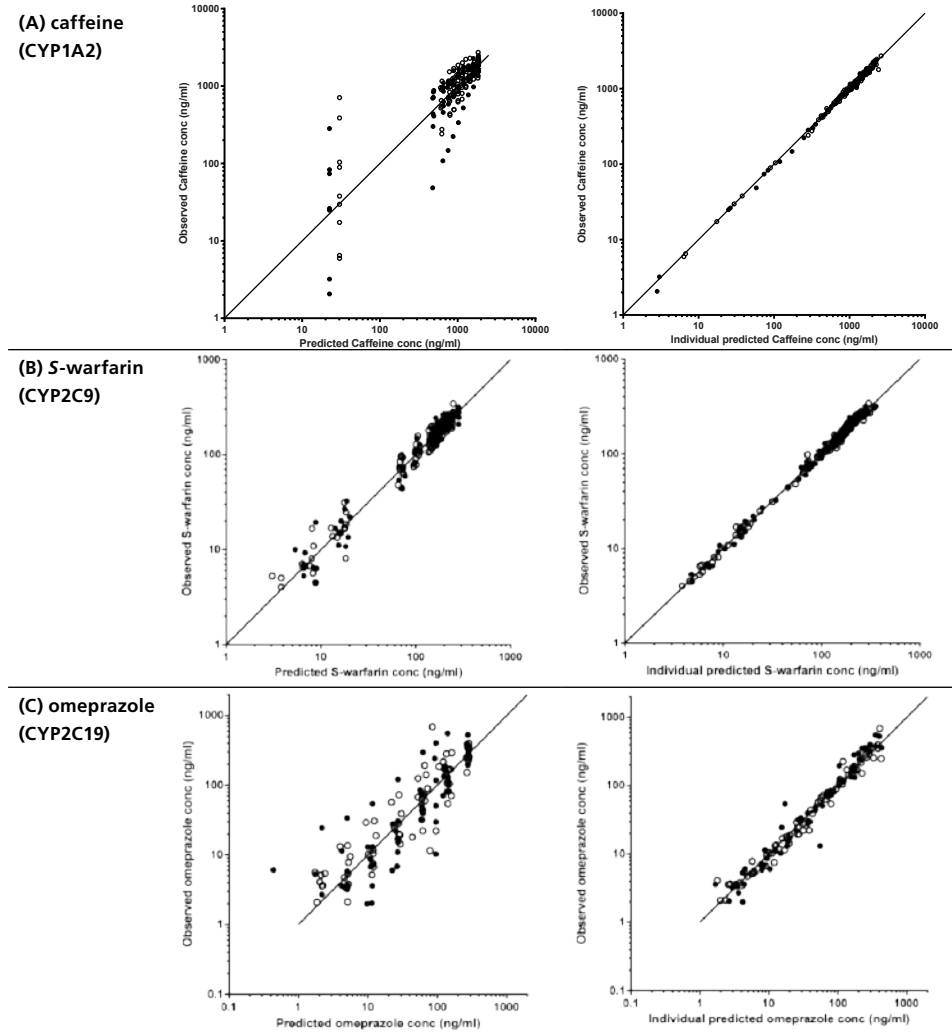
*Midazolam (CYP3A4)*: Fasting did not affect midazolam (CYP3A4) oral clearance ( $Cl/F$ ) or volume of distribution ( $V_d/F$ ) in healthy subjects (Table 3, Figure 2e). After post hoc analysis, the median midazolam clearance ( $Cl/F_{\text{posthoc}}$ ) in the control study was 92 L/h (range: 46-114 L/h) and 78 L/h (range: 49-122 L/h) after 36 hours of fasting.

### Rats

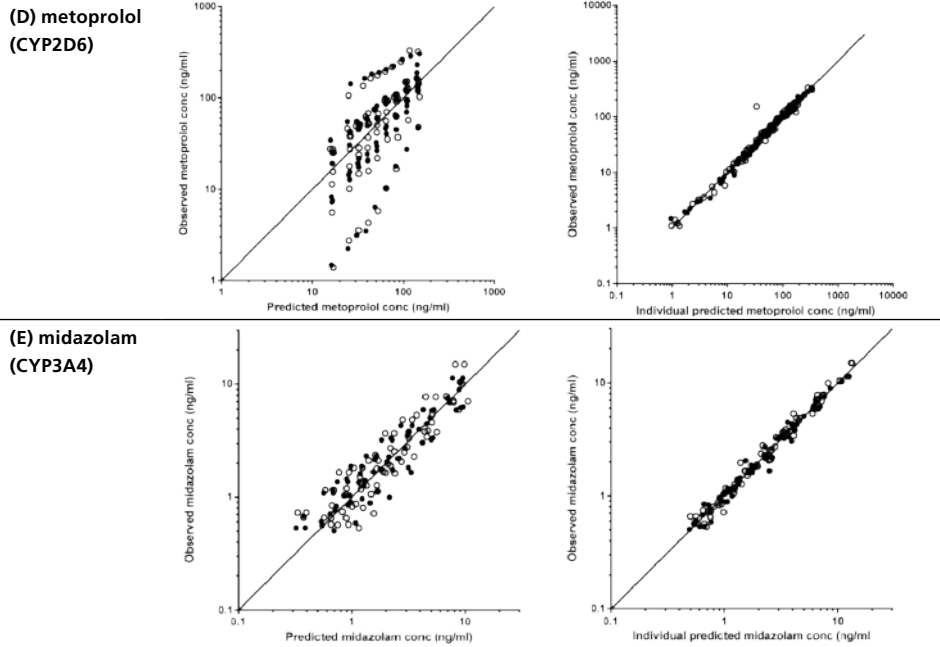
Fasting significantly increased hepatic mRNA expression of the nuclear receptors CAR and PXR (Figure 3a and 3b). Furthermore, 24 hours and 36 hours of fasting increased hepatic mRNA expression of CYP1a2 by 45% ( $p=0.047$ ) and 55% ( $p=0.01$ ) respectively (Figure 3c). Fasting also increased hepatic mRNA expression of CYP2d2 (human orthologs: CYP2D6 and CYP2C19) with 67% ( $p<0.001$ ) after 24 hours and 62% ( $p<0.001$ ) after 36 hours of fasting (Figure 3d). A more than doubled expression of mRNA was seen for CYP3a2 (human ortholog: CYP3A4) after 36 hours of fasting: 154% ( $p<0.001$ ), and although nonsignificant, 77% ( $p=0.24$ ) after 24 hours of fasting (Figure 3e).

Interestingly, the opposite effect was found for the ortholog of human CYP2C9. Fasting significantly decreased hepatic mRNA expression of CYP2c11 by 42% ( $p<0.001$ ) after 24h and 58% ( $p<0.001$ ) after 36h of fasting (Figure 3f).

**Figure 1:** Goodness of fit plots of the five P450-probe drugs: (A) caffeine (CYP1A2), (B) S-warfarin (CYP2C9), (C) omeprazole (CYP2C19), (D) metoprolol (CYP2D6), (E) midazolam (CYP3A4). Observed concentrations versus population predicted (PRED) and individual predicted (IPRED) concentrations. The closed circles represent the 36 hours of fasting intervention, open circles represent the control intervention. The solid line is the line of identity.

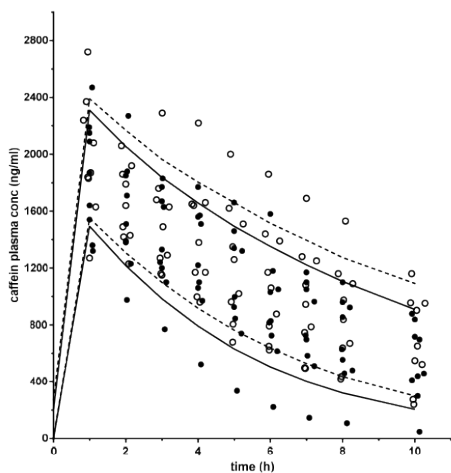


**Figure 1:** (continued)



**Figure 2:** Visual Predictive Check plots of the five CYP450-probe drugs: (A) caffeine (CYP1A2), (B) *S*-warfarin (CYP2C9), (C) omeprazole (CYP2C19), (D) metoprolol (CYP2D6), (E) midazolam (CYP3A4). The closed circles represent observed data points after 36 hours of fasting. Open circles represent the control observations. The solid (fasting) and dashed (control) lines represent the 10<sup>th</sup> and 90<sup>th</sup> percentiles of the simulated data.

**(A) caffeine (CYP1A2)**



**(B) *S*-warfarin (CYP2C9)**

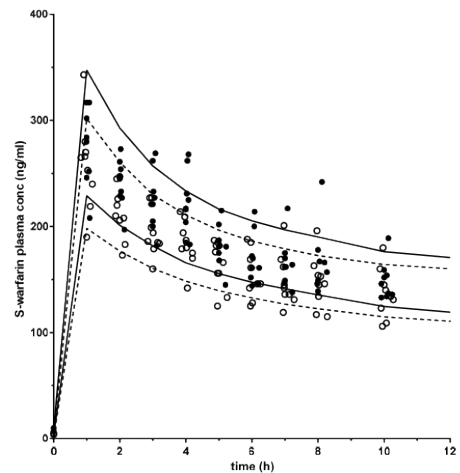
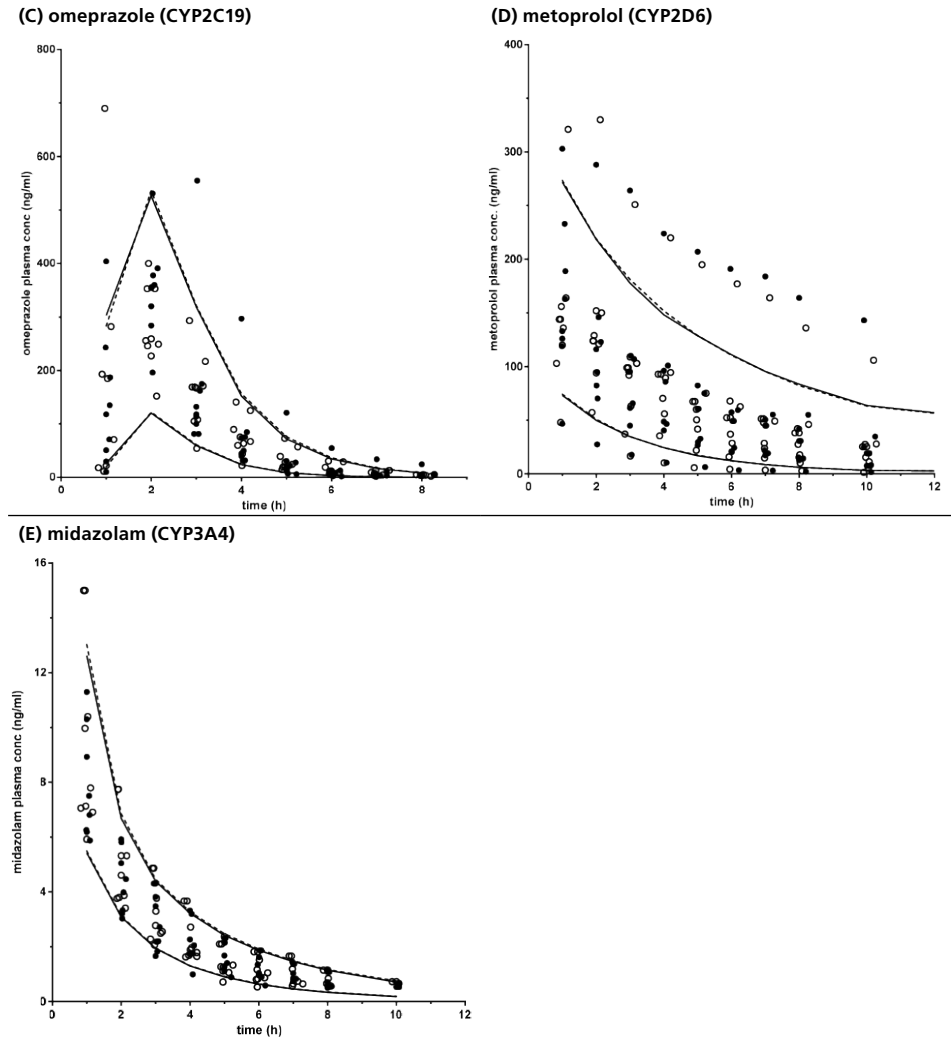




Figure 2: (continued)



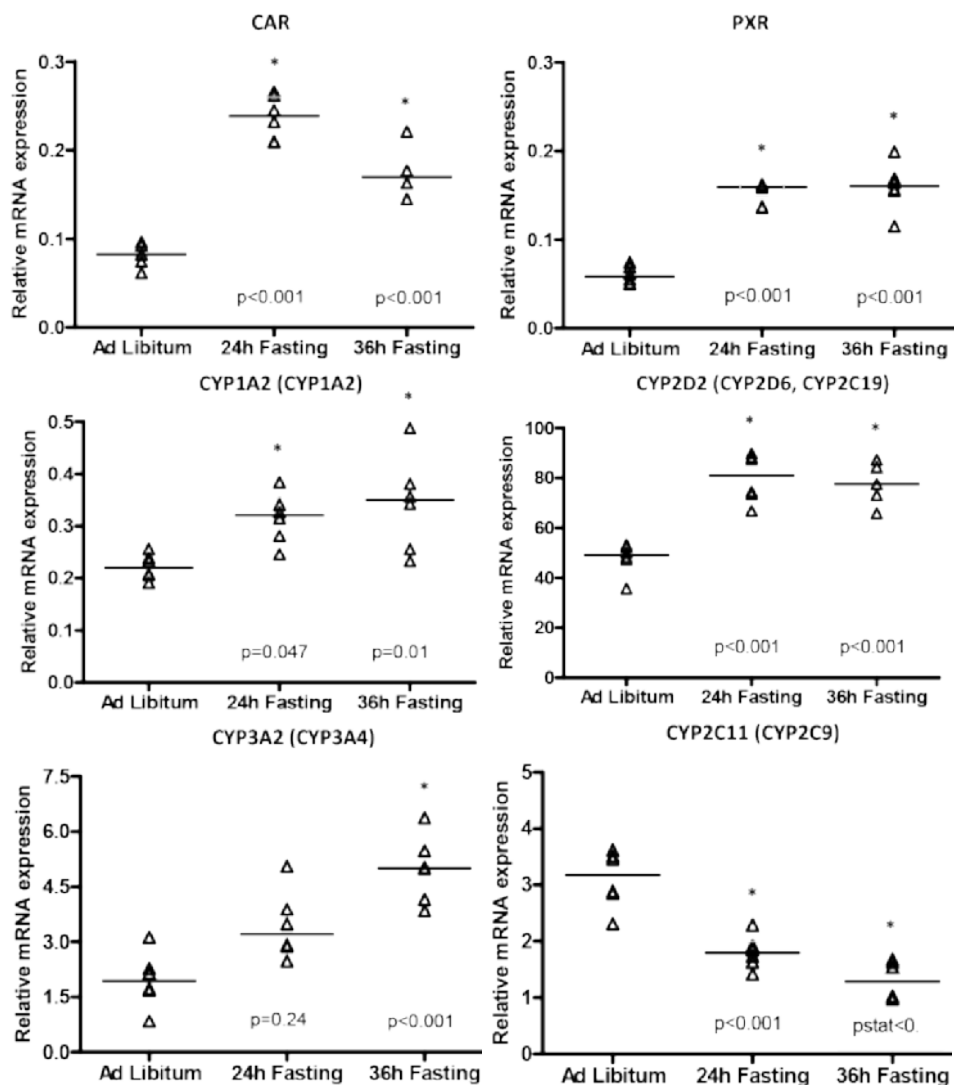
**Table 3:** Pharmacokinetic (PK) parameters of the CYP450 probe cocktail in 9 healthy subjects and median parameter values (2.5–97.5 percentile) of nonparametric bootstrap replicates of the final pharmacokinetic models.

Parameters	Caffeine (CYP1A2)		Metoprolol (CYP2D6)		Midazolam (CYP3A4)		Omeprazole (CYP2C19)		S-Warfarin (CYP2C9)	
	Estimates Typical value (95% CI)	Bootstrap Median (2.5–97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5–97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5–97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5–97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5–97.5%)
$Cl/F$ ( $l\ h^{-1}$ )	6.0 (4.6-7.4)	6.1 (5.0-7.8)	139 (78-200)	131 (64-187)	80 (64-96)	80 (65-95)	31 (22-41)	31 (24-43)	0.17 (0.15-0.20)	0.17 (0.15-0.20)
$-\theta_{Cl/F, \text{Fasting}}$	<b>1.20 (1.03-1.37)</b>	1.23 (1.08-1.39)	-	-	-	-	-	-	0.75 (0.67-0.83)	<b>0.76 (0.64-0.96)</b>
$-\theta_{Cl/F, \text{cup}} (h)$	-	-	-	-	-	-	-	-	23 (0-51)	23 (3.9-36.6)
$-\theta_{Cl/F, \text{Slow metab}}$	-	-	0.80 (0.54-1.06)	0.80 (0.20-1.02)	-	-	-	-	-	-
$V_d/F$ (l)	49 (45-53)	49 (46-53)	536 (430-641)	520 (369-604)	149 (106-191)	147 (114-204)	37 (26-49)	37 (29-52)	9.0 (8.1-9.9)	9.0 (8.3-9.9)
$-\theta_{V_d/F, \text{Fasting}}$	-	-	-	-	-	-	0.97 (0.75-1.18)	0.95 (0.40-1.16)	0.85 (0.79-0.90)	<b>0.84 (0.80-0.89)</b>
$-\theta_{V_d/F, \text{cup}} (h)$	-	-	-	-	-	-	-	-	11.0 (8.6-13.3)	11.0 (9.5-38.1)
Ka (fixed)	6	-	6	-	6	-	-	-	6	-
MTT (h)	-	-	-	-	-	-	1.4 (1.2-1.6)	1.4 (1.2-1.5)	-	-
$Q_2/F$ ( $l\ h^{-1}$ )	-	-	29 (0.0-63)	32 (1.4-283)	40 (23-56)	40 (22-53)	-	-	1.3 (1.1-1.5)	1.3 (1.1-1.5)
$V_d/F$ (l)	-	-	62 (4-119)	66 (25-260)	70 (52-88)	70 (55-91)	-	-	5.9 (4.9-6.9)	5.8 (4.9-6.9)
$Q_3/F$ ( $l\ h^{-1}$ )	-	-	-	-	-	-	-	-	0.084	0.086
$V_d/F$ (l)	-	-	-	-	-	-	-	-	(0.067-0.102)	(0.067-0.102)
$V_d/F$ (l)	-	-	-	-	-	-	-	-	27 (19-36)	25 (18-35)
<b>Inter-individual variability</b>										
$Cl/F$ (%)	37 (20-50)	37 (21-49)	83 (0-140)	61 (24-110)	31 (15-41)	30 (18-41)	46 (0-68)	43 (17-65)	24 (10-33)	23 (13-32)
$V_d/F$ (%)	11 (2.7-15.6)	9.7 (4.1-14.7)	45 (0-69)	37 (12-63)	39 (0-60)	38 (17-59)	-	-	16 (8-22)	14 (7-20)
$Q_3/F$ (%)	-	-	-	-	-	-	-	-	37 (0-59)	38 (14-61)

Parameters	Caffeine (CYP1A2)		Metoprolol (CYP2D6)		Midazolam (CYP3A4)		Omeprazole (CYP2C19)		S-Warfarin (CYP2C9)	
	Estimates Typical value (95% CI)	Bootstrap Median (2.5-97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5-97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5-97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5-97.5%)	Estimates Typical value (95% CI)	Bootstrap Median (2.5-97.5%)
<b>Intra-individual variability</b>										
CL/F (%)	15 (6.8-20.7)	15 (6.8-20.2)	7.9 (0-12)	8.7 (4.4-16.1)	8.8 (0-13.8)	8.6 (3.0-14.0)	17 (10-22)	16 (8-21)	-	-
MTT (%)	-	-	-	-	-	-	21 (11-28)	21 (11-28)	-	-
<b>Residual variability</b>										
Additional error	6.8 (5.3-8.3)	6.7 (5.0-8.2)	18.0 (7.1-29.1)	17 (9-23)	13 (10-17)	13 (10-16)	36 (24-47)	35 (23-46)	8.3 (7.3-9.3)	8.0 (6.9-8.8)

Legend Table 3:  $CL/F$ , elimination clearance;  $\theta$ , fractional change of the parameter due to fasting;  $V_1/F$ , volume of distribution of the central compartment;  $K_a$ , absorption rate constant;  $MTT$ , mean transit time;  $Q_2/F$ , intercompartmental clearance of peripheral compartment 1;  $V_2/F$ , volume of distribution of peripheral compartment 1;  $Q_3/F$ , intercompartmental clearance of peripheral compartment 2;  $V_3/F$ , volume of distribution of peripheral compartment

**Figure 3:** Effect of 24 hours and 36 hours of fasting on the hepatic mRNA expression of PXR, CAR and CYP450-enzymes in rats ( $n=6$  per group): (A) CAR, (B) PXR, (C) CYP1a2 (CYP1A2), (D) CYP2d2 (CYP2D6, CYP2C19), (E) CYP3a2 (CYP3A4), (F) CYP2c11 (CYP2C9). Aligned dot plots with median values of relative mRNA expression normalized by the housekeeping gene values (Cyclophilin). Asterisks indicate values that are statistically different from control (ad libitum).



## Discussion

This study demonstrates that short-term fasting alters P450-mediated drug metabolism in a nonuniform pattern, but similar for rats and humans. In healthy subjects, short-term fasting increased oral caffeine clearance. This indicates increased activity of CYP1A2, considering that caffeine is a probe of CYP1A2 activity. In accordance, fasting induced hepatic mRNA expression of CYP1a2 in rats. Conversely, short-term fasting decreased *S*-warfarin oral clearance in healthy subjects. This indicates decreased activity of CYP2C9, considering that *S*-warfarin is a probe of CYP2C9 activity. In accordance, fasting decreased hepatic mRNA expression of the CYP enzyme corresponding to human CYP2C9 in rats. Furthermore, this study shows that fasting increased hepatic mRNA expression of CAR and PXR in rats, which regulate the activity of many of the drug metabolizing P450 enzymes in animals.<sup>8</sup>

Similar to CYP1A2, fasting increased hepatic mRNA expression of three other key enzymes in drug metabolism in rats: CYP3A4, CYP2C19 and CYP2D6. However, fasting did not affect the pharmacokinetics of the corresponding P450-probe drugs midazolam, omeprazole and metoprolol in humans. This discrepancy may be due to interspecies differences. Although sequence homology of P450 enzymes among species is high, small differences in amino acid sequences at the active sites of P450 enzymes can result in profound differences in isoform-catalyzing metabolism and specificity of the drugs they metabolize.<sup>11</sup> Therefore, fasting may not exhibit the same selectivity for human CYP450 isoforms as for the corresponding rat isoforms. However, our results indicate that CYP1A2 and CYP2C11 in male rats may be used to study the effect of fasting on the corresponding enzymes CYP1A2 and CYP2C9 in humans. Another explanation of this discrepancy may be the relatively short period of fasting (36 hours) in relation to the time necessary to induce some of the P450 enzymes. For example, maximum induction of CYP3A is reached after 3 days of rifampicin treatment, which is a powerful inducer of CYP3A4.<sup>26</sup>

In addition to a time-dependent induction of P450 enzymes by fasting, the recovery of enzyme activity after a period of either induction or inhibition is also time-dependent.<sup>27</sup> Four hours after administration of the drug cocktail, a standardized meal was consumed after which P450-enzyme activity will probably return to the nonfasting state. The time course of this recovery may be determined by either *de novo* synthesis (after inhibition) or degradation (after induction) of the enzyme and the persistent effect of fasting.<sup>27</sup> Because the turnover half-life of P450-enzymes is relatively long, it is unlikely that consumption of this meal would have an immediate effect.<sup>27</sup> In addition, the elimination half-life of nearly all drugs in the cocktail is relatively short such that enzyme activity measured by these probes can be attributed to a reasonably small time frame.

For *S*-warfarin, we show that the effect of fasting on CYP2C9-mediated clearance is only present within the first 23 hours after the 36-hour period of fasting and diminishes when returned to the nonfasting state. This is approximately 2–3 times the half-life of CYP2c11 (ortholog of human CYP2C9), which was found to be approximately 10 hours.<sup>28</sup> Our observations after 36 hours of fasting may therefore be an underestimation of the alterations in P450-mediated drug clearance in subjects or patients who have fasted for a longer period of time. In contrast, the effects of 36 hours of fasting were readily apparent in the changes in plasma concentrations of glucose, ketone bodies, and fatty acids. We cannot exclude the possibility that even shorter periods of fasting alter CYP1A2- and CYP2C9-mediated drug metabolism, as shown for the probe drugs caffeine and warfarin after 36 hours of fasting. With respect to biochemical parameters such as fatty acids, fasting induces a half-maximal response of the total fasting response already within 24 hours.<sup>7</sup> Therefore, periods of fasting shorter than 36 hours, as used in the current study, may already affect drug metabolism. For example, skipping meals, which is common among different types of patients, or chronically reduced oral intake may already influence metabolism.

The drug cocktail used has previously been validated.<sup>16</sup> The absence of a PK interaction between the probes makes this cocktail useful for the *in vivo* evaluation of metabolism-based interactions. Because the drug cocktail was administered orally in this study, the observed differences in exposure (AUC, area under the concentration versus time curve) may be a combined effect of fasting on intrinsic clearance (P450 enzyme activity), bioavailability (*F*), and protein binding, as described by the well-stirred model of drug metabolism:

$$AUC_{\text{oral}} = (F_{\text{abs}} \times F_G \times \text{Dose}) / (f_u \times \text{Clint}) \quad 29$$

(*F*<sub>abs</sub>, fraction of administered drug absorbed into the gut wall; *F*<sub>G</sub>, fraction that gets through the gut wall unchanged; *f*<sub>u</sub>, fraction unbound drug in plasma).

Based on this model, the observed effect of fasting on the orthologs of CYP2D6, CYP2C19 and CYP3A4 in rats may also be present in humans, but was not seen. For metoprolol (CYP2D6) (*F*≅0.45, *f*<sub>u</sub> = 0.90–0.95), omeprazole (CYP2C19) (*F*≅0.40, *f*<sub>u</sub>≅ 0.03) and midazolam (CYP3A4) (*F*≅0.45, *f*<sub>u</sub>≅ 0.03), the effect of fasting on Clint may be masked if fasting simultaneously changes bioavailability (*F*<sub>abs</sub>, *F*<sub>G</sub>) or protein binding.<sup>30 31</sup>

This may also imply that the effects observed for caffeine (CYP1A2) and warfarin (CYP2C9) in humans may not entirely be due to differences in intrinsic clearance but also due to differences in other parameters in the well-stirred model. However, it is unlikely that fasting affects bioavailability, since for both drugs bioavailability is 100% (*F*=1).<sup>31</sup> For plasma protein binding, Vorum *et al.* have shown that the binding affinity of warfarin to human serum albumin increased on addition of plasma fatty acids.<sup>32</sup> As we have shown,

fasting increased the plasma concentration of fatty acids. Therefore, fasting may increase the already high plasma protein binding of warfarin (fraction bound ( $f_b$ )  $\cong 0.99$ ), thereby causing a relatively large decrease in the fraction of unbound warfarin ( $f_{u\text{-warfarin}}$ )<sup>30</sup>, which reduces observed oral clearance.

The decrease of  $f_{u\text{-warfarin}}$  may also reduce the observed volume of distribution (V). V is determined by the fractions unbound drug in plasma and in tissue ( $f_uT$ ), the volume of tissue (VT), and the volume of plasma (VP):<sup>30</sup>

$$V = [f_u/f_uT] VT + VP^{33}$$

Assuming that VT and VP remain constant, a decrease in fraction of unbound warfarin in plasma can decrease the volume of distribution as observed in the present study. In contrast, caffeine has only a limited binding affinity ( $f_b \cong 0.36$ ) to plasma proteins making the volume of distribution much less dependent on changes in protein binding than warfarin.<sup>31</sup> Because fasting did not influence the volume of distribution of caffeine, it is plausible to assume that VT and VP remain unaffected. Otherwise, a possible effect on VP, such as the amount of water intake, or VT resulting in an altered volume of distribution should probably have been seen.

Although the effect of fasting on warfarin (CYP2C9) metabolism may be explained by both increased protein binding and decreased intrinsic clearance, fasting decreased hepatic mRNA expression of the CYP-enzyme corresponding to human CYP2C9 in rats. This implies that fasting affects CYP2C9 intrinsic clearance, but in an opposite way to the findings for the other P450 enzymes studied, and demonstrates the complexity of the underlying molecular and pharmacological mechanisms.

The pathway of P450 enzyme regulation by transcription factors is complex and possibly consists of (multiple) positive and negative feedback loops. Besides PXR and CAR, fasting may affect other

transcription factors, such as the aryl hydrocarbon receptor (AhR). The AhR together with the AhR nuclear translocator are primarily responsible for the regulation of the expression of CYP1A1, CYP1A2, and CYP1B1.<sup>34</sup> Fasting may increase AhR/AhR nuclear translocator activity, leading to enhanced CYP1A2 enzyme activity with, as we have shown, increased caffeine clearance.

Differences in nutritional condition by fasting or malnutrition are common among all types of patients. Due to these differences in nutritional states, patients receiving medication at fixed doses may become either under- or overdosed during progression of their disease. In this study, we showed that fasting decreases and increases the exposure to drugs specifically metabolized by CYP1A2- and CYP2C9, respectively. This may be clinically relevant for drugs with a small therapeutic range and/or metabolized by these specific CYP-enzymes, such as many antidepressants (e.g., amitriptyline, clomipramine),

some atypical antipsychotics (clozapine, olanzapine), flutamide (cytotoxic agent), anticoagulant agents (acenocoumarol, phenprocoumon), phenytoin (anti-epileptic agent), and oral hypoglycemic agents (e.g., tolbutamide, glipizide and sulfonylureas). Furthermore, in addition to short-term fasting, longer periods of fasting might also influence three other key CYP450-enzymes involved in human drug metabolism as we have shown in rats.

Our findings may imply that dose adjustments of drugs metabolized by P450 enzymes could be necessary to improve drug treatment in patients with fasting related consequences such as malnutrition or cachexia. Additional research is necessary to reveal the molecular and pharmacological mechanisms underlying our observations, but also prospective studies to test the clinical implications of our findings.

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## References

1. Ingelman-Sundberg M. Pharmacogenetics: an opportunity for a safer and more efficient pharmacotherapy. *Journal of internal medicine* 2001;250:186-200.
2. Murray M. Altered CYP expression and function in response to dietary factors: potential roles in disease pathogenesis. *Current drug metabolism* 2006;7:67-81.
3. Merrell MD, Cherrington NJ. Drug metabolism alterations in nonalcoholic fatty liver disease. *Drug metabolism reviews* 2011;43:317-34.
4. Qu W, Rippe RA, Ma J, et al. Nutritional status modulates rat liver cytochrome P450 arachidonic acid metabolism. *Mol Pharmacol* 1998;54:504-13.
5. Longo V, Ingelman-Sundberg M, Amato G, Salvetti A, Gervasi PG. Effect of starvation and chlormethiazole on cytochrome P450s of rat nasal mucosa. *Biochem Pharmacol* 2000;59:1425-32.
6. von Haehling S, Anker SD. Cachexia as a major underestimated and unmet medical need: facts and numbers. *Journal of cachexia, sarcopenia and muscle* 2010;1:1-5.
7. Klein S, Sakurai Y, Romijn JA, Carroll RM. Progressive alterations in lipid and glucose metabolism during short-term fasting in young adult men. *The American journal of physiology* 1993;265:E801-6.
8. Hernandez JP, Mota LC, Baldwin WS. Activation of CAR and PXR by Dietary, Environmental and Occupational Chemicals Alters Drug Metabolism, Intermediary Metabolism, and Cell Proliferation. *Current pharmacogenomics and personalized medicine* 2009;7:81-105.
9. Ding X, Lichti K, Kim I, Gonzalez FJ, Staudinger JL. Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha. *The Journal of biological chemistry* 2006;281:26540-51.
10. Buler M, Aatsinki SM, Skoumal R, Hakkola J. Energy sensing factors PGC-1alpha and SIRT1 modulate PXR expression and function. *Biochem Pharmacol* 2011;82:2008-15.
11. Martignoni M, Groothuis GM, de Kanter R. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert opinion on drug metabolism & toxicology* 2006;2:875-94.
12. Waxman DJ, Holloway MG. Sex differences in the expression of hepatic drug metabolizing enzymes. *Mol Pharmacol* 2009;76:215-28.

13. Iber H, Li-Masters T, Chen Q, Yu S, Morgan ET. Regulation of hepatic cytochrome P450 2C11 via cAMP: implications for down-regulation in diabetes, fasting, and inflammation. *The Journal of pharmacology and experimental therapeutics* 2001;297:174-80.
14. Soeters MR, Soeters PB, Schooneman MG, Houten SM, Romijn JA. Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. *American journal of physiology Endocrinology and metabolism* 2012;303:E1397-407.
15. Foster KJ, Alberti KG, Hinks L, et al. Blood intermediary metabolite and insulin concentrations after an overnight fast: reference ranges for adults, and interrelations. *Clinical chemistry* 1978;24:1568-72.
16. Turpault S, Brian W, Van Horn R, et al. Pharmacokinetic assessment of a five-probe cocktail for CYPs 1A2, 2C9, 2C19, 2D6 and 3A. *British journal of clinical pharmacology* 2009;68:928-35.
17. Savic RM, Jonker DM, Kerbusch T, Karlsson MO. Implementation of a transit compartment model for describing drug absorption in pharmacokinetic studies. *Journal of pharmacokinetics and pharmacodynamics* 2007;34:711-26.
18. Jonsson EN, Karlsson MO. Xpose--an S-PLUS based population pharmacokinetic/pharmacodynamic model building aid for NONMEM. *Computer methods and programs in biomedicine* 1999;58:51-64.
19. Keizer RJ, van Benten M, Beijnen JH, Schellens JH, Huitema AD. Pirana and PCluster: a modeling environment and cluster infrastructure for NONMEM. *Computer methods and programs in biomedicine* 2011;101:72-9.
20. Karlsson MO, Savic RM. Diagnosing model diagnostics. *Clinical pharmacology and therapeutics* 2007;82:17-20.
21. Mandema JW, Verotta D, Sheiner LB. Building population pharmacokinetic-pharmacodynamic models. I. Models for covariate effects. *Journal of pharmacokinetics and biopharmaceutics* 1992;20:511-28.
22. Jonsson EN, Karlsson MO. Automated covariate model building within NONMEM. *Pharmaceutical research* 1998;15:1463-8.
23. Ette EI. Stability and performance of a population pharmacokinetic model. *Journal of clinical pharmacology* 1997;37:486-95.
24. Bergstrand M, Hooker AC, Wallin JE, Karlsson MO. Prediction-corrected visual predictive checks for diagnosing nonlinear mixed-effects models. *The AAPS journal* 2011;13:143-51.
25. Lindbom L, Pihlgren P, Jonsson EN. PsN-Toolkit--a collection of computer intensive statistical methods for non-linear mixed effect modeling using NONMEM. *Computer methods and programs in biomedicine* 2005;79:241-57.

26. Tran JQ, Kovacs SJ, McIntosh TS, Davis HM, Martin DE. Morning spot and 24-hour urinary 6 beta-hydroxycortisol to cortisol ratios: intraindividual variability and correlation under basal conditions and conditions of CYP 3A4 induction. *Journal of clinical pharmacology* 1999;39:487-94.
27. Yang J, Liao M, Shou M, et al. Cytochrome p450 turnover: regulation of synthesis and degradation, methods for determining rates, and implications for the prediction of drug interactions. *Current drug metabolism* 2008;9:384-94.
28. Tollet P, Enberg B, Mode A. Growth hormone (GH) regulation of cytochrome P-450IIC12, insulin-like growth factor-I (IGF-I), and GH receptor messenger RNA expression in primary rat hepatocytes: a hormonal interplay with insulin, IGF-I, and thyroid hormone. *Molecular endocrinology* (Baltimore, Md) 1990;4:1934-42.
29. Benet LZ, Hoener BA. Changes in plasma protein binding have little clinical relevance. *Clinical pharmacology and therapeutics* 2002;71:115-21.
30. Imaoka S, Terano Y, Funae Y. Changes in the amount of cytochrome P450s in rat hepatic microsomes with starvation. *Archives of biochemistry and biophysics* 1990;278:168-78.
31. Micromedex. *Micromedex Solutions - Drug Monographs*. 2014 ed. Greenwood Village, U.S.: Truven Health Analytics Inc; 2014.
32. Vorum H, Honore B. Influence of fatty acids on the binding of warfarin and phenprocoumon to human serum albumin with relation to anticoagulant therapy. *The Journal of pharmacy and pharmacology* 1996;48:870-5.
33. Wilkinson GR, Shand DG. Commentary: a physiological approach to hepatic drug clearance. *Clinical pharmacology and therapeutics* 1975;18:377-90.
34. Monostory K, Pascussi JM, Kobori L, Dvorak Z. Hormonal regulation of CYP1A expression. *Drug metabolism reviews* 2009;41:547-72.