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TRANSLATIONAL RESEARCH

Pronounced between-subject and circadian variability in thymidylate synthase and dihydropyrimidine dehydrogenase enzyme activity in human volunteers

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AIMS

The enzymatic activity of dihydropyrimidine dehydrogenase (DPD) and thymidylate synthase (TS) are important for the tolerability and efficacy of the fluoropyrimidine drugs. In the present study, we explored between-subject variability (BSV) and circadian rhythmicity in DPD and TS activity in human volunteers.

METHODS

The BSVs in DPD activity ($n = 20$) in peripheral blood mononuclear cells (PBMCs) and in plasma, measured by means of the dihydrouracil (DHU) and uracil (U) plasma levels and DHU : U ratio ($n = 40$), and TS activity in PBMCs ($n = 19$), were examined. Samples were collected every 4 h throughout 1 day for assessment of circadian rhythmicity in DPD and TS activity in PBMCs ($n = 12$) and DHU : U plasma ratios ($n = 23$). In addition, the effects of genetic polymorphisms and gene expression on DPD and TS activity were explored.

RESULTS

Population mean (± standard deviation) DPD activity in PBMCs and DHU : U plasma ratio were 9.2 (±2.1) nmol mg^{-1} h^{-1} and 10.6 (±2.4), respectively. Individual TS activity in PBMCs ranged from 0.024 nmol mg^{-1} h^{-1} to 0.596 nmol mg^{-1} h^{-1}. Circadian rhythmicity was demonstrated for all phenotype markers. Between 00:30 h and 02:00 h, DPD activity in PBMCs peaked, while the DHU : U plasma ratio and TS activity in PBMCs showed trough activity. Peak-to-trough ratios for DPD and TS activity in PBMCs were 1.69 and 1.62, respectively. For the DHU : U plasma ratio, the peak-to-trough ratio was 1.43.

CONCLUSIONS

BSV and circadian variability in DPD and TS activity were demonstrated. Circadian rhythmicity in DPD might be tissue dependent. The results suggested an influence of circadian rhythms on phenotype-guided fluoropyrimidine dosing and supported implications for chronotherapy with high-dose fluoropyrimidine administration during the night.

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Fluoropyrimidine anticancer drugs target thymidylate synthase (TS) and are metabolized by dihydropyrimidine dehydrogenase (DPD).
- Circadian rhythms in DPD and TS could provide a rationale for fluoropyrimidine chronotherapy.
- However, fluoropyrimidine chronotherapy remains controversial because convincing pharmacological data supporting chronotherapy are lacking.

WHAT THIS STUDY ADDS

- Using mixed-effect modelling, a circadian rhythm of TS activity in peripheral blood mononuclear cells (PBMCs), with peak activity during the afternoon, was demonstrated.
- DPD activity in PBMCs clearly showed circadian rhythmicity, with peak activity during the night. The DPD phenotype in human plasma revealed an opposite, but weak, circadian rhythm.

Introduction

Anticancer drugs belonging to the group of the fluoropyrimidines are extensively used in the treatment of colorectal, breast and gastric cancer. The most frequently applied fluoropyrimidine drugs are 5-fluorouracil (5-FU) and its oral pre-prodrug capecitabine. After oral administration, capecitabine is rapidly absorbed and, via a three-step enzymatic cascade, converted into 5-FU. Approximately 80% of 5-FU is rapidly catabolized by the enzyme dihydropyrimidine dehydrogenase (DPD) to inactive metabolites [1–3]. About 1–3% of 5-FU is anabolized to active metabolites, of which 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) possesses the highest anticancer potency [4]. This metabolite inhibits the enzyme thymidylate synthase (TS). Through inhibition of TS, synthesis of deoxythymidine triphosphate (dTTP) is reduced. Depletion of dTTP disrupts DNA synthesis and cell death ensues.

Approximately 10–30% of all patients treated with fluoropyrimidine-based chemotherapy develop severe, sometimes even lethal, toxicity [5]. Unfortunately, identification of patients at risk of severe toxicity is hampered by the unavailability of sensitive clinical biomarker tests. Genetic polymorphisms in the gene encoding DPD, *DPYD*, may result in DPD deficiency. The polymorphisms *DPYD**2A and *DPYD* 2846A > T are proven risk alleles and are strongly associated with fluoropyrimidine-induced severe toxicity [6]. Given the combined frequency of these two mutations of 2–3% [7, 8], a small but important fraction of patients at risk is identified by these two polymorphisms. Polymorphisms in *TYMS*, the gene encoding TS, have also been associated with fluoropyrimidine-induced toxicity [6, 9]. However, the relevance of these mutations requires clinical validation.

In order to reduce the incidence of fluoropyrimidine-induced toxicity and optimize dosing, more sensitive biomarkers are required. Phenotyping methods could provide increased sensitivity for the identification of patients at risk of fluoropyrimidine-induced toxicity. In order to prevent invasive procedures, DPD activity is often determined in peripheral blood mononuclear cells (PBMCs). Clearance of 5-FU has been shown to correlate well with DPD activity in PBMCs ($DPDA_{pbmc}$) [10]. Furthermore, previous studies have shown that approximately 50% of the patients suffering from severe fluoropyrimidine-induced toxicity had relatively low $DPDA_{pbmc}$ [11–13]. In addition, clearance of 5-FU and fluoropyrimidine-induced toxicity are also associated with pretherapeutic plasma levels of the endogenous DPD substrate uracil (U) in plasma, and the ratio of the reaction product dihydrouracil (DHU) and U in plasma [14–17].

Although application of phenotyping assays seems promising for identifying patients at risk, only few medical centres use these methods in routine clinical practice [18, 19]. Several factors might account for this. The development and validation of accurate and robust phenotyping assays is challenging; this is illustrated by high variability in U and DHU plasma levels among different studies [14]. Importantly, the enzymatic activity of DPD and TS might be subject to circadian rhythms, which impedes interpretation of assay results.

Expression of DPD in PBMCs and the DHU : U plasma ratio have previously been shown to peak at 19:00 h [20]. Others found that human $DPDA_{pbmc}$ peaked at the beginning of or during the night [21, 22]. However, lack of a circadian rhythm has also been suggested [23]. In human oral mucosa, TS activity was found to be lowest during the night [24]. Studies in mice provided evidence that 5-FU-induced toxicity is relatively low when the drug is administered at times of trough TS activity [25, 26].

In patients, a 5-FU dosing regimen that was adapted to anticipated nocturnal peak DPD activity showed excellent tolerability [27]. Nonetheless, fluoropyrimidine chronotherapy remains controversial because convincing pharmacological data supporting its use is lacking. In addition, the implementation of chronotherapy in routine clinical practice is often impractical.

Taken together, DPD and TS phenotype assays are promising for upfront identification of patients at risk of developing fluoropyrimidine-induced toxicity. To facilitate the implementation of pretreatment screening of patients, examination of circadian rhythms in DPD and TS phenotype markers is essential. Moreover, circadian rhythms in DPD and TS activity could provide a rationale for clinical evaluation of fluoropyrimidine chronotherapy in order to improve treatment safety.

The purpose of the current study was to gain insight into the between-subject (BSV) and circadian variability in DPD and TS enzyme activity in human volunteers. Secondary objectives were to explore the effect of genetic polymorphisms and gene expression on DPD and TS enzyme activities. For these reasons, we performed an observational study in healthy volunteers and determined $DPDA_{pbmc}$, U and DHU plasma levels, and TS activity in PBMCs (TSA_{pbmc}) using newly developed and validated bioanalytical methods [28, 29]. We applied a mixed-effect modelling approach to quantify circadian rhythmicity.

Materials and methods

Study design and sample collection

In total, 40 healthy volunteers participated in the study. Included subjects were ≥18 years old, without known cancer, who had not been treated with the investigational drugs in the 30 days prior to study assessments and had not undergone surgery within the previous 6 months. Age, gender, regular sleep and wake-up time were recorded. From 20 volunteers, blood was collected at 09:00 h for determination of $DPDA_{pbmc}$, TSA_{pbmc}, DHU and U plasma levels, and *DPYD* and *TYMS* gene expression. Of these 20 volunteers, 12 underwent additional blood sampling at 13:00 h, 17:00 h, 21:00 h, 01:00 h, 05:00 h and 09:00 h the following day to examine circadian variability in $DPDA_{pbmc}$, TSA_{pbmc}, DHU and U plasma levels. From the second group of 20 volunteers, we collected samples only for the determination of DHU and U plasma levels. For the evaluation of circadian variability, nine of the latter 20 volunteers underwent blood sampling once at 09:00 h and 11 were repeatedly sampled at the previously defined time points. Thus, circadian variability in $DPDA_{pbmc}$ and TSA_{pbmc} was assessed in 12 volunteers, and circadian variability in DHU and U plasma levels in 23 volunteers.

In order to take blood samples at night, volunteers were hospitalized. Intravenous cannulas were used for the volunteers participating in the overnight sampling, to limit the disturbance to the normal day–night rhythm. The study protocol was approved by the Medical Ethics Committee of the Slotervaart Hospital, Amsterdam, The Netherlands. All volunteers provided written informed consent prior to study assessments.

Determination of DPD and TS activity in PBMCs

A volume of 8 ml of peripheral heparinized blood was collected for the assessment of $DPDA_{pbmc}$ and TSA_{pbmc}. PBMCs were isolated using Ficoll–Paque density gradient centrifugation and stored at −80°C until further analysis [28, 29]. $DPDA_{pbmc}$ and TSA_{pbmc} were determined by validated radioassays [28, 29]. DPD activity was expressed as the amount of ^{3}H-dihydrothymine formed per mg of PBMC protein after 1 hour of *ex vivo* incubation (nmol mg^{-1} h^{-1}) with the DPD substrate of ^{3}H-thymine [28]. TS activity was expressed as the amount of 5-^{3}H-2′-deoxyuridine 5′-monophosphate that was metabolized *ex vivo* per mg of PBMC protein per hour of incubation (nmol mg^{-1} h^{-1}) [29]. PBMC protein levels were corrected for haemoglobin contamination using a spectrophotometric method [30].

Quantification of uracil and dihydrouracil plasma levels

A volume of 4 ml of heparinized whole blood was centrifuged at 1500 *g* for 10 min at 4°C. Isolated plasma was stored at −20°C until further analysis. DHU and U were quantified in plasma using mass spectrometry [31]. After defrosting, a volume of 20 µl of internal standard working solution containing 1,3-U-$^{15}N_2$ and 5,6-DHU-$^{13}C_4$,$^{15}N_2$ was added to 300 µl plasma. Protein precipitation was performed using 900 µl of MeOH and acetonitrile (1:1, v/v). Samples were vortex-mixed for 10 s, shaken for 10 min at 1250 rpm (Labinco, Breda, The Netherlands) and centrifuged at 14 000 *g* for 10 min. Clear supernatants were dried under a stream of nitrogen at 40°C and reconstituted in 100 µl 0.1% formic acid in water. U and DHU plasma levels were determined using an ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) system. Chromatographic separation was performed on an Acquity UPLC® HSS T3 (150 × 2.1 mm ID, particle size 1.8 µm; Waters, Milford, MA, USA) column. Mobile phases consisted of 0.1% formic acid in UPLC-grade water (eluent A) and 0.1% formic acid in UPLC-grade acetonitrile (eluent B) at a flow rate of 0.3 ml min^{-1}. The following gradient was used: 0% B at 0–3.0 min, 0–90% B at 3.0–3.2 min, 90% B at 3.2–3.7 min, 0% B at 3.7–5 min. A Qtrap 5500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA) was operated in the negative mode for quantification of U and in the positive mode for quantification of DHU. Selected mass transitions for U and DHU were *m/z* 110.9 ➔ *m/z* 42.0 and *m/z* 114.9 ➔ *m/z* 55.0. Validated concentration ranges for U and DHU were 1–100 ng ml^{-1} and 10–1000 ng ml^{-1}, respectively.

Gene expression of *DPYD* and *TYMS*

PBMCs were isolated from 8 ml of whole blood using Vacutainer® cell preparation (CPT) tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Tubes were centrifuged at 1800 *g* for 20 min at room temperature. The PBMCs were transferred into a clean 50 ml tube and washed twice with NaCl 0.9%. PBMCs were then lysed in RNA-Bee (TelStat, Friendswood, TX, USA) and stored at −80°C until total RNA extraction. Isolation of total RNA was performed according to the RNA-Bee manufacturer's instructions. A quantity of 350 ng of the isolated total mRNA, random primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) was used for the synthesis of cDNA using the following polymerase chain reaction (PCR) protocol: 25°C for 10 min, 42°C for 50 min, 70°C for 15 min, refrigeration at 4°C. Quantification of TYMS and DPYD gene expression was performed in triplicate using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and the 7500 Fast Real-Time PCR (RT-PCR) system (Applied Biosystems). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method [32]. Peptidylprolyl isomerase B (PPIB) was used as the housekeeping gene and Human Reference RNA (Stratagene, La Jolla, CA, USA) as the external calibrator. RT-PCR primer sequences are available upon request.

Genetic polymorphisms in *DPYD* and *TYMS*

Genomic DNA was isolated from 3 ml of ethylenediamine tetraacetic acid-treated blood using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA). The polymorphisms *DPYD**2 and 2846A > T were determined by RT-PCR using allele-specific TaqMan probes (Applied Biosystems, Bleijswijk, The Netherlands) [8]. Polymorphisms within the 5'UTR 28-base pair (bp) tandem repeat (VNTR) in *TYMS* were analysed using Sanger sequencing. Additionally, we screened for the G > C single nucleotide polymorphisms (SNPs) within the first tandem repeat of the 2R allele. Patients were stratified based on the 28-bp VNTR in *TYMS* to either the low *TYMS* expression

group (*2/*2, *2/*3C or *3C/*3C) or high *TYMS* expression group (*2/*3G, *3C/*3G or *3G/*3G). Primer sequences are available upon request.

Mixed-effect modelling of circadian rhythms

Mixed-effect modelling was applied to describe the circadian rhythms in $DPDA_{pbmc}$, U and DHU plasma levels and TSA_{pbmc} using NONMEM (version 7.3.0) [33]. Piraña (version 2.9.1) was used for model management [34]. Circadian rhythms were modelled applying cosine functions as follows:

$$Y(t) = Mesor \times \left\{1 + \sum_{i=1}^{n} \left[AMP_i \times \cos\left(2\pi \times i \times \frac{[t - PS_i]}{24}\right)\right]\right\} \quad (1)$$

where Y represents the studied phenotype biomarker, $Mesor$ is the 24-h mean value, AMP_i is the relative amplitude and PS_i is the phase shift (time of peak) for cosine function i. The time of day is represented by t. BSV on model parameters was described using log-normal models. Residual unexplained variability (RUV) was estimated using additive residual error models. Circadian variability in U and DHU plasma levels was estimated using a single set of parameters for AMP_i and PS_i. Model evaluation was guided by goodness-of-fit (GOF) plots, visual predictive checks (VPC), drop in objective function value (dOFV) and precision of obtained parameter estimates. Model management and diagnostics were carried out using R [35], the Xpose4 package (version 4.5.3) for R and PsN (version 4.2) [34].

Statistical analyses

Statistical analyses were performed in R (version 3.1.2) [35]. Descriptive statistics were used to describe $DPDA_{pbmc}$, TSA_{pbmc}, U and DHU plasma levels and DHU : U plasma ratios. Assessments of normality were done using the Shapiro–Wilk test. Mann–Whitney U tests were applied for comparing continuous variables between subgroups. Pearson correlations were estimated to examine the relationship between $DPDA_{pbmc}$ and U plasma levels and the DHU : U plasma ratio. The Pearson and Filon's statistical test was applied to explore the difference between the overlapping correlations of $DPDA_{pbmc}$ and U plasma level and of $DPDA_{pbmc}$ and DHU:U ratio using the cocor package for R [36]. Associations between gene expression and $DPDA_{pbmc}$ and TSA_{pbmc} were explored using the Pearson correlation test. Intraday variability in $DPDA_{pbmc}$, TSA_{pbmc}, DHU : U plasma ratio and U and DHU plasma levels were assessed using repeated measures analysis of variance tests with Tukey *post hoc* analysis, unless stated differently. *P*-values <0.05 were considered statistically significant.

Results

Volunteer characteristics

A total of 40 volunteers (21 females) with a mean age of 28.8 years (range: 20.3–49.7 years) were included. Of the 40 volunteers, 39 were Caucasian. Mean (range) waking and sleep times were 07:00 h (06:00–8:30 h) and 23:25 h (21:45–01:50 h), respectively. Sample collection for baseline assessment of $DPDA_{pbmc}$, TSA_{pbmc}, DHU and U plasma levels and *DPYD* and *TYMS* gene expression took place, on average, at 09:25 h (range: 08:45–11:15 h).

Baseline characteristics of DPD and TS phenotype markers

Mean and BSV in TSA_{pbmc}, $DPDA_{pbmc}$, DHU : U plasma ratio and U and DHU plasma levels are summarized in Table 1. For one volunteer, the amount of isolated PBMC protein was insufficient for TSA_{pbmc} analysis. TSA_{pbmc} followed a non-normal distribution ($P < 0.001$), whereas for the other variables this test was not significant. Large BSV in baseline TSA_{pbmc} was found as illustrated by a factor of 25 difference between the maximum and minimum observed values.

Intraday variability in markers for TS and DPD activity

Individual and population average TSA_{pbmc} and $DPDA_{pbmc}$ are shown in Figure 1. Trough TSA_{pbmc} and peak $DPDA_{pbmc}$ levels were observed at 01:00 h, while peak TSA_{pbmc} and trough $DPDA_{pbmc}$ levels were both detected at 17:00 h and 13:00 h, respectively. The peak-to-trough ratios of $DPDA_{pbmc}$ and TSA_{pbmc} were 1.69 and 1.62, respectively. Figure 2 displays the intraday variability in U, DHU and DHU : U in plasma. Peak and trough U plasma levels occurred at 05:00 h and 17:00 h, respectively. Throughout the day, DHU plasma levels showed significant differences, with peak levels at 09:00 h and trough levels at 01:00 h. Peak-to-trough ratios for U and DHU plasma levels were 1.39 and 1.22, respectively. Intraday variability in U and DHU plasma levels also resulted in clear intraday variability in DHU : U plasma ratios. Peak and trough DHU : U ratios were detected at 17:00 h and 05:00 h, respectively, with a peak-to-trough ratio of 1.43. All examined phenotype markers revealed significant intraday variability (Table 2).

Table 1

Descriptive statistics for baseline values of thymidylate synthase and dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells and plasma levels of uracil (U) and dihydrouracil (DHU), including the DHU : U plasma ratio, in healthy volunteers. The mean time of sample collection was 09:25 h (range: 08:45–11:15 h)

Variable	*n*	Mean ± SD	CV (%)
TS activity in PBMCs (nmol mg^{-1} h^{-1})	19	0.072 (0.024–0.596)*	–
DPD activity in PBMCs (nmol mg^{-1} h^{-1})	20	9.2 ± 2.1	23
DHU : U plasma ratio	40	10.6 ± 2.4	22
U concentration (ng ml^{-1})		9.8 ± 2.9	29
DHU concentration (ng ml^{-1})		98 ± 23.8	24

CV, coefficient of variation; DHU, dihydrouracil; DPD, dihydropyrimidine dehydrogenase; *n*, number of subjects; PBMCs, peripheral blood mononuclear cells; SD, standard deviation; TS, thymidylate synthase; U, uracil. *Median and range are shown for TS activity as non-normal distribution was demonstrated.

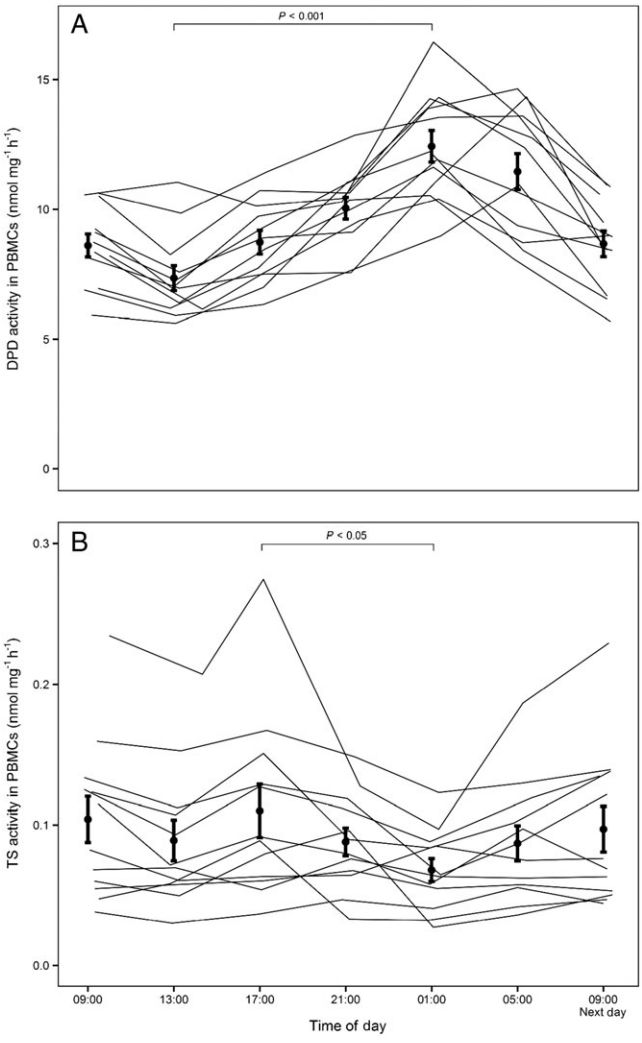

Figure 1

Intraday variability in dihydropyrimidine dehydrogenase (A) and thymidylate synthase activity (B) in peripheral blood mononuclear cells from volunteers (n = 12). Grey lines represent individual lines for DPD and TS activity. Mean ± standard error per time point are shown by black points and error bars. Differences between peak and trough activities are supported by *P*-values. DPD, dihydropyrimidine dehydrogenase; PBMCs, peripheral blood mononuclear cells; TS, thymidylate synthase

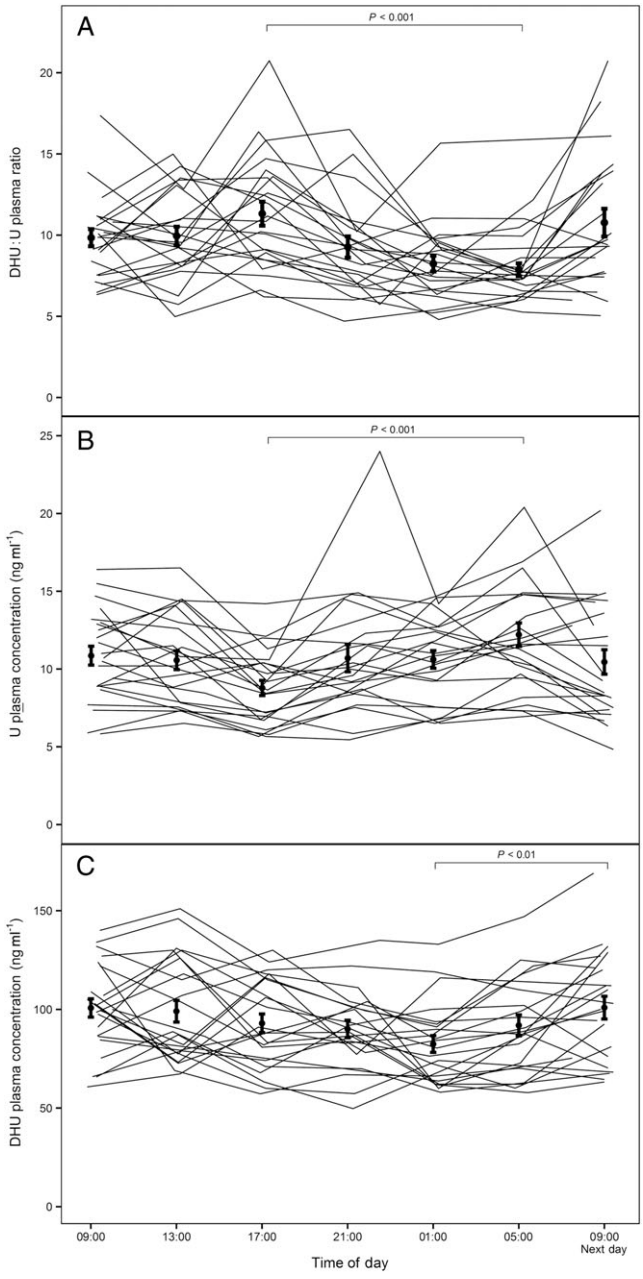

Figure 2

Intraday variability in the dihydrouracil : uracil plasma ratio (A), uracil plasma levels (B) and dihydrouracil plasma levels (C) in 23 healthy volunteers. Grey solid lines represent individual curves. Mean ± standard error per time point is shown by black points and error bars. Differences between peak and trough levels are supported by *P*-values. DHU, dihydrouracil; U, uracil

Mixed-effect modelling of circadian rhythms

A mixed-effect modelling approach was applied to quantify circadian variability. A combined model for circadian rhythms in U and DHU plasma levels and $DPDA_{pbmc}$ was developed. Base models included BSV terms for mesors of U ($Mesor_U$) and DHU ($Mesor_{DHU}$) plasma levels and $DPDA_{pbmc}$ ($Mesor_{DPD}$). One cosine function adequately described circadian rhythms in U and DHU plasma levels.

Equation (2) illustrates the function for model prediction of U and DHU plasma levels as a function of the time of day (0–24 h):

$$\mathrm{Rhythm}_{U\&DHU}(t) = \mathrm{AMP}_{U\&DHU} \times \cos\left(2\pi \frac{[t - \mathrm{PS}_{U\&DHU}]}{24}\right) \quad (2)$$

where circadian variability in U and DHU plasma levels is represented by $Rhythm_{U\,\&\,DHU}$, the amplitude $AMP_{U\,\&\,DHU}$, phase shift by $PS_{U\,\&\,DHU}$ and time of day by t.

Equation (2) was incorporated in the population model for circadian variability in U and DHU plasma levels, as illustrated by Equations (3) and (4):

$$U(t) = Mesor_U \times (1 + Rhythm_{U\&DHU}(t)) + \varepsilon_U \quad (3)$$

Table 2

Results of the repeated measures anova of thymidylate synthase and dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells, the dihydrouracil : uracil plasma ratio, and uracil and dihydrouracil plasma levels in healthy volunteers

Variable	*n*	Trough ± SEM	Peak ± SEM	Time of trough (hh : mm)	Time of peak (hh : mm)	Peak: Trough	*P*-value of repeated measures anova
TS activity in PBMCs (nmol mg^{-1} h^{-1})	12	0.068 ± 0.008	0.110 ± 0.019 *	01:00 h	17:00 h	1.62	0.012†
DPD activity in PBMCs (nmol mg^{-1} h^{-1})	12	7.35 ± 0.48	12.4 ± 0.61 ***	13:00 h	01:00 h	1.69	<0.0001
DHU : U plasma ratio	23	7.87 ± 0.40	11.32 ± 0.74 ***	05:00 h	17:00 h	1.43	<0.0001
U concentration (ng ml^{-1})	23	8.78 ± 0.49	12.21 ± 0.75 ***	17:00 h	05:00 h	1.39	0.0002
DHU concentration (ng ml^{-1})	23	82.4 ± 4.2	100.9 ± 8.8 **	01:00 h	09:00 h	1.22	0.0009

anova, analysis of variance; DHU, dihydrouracil; DPD, dihydropyrimidine dehydrogenase; *n*, number of subjects; PBMCs, peripheral blood mononuclear cells; SEM, standard error of the mean; TS, thymidylate synthase; U, uracil. Differences between peak and trough levels were based on a *post hoc* test: *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$, †In the case of TS activity in PBMCs, the nonparametric repeated measures anova (Friedman test) with *post hoc* analysis was applied.

$$DHU(t) = Mesor_{DHU} \times \left(1 - Rhythm_{U\&DHU}(t)\right) + \varepsilon_{DHU} \qquad (4)$$

In these equations, additive residual errors for U and DHU plasma levels are represented by ε_U and ε_{DHU}, with mean zero and variance σ^2.

The circadian rhythm of $DPDA_{pbmc}$ was adequately described using a single cosine function. Covariance between BSV of $Mesor_U$ and $Mesor_{DHU}$ was assessed. The estimated correlation coefficient was 0.68 (dOFV = −17.2). In addition, there was a negative correlation between the BSV of $Mesor_U$ and $Mesor_{DPD}$. The correlation coefficients between BSV of $Mesor_U$ and $Mesor_{DHU}$ and BSV of $Mesor_U$ and $Mesor_{DPD}$ were included in the final model. Furthermore, there was a positive correlation between RUV in U and DHU plasma levels, which was also included in the final model (dOFV = −9.9).

The optimal model for TSA_{pbmc} contained two cosine functions to describe circadian variability. This model was further improved by the addition of a single BSV term on both amplitudes (dOFV = −16.7).

VPCs of the final circadian models are shown in Figure 3. The parameter estimates are given in Table 3. The typical circadian rhythms of the studied phenotype markers are shown in Figure 4. GOF plots are provided in Figures S1–4).

Associations between DPD activity in PBMCs and in plasma

There was a modest but significant negative correlation between $DPDA_{pbmc}$ and U plasma levels (r^2 = 0.26; P = 0.02; Figure S5). There was a trend toward a positive association between $DPDA_{pbmc}$ and the DHU : U plasma ratio (r^2 = 0.17; P = 0.07). The difference in overlapping correlations between $DPDA_{pbmc}$ and the U plasma level and of $DPDA_{pbmc}$ and the DHU : U ratio was not statistically significant.

Associations between TYMS and *DPYD* genotypes and phenotypes

There were 10 volunteers with the *TYMS* low-expression genotype and nine volunteers with the *TYMS* high-expression genotype. Although the average TSA_{pbmc} was, as expected, higher in volunteers with the high-expression genotype, the difference was not significant (P = 0.32; Figure S6). Among the volunteers, we identified one with the very uncommon 2RC/3RC expression genotype. The proportion of individuals with this genotype was previously reported to be 2% [37]. TSA_{pbmc} in this volunteer was relatively low (0.036 nmol mg^{-1} h^{-1}; Figure S6). None of the 40 volunteers were carriers of the *DPYD*2A* allele, and one was heterozygous for *DPYD* 2846A > T.

Associations between TS and DPD enzyme activity and gene expression in PBMCs

To assess whether TS and DPD enzyme activity in PBMCs are regulated at a transcriptional level, we explored associations between gene expression and TSA_{pbmc} and $DPDA_{pbmc}$ (Figure S7). There was a strong positive correlation between TSA_{pbmc} and *TYMS* gene expression (r^2 = 0.84; P < 0.001). By contrast, a weak, but significant negative correlation was found for $DPDA_{pbmc}$ and *DPYD* gene expression (r^2 = 0.22; P = 0.04).

Discussion

The present study clearly identified and quantified wide BSV and circadian rhythmicity in phenotypic markers of TS and DPD activity. Intraday variability was noticeable in the presented data. Eventually, we were able to describe circadian rhythms in $DPDA_{pbmc}$, U and DHU plasma levels using a combined model, and rhythmicity in TSA_{pbmc} by the sum of two cosine functions. Model parameters were estimated with adequate precision and model diagnostics did not suggest structural deviations. Therefore, this is the first report to describe successfully and quantify fully the circadian rhythmicity of TSA_{pbmc}, $DPDA_{pbmc}$, U and DHU plasma levels using the mixed-modelling approach.

Although there was a moderate correlation between baseline $DPDA_{pbmc}$ and U plasma levels, circadian rhythmicity in $DPDA_{pbmc}$ and U and DHU plasma levels clearly suggested two different patterns in DPD activity. The time of peak

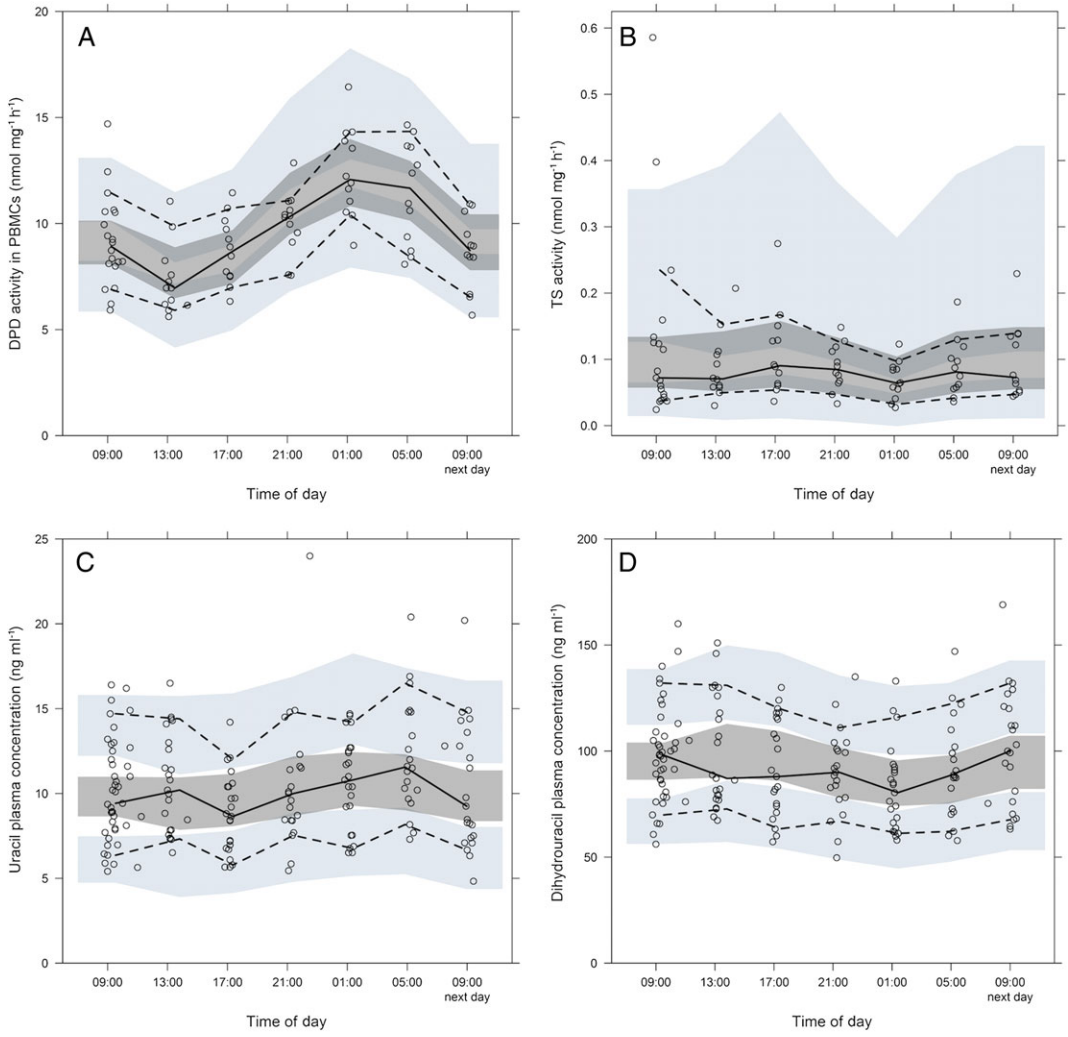

Figure 3

Visual predictive checks for final models for circadian rhythms in dihydropyrimidine dehydrogenase (A) and thymidylate synthase (B) activity in peripheral blood mononuclear cells, and uracil (C) and dihydrouracil (D) plasma levels. Lines represent the 10th percentile, median and 90th percentile of observed data. Shaded areas represent corresponding 95% confidence intervals of simulated data. DPD, dihydropyrimidine dehydrogenase; PBMCs, peripheral blood mononuclear cells; TS, thymidylate synthase

$DPDA_{pbmc}$ was estimated at 01:46 h. However, at that time, we also discovered peak U and trough DHU plasma levels, suggestive of trough DPD activity at the same time point. The magnitude of circadian variation was more pronounced for $DPDA_{pbmc}$ than for U and DHU plasma levels, which was illustrated by relative amplitudes of 0.245 for $DPDA_{pbmc}$ and 0.082 for U and DHU plasma levels. Residual unexplained variability was relatively large in the circadian model for U and DHU in plasma. This indicates that there are unidentified variables, other than circadian rhythmicity, accounting for variability in U and DHU plasma levels.

Others have also reported circadian rhythmicity on surrogate markers for systemic DPD activity [20, 21, 38]. The results from these studies show large differences. Jiang *et al*. [20] determined circadian rhythmicity in DHU : U plasma ratios and DPD protein expression in PBMCs in human volunteers and found peak levels for both markers at approximately 18:40 h. They found an average DHU : U plasma ratio in their study of 5.1 and found a large effect of circadian rhythmicity on this marker, considering the observed peak-to-trough ratio of 3.4 [20]. Compared with the present study, these authors showed more pronounced circadian variability in U and DHU and earlier peak expression of DPD protein in PBMCs. Their average DHU : U plasma ratio was remarkably lower than the average DHU : U plasma ratio that we observed. Zeng *et al*. reported peak DHU : U plasma ratios in patients at 02:30 h and with a relative amplitude of 0.13 [38]. Although the magnitude of circadian variability of their findings was in line with ours, the time of peak was completely opposite to that found in the present study. Peak $DPDA_{pbmc}$ in patients who were treated with 5-FU was detected at around midnight by Harris *et al*. [21]. Besides the time of peak activity, the magnitude of rhythmicity was also similar to our results on $DPDA_{pbmc}$.

DPD activity in PBMCs and the DPD phenotype in plasma might be regulated by different mechanisms. Circadian

Table 3

Parameter estimates of the final models of circadian rhythms in uracil and dihydrouracil plasma levels, and thymidylate synthase and dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells. The circadian rhythm in thymidylate synthase activity was described by two cosine functions, for which separate amplitudes and phase shifts were estimated

Parameter	Estimate	RSE (%)
Structural model parameter		
$Mesor_U$ (ng ml^{-1})	9.95	4.3
$Mesor_{DHU}$ (ng ml^{-1})	91.4	2.9
$Amplitude_{U\&DHU}$	0.082	15.5
Phase $shift_{U\&DHU}$ (hh : mm)	01:56	21.4
$Mesor_{DPD}$ (nmol mg^{-1} h^{-1})	9.94	4.0
$Amplitude_{DPD}$	0.245	9.3
Phase $shift_{DPD}$ (hh : mm)	01:46	18.4
$Mesor_{TS}$ (nmol mg^{-1} h^{-1})	0.080	19.4
$Amplitude_{TS}$ 1	0.129	28.9
$Amplitude_{TS}$ 2	0.106	29.6
Phase $shift_{TS}$ 1 (hh : mm)	13:24	3.1
Phase $shift_{TS}$ 2 (hh : mm)	18:42	1.1
Between-subject variability		
$Mesor_U$ (CV%)	24.3	10.7
$Mesor_{DHU}$ (CV%)	17.8	14.3
$Mesor_{DPD}$ (CV%)	17.5	17.8
$Mesor_{TS}$ (CV%)	92.3	16.8
$Amplitude_{TS}$ 1 and 2 (CV%)	56.5	33.2
Residual unexplained variability		
$\sigma_{additive,\ U}$ (ng ml^{-1})	2.05	21.5
$\sigma_{additive,\ DHU}$ (ng ml^{-1})	16.2	11.8
$\sigma_{additive,\ DPD}$ (nmol mg^{-1} h^{-1})	1.14	19.5
$\sigma_{additive,\ TS}$ (nmol mg^{-1} h^{-1})	0.014	20.3
Correlations		
ρ ($BSV\ Base_U$, $BSV\ Base_{DHU}$)	0.68	–
ρ ($BSV\ Base_U$, $BSV\ Base_{DPD}$)	−0.16	–
ρ (σ_U, σ_{DHU})	0.26	–

BSV, between-subject variability; CV, coefficient of variation; DHU, dihydrouracil; DPD, dihydropyrimidine dehydrogenase; Mesor, rhythm-adjusted mean; Phase shift, time of peak; RSE, relative standard error; TS, thymidylate synthase; U, uracil; σ, residual variability; ρ, correlation coefficient.

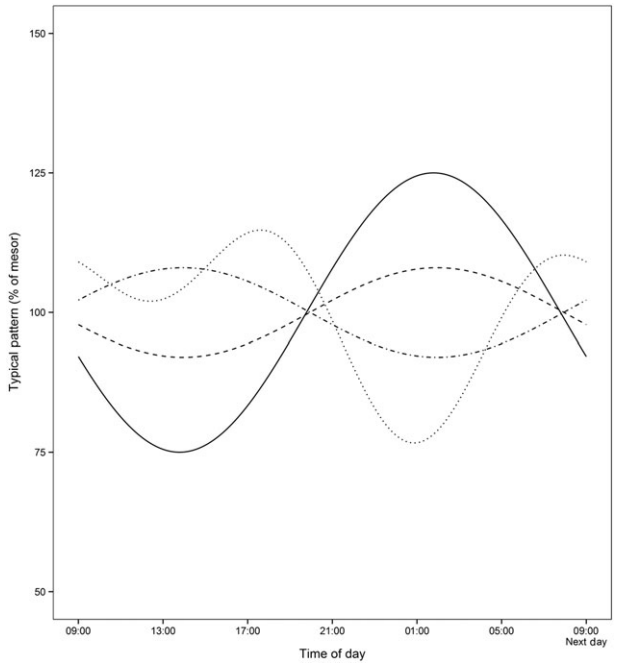

Figure 4

Typical patterns of circadian variability in dihydropyrimidine dehydrogenase and thymidylate synthase enzyme activity in peripheral blood mononuclear cells, and uracil and dihydrouracil plasma levels in healthy volunteers. Circadian variability is expressed as a percentage of the mesor (rhythm-adjusted mean). DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase. - - Uracil (ng ml^{-1}), ·-· dihydrouracil (ng ml^{-1}), —— DPD activity in PBMCs (nmol mg^{-1} h^{-1}), ···· TS activity in PBMCs (nmol mg^{-1} h^{-1})

rhythms are generally maintained by a central circadian clock located in the hypothalamus [39]. The circadian clock consists of a transcription–translation-based oscillatory feedback mechanism involving a set of genes, called clock genes. Clock proteins control the circadian rhythms of other genes by regulating transcriptional processes. However, circadian clocks have been found in several peripheral tissues. This allows for tissue-specific regulation of rhythmicity. Surprisingly, circadian clock genes in PBMCs show expression in a circular manner which has been shown to be conserved in cultured PBMCs [40, 41]. Previous experiments suggested that DPD activity is coordinated by the period 1 (*PER1*) clock gene [42], which is one of the genes that show circadian rhythmicity in PBMCs [40, 41]. Tissue-specific regulation of clock genes might cause DPD activity in PBMCs to deviate from DPD activity in other body compartments, which could lead to differences in circadian rhythms for DHU : U in plasma and $DPDA_{pbmc}$. Alternatively, other enzymatic processes might contribute to rhythmicity in U and DHU plasma levels. The enzyme uridine phosphorylase (UP), which degrades uridine to U, and the enzyme DPD show opposite circadian rhythms in mice [43]. If these two enzymes also show opposite rhythms in humans, this could contribute to circadian variability in U plasma levels. As $DPDA_{pbmc}$ and the DHU : U plasma ratio show opposing circadian rhythms, patient samples for DPD phenotyping or therapeutic drug monitoring of 5-FU should preferably be collected at the intersect of both rhythms, which is at approximately 08:00 h and 20:00 h.

The correlations between $DPDA_{pbmc}$ and the U plasma level, and between $DPDA_{pbmc}$ and the DHU : U ratio were weak. These weak correlations could be caused by differences in analytical methods and biological sample matrices. The method for measuring DPD activity in PBMCs is based on *ex vivo* conversion of the radiolabelled DPD substrate ^{3}H-dihydrothymine and has been shown to be highly selective for the enzyme DPD [28]. Conversely, enzymes, other than DPD, might contribute to the regulation of U and DHU plasma levels. The enzyme UP is involved in the formation of U, and dihydropyrimidinase catalyses the degradation of DHU. Consequently, BSV in UP and dihydropyrimidinase activity could affect U and DHU plasma levels, which, in turn, could contribute to the weak correlation with $DPDA_{pbmc}$.

DPD-based adaptive dosing of fluoropyrimidines using the DHU : U plasma ratio could be biased by the involvement of other enzymes. Additional clinical research is needed to evaluate the applicability of the DHU : U plasma ratio for DPD-based adaptive dosing of fluoropyrimidines.

TSA_{pbmc} displayed circadian variability and a particularly large BSV. Smaaland *et al*. [44] discovered a circadian rhythm in bone marrow DNA synthesis in healthy volunteers. They observed trough DNA synthesis at midnight, a minor peak at 08:00 h and maximum DNA synthesis at 16:00 h [44]. This is in agreement with the pattern we observed in TSA_{pbmc}. As TS activity is required for DNA synthesis, it might be anticipated that these processes are dependent on each other and, therefore, show similar patterns.

A strong correlation between TSA_{pbmc} and *TYMS* gene expression in PBMCs clearly suggests that BSV in TS activity is regulated at a transcriptional level. Within cancer tissue, others have also found correlations between TS protein and gene expression [45, 46]. One volunteer carried the G > C SNP in the 2R allele and was identified with the 2RC/3RC genotype of *TYMS*. The 2RC allele has been associated with low TS expression *in vitro* [37, 47]. We also found that the TSA_{pbmc} for this subject was relatively low. Moreover, we recently showed that patients carrying the 2RC allele are at a strongly increased risk of developing fluoropyrimidine-induced toxicity [9].

DPD_{pbmc} showed a weak negative correlation with *DPYD* gene expression. A trend toward negative correlation between DPD activity and gene expression was also found in human mucosa tissue [48]. It seems plausible that DPD activity is regulated at a post-transcriptional level, which indicates that *DPYD* gene expression cannot be used for the prediction of the DPD phenotype in PBMCs.

Although various pharmacological methods were used to measure TS and DPD activity patterns, a drawback of the present study might be the relatively small number of individuals participating. For this reason, we performed mixed-effect modelling, to use all available data for a full quantitative description of the time course of changes in the markers. Larger studies are needed to assess the associations between *DPYD* and *TYMS* polymorphisms and DPD and TS phenotypes more extensively.

Taken together, multiple studies have been performed in order to assess circadian rhythms in $DPDA_{pbmc}$, U and DHU. These studies have shown differences in the timing of peak levels and magnitude of circadian rhythms. This might be due to limited sample size, variability among populations and differences in bioanalytical methods. Other factors that are subject to circadian variation, such as exposure to light, intake of food, liver blood flow, body temperature, physical activity and the actions of other enzymes or mediators, may additionally play a role in the regulation of circadian rhythmicity of U and DHU plasma levels, TSA_{pbmc} and $DPDA_{pbmc}$. However, further research is warranted to identify factors that are associated with rhythmicity in DPD and TS.

By extensively validating our phenotyping assays, we maximized our effort to obtain accurate and representative values for the phenotype markers. We discovered substantial BSV in baseline $DPDA_{pbmc}$, and U and DHU plasma levels, and large BSV in baseline TSA_{pbmc}. Circadian rhythmicity in $DPDA_{pbmc}$ was opposite and more pronounced to circadian rhythmicity in U and DHU plasma levels. In addition, TSA_{pbmc} showed peak activity in the afternoon and trough activity during the night. These results are highly suggestive of a possible influence of circadian rhythms on phenotype-guided fluoropyrimidine dosing. Moreover, there might be implications for fluoropyrimidine chronotherapy. The tolerability of fluoropyrimidines in mice has been shown to be highest at the time of trough TS and peak DPD activity [26], which should occur during the night, according to our results. Levi *et al*. showed superior 5-FU tolerability when the drug was administered at night [27]. The results of the current study support this clinical observation that fluoropyrimidine chronotherapy, with a relatively high dose intensity during the night, might show better tolerability.

Capecitabine is usually administered twice daily at 12-h intervals, with equal morning and evening doses. This dosing regimen could cause the circadian rhythms in DPD and TS activity to affect the tolerability of capecitabine. We are currently performing a pharmacological study of chronomodulated capecitabine therapy in patients to explore the role of circadian rhythms in DPD and TS activity on treatment tolerability and drug exposure. In this pharmacological study, the morning : evening ratio of the daily capecitabine dose is 3:5 in order to administer high-dose capecitabine in the late evening (http://www.trialregister.nl; study identifier: NTR4639). In addition, we are exploring the applicability of our phenotype markers with regard to phenotype-adjusted fluoropyrimidine treatment in a prospective study (http://www.clinicaltrials.gov; study identifier: NCT02324452). In conclusion, markers for TS and DPD show circadian variability, which offers opportunities for improved fluoropyrimidine treatment safety.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization for the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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Contributors

BJ, MD, RvG, AC, JB and JS were responsible for the study concept and protocol. BJ, MD, DP, MK, RvG, DM, NdV and AB collected the data. BJ, CvH and AH carried out the data analysis and interpretation. BJ wrote the manuscript. BJ, MD, DP, CvH, MK, RvG, NdV, HR, DM, AB, AC, JB, AH and JS approved the final manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://onlinelibrary.wiley.com/doi/10.1111/bcp.13007/suppinfo.

Figure S1 Goodness-of-fit plots for circadian model-predicted dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells. Grey dots represent observed data and black lines represent the trend of the data

Figure S2 Goodness-of-fit plots for circadian model-predicted uracil plasma levels. Grey dots represent observed data and black lines represent the trend of the data

Figure S3 Goodness-of-fit plots for circadian model-predicted dihydrouracil plasma levels. Grey dots represent observed data and black lines represent the trend of the data

Figure S4 Goodness-of-fit plots for circadian model-predicted thymidylate synthase activity in peripheral blood mononuclear cells. Grey dots represent observed data and black lines represent the trend of the data

Figure S5 Correlation between baseline dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells and uracil plasma levels ($r^2 = 0.26$, $P = 0.02$) in 20 healthy volunteers. The shaded area represents the 95% confidence interval of the line

Figure S6 Baseline thymidylate synthase activity in peripheral blood mononuclear cells for low (n = 10) and high (n = 9) TYMS expression genotypes. Median TS activity per group is shown in the plot. The range of TS activity was highest for the TYMS high-expression group. The difference in TS activity between groups was not statistically significant ($P = 0.32$). TS activity in the volunteer with the 2RC/3RC genotype is shown separately (♦)

Figure S7 Strong positive correlation between thymidylate synthase activity and gene expression (r^2 = 0.84; $P < 0.001$) (left panel) and weak negative correlation between dihydropyrimidine dehydrogenase activity and gene expression (r^2 = 0.22; P = 0.04) (right panel) in human peripheral blood mononuclear cells obtained from 20 healthy volunteers. Shaded areas represent 95% confidence intervals of the lines