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Intestinal CYP3A4 and midazolam disposition *in vivo* associate with *VDR* polymorphisms and show seasonal variation

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ABSTRACT

Vitamin D, whose levels vary seasonally with sunlight, is activated to 1α,25-dihydroxyvitamin D_3 that binds the vitamin D receptor (VDR) and transcriptionally regulates intestinal CYP3A4 expression. We genotyped *VDR* polymorphisms and determined their associations with intestinal CYP3A4 and with midazolam pharmacokinetics, and whether intestinal CYP3A4 levels/activity varied seasonally. The *VDR BsmI*G>A (rs1544410) polymorphism was significantly associated with CYP3A4 jejunal expression/activity, with CYP3A4 duodenal mRNA, and with midazolam area under the curve (AUC). Intestinal CYP3A4 expression/activity was significantly higher in biopsies with the *VDR* promoter polymorphisms Cdx2-3731G>A and GATA-1012A>G that increase VDR activation of target genes. Duodenal CYP3A4 mRNA was significantly higher between April and September than between October and March. Midazolam p.o. AUC and oral bioavailability trended higher October through March compared to April through September. These data suggest *VDR* polymorphisms are predictors of intestinal CYP3A4, and that CYP3A4 intestinal expression varies seasonally – likely related to annual changes in UV sunlight and vitamin D levels.

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1. Introduction

CYP3A4 is highly expressed in human duodenal and jejunal mucosal epithelium [1,2]. Inter-individual differences in intestinal CYP3A4 expression and activity contribute significantly to the low and variable oral bioavailability for many CYP3A4 drug substrates. For example, it was estimated that intestinal CYP3A4 catalyzes the first pass loss of >40% (on average) of an oral dose of midazolam [3,4]. Moreover, for midazolam, the extent of intestinal first-pass extraction can vary between negligible to as much as 75% [4]. This result is consistent with the more than 20-fold inter-individual differences in CYP3A4 content in human duodenal enterocytes [5–7]. These findings have important therapeutic implications because the large inter-patient differences in first-pass intestinal extraction efficiency leads to differences in systemic exposure to the parent drug and its metabolites. These differences are important determinants of drug efficacy and toxicity, and hence therapeutic outcome after oral drug administration.

Inter-individual differences in intestinal CYP3A4 expression are likely governed by both genetic and environmental factors. However, numerous studies have revealed that CYP3A4 cis-nucleotide variation cannot fully explain variable CYP3A4 expression in liver, or the oral or intravenous clearance of the CYP3A4 substrate midazolam [8]. Genetic variation in liver-enriched transcription factors (*e.g.*, PXR and FoxA2) regulating CYP3A4 expression appears to explain some of the variability in hepatic CYP3A4 expression [9,10]. This suggests that genetic variation in transcription factors governing intestinal CYP3A4 expression might contribute to inter-individual differences in gut CYP3A4 expression.

There are multiple lines of evidence supporting the hypothesis that CYP3A4 intestinal expression is highly regulated by the biologically active form of vitamin D3 (1α,25-dihydroxyvitamin D3 (VD3)). Treatment of intestinal cells with VD3 increases CYP3A4 expression [11]. CYP3A4 transcription is induced by VD3 activating the vitamin D receptor (VDR) which binds to proximal elements in the CYP3A4 promoter [12] and distal ER6 and CLEM4 ER6 response element [13,14] to induce CYP3A4 transcription. *In vivo*, treatment of rats and mice with VD3 increases intestinal CYP3A protein expression and activity [7]. Likewise, humans treated with vitamin D supplementation show a decreased AUC and increased clearance of the CYP3A substrate atorvastatin [15]. Finally, it was recently shown that blood levels of tacrolimus and sirolimus showed striking seasonal variation that correlated with documented levels of ultraviolet light and serum vitamin D3 levels [16] further supporting the role of the vitamin D receptor as an important regulator of human intestinal CYP3A expression.

In the present study we tested the hypothesis that common *VDR* polymorphisms, each previously associated with VDR expression/function *in vitro* and *in vivo*, would be associated with intestinal CYP3A4 expression and activity, and further that intestinal CYP3A4 expression and midazolam disposition *in vivo* would show seasonal variation correlating with the documented seasonal availability of ultraviolet light and vitamin D levels.

2. Materials and methods

2.1. Study populations

Institutional Review Boards at the University of Washington approved the use of tissue samples from organ donors, and at the University of Indiana approved the midazolam study protocols, and at St. Jude Children's Research Hospital approved genotyping of DNA from anonymous subjects. The Health Sciences Research Ethics Board at the University of Western Ontario approved the study protocol.

2.2. Human jejunal mucosa cohort

Human jejunal mucosa cohort (n = 30) from White donors was obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA). Both intestinal and hepatic tissues (below) were obtained through the solid organ donation program operated by Life Center Northwest, following informed consent by the family for use of the tissues for research. Demographic information for the jejunal and liver donors (below) and detailed methods of CYP3A4 protein immunoquantitation and activity, as measured by midazolam hydroxylation, have been described earlier [17]. Unfortunately, it was not possible to measure VDR protein in these samples. The considerable measures taken to ensure cellular viability and preserve CYP protein and activity from tissue harvest to freezing have been extensively detailed previously [1,17]. Anecdotally, we have found no evidence of CYP3A4 protein and mRNA degradation in the frozen, banked tissue samples when measured periodically over the last 15 years.

2.3. Human liver cohort

Human liver cohort (n = 54) from White donors was obtained from the University of Washington School of Pharmacy Human Tissue Bank (Seattle, WA). All subjects' families provided written informed consent prior to tissue procurement. The considerable measures taken to ensure cellular viability and preserve CYP protein and activity from tissue harvest to freezing have been extensively detailed previously [17]. The amount of CYP3A4 protein, midazolam hydroxylase activity, and CYP3A5 genotypes have been previously described [17].

2.4. Human duodenal biopsy cohort

Human duodenal biopsy cohort from White donors consisted of 45 subjects. The mean age was 53.1 ± 14.7 years (range 18–78). A single 4-mL blood sample was collected on the day of the procedure for DNA extraction using a DNA blood midi extraction kit (Qiagen, Valencia, CA). Duodenal biopsies were obtained from healthy subjects undergoing diagnostic esophagogastro-duodenoscopy at the London Health Sciences Centre – Victoria Campus as part of their medical care, and who were invited to participate in the study. No subject gave more than a single biopsy. All subjects provided written informed consent prior to sample procurement. During the subject's scheduled endoscopic procedure, an additional pinch biopsy was collected from the duodenum at or slightly distal to the ampulla of Vater. The Pathologists reviewing the slides of these biopsies indicated they are all histologically normal.

2.5. Duodenal CYP3A4 expression

The duodenal specimen was immediately placed in RNA*later* according to the manufacturer's instructions (Qiagen, Valencia, CA) and stored at −80 °C until analysis. Tissue was homogenized and RNA extracted in Trizol (Invitrogen, Carlsbad, CA) following standard methods. The cDNA synthesis was performed with 500 ng of RNA. Quantitative RT-PCR for CYP3A4 was performed using a SYBR green assay (Applied Biosystems, Foster City, CA) with the following primers: 5′-CAGGAGGAAATTGATGCAGTTTT-3′ (forward), and 5′-GTCAAGATACTCCATCTGTAGCACAGT-3′ (reverse). All samples were compared to a standard curve of the CYP3A4 amplicon, which was sub-cloned into pCR2.1 TOPO® (Invitrogen, Carlsbad, CA) for quantitative determination of copy number.

Table 1
Methods used to genotype *VDR* polymorphisms.

SNP ID	Genotyping method	Assay detail
rs2228570 *Fok*I T>C g.30920T>C E2-T4C	RFLP	FW 5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ RV 5′-ATGGAAACACCTTGCTTCTTCTCCCTC-3′ Annealing: 58 °C Digest: *Fok*I, 37 °C for 2 h
rs1544410 *Bsm*I G>A g.63980G>A E8-G + 284A	RFLP	FW 5′-CAACCAAGACTACAAGTACCGCGTCAGTG-3′ RV 5′-AACCAGCGGGAAGAGGTCAAGGG-3′ Annealing: 60 °C Digest: *Bsm*I, 65 °C for 2 h
rs7975232 *Apa*I T>G g.64978T>G E9-T-48G	RFLP	FW 5′-CAACCAAGACTACAAGTACCGCGTCAGTG-3′ RV 5′-CACTTCGAGCACAAGGGGCGTTAGC-3′ Annealing: 65 °C Digest: *Apa*I, 37 °C for 2 h
rs731236 *Taq*I T>C g.65058T>C E9-T32C	RFLP	FW 5′-CAACCAAGACTACAAGTACCGCGTCAGTG-3′ RV 5′-CACTTCGAGCACAAGGGGCGTTAGC-3′ Annealing: 65 °C Digest: *Taq*I, 65 °C for 2 h
rs4516035 GATA-1012A>G	TaqMan assay	Assay ID C___2880805_10
rs11568820 Cdx2-3731G>A	TaqMan assay	Assay ID C___2880808_10

Genomic positions refer to *VDR* reference sequence NG_008731.

2.6. Midazolam (MDZ) clearance cohort

A total of 86 MDZ levels were available from 62 Whites (complete demographics described previously [8]). Forty-three Whites had a single measure, and nineteen Whites had repeated measures. Study design for *in vivo* midazolam phenotyping from multiple clinical trials has previously been described in detail [8]. Briefly, in each study single-dose MDZ was administrated both i.v. (intravenously) and p.o. (by mouth). In five studies, subjects were simultaneously administered a single i.v. dose (0.05 mg/kg over 30 min) of MDZ and an oral dose of ^{15}N-MDZ (3 mg) after an overnight fast. In the additional two studies, oral MDZ (4 mg) was administered 24 h after the i.v. dose. All drugs and food known to affect CYP3A activity were prohibited before and for the duration of the studies. For each subject, blood samples for MDZ concentrations were obtained over a period of 12–24 h. Weight-corrected p.o. clearance, dose adjusted p.o. area under the concentration–time curve (AUC), and bioavailability (*F*) were used as MDZ PK parameters, since dosages were different in these clinical trials and body size appears to be an important determinant of between-subject variability.

2.7. Genotyping for VDR polymorphisms

We genotyped *VDR* polymorphisms rs1544410 (intron 8 *Bsm*I-G>A), rs7975232 (intron 8 *Apa*I-T>G), rs731236 (exon 9 *Taq*I-T>C), rs2228570 (exon 2 *Fok*IT>C), rs11568820 (Cdx2-3731G>A) and rs4516035 (GATA-1012A>G). Genomic DNA was extracted from blood using the DNA blood midi kit (Qiagen, Valencia, CA) and quantified by Pico Green assay (Invitrogen, Carlsbad, CA) and was utilized to determine genotypes. Many of the genotypes were determined by direct DNA sequencing (Hartwell Center) and Taqman Allelic Discrimination Assays. Alternatively, genomic DNA was amplified using primers (St Jude Hartwell Center, Memphis, TN) and conditions listed in Table 1. The PCR products were digested with the selective restriction enzymes.

2.8. Statistical analysis

Only White subjects were used in all studies. Samples from the duodenum and jejunum were not gender stratified before genotype/CYP3A phenotype analysis because gender had no effect on intestinal CYP3A4 protein expression or activity [6]. Midazolam PK measures were not gender stratified because oral dose-adjusted AUC and bioavailability were not different between men and women [8]. *In vivo* MDZ PK data was not corrected for CYP3A5 expressor/non-expressor or *CYP3A4*1B* genotypes because it was previously determined, in these exact same samples, that there were no significant differences in MDZ PK parameters between genotypes [8]. To date, there is no good explanation for the lack of genotype–phenotype concordance for the effect of CYP3A5 expression on MDZ metabolism *in vitro* but not *in vivo* [8]. In the midazolam cohort, if a subject contributed more than one sample, the sample average value was used to avoid over-weighting of subjects with repeated sampling. The Chi-squared test for deviation from Hardy–Weinberg equilibrium was used to calculate the observed *versus* expected distribution of genotypes. Because the phenotypic markers were not normally distributed, group differences were analyzed nonparametrically using the Wilcoxon rank sum test, which is more robust against outliers, to compare binary groups (*e.g.*, GG + GT *versus* TT). The Kruskal–Wallis test was used to compare three groups of genotype for each polymorphism (*e.g.*, GG *versus* GT *versus* TT) with the phenotype.

To test for seasonal differences, the months were grouped into the six months with the highest (April through September) and the lowest (October through March) historical average daily UV-B Index in Western Ontario and Indiana regions [18,19]. The Wilcoxon rank sum test was used to test for seasonal differences in duodenal CYP3A4 mRNA expression. To evaluate seasonal effects for the midazolam cohort, if the subject had more than one observation in the same season, the median value was used, an accepted statistical approach [16]. If the subject had more than one observation, but in different seasons, all individual values were included. Thus, each subject would have one unique measurement for each phenotype, unless he/she was measured in both seasons. We then applied the Wilcoxon rank sum test to evaluate the seasonal difference. All the statistical calculations were performed using program R (version 2.8.1): A Language and Environment for Statistical Analysis (http://www.Rproject.org). Unfortunately, neither the duodenum nor midazolam cohorts were large enough to stratify by VDR genotype first in order to test for significant gene/environment (season) interactions.

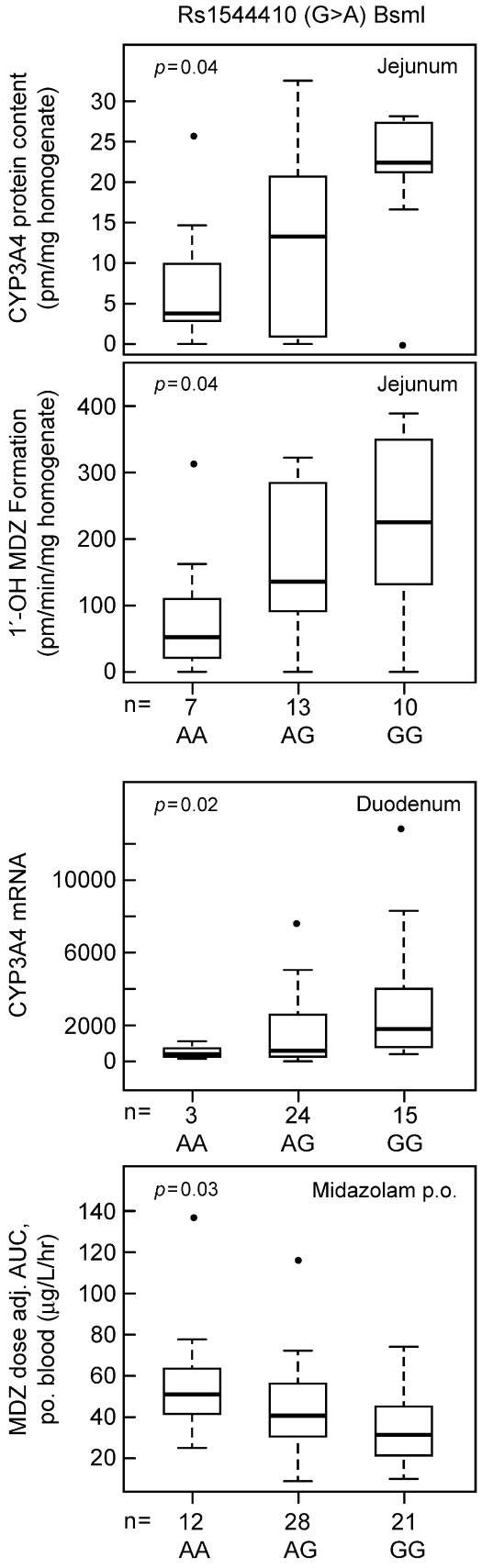

Fig. 1. The *VDR BsmI* polymorphism is associated with intestinal CYP3A4 phenotypes in White subjects. Jejunal CYP3A4 protein expression (expressed as pmoles CYP3A4 protein normalized for total protein content in a homogenate of jejunal mucosa) and midazolam1′-OH hydroxylation activity, duodenal CYP3A4 mRNA expression, and MDZ AUC association with VDR *Bsm*I G>A rs1544410 genotypes (listed at the bottom of each cohort panel). *Box plots* indicate 1st and third quartiles, bold interior *line within boxes* represent median values, *bars* represent ranges after outliers were excluded, and *circles outside of boxes* represent outliers. The *p*-value results from the Kruskal–Wallis nonparametric test is shown for comparing the significance between the three genotypes. *n* = number of subjects.

3. Results

3.1. The VDR BsmI polymorphism is significantly associated with intestinal CYP3A4 expression and activity in multiple White cohorts

We genotyped *VDR* polymorphisms rs1544410 (intron 8 *Bsm*I-G>A), and rs7975232 (intron 8 *Apa*I-T>G), and rs731236 (exon 9 *Taq*I-T>C) because these SNPs, and their associated RFLPs, have been extensively tested for their association with multiple VDR regulated traits [20]. The *VDR Bsm*I genotype was significantly associated with jejunal CYP3A4 protein levels (AA: 3.8 ± 9.1 < AG: 12.3 ± 11.5 < GG: 21.2 ± 8.0 pmol/mg homogenate; Kruskal–Wallis $p = 0.04$) (Fig. 1); and with CYP3A4 midazolam hydroxylation (AA: 67.4 ± 65.1 < GA: 154.8 ± 117.5 < GG: 230.6 ± 126.8 pmol/mg homogenate; Kruskal–Wallis $p = 0.04$) (Fig. 1). CYP3A5 is polymorphically expressed in human intestine [17]. VDR is known to induce intestinal CYP3A4 [12], but is not expected to regulate CYP3A5 since CYP3A5 lacks many of the VDR response elements, and is not an inducible member of the CYP3A family [12]. Hence, we did not test for association of VDR genotype with CYP3A5 expression. However, since CYP3A5 expression clearly affects midazolam hydroxylation *in vitro* [12], we determined the effect of VDR polymorphisms on MDZ hydroxylation in jejunum homogenates with and without CYP3A5 in our analysis. The association between CYP3A4 activity and *VDR* genotype remained significant even after removing CYP3A5 expressers ($p = 0.04$).

A similar result was seen in duodenal biopsies from White donors (Fig. 1) with CYP3A4 mRNA expression levels corresponding with *Bsm*I genotype (GG > GA > AA). Subjects with at least one *Bsm*I-A allele had significantly lower intestinal CYP3A4 mRNA levels as compared to *Bsm*I-G homozygous subjects (Fig. 1). The *Taq*I and *Apa*I polymorphisms, in partial LD with the *Bsm*I polymorphism [21], showed a less significant association with intestinal CYP3A4 expression and activity ($p = 0.05$).

Next we took advantage of unique samples in which midazolam, a drug selectively metabolized by CYP3A to its primary metabolite, 1′-hydroxymidazolam, was administered orally and intravenously. The apparent clearance of orally administered MDZ is recognized as a biomarker of intestinal CYP3A and hepatic CYP3A activities, while the intravenous MDZ reflects predominantly hepatic CYP3A. The simultaneous p.o./i.v. administration of MDZ was previously used to examine the individual contribution of intestinal CYP3A to metabolism [8].

Consistent with *Bsm*I GG subjects having *higher* intestinal levels of CYP3A (Fig. 1) and a more rapid weight adjusted oral clearance of midazolam ($p = 0.05$), the dose adjusted midazolam oral AUC was *lower* in subjects with the *VDR Bsm*I-G genotype. This inverse relationship is exactly what would be expected since higher intestinal CYP3A4 would be associated with higher CL/F, but lower bioavailability and AUC.

3.2. VDR genotypes are not associated with hepatic CYP3A4

We hypothesized that the *VDR* genotypes would be associated with intestinal and not hepatic CYP3A4 expression because VDR is not highly expressed in human hepatocytes [22]. We *VDR* genotyped human livers from 54 White donors that had previously been phenotyped for CYP3A4 protein expression and activity, and that were all CYP3A5 non-expressors [17]. There was no

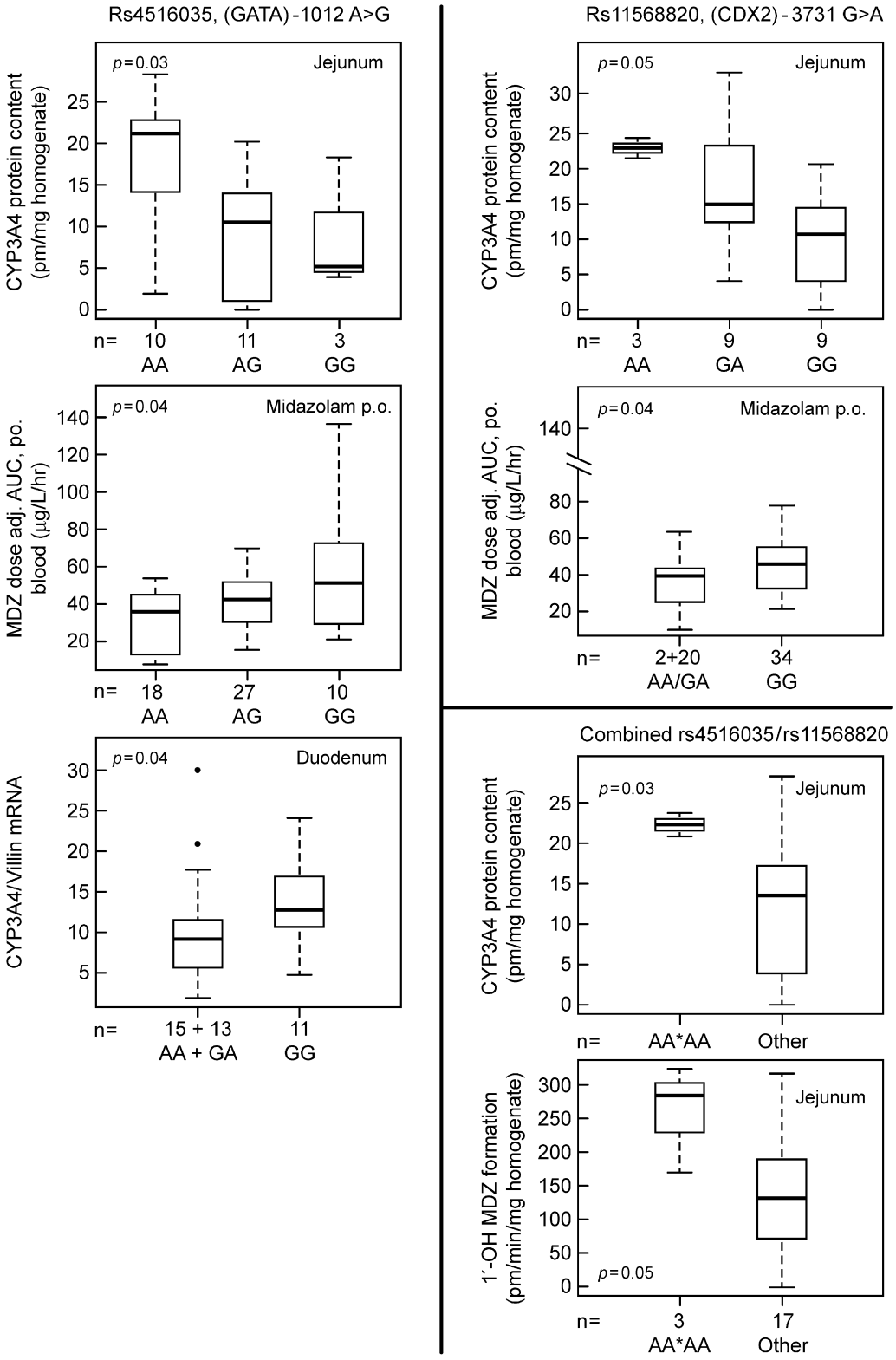

Fig. 2. *VDR* promoter polymorphisms are associated with intestinal CYP3A4 phenotypes in White subjects. Left panel: jejunal CYP3A4 protein, MDZ AUC and duodenal CYP3A4 mRNA expression associated with the VDR −1012 promoter (GATA binding site) genotypes (listed at the bottom of the each panel). Right top panel: jejunal CYP3A4 protein and MDZ AUC association with VDR −3731 promoter (CDX2 binding site) genotypes. Right bottom panel: jejunal CYP3A4 protein and 1′-OH MDZ activity associated with the combined VDR −3731/−1012 genotypes. Box plots are described in Fig. 1, the *p*-value results from the Kruskal–Wallis nonparametric test is shown when comparing the significance of three genotypes, and the *p*-value results from the Wilcoxon test is shown when comparing the significance of two genotypes. *n* = number of subjects.

association of the *VDR Bsm*I genotype with hepatic CYP3A4 protein expression or midazolam 1′-hydroxylase activity ($p = 0.51$). In addition, we tested for but found no significant association between the intravenous midazolam clearance and VDR *Bsm*I genotype ($p > 0.05$). This result is consistent with the limited expression of VDR in the liver [23] and the lack of an association between hepatic CYP3A4 content measures and VDR *Bsm*I genotype.

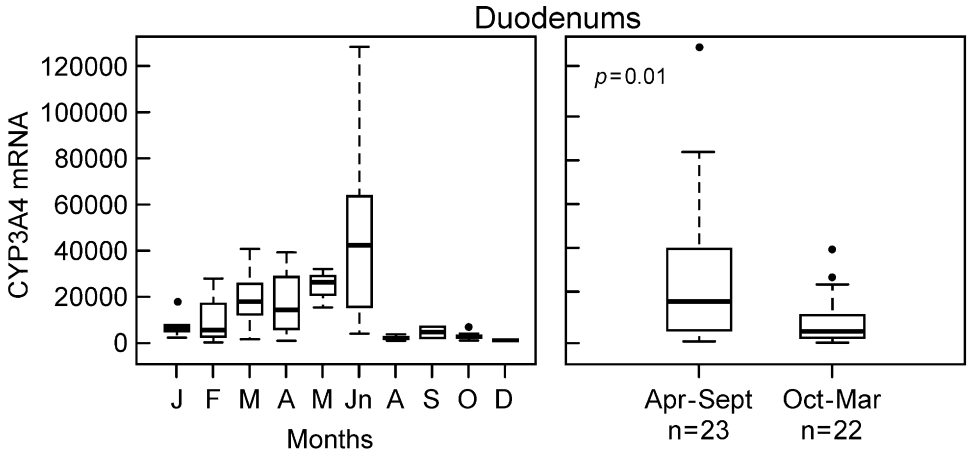

Fig. 3. Seasonal variation in CYP3A4 duodenum expression in White donors. Box plots (described in Fig. 1) indicate the CYP3A4 mRNA expression in human duodenums grouped monthly (July and November had no individuals) (left panels) and grouped seasonally from April–September *versus* October–March (right panels). The *p*-value results from the Wilcoxon test are shown for the right panel comparing CYP3A4 mRNA expression in April–September *versus* October–March.

3.3. The FokI polymorphism was not associated with any CYP3A4 traits

The common rs2228570 (exon 2 *Fok*IT>C) was genotyped because it results in a three amino acid shorter VDR protein with reported higher transcriptional activity [20]. The *Fok*I RFLP has no LD with any of the other *VDR* SNPs or LD blocks [21], and was not associated with intestinal CYP3A4 ($p > 0.05$).

3.4. VDR promoter polymorphisms are associated with intestinal CYP3A4 expression and activity in White subjects

We genotyped for *VDR* promoter polymorphisms rs11568820 (Cdx2-3731G>A) and rs4516035 (GATA-1012A>G) because these affect binding sites for Cdx2 and GATA, respectively, and *VDR* transcriptional activity [24,25]. CYP3A4 jejunal protein (Fig. 2) and activity ($p = 0.03$) were higher in subjects with the GATA-A genotype (that creates a *VDR* promoter GATA binding site) [24] and were higher in subjects with the Cdx2-A alleles (that increases Cdx2 binding to the *VDR* promoter) [25] (Fig. 2), and with the combined GATA/Cdx2 A/A genotype. Consistent with this finding, the midazolam AUC was lower in those subjects who had the GATA-A and Cdx2-A promoter genotypes (Fig. 2). Surprisingly, the CYP3A4 mRNA showed the opposite trend with higher mRNA in subjects with the GATA-G genotype (Fig. 2).

3.5. Duodenal CYP3A4 mRNA expression and midazolam in vivo PK parameters show seasonal variation in expression

Because circulating levels of vitamin D are known to be directly related to seasonal levels of UV sunlight, we compared CYP3A4 expression monthly and by seasons in the subjects sampled in London, Ontario, Canada (latitude 42.97°N). Duodenal CYP3A4 mRNA levels showed month-to-month variation (Fig. 3). Based on data published for historical UV-B levels across the upper mid-West and in Toronto [18,19], the mean CYP3A4 levels were compared between the six months when surface UV-B irradiance was historically between 55% and 100% of maximum (April through September), and the six months when the UV-B surface irradiance levels were below these values (October through March). The mean CYP3A4 levels in the duodenum were highest in the six months with the highest documented levels of UV-B (Fig. 3) and the difference was significant ($p = 0.01$).

There was a trend toward a decrease in midazolam dose adjusted AUC and oral bioavailability (Fig. 4) in the six months with the highest UV-B levels in the upper Midwest [18] (April through September) compared to the six months with the lowest UV-B levels (October through March) in the subjects analyzed in Indianapolis, IN (latitude 39.77°N), although the difference did not reach statistical significance. Unfortunately, neither the duodenum nor midazolam cohorts were large enough to stratify by VDR genotype first in order to test for significant gene/environment (season) interactions.

4. Discussion

Although it has been suggested that nearly all of the observed differences in CYP3A catalyzed activity (~90%) can be attributed to genetic variation [26], there are relatively few examples where CYP3A cis-genetic variation has been definitively linked to differences in CYP3A dependent drug clearances *in vivo* [9,10,27]. In this study we tested the hypothesis that VDR genetic variation was related to CYP3A4 intestinal expression and activity. Our investigation revealed that the same *VDR* genotypes predictive of CYP3A4 expression in human duodenum and jejunum were also associated with oral disposition of the CYP3A4 substrate midazolam. These results add to our growing understanding of the genetic determinants of intestinal CYP3A4 expression and individual differences in first pass intestinal extraction efficiency following oral administration of CYP3A4 substrates.

The *VDR Bsm*I polymorphism, in particular, showed association with CYP3A4 intestinal expression/activity (*Bsm*I-G allele = higher CYP3A4 expression). The *VDR Bsm*I RFLP, in combination with the *Apa*I, *Taq*I RFLPs, that are in partial LD in Whites [21], have been used extensively in association studies with multiple VDR regulated traits, including bone mineral density and risk of osteoporosis [20,28]. The relationship of the *Bsm*I RFLP to bone density is controversial with some groups finding increased bone density while others have found reduced bone density [28].

The Cdx2-3731A and GATA-1012A (upstream of exon 1a) promoter genotypes, particularly in combination, were associated with higher CYP3A4 intestinal expression/activity. Although *VDR* uses multiple promoters, the 1a promoter is transcriptionally the most active [29]. Both Cdx2 and GATA are intestinal transcription factors important for regional differences in expression of genes along the small intestine [30]. Cdx2 is expressed at higher levels in the distal compared with the proximal small intestine. In contrast, GATA4 is expressed as a gradient from highest in duodenum to absent in ileum [31]. The regional differences in the ratios of Cdx2/GATA4 in duodenum and jejunum may help explain the surprising

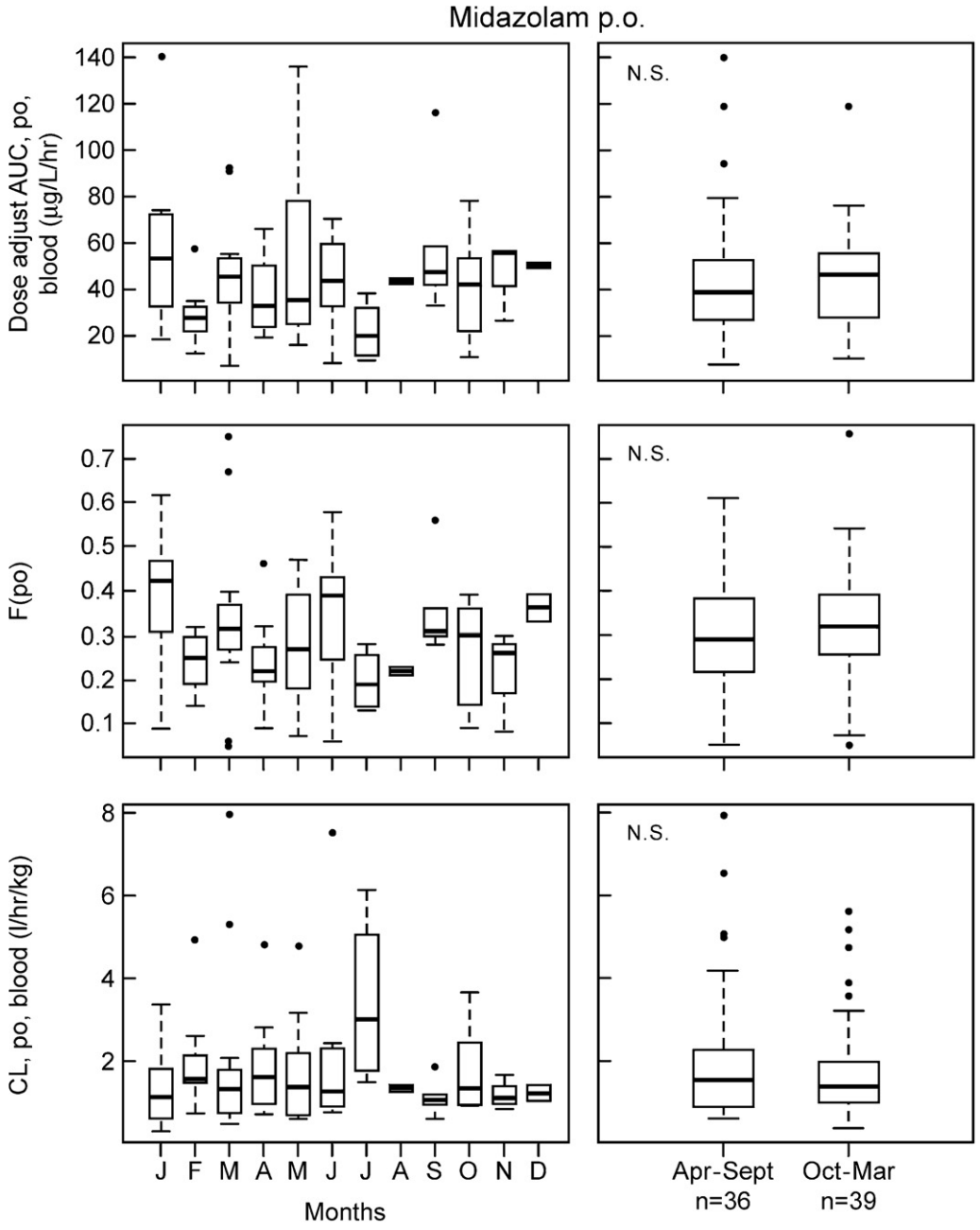

Fig. 4. Seasonal variation in oral midazolam pharmacokinetic parameters in White subjects. Box plots (described in Fig. 1) indicate the midazolam PK parameters grouped monthly (left panels) and grouped seasonally from April–September *versus* October–March (right panels). AUC is the midazolam dose adjusted area under the concentration-time curve, *F* is the midazolam bioavailability, and CL is the weight-corrected p.o. clearance of midazolam. The *p*-value results from the Wilcoxon test are shown for the right panels comparing the significance of CYP3A4 traits in April–September *versus* October–March. N.S. = no significant difference observed under the 5% significance level.

finding that the *VDR* Cdx2 promoter SNP was associated with higher jejunal, but lower duodenal, CYP3A4 expression (Fig. 2). These promoter genotypes appear directly associated with VDR expression levels because, compared with the -3731 Cdx2-A allele, the -3731Cdx2-G allele leads to a decrease in Cdx transactivation of the *VDR* exon 1a promoter and is associated with a 10% decrease in spinal bone mineral density in Japanese women [24]. Similarly, we found that CYP3A4 jejunal levels were lower in intestines with the Cdx2-G allele. The VDR -1012A GATA+ (but not -1012G GATA−) genotype binds GATA in electromobility shift assays [21]. Correspondingly, in transfected cells, *VDR* promoter-reporter constructs containing the GATA− binding site had a 50% lower transcription rate compared with the GATA+ reporter plasmid [21]. These results suggest that the higher CYP3A4 expression in intestines with the *VDR*/GATA+ allele would correspond to greater GATA4 transactivation of the *VDR* promoter and correspondingly higher VDR levels in these intestines, although this was not formally tested. Importantly, the GATA− and -3731Cdx-2-G promoter polymorphisms are combined in a risk haplotype for fracture [21], and we found that CYP3A4 expression/activity was significantly lower in jejunums with the combined GATA−/-3731G genotype (Fig. 2). The differences observed for midazolam clearance (50% higher CL/F for the *VDR Bsm*I variant and 50% lower CL/F for the GATA variant) may not have clinical significance for midazolam because the drug is generally dosed to a desired pharmacological effect (sedation). However, these differences would be clinically meaningful for a narrow therapeutic index CYP3A substrate like tacrolimus, particularly if the extent of intestinal first-pass extraction is much higher than that of midazolam.

Although there is considerable data supporting the role of VDR in regulating intestinal CYP3A4, the role for VDR in regulating hepatic CYP3A4 is more controversial and weak. Early reports found that VDR was only expressed in non-parenchymal cells and biliary epithelial cells and not in hepatocytes [22]. Some investigators have reported that VDR is expressed in isolated human hepatocytes [32,33]. However, the expression level of VDR in rat liver is very low, about 0.1% compared to that in rat intestine [23]. Even greater differences in VDR mRNA content are found in intact human liver *versus* intestine [7,34]. Moreover, the magnitude of CYP3A4 mRNA induction reported in human liver slices by VD3 was only 2-fold (compared to 10-fold by dexamethasone, a relatively weak inducer of human CYP3A4) [23]. Finally, the *VDR BsmI* polymorphism had no association with hepatic CYP3A4 expression ($p = 0.51$).

Lindh et al. [16] recently demonstrated that dose adjusted blood levels of sirolimus and tacrolimus (CYP3A substrates) in patients in Stockholm, Sweden (59.17°N) were higher in winter months and lower in summer months, directly correlating with seasonal differences in UV sunlight levels and systemic vitamin D3 levels in Finland (64°N) and Sweden, respectively. However, it remained to be determined whether intestinal (or hepatic) CYP3A4 levels were similarly related to seasonal variation in UV sunlight. In addition, because the seasonal variation in the availability of UV light is much greater at the northern latitudes, and diminishes as one moves closer to the equator, it was unclear whether the results would be the same among persons living in lower latitudes. The results from this exploratory retrospective study clearly demonstrate that documented seasonal changes in UV sunlight around London, Ontario, Canada (latitude 42.97°N) [18,19] correlated with human duodenal CYP3A4 levels. CYP3A4 intestinal expression was significantly higher in the six months with the highest historical UV-B levels compared to the six months with the lowest UV-B levels. The trend toward decreased midazolam oral bioavailability in the months with high UV-B levels around Indianapolis, IN (latitude 39.77°N) [18], and with higher intestinal CYP3A4, demonstrates that it is variation in intestinal CYP3A4, and not seasonal variation in CYP3A4 systemic/hepatic clearance, that is driving the apparent seasonal differences in midazolam oral bioavailability.

We acknowledge that a large prospective clinical study to simultaneously measure serum 25(OH)D blood levels and CYP3A4 pharmacokinetics is the gold standard. However, the availability of these unique retrospective samples from three separate human clinical studies of extensively CYP3A phenotyped samples allowed us to move immediately and rapidly forward in testing our hypothesis. We think this is a distinct advantage since, realistically, a prospective clinical trial would require 2+ years to run and cost hundreds of thousands of dollars. The significant association of intestinal CYP3A4 expression with seasonal variation in UV sunlight in the upper Midwest [18,19] is notable since none of these studies was designed to test for this relationship and hence did not control for all of the potential confounders (*e.g.*, vitamin D supplements or travel to areas with higher UV sunlight) that would affect average UV exposure/vitamin D levels.

In summary, our results demonstrate that genetic variation in *VDR*, a direct transcriptional activator of intestinal CYP3A4, is associated with CYP3A4 duodenal and jejunal expression. Moreover, CYP3A4 intestinal expression varied seasonally, correlating with the documented levels of UV sunlight and reported seasonal levels of vitamin D. Hence, variation in VDR and its ligand appear to be primary determinants of individual variation in intestinal CYP3A4 expression. These results suggest that *VDR* genotypes may help explain individual differences in first pass intestinal extraction efficiency following oral administration of drugs that are substrates of CYP3A4, and perhaps of other intestinal VDR regulated drug detoxification genes including P-glycoprotein and CYP2C9 [35,36]. Our results support further testing for how seasonal, regional, racial and ethnic differences in vitamin D levels correlate with CYP3A4 mediated drug clearance. Comprehensive testing for *VDR* genotype/UV sunlight-vitamin D levels will require inclusion of larger populations but also more comprehensive, standardized, and precise approaches to capturing environmental information.

Conflict of interest

The authors have no conflict of interest to disclose.

Acknowledgments

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References

[1] Paine MF, Khalighi M, Fisher JM, Shen DD, Kunze KL, Marsh CL, et al. Characterization of interintestinal and intraintestinal variations in human CYP3A-dependent metabolism. J Pharmacol Exp Ther 1997;283:1552–62.
[2] Watkins PB, Wrighton SA, Schuetz EG, Molowa DT, Guzelian PS. Identification of glucocorticoid-inducible cytochromes P-450 in the intestinal mucosa of rats and man. J Clin Invest 1987;80:1029–36.
[3] Gorski JC, Jones DR, Haehner-Daniels BD, Hamman MA, O'Mara Jr EM, Hall SD. The contribution of intestinal and hepatic CYP3A to the interaction between midazolam and clarithromycin. Clin Pharmacol Ther 1998;64:133–43.
[4] Thummel KE, O'Shea D, Paine MF, Shen DD, Kunze KL, Perkins JD, et al. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. Clin Pharmacol Ther 1996;59:491–502.
[5] Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA, et al. Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test. Drug Metab Dispos 1994;22:947–55.
[6] Paine MF, Ludington SS, Chen ML, Stewart PW, Huang SM, Watkins PB. Do men and women differ in proximal small intestinal CYP3A or P-glycoprotein expression. Drug Metab Dispos 2005;33:426–33.
[7] Xu Y, Iwanaga K, Zhou C, Cheesman MJ, Farin F, Thummel KE. Selective induction of intestinal CYP3A23 by 1alpha,25-dihydroxyvitamin D3 in rats. Biochem Pharmacol 2006;72:385–92.
[8] Miao J, Jin Y, Marunde RL, Kim S, Quinney S, Radovich M, et al. Association of genotypes of the CYP3A cluster with midazolam disposition in vivo. Pharmacogenomics J 2009;9:319–26.
[9] Lamba J, Lamba V, Strom S, Venkataramanan R, Schuetz E. Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. Drug Metab Dispos 2008;36:169–81.
[10] Lamba V, Panetta JC, Strom S, Schuetz EG. Genetic predictors of interindividual variability in hepatic CYP3A4 expression. J Pharmacol Exp Ther 2010;332:1088–99.
[11] Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS, Watkins PB. Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alpha,25-dihydroxyvitamin D3. Mol Pharmacol 1997;51:741–54.
[12] Thummel KE, Brimer C, Yasuda K, Thottassery J, Senn T, Lin Y, et al. Transcriptional control of intestinal cytochrome P-4503A by 1alpha,25-dihydroxy vitamin D3. Mol Pharmacol 2001;60:1399–406.
[13] Pavek P, Pospechova K, Svecova L, Syrova Z, Stejskalova L, Blazkova J, et al. Intestinal cell-specific vitamin D receptor (VDR)-mediated transcriptional regulation of CYP3A4 gene. Biochem Pharmacol 2010;79:277–87.
[14] Thompson PD, Jurutka PW, Whitfield GK, Myskowski SM, Eichhorst KR, Dominguez CE, et al. Liganded VDR induces CYP3A4 in small intestinal and colon cancer cells via DR3 and ER6 vitamin D responsive elements. Biochem Biophys Res Commun 2002;299:730–8.

[15] Schwartz JB. Effects of vitamin D supplementation in atorvastatin-treated patients: a new drug interaction with an unexpected consequence. Clin Pharmacol Ther 2009;85:198–203.
[16] Lindh JD, Andersson ML, Eliasson E, Bjorkhem-Bergman L. Seasonal variation in blood drug concentrations and a potential relationship to vitamin D. Drug Metab Dispos 2011;39:933–7.
[17] Lin YS, Dowling AL, Quigley SD, Farin FM, Zhang J, Lamba J, et al. Co-regulation of CYP3A4 and CYP3A5 and contribution to hepatic and intestinal midazolam metabolism. Mol Pharmacol 2002;62:162–72.
[18] Hicke JA, Slusser J, Lantz K, Pascual FG. Trends and interannual variability in surface UVB radiation over 8 to 11 years observed across the United States. J Geophys Res D Atmos 2008;113:1–11.
[19] Tarasick DW, Fioletov VE, Wardle DI, Kerr JB, McArthur LJB, McLinden CA. Climatology and trends of surface UV radiation. Atmos Ocean 2003;41:121–38.
[20] Uitterlinden AG, Fang Y, Van Meurs JB, Pols HA, Van Leeuwen JP. Genetics and biology of vitamin D receptor polymorphisms. Gene 2004;338:143–56.
[21] Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, et al. Promoter and 3′-untranslated-region haplotypes in the vitamin D receptor gene predispose to osteoporotic fracture: the rotterdam study. Am J Hum Genet 2005;77:807–23.
[22] Gascon-Barre M, Demers C, Mirshahi A, Neron S, Zalzal S, Nanci A. The normal liver harbors the vitamin D nuclear receptor in nonparenchymal and biliary epithelial cells. Hepatology 2003;37:1034–42.
[23] Khan AA, Chow EC, van Loenen-Weemaes AM, Porte RJ, Pang KS, Groothuis GM. Comparison of effects of VDR versus PXR, FXR and GR ligands on the regulation of CYP3A isozymes in rat and human intestine and liver. Eur J Pharm Sci 2009;37:115–25.
[24] Yamamoto H, Miyamoto K, Li B, Taketani Y, Kitano M, Inoue Y, et al. The caudal-related homeodomain protein Cdx-2 regulates vitamin D receptor gene expression in the small intestine. J Bone Miner Res 1999;14:240–7.
[25] Halsall JA, Osborne JE, Potter L, Pringle JH, Hutchinson PE. A novel polymorphism in the 1A promoter region of the vitamin D receptor is associated with altered susceptibilty and prognosis in malignant melanoma. Br J Cancer 2004;91:765–70.
[26] Ozdemir V, Kalow W, Tang BK, Paterson AD, Walker SE, Endrenyi L, et al. Evaluation of the genetic component of variability in CYP3A4 activity: a repeated drug administration method. Pharmacogenetics 2000;10:373–88.
[27] Lamba JK, Lin YS, Schuetz EG, Thummel KE. Genetic contribution to variable human CYP3A-mediated metabolism. Adv Drug Deliv Rev 2002;54:1271–94.
[28] Whitfield GK, Remus LS, Jurutka PW, Zitzer H, Oza AK, Dang HT, et al. Functionally relevant polymorphisms in the human nuclear vitamin D receptor gene. Mol Cell Endocrinol 2001;177:145–59.
[29] Crofts LA, Hancock MS, Morrison NA, Eisman JA. Multiple promoters direct the tissue-specific expression of novel N-terminal variant human vitamin D receptor gene transcripts. Proc Natl Acad Sci USA 1998;95:10529–34.
[30] Gao N, White P, Kaestner KH. Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. Dev Cell 2009;16:588–99.
[31] Battle MA, Bondow BJ, Iverson MA, Adams SJ, Jandacek RJ, Tso P, et al. GATA4 is essential for jejunal function in mice. Gastroenterology 2008;135:1676–86 [e1].
[32] Drocourt L, Ourlin JC, Pascussi JM, Maurel P, Vilarem MJ. Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. J Biol Chem 2002;277:25125–32.
[33] Han S, Li T, Ellis E, Strom S, Chiang JY. A novel bile acid-activated vitamin D receptor signaling in human hepatocytes. Mol Endocrinol 2010;24:1151–64.
[34] Zhou C, Assem M, Tay JC, Watkins PB, Blumberg B, Schuetz EG, et al. Steroid and xenobiotic receptor and vitamin D receptor crosstalk mediates CYP24 expression and drug-induced osteomalacia. J Clin Invest 2006;116:1703–12.
[35] Chen Y, Goldstein JA. The transcriptional regulation of the human CYP2C genes. Curr Drug Metab 2009;10:567–78.
[36] Saeki M, Kurose K, Tohkin M, Hasegawa R. Identification of the functional vitamin D response elements in the human MDR1 gene. Biochem Pharmacol 2008;76:531–42.