Endogenous Biomarkers of CYP3A Activity

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Abstract

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The activity of the drug-metabolizing enzyme cytochromes P450 3A (CYP3A) varies up to 20-fold between individuals. The interindividual variability may be due to both genetic variations and factors such as age, diet, concurrent use of multiple medications, disease, pregnancy, and environmental constituents. Phenotyping using endogenous CYP3A biomarkers would allow for direct individual assessments of CYP3A activity. Our goal was to explore the utility of endogenous biomarkers (metabolites of cortisol, cholesterol and vitamin D_3) in predicting CYP3A activity as determined by oral midazolam clearance. We found moderate correlations between midazolam oral clearance and plasma 4β-hydroxycholesterol/cholesterol, urinary 6β-hydroxycortisol/cortisol ratios, and urinary 6β-hydroxycortisone/cortisone ratios. We found a poor correlation between midazolam oral clearance and plasma 4β,25-dihydroxyvitamin D_3/25-hydroxyvitamin D_3 ratios. Although these markers were sensitive to CYP3A induction, they were poorly predictive of the fold-change of midazolam oral clearance following rifampin treatment. A better endogenous marker for CYP3A activity is needed.

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Chapter 1

Introduction

1.1 Background and Importance of CYP3A

Drug metabolizing enzymes are the major determinant of non-renal drug clearance in humans. Cytochrome P450 enzymes (P450) play an important role the in the elimination of drugs. These enzymes convert a wide range of drugs into metabolites by oxidation, peroxidation, and reduction changes to the substrate. (1) In humans, the P450 superfamily of enzymes contains 57 different genes and 58 pseudogenes. Enzymes are further organized into 18 families that share at least 40% amino acid identity, and 44 subfamilies that share 55% amino acid identity. (2)

1.2 Cytochrome P450 3A (CYP3A)

CYP3A is the most abundant and possibly the most important subfamily of human cytochrome P450 enzymes, accounting for 25% of the total P450 content in the liver, and 50 to 70% of total P450 content in the human small intestine. (3, 4) The CYP3A subfamily is comprised of 4 isoforms: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 is the dominant CYP3A enzyme expressed in the liver and small intestine of adults. In addition to similar tissue expression as CYP3A4, CYP3A5 can be expressed in the kidney, but expression levels at that site and all others including the liver, vary due to genetic polymorphisms. CYP3A7 is expressed in fetal livers but is down-regulated after birth. (5) CYP3A43 has very low activity and little is known about its expression and substrate profile. (6, 7)

1.3 CYP3A Substrates

The CYP3A subfamily of enzymes is responsible for the metabolism of a large number of structurally diverse endogenous and exogenous substrates. (8) In fact, it is estimated that 40 to 50% of currently used drugs are substrates for CYP3A. (9) Substrates of CYP3A include:

cyclosporine, erythromycin, cortisol, testosterone, quinidine, midazolam, and many others. (10) There is some substrate overlap between CYP3A4 and CYP3A5, but CYP3A5 generally has fewer known substrates of significance than CYP3A4. (11, 12)

1.4 CYP3A Variability

Interindividual variability of CYP3A has been studied extensively. Levels of CYP3A enzyme vary as much as 40-fold among liver and intestinal tissues. (13-15) For instance, CYP3A4 content as determined by Western blot varied by 37-fold in liver biopsy tissue obtained from subjects following organ transplantation. (16) In addition, Lown et al. found 6-fold variation in CYP3A catalytic activity and 11-fold variation in CYP3A4 protein content in small intestine samples. (17) It has also been reported that between 10 and 97% of human livers express the CYP3A5 isoform, with levels varying from negligible to higher than that of CYP3A4. (18-22) Because CYP3A isoforms are located in both the small intestine and liver, interindividual variability in systemic drug exposure is particularly apparent after oral administration.

Overall, a 5- to 20-fold variation in CYP3A-mediated drug clearance is observed in vivo. (23) The coefficient of variation (CV) for the oral AUC of midazolam is 80%, (24) whereas the CV for buspirone, a drug with very low oral bioavailability, is 112%. (25) Alprazolam, a drug with high oral bioavailability, has an AUC CV of 11%. (26) The literature suggests that there is substantial variability in CYP3A-substrate exposure between patients, particularly for substrates that undergo significant first-pass CYP3A-mediated metabolism.

1.5 Induction of CYP3A

CYP3A activity can be modulated by exposure to other drugs that act as CYP3A inhibitors or inducers. CYP3A intrinsic clearance can be increased by enzyme induction via the pregnane X receptor (PXR) and to a lesser extent via the constitutive androstane receptor (CAR). Both contain a ligand-binding domain and a DNA-binding domain. When a PXR ligand binds to the ligand-binding domain of PXR, retinoid X receptor (RXR) will heterodimerize with PXR, and the complex will bind to DNA response elements upstream of the CYP3A transcription start site, activating transcription. The signal transduction by CAR is more complex and will not be discussed further because of its secondary role. According to X-ray crystallography analysis, the ligand-binding pocket of PXR is much larger than other nuclear receptors, which theoretically enables it to bind a wide variety of ligands. (27) Many ligands of PXR have been identified since its discovery, including glucocorticoids, antiglucocorticoids, antibiotics, antifungals, pesticides, and herbal extracts. (28, 29) Dexamethasone and rifampicin are the gold standards used to induce CYP3A in vitro and in vivo. (30-33) These drugs can have profound effects on the pharmacokinetics of ‘sensitive’ substrates. For example, rifampin induction of CYP3A in the liver and small intestine has been shown to decrease the plasma-concentration time curve of midazolam by 96%, and the elimination half-life by 58% following oral administration of 600 mg of rifampin daily for 5 days. (34)

1.6 Sulforaphane

Sulforaphane, a biologically active chemical found in broccoli, is a PXR antagonist. It was shown that sulforaphane downregulates CYP3A4 transcription and reduces mRNA content in human hepatocytes. (35, 36) Further studies found that the mechanism by which sulforaphane downregulates CYP3A is by inhibiting ligand binding to PXR. (37) This suggests that sulforaphane may be capable of preventing PXR induction in vivo. A recent study tested whether sulforaphane was able to block induction of CYP3A by hindering PXR activation. The investigators found that there was no significant difference in CYP3A induction with rifampin when compared to CYP3A induction with rifampin in the presence of sulforaphane and attributed this to a failure to achieve inhibitory concentrations within the enterocytes and hepatocytes where induction occurs. (38)

1.7 Age, Gender, and Disease

CYP3A activity can be altered by many environmental and intrinsic factors. For example, in the elderly population, P450 expression is decreased compared to younger adults. (39) (40) Greenblatt et al. determined that mean intravenous midazolam clearance was 7.8 mL/min/kg in healthy volunteers aged 24 to 37 years compared to an average of 4.4 mL/min/kg in those aged 60 to 79 years ($p < 0.01$). (41) Similarly, Albrecht et al. determined that mean intravenous midazolam clearance was 5.74 mL/min/kg in patients aged 20 to 34 years, compared to 3.88 mL/min/kg in patients aged 67 to 81 years ($p < 0.01$). (42) These data indicate that elderly individuals have lower CYP3A expression and/or activity compared to younger individuals.

It is also important to consider CYP3A expression in neonates and children. Neonates have significantly lower midazolam clearance (1.83 to 2.17 mL/min/kg) when compared to that of adults (6.33 to 11 mL/min/kg). (43) Preterm infants had even lower rates of midazolam systemic clearance (1.2 to 1.6 mL/min/kg). (43) Because midazolam is poor substrate for CYP3A7, the decreased clearance in neonates and preterm infants is attributed to lower CYP3A4 expression during the perinatal period. Hughes et al. found lower midazolam systemic clearances in children until 2 years of age (2.33 to 3 mL/min/kg). However, from ages 3 to 13, systemic midazolam clearances were significantly higher (13 mL/min/kg) than adults (6.33 to 11 mL/min/kg). (44)

The influence of sex on CYP3A-mediated metabolism is an important issue especially as CYP3A is not only critical for xenobiotic metabolism, but also for the metabolism of endogenous compounds. CYP3A plays a major role in the metabolism of sex steroids such as estradiol, progesterone, and testosterone. (45, 46) Many studies have reported no differences between men and women in regards to CYP3A clearance when using intravenous midazolam clearance as a probe. (41, 47, 48) In contrast, a few studies did show sex-dependent differences. Moreover, intramuscular midazolam was cleared at a higher rate in elderly women compared to elderly men. (47) Although Gorski et al. (49) found that the mean apparent oral midazolam clearance was 1.9 and 1.0 L/hr/kg among female and male subjects, respectively ($p < 0.05$), the systemic midazolam clearances in the same subjects were not significantly different between men and women. (49, 50) However, when clarithromycin, a CYP3A inhibitor, was co-administered with midazolam, female subjects exhibited a significantly greater extent of interaction after oral and intravenous administration compared to male subjects. (49)

CYP3A activity can be reduced in certain disease states. McConn et al. saw significantly lower duodenal CYP3A expression in patients with liver cirrhosis compared to healthy patients. Patients with the most severe level of liver disease, in accordance with the Child-Pugh ratings system, express 2.8-fold lower duodenal CYP3A content compared to control subjects. (51) CYP3A protein expression in the small intestine was also shown to be decreased in patients with celiac disease. (52) With regard to the liver, patients with non-alchoholic fatty liver disease also have decreased hepatic mRNA expression and CYP3A protein. (53) Liver disease has been shown to downregulate hepatic CYP3A activity and result in decreased CYP3A-dependent ironotecan oxidation. (54) Liver CYP3A has also been shown to decrease during end-stage renal disease in murine models. (55)

All of these factors, and many more that may remain unstudied, can impact CYP3A activity. For this reason, phenotyping would be a more accurate measure of enzyme activity because it would take all these modulating factors into account.

1.8 CYP3A Probes

Probe substrates are selected based on characteristics that allow determination of specific in vitro or in vivo activity. Probe drugs should be non-toxic substances so that they can be administered in vivo (i.e., an easily available substance that is registered as a therapeutic drug). Ideally, probes of any enzyme should be metabolized solely by the enzyme in question. (56) Probe drugs should be not only able to detect induction and inhibition of an enzyme, but the magnitude of change in enzyme content in the body and probe clearance. (57)

1.8.1 Midazolam as a CYP3A Probe

Midazolam is a short-acting benzodiazepine drug that is considered the gold standard probe drug for CYP3A function. Midazolam can be either administered orally or intravenously and is used therapeutically in this country to provide sedation prior to potentially uncomfortable medical and dental procedures. It is extensively metabolized with less that 0.3% excreted unchanged in the urine. (58) Evidence for the use of midazolam as a CYP3A probe was provided by Thummel et al. who studied ten patients undergoing liver transplant surgery. (59) Patients were given an intravenous dose of midazolam post-surgery. The in vitro midazolam clearance determined from liver biopsies and in vivo midazolam clearance were strongly correlated ($r = 0.93$, $p < 0.001$) as was the formation of the major metabolite, 1'OH-midazolam, and hepatic CYP3A protein ($r = 0.84$, $p < 0.01$). (59) They found that the mean hepatic enzyme content was 4.7-fold higher in inducer-treated donors compared to non-exposed donors. (59) The association between liver CYP3A expression and systemic midazolam clearance offer supporting evidence that midazolam is a useful probe for in vivo CYP3A activity.

1.8.2 Erythromycin as a CYP3A Probe

Another well established probe drug is erythromycin, a macrolide antibiotic that is metabolized by CYP3A. CYP3A demethylates erythromycin, and the methyl group is converted to and subsequently exhaled through the lungs as carbon dioxide. In vivo, subjects are given a trace dose of ^{14}C-labeled erythromycin and CYP3A activity is estimated by the amount of radiolabeled carbon dioxide exhaled into a balloon. A study conducted in rats analyzed the percentage of labeled carbon dioxide exhaled over the first 135 minutes following IV erythromycin administration. Subsequently, the immunoreactive CYP3A and holochrome CYP3A expression were measured and a strong correlation was found between enzyme expression and radiolabeled carbon dioxide ($r = 0.71$ and 0.74, respectively). (60) In a human study, the exhaled carbon dioxide was collected from subjects for one hour following IV administration of erythromycin. (61) Subjects were also given midazolam and the correlation between unbound clearance of midazolam and percent radiolabeled carbon dioxide exhaled was $r = 0.6$ in all subjects, and 0.71 amongst the males in the study. (60, 61)

1.8.3 Drawbacks to Probe Administration

The unfortunate drawback to probe substrate administration is that certain populations may not want or be able to participate for a number of reasons. Young children, pregnant women, and the elderly may be unwilling to take a drug or radiolabeled probe for phenotyping purposes. Besides those limitations, there are other factors that complicate the predictability of CYP3A activity using erythromycin. Erythromycin is a substrate of uptake transporter organic anion transporter 1B1 (OATP1B1) and efflux transporter P-glycoprotein (P-gp), both of which vary in activity. (62) Therefore, changes in the percent of radiolabeled carbon dioxide exhaled following erythromycin administration could be attributed to factors other than changes in metabolism. For example, Frassetto et al. conducted erythromycin breath tests under three conditions: baseline, with rifampin and with lansoprazole concomitant treatment. Rifampin and lansoprazole were used to inhibit OATP1B1 and P-glycoprotein, respectively. Compared with baseline, inhibition of P-glycoprotein increased ^{14}C excretion by 0.257 met/h ($p < 0.07$), whereas inhibition of OATP1B1 decreased ^{14}C excretion by 0.44 met/h ($p < 0.001$). They concluded that relying solely on erythromycin breath test results without taking transporter activity into account could lead to errors in estimating CYP3A activity. (62) In contrast, although midazolam appears to be a better predictor of enzyme activity, phenotyping with midazolam is more time-consuming, labor-intensive and invasive. (63) Midazolam clearance can only be determined in a pharmacokinetic study where multiple plasma samples are drawn or estimated using a single time point analysis, which requires at least one blood draw.

1.9 Endogenous Markers of CYP3A

Even though phenotyping with probe substrates do take intrinsic and extrinsic factors into account, they are still not an ideal measure of enzyme activity because of their invasiveness and are problematic for certain populations. The usefulness and importance of acquiring enzyme activity information prior to drug administration prompts the development of alternative methods. The major initiative that is currently being studied is the use of endogenous CYP3A substrate probes. The remainder of this introduction will be devoted to a summary of the use of metabolites of cortisol, cholesterol and vitamin D_3 as potential endogenous CYP3A biomarkers. Listed in Tables 1.1 and 1.2 are in vitro and in vivo studies that compare midazolam clearance with endogenous biomarker measurements, and changes to biomarker measurements following treatment with CYP3A inhibitors or inducers, respectively.

1.9.1 Cortisol as an Endogenous Marker of CYP3A

Cortisol is a steroid hormone produced by the adrenal glands and released into blood in response to stress and/or low levels of glucocorticoids. Its function is to increase blood sugar levels by aiding in gluconeogenesis and fat metabolism. Cortisol is a hormone regulated by the hypothalamus. Healthy individuals without hypothalamic dysfunction have very low nocturnal levels of cortisol that build up and peak in the morning. Levels then decline slowly throughout the day. During the morning peak, cortisol concentrations range typically from 5 to 25 ug/dL. At midnight, cortisol concentrations range from 2.9 to 12 ug/dL. (64, 65)

Cortisol is converted to 6β-hydroxycortisol (6βOHF) by CYP3A. Although this is a minor pathway of total cortisol metabolism, previous investigators have shown it to be a sensitive marker of CYP3A activity, particularly following enzyme induction. (66) The diurnal variation in cortisol formation can influence metabolite formation, and as such could make 6βOHF an unreliable biomarker for determining CYP3A activity. Many investigators have corrected for this circadian variability by using the urinary 6β-hydroxycortisol/cortisol ratio (6βOHF/F) as a biomarker instead of 6βOHF alone.

In a study by Ged et al., urinary 6βOHC was measured in 14 patients before and after rifampicin treatment. In the same patients, liver biopsies were taken and CYP3A protein and activity were determined. They found that the urinary 6βOHF/F ratio was correlated with hepatic CYP3A expression ($r = 0.83$) and rifampicin treatment significantly increased 6βOHF/F ratios. (66)

Cortisol and 6βOHF/F ratios have been used to predict CYP3A activity in a number of pharmacokinetic studies. (67, 68) However, others have shown that 6βOHF or 6βOHF/F are not ideal CYP3A biomarkers. For example, Chen et al. compared 6βOHF/F ratios with midazolam systemic clearance before and after treatment with oral doses of fluvoxamine, a CYP3A inhibitor. (69, 70) They found that at baseline, midazolam clearance was poorly correlated with 6βOHF/F ratios ($r = 0.04$) and the correlation was still weak following CYP3A inhibition ($r = 0.39$). They also found no correlation between the change in 6βOHF/F ratio and the change in midazolam plasma clearance. This suggests that urinary 6βOHF and/or 6βOHF/F ratios are not predictive of CYP3A activity or inhibition of CYP3A activity. (69) Other studies have shown that the urinary 6βOHF/F ratio does not predict clearance of the prototypical CYP3A markers, erythromycin and midazolam. (71-73) or CYP3A activity following inhibition with ritonavir or amprinavir (74-76)

There are a couple of different theories regarding the reason for the lack of correlation between urinary 6βOHF/F ratio and CYP3A activity. It was noted that the urinary 6βOHF/F ratio depends on the urinary clearance of cortisol in addition to the formation clearance of 6βOHF, potentially confounding interpretation of 6βOHF/F ratio results. (77) Another complicating factor is that in vivo, cortisol and 6β-hydroxycortisol can be interconverted to cortisone and 6β-hydroxycortisone by renal 11β-hydroxysteroid dehydrogenase. However, CYP3A has been found to be the major contributor to 6β-hydroxycortisol formation from cortisol, and 6β-hydroxycortisone formation from cortisone. Thus, one possible way to use cortisol as a CYP3A biomarker, while accounting for this interconversion, would be to use the sum of 6β-hydroxycortisol and 6β-hydroxycortisone formation clearances. When subjects were treated with itraconazole, a CYP3A inhibitor, the observed itraconazole dose-dependent change in the sum of these formation clearances was consistent with expected decrease in enzyme activity. (78, 79)

1.9.2 4β-hydroxycholesterol as a Marker for CYP3A Activity

Cholesterol is a sterol produced in the liver. It is an essential structural component of mammalian cell membranes and is required to establish proper membrane fluidity. It is also an important component in the synthesis of bile acids, steroid hormones, and vitamin D. Cholesterol is metabolized by various enzymes to form different oxysterols. The most prominent oxysterols found circulating in human blood are 27-hydroxycholesterol, 24-hydroxycholesterol, and 7α-hydroxycholesterol, formed by CYP27, CYP46, and CYP7A, respectively. (80) Recently, two other oxysterols, 4α-hydroxycholesterol and 4β-hydroxycholesterol (4βOHC), were identified and plasma concentrations ranged from 15 to 500 ng/mL. (81) In most individuals, the circulating concentrations of 4α-hydroxycholesterol and 4β-hydroxycholesterol are approximately 1:1. (82) Of note though, 4α-hydroxycholesterol is formed by cholesterol auto-oxidation. In contrast, recombinant CYP3A4 was shown to metabolize cholesterol to 4βOHC, and strong CYP3A inducers including antiepileptic drugs, were shown to greatly increase 4βOHC circulating concentrations in blood. (83) Also, with increasing doses of rifampin from 20 to 500 mg, 4βOHC concentrations increased from 46 to 141 ng/mL, respectively. (84) Treatment with ursodeoxycholic acid, a weak inducer of CYP3A, has been shown to correlate with slight, but significant, increases in 4βOHC circulating concentrations. (85) Likewise, in a study where patients were treated with itraconazole, a CYP3A inhibitor, circulating 4βOHC concentrations decreased significantly. (86) The increase in 4βOHC after CYP3A induction was found to continue for several weeks following completion of enzyme induction. (87) After termination of rifampicin treatment, plasma 4βOHC concentrations decreased with a half-life of 17 days, with little variation among subjects. This long half-life results in stable plasma concentrations over time, which may indicate that 4βOHC is not an ideal marker for short-term changes in enzyme activity. (88)

Despite this potential limitation, tracking changes in 4βOHC concentrations has found some research utility. For example, Diczfalusy et al. determined the association of 4βOHC concentrations with CYP3A-mediated quinine hydroxylation and CYP3A5 genotype in Koreans, Swedes, and Tanzanian subjects. They found that in all three populations, there was significantly higher 4βOHC concentrations in subjects carrying at least one CYP3A5 wild-type allele compared to non-carriers. This indicates that CYP3A5 may be an important contributor to the circulating concentrations of 4βOHC. They also found a negative correlation between the quinine metabolic ratio and 4βOHC concentrations in CYP3A5 non-carriers in Tanzanians and Koreans. (89)

It has also been suggested that plasma 4β-hydroxycholesterol/cholesterol (4βOHC/C) ratios can be used as a more accurate measure of CYP3A activity. Tomalik-Scharte et al. compared midazolam systemic and oral clearances with 4βOHC and 4βOHC/C in 52 healthy subjects and 27 human immunodeficiency virus-infected subjects. A slightly better correlation was found between the 4βOHC/C ratio and midazolam systemic clearance, compared to the correlation with 4βOHC, and even stronger correlations were observed with oral midazolam clearance data. (90)

1.9.3 25-hydroxycholesterol as a Marker for CYP3A Activity

Other studies have explored the use of 25-hydroxycholesterol (25OHC) as a marker for CYP3A activity. Circulating concentrations of 25OHC range from 0 to 15 ng/mL. In vitro, cholesterol is converted to 25OHC by CYP27A1 and CYP46A1, and by autoxidation. (81) The origin of 25OHC in circulation may be due to a specific dioxygenase that has recently been characterized and discovered to have broad tissue distribution. (91) Additionally, cells expressing recombinant CYP3A4 had significant 25-hydroxylase activity toward cholesterol. In mouse liver cells, induction of CYP3A by pregnenolone-16α-carbonitrile was associated with 25OHC accumulation whereas treatment with troleandomycin, a CYP3A inhibitor, reduced 25-hydroxylase activity. Finally, 25OHC levels were well correlated with 4BOHC levels. Altogether, these data suggest that CYP3A may be involved in the 25-hydroxylation of cholesterol. (92)

1.9.4 4 β,25-dihydroxyvitamin D_3 as a Marker for CYP3A Activity

Vitamin D_3 is the major source of vitamin D in humans and is responsible for maintaining calcium and phosphate homeostasis. (93) $25OHD_3$ is the most abundant circulating form of Vitamin D_3 , found typically at concentrations ranging from 20 to 50 ng/mL in plasma. (93) $25OHD_3$ is metabolized to the biologically active form, 1α,25-hydroxyvitamin D_3 by mitochondrial CYP27B1. (94, 95) It has recently been reported that $25OHD_3$ is also hydroxylated at the 4-position, resulting in two diastereomers, 4α,25-hydroxyvitamin D_3 ($4\alpha,25OHD_3$) and 4β,25-hydroxyvitamin D_3 ($4\beta,25OHD_3$) . This metabolic pathway was mediated exclusively by CYP3A. In addition, Wang et al. found that in human liver microsomes both $4\alpha,25OHD_3$ and $4\beta,25OHD_3$ formation were correlated with CYP3A content and activity as determined by midazolam hydroxylation and reduced by selective CYP3A inhibitors. There was also a significant increase in plasma $4\beta,25OHD_3$ levels after rifampin treatment. Together, the data indicate that $4\beta,25OHD_3$ is sensitive to changes in CYP3A activity. (96)

1.10 Research Directions

In conclusion, there is no doubt that the discovery and validation of a reliable biomarker of CYP3A activity is critical for accurately assessing the individual phenotype in clinical and research applications. In particular, an endogenous marker that does not have to be administered like a probe substrate and that can be measured with minimally invasive techniques would be very desirable to clinicians and researchers alike. Though several endogenous probes have been proposed (cortisol, cholesterol, and 25-hydroxyvitamin D) further work needs to be done to determine how well these correlate with oral clearance of midazolam, the prototypical CYP3A probe drug. In the following chapter, we explored the association of these proposed endogenous markers of CYP3A activity and oral midazolam clearance data.

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Table 1.1 A list of studies comparing endogenous biomarkers of CYP3A activity with CYP3A content, midazolam clearance or erythromycin breath test results. The associations of the parameters are listed, with study information and number of participants, if relevant.

Parameters	**r^2**	**Study Information**	**Reference**
Cortisol Biomarkers			
6βOHF/F vs. CYP3A protein content	0.83	in vitro, human liver microsomes	(66)
6βOHF/F vs. ERMBT	-0.12	Clinical Study, n=30	(71)
6βOHF/F vs. ERMBT	0.59	Clinical Study, n=47	(73)
6βOHF/F vs. MDZ CL, no treatment	0.04	Clinical Study, n=20	(97)
6βOHF/F vs. MDZ CL, post fluvoxamine treatment	0.39	Clinical Study, n=20	(98)
6βOHF/F vs. MDZ systemic clearance	0.7	Clinical Study, n=8	(98)
6βOHF/F vs. 1-OH-MDZ AUC/MDZ AUC	0.05	Clinical Study, n=8	(98)
6βOHF/F vs. 1-OH-MDZ C 30min/MDZ C 30 m in	0.04	Clinical Study, n=8	(98)
6βOHF/F vs. 1-OH-MDZ/MDZ ratio post-clarithromycin	0.0373	Clinical Study, n=24	(99)
6βOHF/F vs. 1-OH-MDZ/MDZ ratio pre-clarithromycin	0.0052	Clinical Study, n=24	(99)
Cholesterol Biomarkers			
25OHC vs. 4βOHC	0.65	Clinical Study, n=78	(92)
4βOHC vs. MDZ CL	0.239	Clinical Study, n=78	(90)
4βOHC vs. MDZ CL/F	0.267	Clinical Study, n=78	(90)
4βOHC/C vs. MDZ CL	0.353	Clinical Study, n=78	(90)
4βOHC/c vs. MDZ CL/F	0.353	Clinical Study, n=78	(90)
Vitamin D Biomarkers			
4-hydroxylation of 25OHD3 vs. total MDZ hydroxylation	0.86	in vitro, human liver microsomes	(96)
4β-hydroxylation of 25OHD$_3$ vs. CYP3A4 liver content	0.59	in vitro, human liver microsomes	(96)

Table 1.2 A list of studies where endogenous markers of CYP3A activity were quantified prior to and following administration of CYP3A inducers or inhibitors.

Marker	Treatment	Mean before treatment	Mean after treatment	Mean % Change	p-value	Study Information	Ref
Cortisol Biomarkers							
6βOHF/F	Ritonavir	4.72 ± 4.36	3.29 ± 1.73	30%	0.1298	clinical study, n=18	(76)
6βOHF/F	Amprenavir	3.94 ± 2.1	2.56 ± 1.12	35%	0.1406	clinical study, n=18	(76)
6βOHF	Rifampin, 600 mg	159 ±142 ng/mL	499 ± 199 ng/mL	213%		clinical study, n=60	(100)
6βOHF/F	Fluvoxamine, 150 mg/day	10 ± 6.8	5.3 ± 3.1	47%	< 0.05	clinical study, n=20	(69)
6βOHE CL_f	Itraconazole	56 ±14 mL/h	31 ± 6 mL/h	45%	0.006	in vitro, HLM	(79)
6βOHF CL_f	Itraconazole	125 ± 65 mL/h	61 ± 30 mL/h	51%	0.017	in vitro, HLM	(79)
(6βOHF + 6βOHE) CL_f	Itraconazole	107 ± 41 mL/h	55 ± 25 mL/h	48%	0.006	in vitro, HLM	(79)
cortisol AUC	Midazolam	1460 ± 696 ug·h/L	1609 ± 358 ug·h/L	19%	0.04	clinical study, n=24	(99)
cortisol AUC	Clarithromycin	1460 ± 696 ug·h/L	1919 ± 620 ug·h/L	31%	0.22	clinical study, n=24	(99)
Cholesterol Biomarkers							
4βOHC	Rifampin, 20 mg	31 ng/mL	46 ng/mL	33%	<0.01	clinical study, n=8	(81)
4βOHC	Rifampin, 100 mg	34 ng/mL	81 ng/mL	138%	<0.001	clinical study, n=8	(81)
4βOHC	Rifampin, 500 mg	37 ng/mL	141 ng/mL	281%	<0.001	clinical study, n=8	(81)

Chapter 2

Endogenous Markers of CYP3A:

Correlation with Midazolam Oral Clearance and Changes Following Induction

2.1 Abstract

The activity of the drug-metabolizing enzyme cytochromes P450 3A (CYP3A) varies up to 20-fold between individuals. Midazolam is frequently used to determine in vivo CYP3A activity. However numerous factors, such as invasiveness, and the time and cost-associated with a pharmacokinetic study render midazolam administration impractical, especially in vulnerable populations. Endogenous CYP3A biomarkers have been proposed to assess CYP3A activity. Our goal was to explore the utility of these endogenous biomarkers (metabolites of cortisol, cholesterol and vitamin D_3) in predicting CYP3A activity as determined by oral midazolam clearance. We determined the intraindividual variability of these biomarkers and ascertained whether there were gender differences in the biomarker measurements. Finally, we evaluated whether these endogenous markers were correlated with midazolam oral clearance and how well they predicted CYP3A induction by rifampin. The interday variability for plasma 4β-hydroxycholesterol and plasma 4β-hydroxycholesterol/cholesterol ratio ranged from 3 to 57% and 12 to 75%, respectively. Interestingly, only baseline plasma 4β-hydroxycholesterol and 4β-hydroxycholesterol/cholesterol were significantly higher among women compared to men. We found moderate correlations between the midazolam oral clearance and urinary 6β-hydroxycortisol/cortisol ratios, urinary 6β-hydroxycortisone/cortisone ratios, plasma 4β-hydroxycholesterol and plasma 4β-hydroxycholesterol/cholesterol ratios. We found a poor correlation between midazolam oral clearance and plasma 4β,25-dihydroxyvitamin D_3/25-hydroxyvitamin D_3 ratios. Following rifampin treatment, all of these biomarkers were significantly increased. However, the change in midazolam oral clearance following rifampin treatment was not correlated with the change in any of the biomarkers. Further work needs to be done to identify a more predictive endogenous marker for CYP3A activity.

2.2 Introduction

Drug metabolizing enzymes are the major determinant of non-renal drug clearance in humans. Cytochrome P450 enzymes play an important role in elimination of drugs by performing oxidation, peroxidation, and reduction reactions. (1) The cytochrome P450 3A (CYP3A) subfamily is the most abundant and possibly the most important subfamily of human cytochrome P450 enzymes, accounting for 40% of the total P450 in the liver and 82% of total P450 in the human small intestine. (2)

The interindividual variability of CYP3A has been studied extensively. CYP3A protein expression in liver and small intestine varies considerably. (2, 3) In parallel, there is substantial interindividual variability in the clearance of CYP3A-metabolized drugs following intravenous (IV) and oral administration. Although an estimated 90% of interindividual variability in hepatic CYP3A activity is attributed to genetics (4), few genetic variants have been identified which adequately explain the interindividual variability. Besides genetic variability, there are other factors that can influence CYP3A activity. CYP3A expression can vary due to age and disease states. (5-7) Gender has also been shown to impact CYP3A activity, with studies showing that females typically having higher CYP3A activity than males. (7-9)

Because CYP3A plays a major role in drug elimination, estimation of an individual's CYP3A activity would allow dosing to be tailored to the individual's CYP3A activity level. For drugs with a narrow therapeutic range, personalizing the dose would ensure that patients achieve an optimal plasma drug concentration for efficacy that minimizes toxicity. Currently, midazolam is the gold standard probe drug used to phenotype for CYP3A activity. CYP3A metabolizes midazolam to 1'-hydroxymidazolam, its major metabolite, and to 4-hydroxymidazolam (Figure 2.1A). (10) Midazolam is extensively metabolized with less than 0.03% excreted unchanged in urine. (11) Upon oral administration, midazolam is highly metabolized in both the small intestine and liver, and the bioavailability ranges from 12 to 50% in healthy volunteers. However, phenotyping with midazolam is not always an ideal option. Using exogenous probes to assess CYP3A may not be feasible in young children, elderly individuals, or pregnant women because of a number of concerns including administering a drug that is not therapeutically warranted, intensive blood sampling to determine the probe drug's pharmacokinetics, and the barriers to participation due to the "vulnerable" status of the group. Using an endogenous biomarker would be a simpler approach to ascertaining CYP3A activity in these populations. (12)

In addition to measuring the basal CYP3A activity, the endogenous biomarker should reflect alterations in CYP3A activity due to disease or as a result of drug treatment. For example, rifampin is a potent inducer of CYP3A. Rifampin is a ligand of the pregnane X receptor (PXR), which activates gene transcription. (13, 14) Rifampin has been shown to decrease the midazolam exposure by up to 96% due to induction of CYP3A in the liver and intestine. (15)

Several endogenous CYP3A substrates have been studied. CYP3A is known to metabolize cortisol to 6β-hydroxycortisol (Figure 2.1C). Cortisol (F) and cortisone (E) are interconverted by the activity of 11β-hydroxysteroid dehydrogenase, as are 6β-hydroxycortisol (6βOHF) and 6β-hydroxycortisone (6βOHE). Due to circadian rhythms in cortisol synthesis, the urinary metabolite-to-parent ratios can be used to evaluate CYP3A activity (i.e., urinary 6βOHF/F and 6βOHE/E ratios). (16) Ged et al. found a very strong correlation (r^2=0.84) between the urinary 6βOHF/F ratio and hepatic CYP3A levels. (17) Moreover, they found that patients treated with rifampin had significantly higher 6βOHF/F ratios, implying that the 6βOHF/F ratio reflected increased CYP3A expression due to induction. (17) Although the urinary 6βOHF/F ratios were predictive of CYP3A activity in a number of pharmacokinetic studies, (18, 19) other studies found that the urinary 6βOHF/F ratio did not mirror the oral clearance of midazolam, possibly due to extrahepatic CYP3A contribution to midazolam elimination. (20-22)

More recently, a hydroxylated metabolite of cholesterol has been proposed as a CYP3A biomarker. Tomalik-Scharte et al. determined using recombinant enzymes, that CYP3A4 is the major enzyme that converts cholesterol to 4β-hydroxycholesterol (4βOHC) (Figure 2.1B). (12) Moreover, strong CYP3A inducers, such as antiepileptic drugs, were shown to greatly increase 4βOHC circulating concentrations. (23) The 4β-hydroxycholesterol/cholesterol ratio (4βOHC/C) has been suggested as an alternative 4β-hydroxycholesterol measure, which might eliminate potential bias caused by plasma cholesterol concentration variability. (12)

Finally, a newly identified metabolite of 25-hydroxyvitamin D_3 (25OHD_3) has been proposed as a biomarker for CYP3A activity (Figure 2.1D). 25OHD_3 is the most abundant circulating form of vitamin D_3,with concentrations ranging from 20 to 50 ng/mL in plasma from healthy individuals. (24) It has recently been reported that 25OHD_3 is hydroxylated at the 4-position, resulting in two stereoisomers, 4α,25-hydroxyvitamin D_3 (4α,25OHD_3) and 4β,25-hydroxyvitamin D_3 (4β,25OHD_3) . Wang et al. (25) found a strong correlation between both 4α,25OHD_3 and 4β,25OHD_3 formation and midazolam hydroxylation in a panel of human liver microsomes and that only CYP3A, among the human P450s screened, catalyzed the reaction. They also found that the formation of both metabolites was correlated with CYP3A content as determined by Western blot analysis and was inhibited extensively by selective CYP3A inhibitors. Both plasma 4β,25OHD_3 concentrations in healthy volunteers and 4β,25OHD_3 levels in primary human hepatocytes were increased upon rifampin treatment, suggesting that 4β,25OHD_3 is sensitive to changes in CYP3A activity. (25)

Our goal in this investigation was to compare the utility of these endogenous biomarkers in predicting CYP3A activity, as determined by oral midazolam clearance. We analyzed samples collected as part of a midazolam pharmacokinetic study in which healthy volunteers were studied at baseline and following treatment with rifampin, sulforaphane or combination (rifampin + sulforaphane). We determined the intraindividual and interindividual variability of these biomarkers and ascertained whether there were gender differences in biomarker levels. Finally, we evaluated whether these endogenous markers were correlated with oral midazolam clearance and how well they predicted CYP3A induction by rifampin.

2.3 Materials and Methods

2.3.1 Chemicals and Reagents

4β-hydroxycholesterol, d4-4β-hydroxycholesterol, d7-4β-hydroxycholesterol, cholesterol, and d7-cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). Blank human plasma was obtained from the Puget Sound Blood Bank (Seattle, WA). Butylated hydroxytoluene in ethanol and ethanolic KOH were obtained from Sigma Aldrich (St. Louis, MO). The derivatization chemicals were purchased from Sigma Aldrich. They consisted of 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, picolinic acid, triethylamine and pyrindine. Triethylamine and picolinic acid were stored over molecular sieves (Type 3A, 8-12 mesh, activated at 220 °C for two hours) to absorb water and prevent ionization. All derivatization chemicals were stored in a desiccator at room temperature. All other chemicals and solvents used were of highest purity available and purchased through commercial vendors.

2.3.2 Study Design

As reported by Poulton et al. (26), a double-blind crossover study was conducted to assess the effects of rifampin, sulforaphane and the combination treatment on CYP3A activity. The study was approved by the University of Washington Human Subjects Review Board. A total of 23 subjects participated in the study. Patient demographics are reported in Table 2.1. The study design and midazolam pharmacokinetic results are presented in further detail elsewhere (26). In brief, subjects were randomized to the treatment order of receiving rifampin, sulforaphane or combination (rifampin and sulforaphane). A washout period of at least 2 weeks separated each of the treatment arms. On each visit (Day 1 and Day 8), subjects were phenotyped with a 1 mg oral dose of midazolam. For the rifampin and combination treatment arms, subjects received 300 mg rifampin daily for 7 days followed by midazolam phenotyping on Day 8. For the sulforphane and combination treatment arms, subjects received 450 μmol sulforaphane daily for 7 days followed by midazolam phenotyping on Day 8. We used predose plasma samples and urine collected for 24 hrs following midazolam phenotyping for our analyses of endogenous biomarkers.

2.3.3 Cholesterol and 4βOHC Sample Preparation

We developed an assay for the analysis of cholesterol and 4βOHC. We used d7-deuterated compounds as internal standards (d7-cholesterol and d7-4βOHC) and a deuterated compound for the 4βOHC calibration curve (d4-4βOHC). To prepare samples, we added 425 μL of 1 mg/mL butylated hydroxytoluene in EtOH and 250 μL of ethanolic KOH to 50 μL of plasma (standard or unknown samples). Samples were saponified for one hour at 37°C. We added 0.14 mL of nanopure water followed by 0.5 mL hexane to the samples. Samples were vortexed for 10 min, centrifuged at 11,400 g for 20 min at 4°C and 300 µL of the supernatant was removed and transferred to clean 2 mL microcentrifuge tubes. Samples were dried under nitrogen at 40°C.

Since oxysterols generally show very low sensitivity on mass spectrometry, we used a derivatization technique to replace the hydroxy groups with picolinic acid (see Figure 2.2). The derivatization reagent was prepared 2 hr prior to use and contained: 1244.5 mg of 2-methyl-6-nitrobenzoic anhydride, 373.3 mg of 4-dimethylaminopyridine, 995.5 mg of picolinic acid, 2.5 mL of triethylamine and 18.7 mL pyridine. Samples were derivatized by adding 170 µL of the derivatization reagent and samples were incubated for 30 min at room temperature and vortexed at 15 min intervals. To the derivatized samples, we added 800 µL hexane and vortexed for 10 minutes. Samples were centrifuged at 11,400 g for 20 min at 4°C. From each sample, 200 µL of the supernatant was removed, transferred to 1.5 mL plastic microcentrifuge tubes and dried under nitrogen at 37°C. We added 200 µL of acetonitrile to the dried, derivatized samples and centrifuged at 11,400 g for 5 min. We transferred 125 µL to HPLC vial for LC/MS/MS analysis for 4βOHC, d4-4βOHC, and d7-4βOHC. We diluted 10 µL of the derivatized samples with 990 µL of acetonitrile for analysis by LC/MS/MS for cholesterol and d7-cholesterol. Standards, quality controls (QCs), and clinical samples were analyzed in duplicate.

2.3.4 Mass Spectrometry Methods for 4βOHC and Cholesterol

Chromatographic separation was performed using an Acquity UPLC BEH C18 column (100x2.1 mm, 1.7 µm) from Waters (Milford, MA) on an Agilent 1290 Infinity HPLC. The mobile phase consisted of: A = 98:2 methanol/water containing 0.1% formic acid and B = 1:1 acetonitrile/methanol containing 0.1% formic acid. For the cholesterol assay, 1 uL of sample was injected and analyzed using an isocratic method of 50% B with a 0.3 mL/min flow rate. The total run time was 7 min. For the 4βOHC assay, 5 uL of sample was injected and analyzed using an isocratic method of 90% B with a 0.5 mL/min flow rate. The total run time was 16 min. Data were collected in multiple reaction monitoring (MRM) in ESI positive mode using an Agilent 6460 triple quadrupole mass spectrometer. The transitions that were monitored and mass spectrometry settings are listed in Table 2.2.

2.3.5 Quantification of Cholesterol

Cholesterol and d7-cholesterol (internal standard) stock solutions were prepared in 100% ethanol at an initial concentration of 5 mg/mL. Average plasma cholesterol levels are approximately 200 mg/dL or 2 mg/mL and the calibration curve concentrations were: 0, 0.1, 0.5, 1, 2, 3, and 4 mg/mL. d7-Cholesterol was diluted to 1 mg/mL. A 50 μL aliquot of cholesterol calibration standards and 50 μL of d7-cholesterol diluted stock were spiked into 50 μL of blank plasma for the calibration curve. The peak area ratio for the 0 mg/mL point (plasma blank) was subtracted from the peak area ratios for the rest of the calibration curve. These adjusted peak area ratios were used to construct a calibration curve by fitting a linear regression line to the data. The calibration curve was used to estimate the unknown cholesterol concentrations in the clinical samples.

2.3.6 Quantification of 4βOHC

When using blank plasma for preparing the 4βOHC calibration curve, large variations were observed in the low calibration standards due to moderate endogenous concentrations of 4βOHC in the blank plasma samples. Thus, we used d7-4βOHC to construct the calibration curve and d4-4βOHC for the internal standard. We prepared calibration curves with d7-4βOHC and 4βOHC at the same concentrations on the same day to compare the response of the d7-4βOHC to 4βOHC. The d7-4βOHC/d4-4βOHC peak area ratios were used to construct a calibration curve by fitting a linear regression line to the data. The calibration curve was used to estimate the unknown 4βOHC concentrations in the clinical samples.

2.3.7 Cortisol Assay

Urine samples were prepared according to previously published protocols. (27) Details are presented elsewhere.

2.3.8 Vitamin D Assays

Vitamin D assay results for the plasma samples were previously reported by Wang et al. (28)

2.3.9 Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.0 B (La Jolla, CA). As each subject had three baseline measurements, we calculated the mean baseline measurement for each subject. The normality of the distribution of the mean baseline biomarker measurements was assessed using the D'Agostino and Pearson normality test. We used an unpaired, non-parametric, two-tailed t-test to assess whether the mean biomarker measurements differed between male and female subjects. We used a paired, two-tailed, non-parametric t-test to determine the difference in endogenous biomarker measurements before and after CYP3A induction by rifampin. We used Spearman's rank correlation to determine the strength of association between oral midazolam clearance and the endogenous biomarkers. We considered Spearman's correlation coefficients (ρ) between 0.7 and 1 to be strong, between 0.3 and 0.69 to be moderate, and less than 0.3 to be weak. We used a Pearson's correlation to determine the correlation between change in midazolam clearance and change in biomarker measurements. Multivariate regression analysis was used to determine whether combinations of the endogenous biomarker measurements were able to better predict oral midazolam clearance. P-values less than 0.05 were considered statistically significant.

2.4 Results

2.4.1 Cholesterol and 4 β OHC Assay Results

Peaks for the cholesterol and 4βOHC (Figure 2.3) were well resolved from interfering peaks in plasma samples. Peak area ratios of cholesterol/d7-cholesterol were plotted against cholesterol concentration to create a calibration curve (Figure 2.4). We confirmed that calibration curves prepared with d7-4βOHC and 4βOHC at the same concentrations on the same day resulted in identical slopes (Figure 2.5 and 2.6), suggesting that the response of d7-4βOHC was equivalent to 4βOHC. The QC samples for 4βOHC were prepared at 0 and 100 ng/mL on each of the assay days and the interday variability was below 10% (Table 2.3).

2.4.2 Cholesterol Results

There was a large amount of interday variation in cholesterol levels at the three baseline measurements. The mean coefficient of variation (CV) was 17.3%, ranging from approximately 3 to 57% in subjects (Table 2.4). This could be due to a variety of factors, including subjects' diet. We compared cholesterol levels between Day 1 and Day 8 of rifampin, sulforaphane, and combination treatment arms. In order to mask the taste of sulforaphane, sulforaphane was mixed into a high-fat cheese soup and consumption of the soup could impact overall cholesterol levels. We found that when looking at all three arms of treatment collectively, there was a significant difference in cholesterol levels on Day 1 vs. Day 8 ($p = 0.0056$). However, when stratified by the treatment arms, no difference was seen following rifampin treatment (Figure 2.7), although a significant difference in plasma cholesterol levels was observed following combination treatment ($p = 0.0085$).

2.4.3 4β-hydroxycholesterol Results

The average baseline plasma 4βOHC concentrations ranged from 6.42 to 69.71 ng/mL (Table 2.3) and were normally distributed (Figure 2.8). In addition, female subjects had higher 4βOHC concentrations compared to male subjects (Figure 2.9). The interday CV in baseline 4βOHC concentrations ranged from 12 to 75%. Average 4βOHC concentrations increased from 32.99 ± 4.62 ng/mL to 57.15 ± 5.99 ng/mL following rifampin treatment (Figure 2.10, $p = 0.0028$). There was a moderate correlation, but significant association between 4βOHC plasma concentrations and midazolam oral clearance values (Figure 2.11, $\rho = 0.535$, $p < 0.0001$). Finally, the change in 4βOHC concentrations after rifampin treatment was not correlated with the change in midazolam oral clearance (Figure 2.13, $r^2 = 0.306$, $p = 0.114$).

2.4.4 4βOHC/Cholesterol Ratio

The molar ratios of 4βOHC/C ranged from 0.17×10^4 to 1.057×10^4 and were normally distributed (Table 2.4 and Figure 2.13). Women had higher 4βOHC/C ratios compared to men (Figure 2.14, $p=0.033$). Following rifampin treatment, the average 4βOHC/C ratio was 0.291 ±

0.032×10^4 compared to $0.186 \pm 0.024 \times 10^4$ for baseline values (Figure 2.15, $p = 0.0025$). The 4βOHC/C ratios were moderately correlated with oral midazolam clearance (Figure 2.16, $\rho = 0.48$). Moreover, the change in 4βOHC/C ratio after rifampin treatment was weakly correlated with changes in midazolam oral clearance after rifampin treatment (Figure 2.17, $r^2 = 0.306$).

2.4.5 Cortisol Results

Urinary 6β-hydroxycortisol/cortisol ratios (6βOHF/F) ratios ranged from 0.031 to 65.02, with some ratios being excluded due to erroneous values. The average baseline 6βOHF/F ratios were normally distributed (Figure 2.18). When comparing male and female baseline 6βOHF/F ratios, there were no significant gender-related differences (data not shown). Following rifampin treatment, 6βOHF/F ratios were significantly increased by approximately 4-fold compared to baseline (Figure 2.19, $p < 0.0001$). Urinary 6βOHF/F ratios were moderately correlated with midazolam oral clearance (Figure 2.20, $\rho=0.66$). In contrast, the change in 6βOHF/F ratio following rifampin treatment did not correlate with the change in midazolam oral clearance (Figure 2.21).

The average urinary 6β-hydroxycortisone/cortisone ratios (6βOHE/E) were also normally distributed (Figure 2.22). There was no significant difference in average baseline 6βOHE/E ratios between male and female subjects (data not shown). There was a significant increase in the 6βOHE/E ratio following CYP3A induction by rifampin (Figure 2.23). Overall, the 6βOHE/E was moderately correlated with midazolam oral clearance (Figure 2.25, $\rho = 0.57$). Finally, the change in 6βOHE/E ratio following rifampin treatment was very poorly correlated with the change in midazolam oral clearance following rifampin treatment much like results seen with

6βOHF/F (Figure 2.25).

2.4.6 Vitamin D_3 Results

Before rifampin treatment, the mean 4β,25-dihydroxyvitamin D_3/25-hydroxyvitamin D_3 ratio (4β,25OHD_3/25OHD_3) among subjects was 2.12 ± 0.16. After treatment, the 4β,25OHD_3/25OHD_3 ratio was significantly increased to 3.8 ± 0.44 (Figure 2.26, p = 0.0002). We found that there was no correlation between 4β,25OHD_3/25OHD_3 ratios and oral midazolam clearance (Figure 2.27, ρ=0.1). Not surprisingly, the change in 4β,25OHD_3/25OHD_3 ratios following rifampin treatment did not correlate with the change in midazolam oral clearance following rifampin treatment (Figure 2.28, $r^2 = 0.0026$).

2.4.7 Multivariate Analyses Results

Multivariate analyses were performed to test if a combination of endogenous biomarker measurements would better predict midazolam oral clearance. Using 4βOHC/C, 6βOHF/F, 6βOHE/E, and 4β25OHD3/25OHD3, only 36% of the variability in oral midazolam clearance was explained and the only parameter that made a significant contribution was the urinary 6βOHE/E ratio.

2.5 Discussion

Basal CYP3A activity as assessed by these biomarkers was characterized by a large degree of intraindividual and interindividual variability. Interestingly, only plasma 4βOHC and 4βOHC/C were significantly higher among women compared to men at baseline. Several reports describe higher CYP3A activity in females compared to males. (7-9) Due to the small sample size (or for other reasons), we did not see a significant difference in midazolam oral clearance, cortisol ratios or vitamin D ratios.

Unlike a previous report by Tomalik-Scharte (12) where 4βOHC was weakly correlated with midazolam clearance ($r^2 = 0.239$), we observed far a weaker correlation ($\rho = 0.535$). Our value was considerably lower, perhaps because our comparison was made to oral midazolam clearance instead of systemic midazolam clearance. There may be less of a correlation between 4βOHC concentration and oral midazolam clearance because of the intestinal CYP3A first-pass activity, which does not play a role in endogenous 4βOHC formation. Similarly, using the plasma 4βOHC/C ratio did not improve our estimate of midazolam clearance ($\rho = 0.4832$) for our study; $r^2 = 0.348$ for Tomalik-Scharte et al. (12)) Tomalik-Scharte et al. also found that 4βOHC was not considerably influenced by cholesterol concentrations. (29, 30)

Although 4βOHC and 4βOHC/C were not associated with oral midazolam clearance, both 4βOHC and 4βOHC/C were increased by 58.1% and 56.4%, respectively, following rifampin treatment. In subjects receiving 100 mg of rifampin daily for two weeks, 4βOHC concentrations were increased by an average of 138%. (29) Though treatment with CYP3A inducers result in a significant increases in 4βOHC and 4βOHC/C ratios, the magnitude of CYP3A induction may be more difficult to predict with these markers, as we did not see a good correlation between the change in 4βOHC or 4βOHC/C and the change in oral midazolam clearance following rifampin treatment.

Previous studies assessing the ability of 6βOHF/F to predict midazolam clearance have reported r^2 values ranging from 0.04 to 0.39. (31-33) We observed a correlation of 0.28 with

6βOHF/F and a similar correlation of 0.32 with 6βOHE/E, which has no comparative values in the literature. In our study, the urinary 6βOHF/F and 6βOHE/E ratios had the strongest correlations with oral midazolam clearance of all of the biomarkers we studied. We found that after rifampin treatment, urinary 6βOHF/F and 6βOHE/E ratios increased by 297% and 307%, respectively, which was substantially higher than observed increases in 4βOHC or 4βOHC/C. Kovacs et al. (34) demonstrated a similar increase (213%) in urinary 6βOHF concentrations after subjects received 500 mg of rifampin daily for one week. Tran et al. (35) found that after 600 mg of rifampin daily for two weeks, the mean urinary 6βOHF/F ratio increased by 320% ± 73% in 15 subjects. We are the first to report a similar induction of 6βOHE/E ratios following rifampin treatment. Altogether, 6βOHF/F and 6βOHE/E seem to be the best options of all of the endogenous markers we studied. However, these ratios still only account for approximately 28 to 30% of the variability in CYP3A activity. The limited ability of 6βOHF/F and 6βOHE/E to predict oral midazolam clearance may reflect activity of the 11β-hydroxysteroid dehydrogenase, renal transporter effects, or that 6βOHF/F and 6βOHE/E are not able to account for the intestinal CYP3A first-pass effect.

Finally, the least successful biomarker in predicting oral midazolam clearance was $4\beta25OHD_3/25OHD_3$ ($r^2 = 0.0078$). Although $4\beta25OHD_3/25OHD_3$ was increased significantly by 80% following rifampin treatment, it was not correlated with the change in oral midazolam clearance. As $4\beta25OHD_3$ is a newly discovered metabolite, further work will need to be done to confirm these results; specifically, clearance of the hydroxy metabolite through conjugation may need to be taken into consideration.

In the first comparison of CYP3A endogenous biomarkers, we found that 4βOHC/C, 6βOHF/F, 6βOHE/E, and 4β,25OHD$_3$/25OHD$_3$ were at best weakly correlated with oral midazolam clearance. Although these markers were sensitive to CYP3A induction, they were poorly predictive of the fold-change of midazolam oral clearance following rifampin treatment. Though the urinary 6βOHF/F and 6βOHE/E ratios were barely adequate in predicting midazolam oral clearance, the search for a suitable endogenous marker of CYP3A activity is ongoing. Identification of an endogenous biomarker that can accurately predict CYP3A activity would not only allow personalization of dosing for CYP3A substrates, but could potentially inform whether new drug entities are CYP3A inhibitors or inducers based on changes to endogenous metabolites. Using new techniques such as metabolomics may facilitate the identification of potential new CYP3A biomarkers in urine or plasma samples. Further research on endogenous steroids and other molecules, and how they are eliminated, would certainly help to move the search forward.

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Table 2.1 Patient demographics.

Subject	Sex	Age (years)	Race/Ethnicity
1	F	24	Caucasian
2	F	29.1	Caucasian
3	M	28.3	Asian
4	F	28	Caucasian
5	M	22.8	Caucasian
6	M	23.6	Caucasian
7	M	21.3	Caucasian
8	F	23.6	Asian
9	M	21.6	Asian
10	M	25.3	Caucasian
11	F	27.7	Caucasian
12	F	34.7	Caucasian
13	F	22.4	Caucasian
14	M	20.5	Asian
15	F	21.4	Caucasian
16	M	21.1	Caucasian
17	F	20.2	Caucasian
18	F	22	Caucasian
19	M	21	Black
20	F	21.4	Caucasian
21	M	22.6	Caucasian
22	M	22.7	Caucasian
23	M	28.4	Caucasian

Table 2.2 The mass spectrometry parameters for 4β-hydroxycholesterol and cholesterol assay including parent and daughter ion monitored, dwell time, and collision energy.

Derivatized compound	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**Dwell time (ms)**	**Fragmentor Voltage (V)**	**Collision Energy (V)**
Cholesterol	369.4	147.1	200	174	20
*d*7-cholesterol	376	147.1	200	179	20
4βOHC	613.4	490.4	200	114	14
*d*4-4βOHC	617.4	494.4	200	109	10
*d*7-4βOHC	620.4	374.4	200	124	14

Table 2.3 The 4βOHC quality control standards were prepared in duplicate at 0 ng/mL (low) and 100 ng/mL (high) on each of the assay days.

	Low QC (ng/mL)	**High QC (ng/mL)**
Day 1	4.59	114.64
	4.57	116.86
Day 2	5.86	112.71
	5.38	112.28
Day 3	4.95	121.44
	4.7	119.07
Day 4	5.0	119.55
	4.67	113.25
Day 5	5.49	118.4
	5.33	121.49
Mean ± SD	5.05 ± 4.57	116.97 ± 3.54
Interday Variability (%)	9.05	3.03

Table 2.4 Interday variability in baseline plasma cholesterol and 4β-hydroxycholesterol concentrations taken on three separate occasions.

Subject	Cholesterol (mg/mL)	% CV	4βOHC (mg/mL)	% CV	4βOHC/C Ratio (*10^4)	% CV
1	1.63 ± 0.22	13.42	26.56 ± 8.87	33.41	0.16 ± 0.03	19.17
2	1.3 ± 0.05	3.78	16.85 ± 2.26	13.42	0.12 ± 0.02	16.07
3	1.4 ± 0.29	20.77	31.43 ± 6.68	21.25	0.22 ± 0.02	9.28
4	1.68 ± 0.18	10.88	31.03 ± 3.57	11.52	0.18 ± 0.02	11.28
5	1.9 ± 0.00	0	22.33 ± 0.00	0	0.11 ± 0.03	26.58
6	1.57 ± 0.18	11.21	23.63 ± 6.21	26.29	0.14 ± 0.02	13.84
7	2.44 ± 1.32	54.16	6.42 ± 0.77	11.96	0.03 ± 0.01	39.58
8	1.47 ± 0.36	24.57	69.71 ± 51.94	74.51	0.46 ± 0.05	10.98
9	1.59 ± 0.17	10.68	30.27 ± 6.27	20.73	0.18 ± 0.05	27.35
10	1.93 ± 0.29	14.78	28.65 ± 10.5	36.64	0.14 ± 0.02	14.03
11	1.84 ± 0.25	13.62	50.32 ± 22.96	45.62	0.26 ± 0.06	22.85
12	1.64 ± 0.16	9.8	48.84 ± 30.72	62.88	0.29 ± 0.17	59.45
13	1.5 ± 0.25	16.68	57.11 ± 30.61	53.6	0.37 ± 0.24	65.65
14	1.21 ± 0.08	6.86	36.28 ± 4.42	12.18	0.29 ± 0.04	13.89
15	1.39 ± 0.65	46.97	36.92 ± 14.11	38.21	0.26 ± 0.12	47.05
16	1.6 ± 0.31	19.21	17.82 ± 11.08	62.16	0.11 ± 0.01	9.35
17	2.03 ± 0.06	3.17	17.92 ± 3.23	18.05	0.08 ± 0.01	11.80
18	1.73 ± 0.22	12.91	25.89 ± 8.02	30.97	0.14 ± 0.04	27.84
19	1.18 ± 0.24	20.34	14.17 ± 1.88	13.28	0.12 ± 0.02	17.35
20	1.34 ± 0.25	18.77	29.29 ± 6.35	21.68	0.21 ± 0.05	23.82
21	1.59 ± 0.57	35.58	29.58 ± 6.02	20.35	0.18 ± 0.06	33.59
22	1.43 ± 0.24	16.86	19.16 ± 4.16	21.74	0.13 ± 0.04	31.09
23	1.6 ± 0.22	13.64	36.17 ± 4.73	13.09	0.22 ± 0.04	18.43
Mean ± SD	1.61 ± 0.29		30.71 ± 14.65		0.19 ± 0.1	
Range		0 to 54		0 to 75		9 to 66

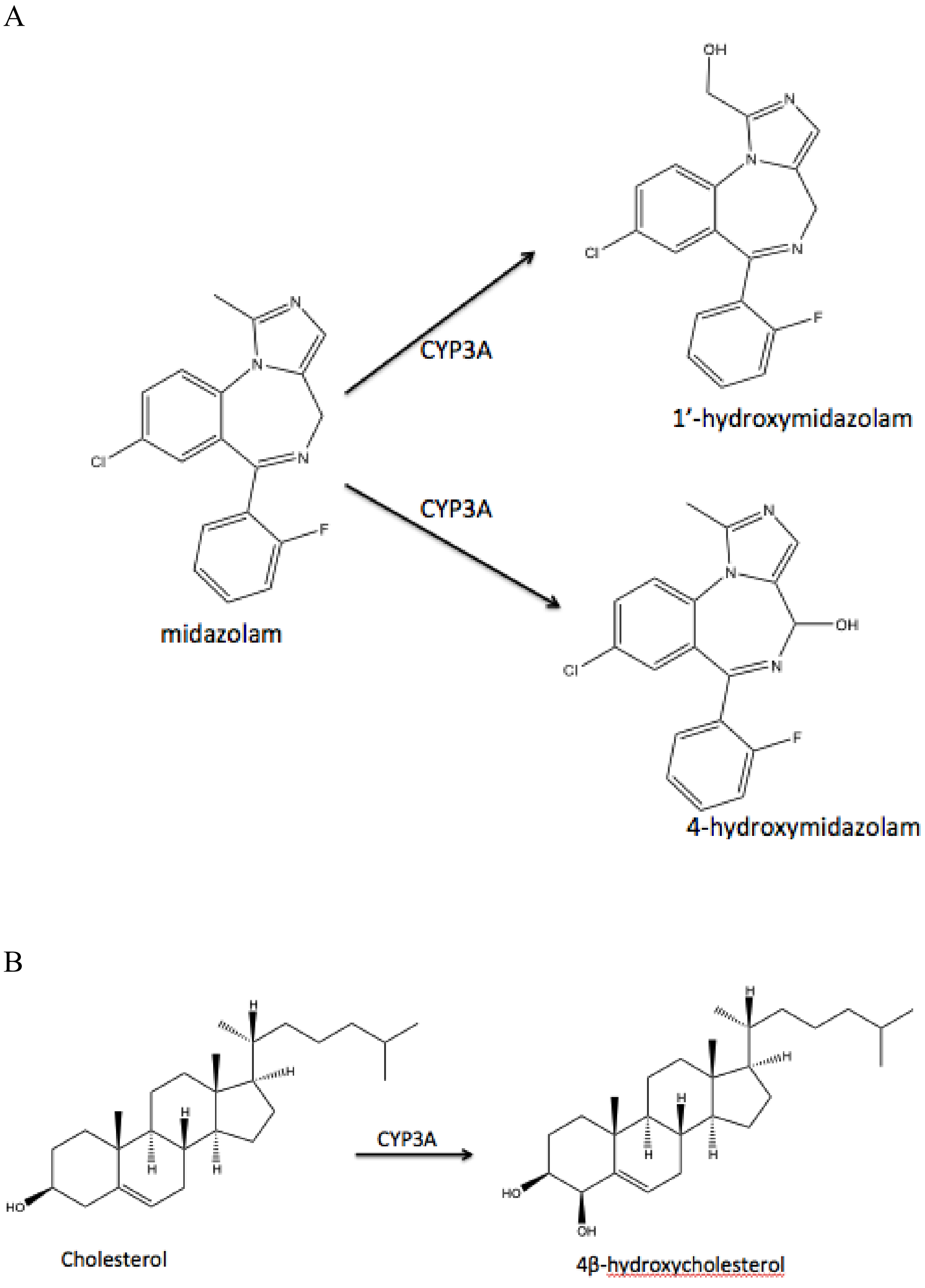
Table 2.5 Mean biomarker concentrations before and after CYP3A induction by rifampin

Marker	Mean before RIF	Mean after RIF	% Change	p-value
4βOHC	32.99 ± 4.62	57.15 ± 5.99	58.1	0.0028
4βOHC/C * 10^4	0.186 ± 0.24	0.291 ± 0.32	56.4	0.0048
6βOHF/F	7.04 ± 0.88	28.68 ± 3.25	306.8	<0.0001
6βOHE/E	0.32 ± 0.05	1.27 ± 0.16	296.9	<0.0001
4β25OHD3/25OHD3	2.12 ± 0.16	3.8 ± 0.44	79.2	0.0002

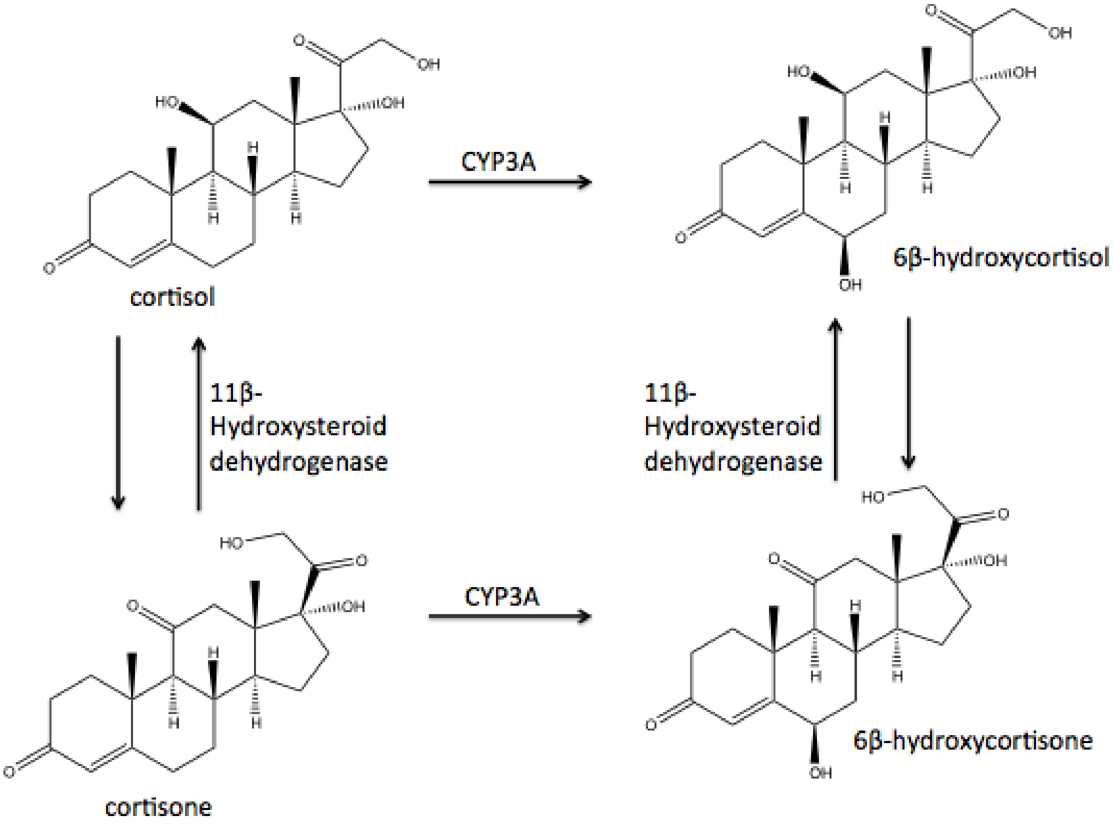
Table 2.6 Correlation between endogenous biomarkers and midazolam oral clearance.

Endogenous Marker	Spearman's correlation (ρ)
4βOHC	0.53
4βOHC/C	0.48
6βOHF/F	0.66
6βOHE/E	0.60
4β25OHD3/25OHD3	0.10

Figure 2.1 CYP3A-mediated metabolism of midazolam and various endogenous substrates. A: midazolam; B: cholesterol; C: cortisol; D: 25-hydroxyvitamin D_3.



C


cortisol
CYP3A
6β-hydroxycortisol
11β-
Hydroxysteroid
dehydrogenase
11β-
Hydroxysteroid
dehydrogenase
cortisone
CYP3A
6β-hydroxycortisone

D

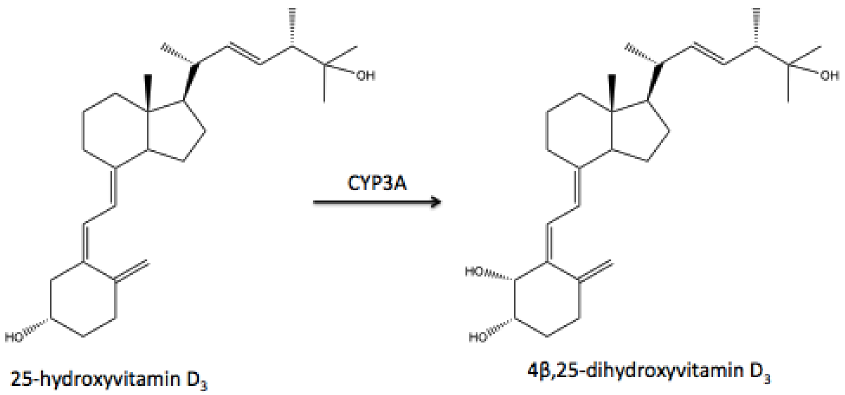

CYP3A
25-hydroxyvitamin D_3
4β,25-dihydroxyvitamin D_3

Figure 2.2 An illustration of derivatization of d7-4β-hydroxycholesterol (left), which replaces hydroxy-groups with picolinic acid, resulting in a derivatized d7-4β-hydroxycholesterol (right).

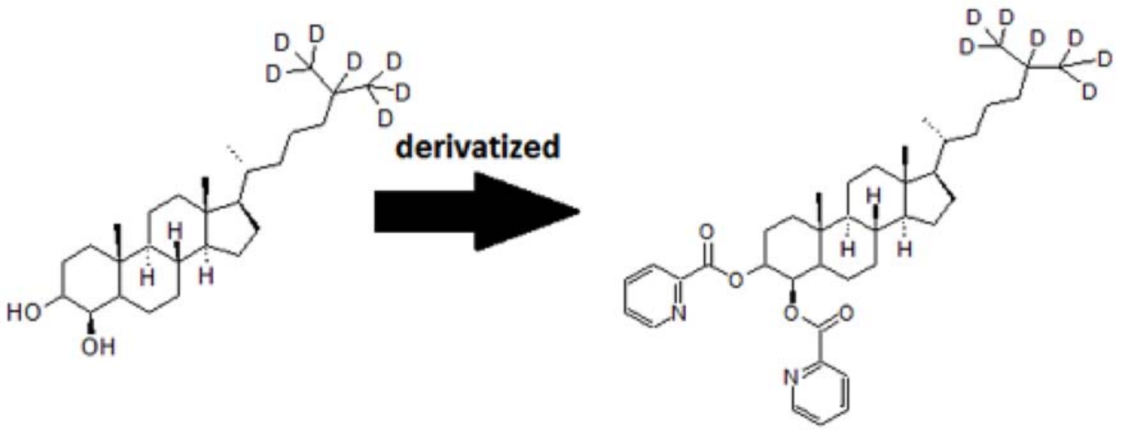
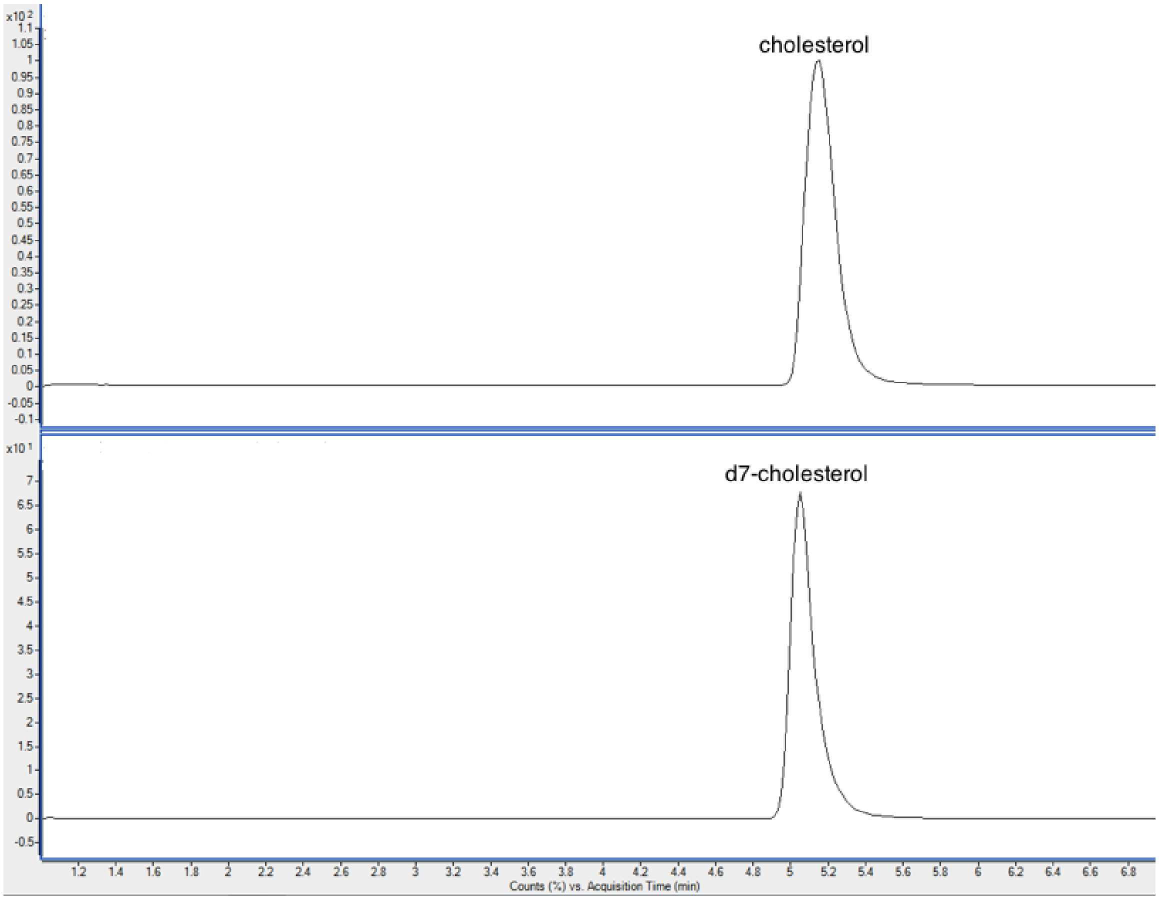


Figure 2.3 Representative chromatograms of the cholesterol and 4βOHC assays.
A: Cholesterol and d7-cholesterol; B: 4βOHC, d4-4βOHC, and d7-4βOHC

A



B

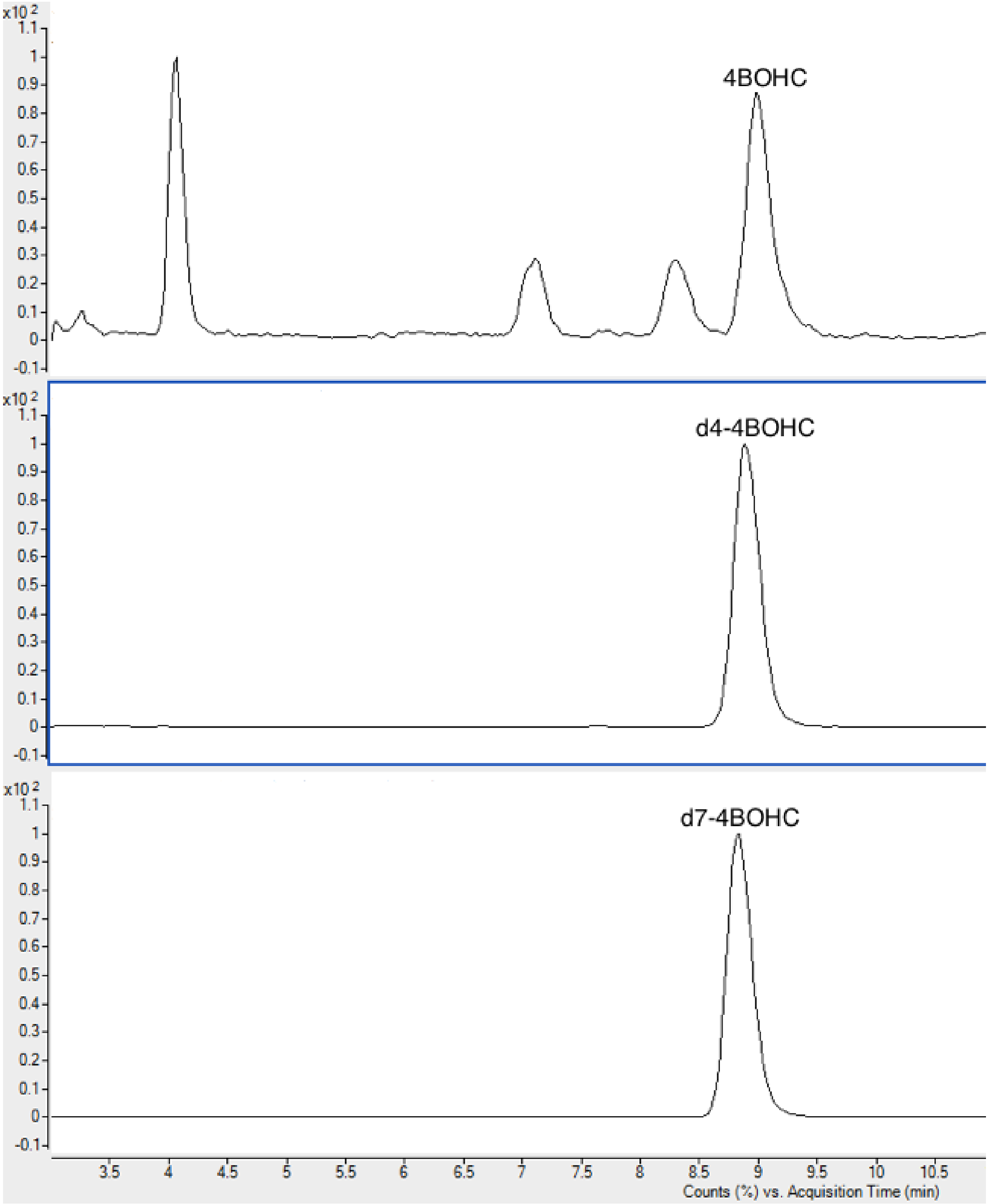

x10 2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
4BOHC
x10 2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
d4-4BOHC
x10 2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
d7-4BOHC
3.5
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
10.5
Counts (%) vs. Acquisition Time (min)

Figure 2.4 Cholesterol concentration (x-axis) versus cholesterol:d7-cholesterol peak area ratio (y-axis). This curve was adjusted so that the peak area ratio for the 0 mg/mL cholesterol concentration was subtracted from all peak area ratios so the standard curve passes through the origin.

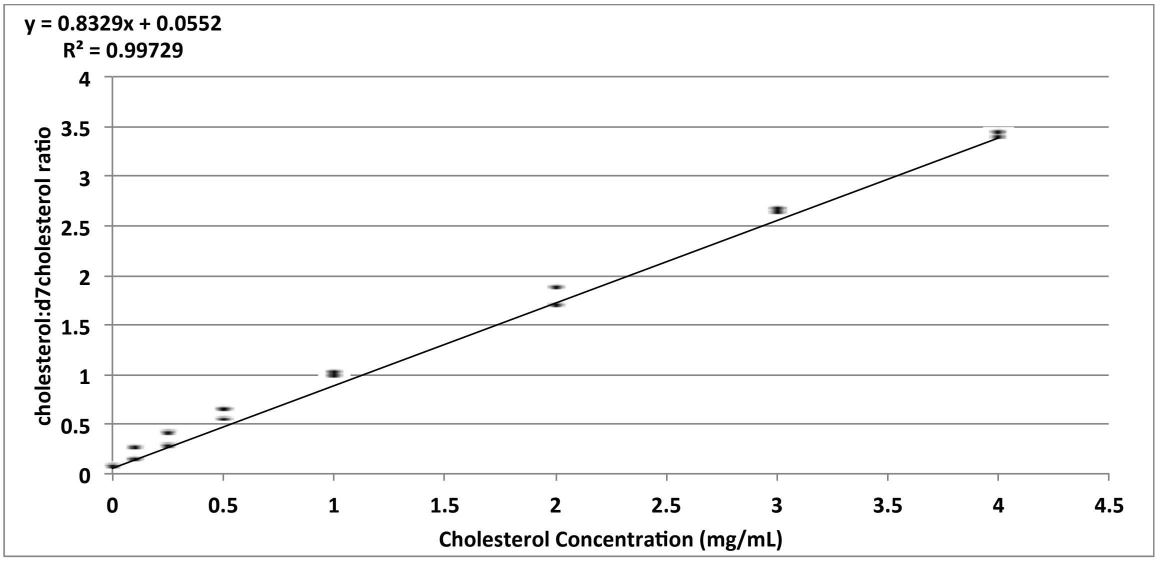


Figure 2.5 D7-4β-hydroxycholesterol concentration (x-axis) versus d7-4β-hydroxycholesterol/d4-4β-hydroxycholesterol cholesterol peak area ratio (y-axis).

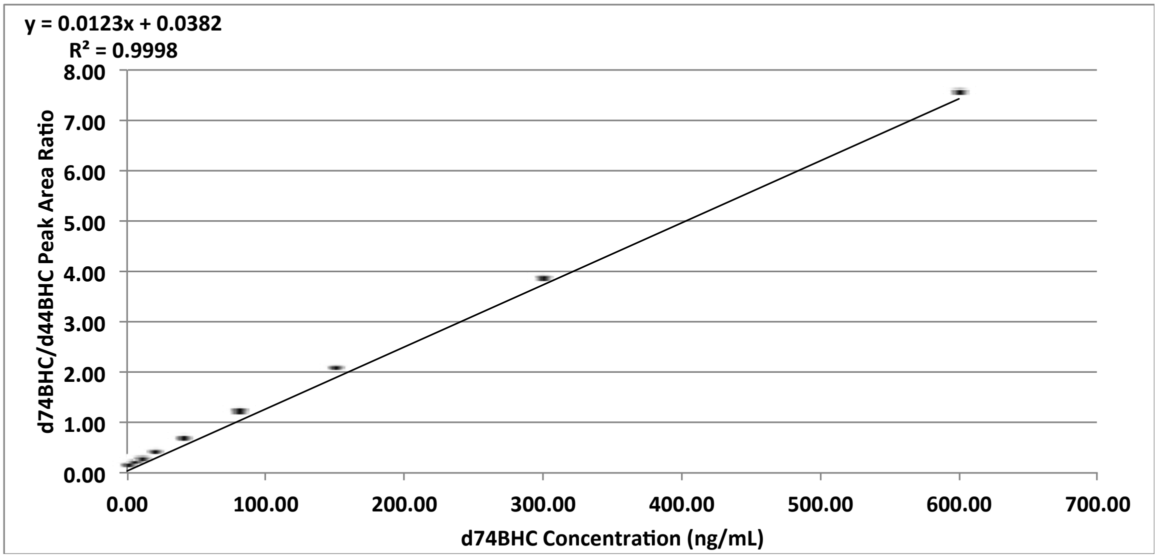


Figure 2.6 4β-hydroxycholesterol concentration (x-axis) versus 4β-hydroxycholesterol:d4-4β-hydroxycholesterol peak area ratio (y-axis). Blank plasma 4β-hydroxycholesterol levels were subtracted from each calibration curve sample.

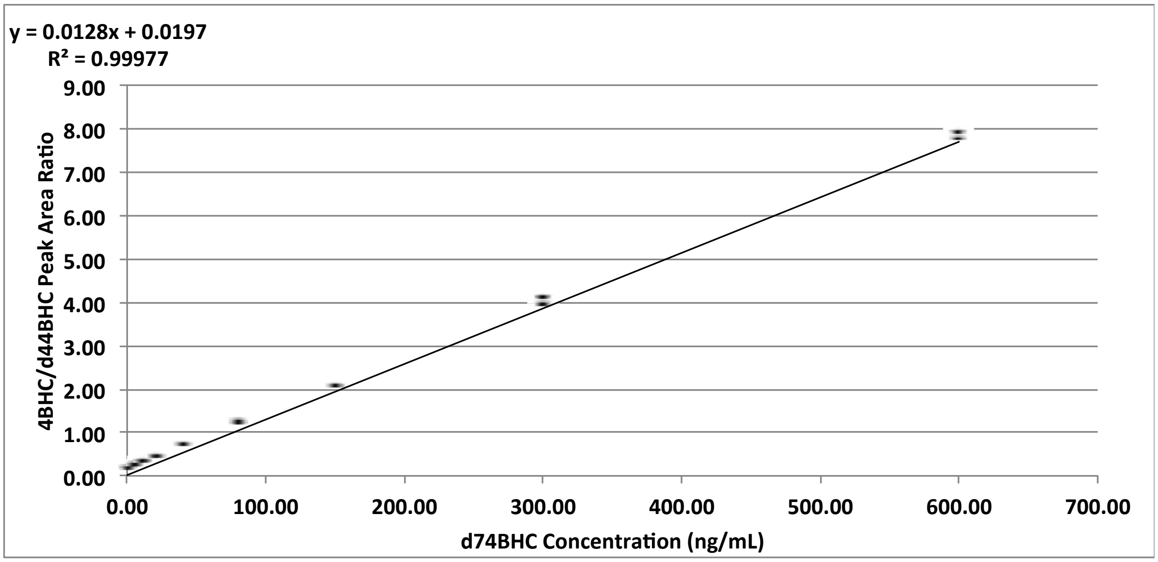


Figure 2.7 There was no significant difference in cholesterol levels before (BT) and after rifampin treatment (AT) (n = 23, p = 0.1474).

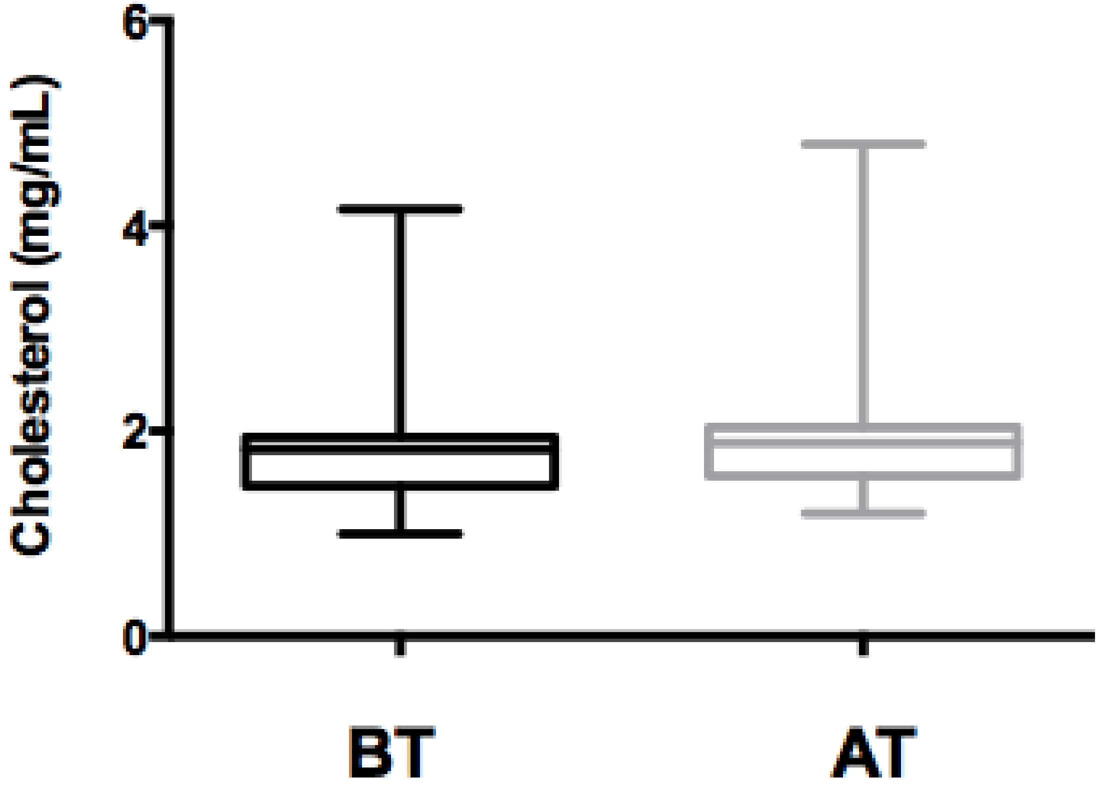


Figure 2.8 4βOHC plasma concentrations were normally distributed at baseline (n = 23, p = 0.11).

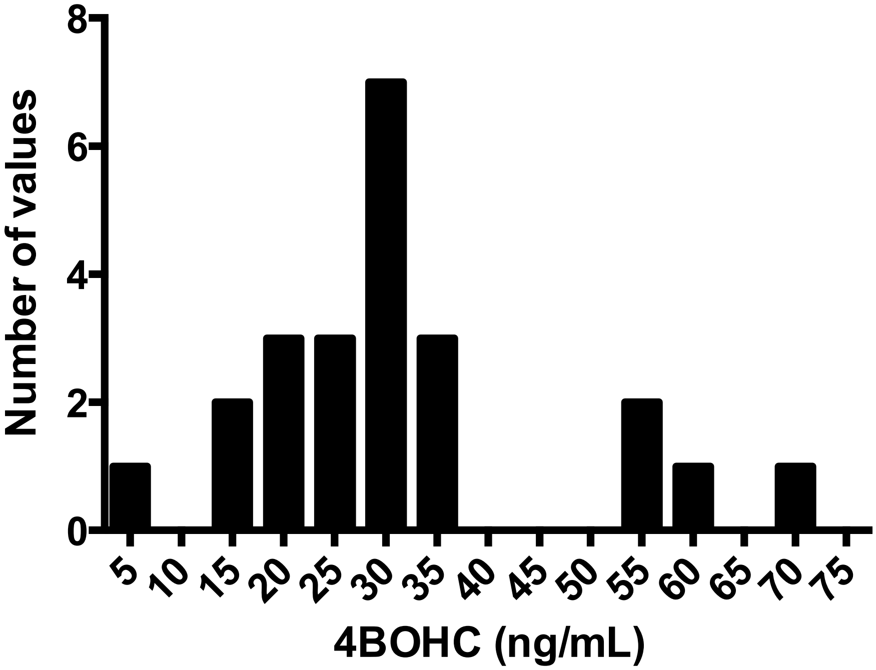


Figure 2.9 4βOHC concentrations were significantly higher in female subjects compared to male subjects at baseline (p = 0.0179).

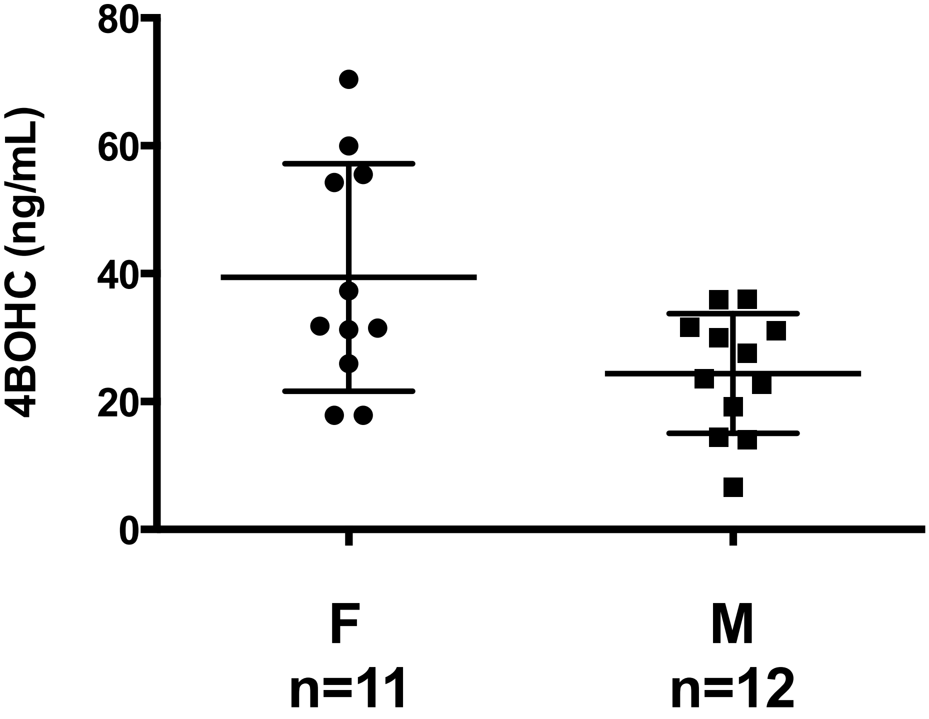


Figure 2.10 4βOHC concentrations were significantly higher after rifampin treatment (AT) compared to before treatment (BT) (57.15 ± 5.99 ng/mL vs. 32.99 ± 4.62 ng/mL, p = 0.0028).

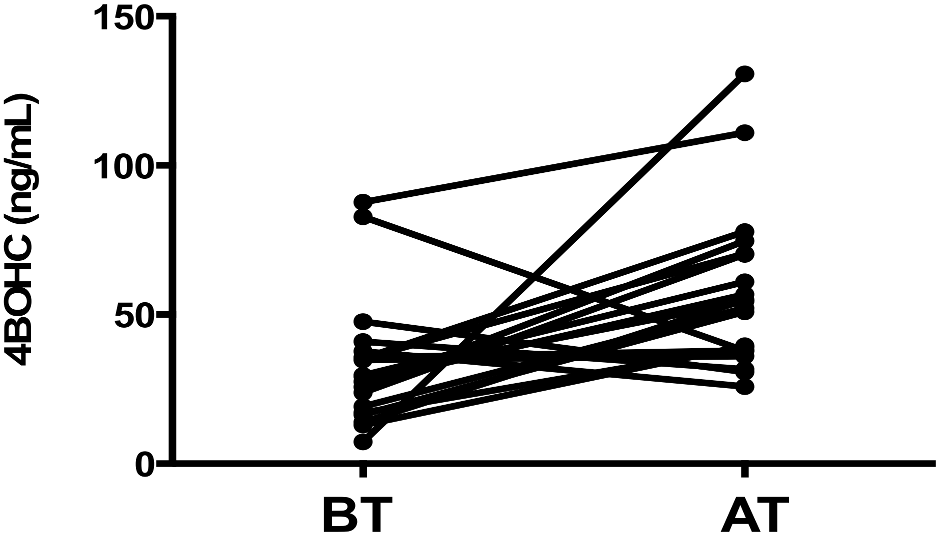


Figure 2.11 Spearman rank correlation (ρ) was performed to determine correlation coefficient between midazolam oral clearance (MDZ CL/F) and 4β-hydroxycholesterol concentrations (4βOHC). There was a moderate correlation between MDZ CL/F and 4βOHC (n = 138, ρ = 0.535, p < 0.0001).

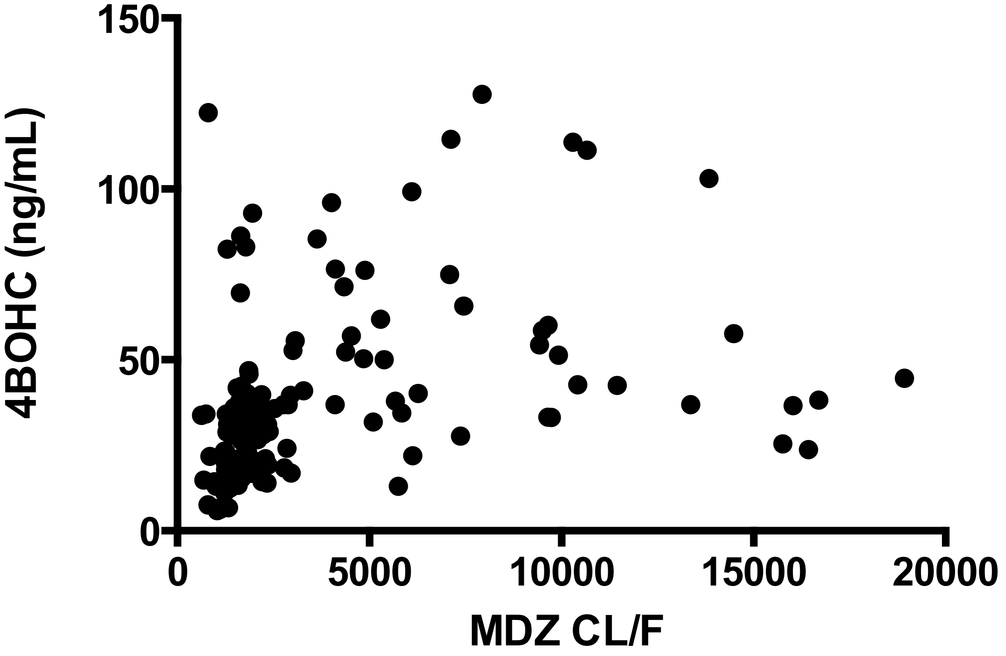


Figure 2.12 Change in midazolam clearance after rifampin treatment (MDZ'/MDZ) vs. change in 4βOHC concentration after rifampin treatment (4βOHC'/4βOHC) (r^2 = 0.306. p = 0.114).

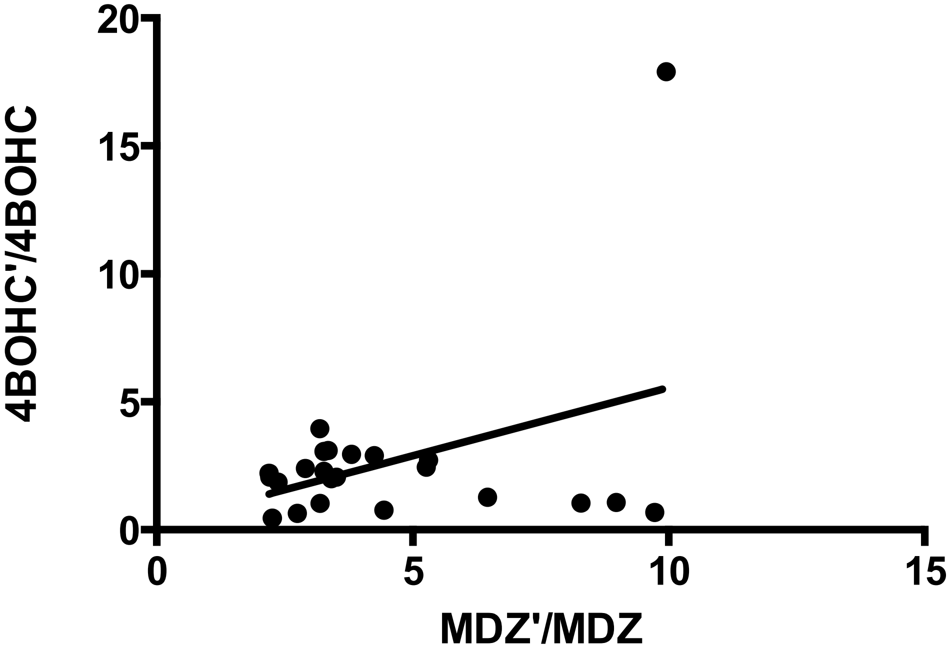


Figure 2.13 4βOHC/C ratios were normally distributed at baseline (p = 0.11, n = 23)

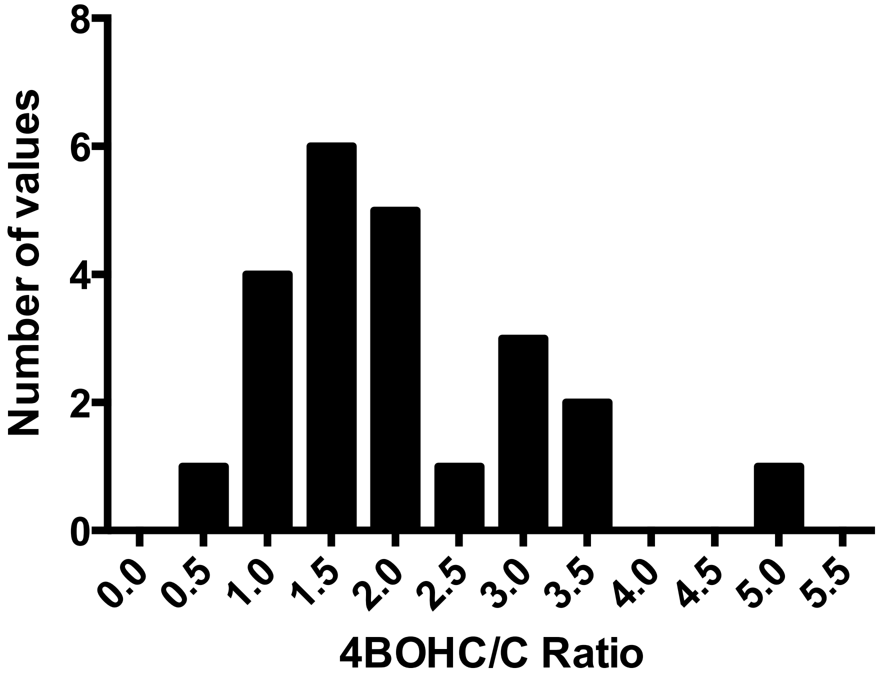


Figure 2.14 Female subjects had higher 4βOHC/C ratios at baseline compared to men (0.25 ± 0.038 vs. 0.155 ± 0.02, p = 0.033).

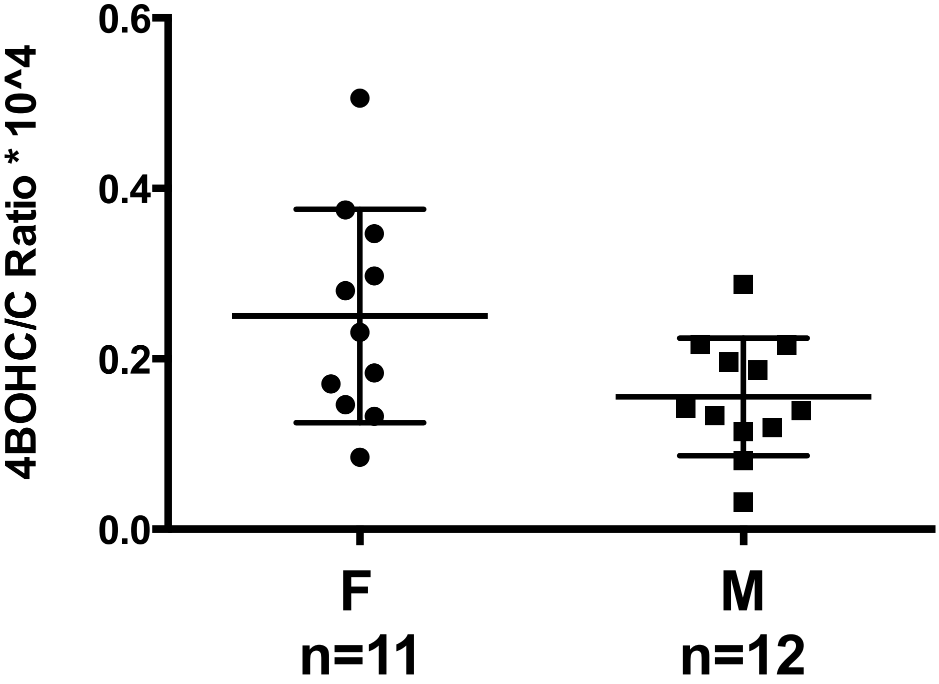


Figure 2.15 4βOHC/C molar ratios were higher after rifampin treatment (AT) compared to before treatment (BT) (0.291 ± 0.032 * 10^4 vs 0.186 ± 0.024 * 10^4, p = 0.0025).

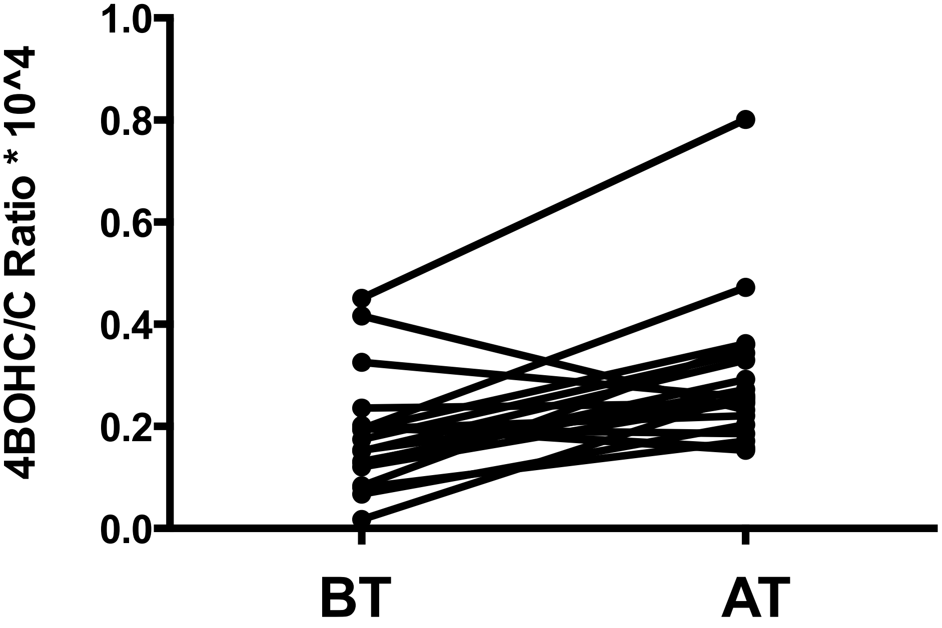


Figure 2.16 The 4β-hydroxycholesterol/cholesterol ratios (4βOHC/C) were moderately correlated with midazolam oral clearance (ρ = 0.4832, p < 0.0001).

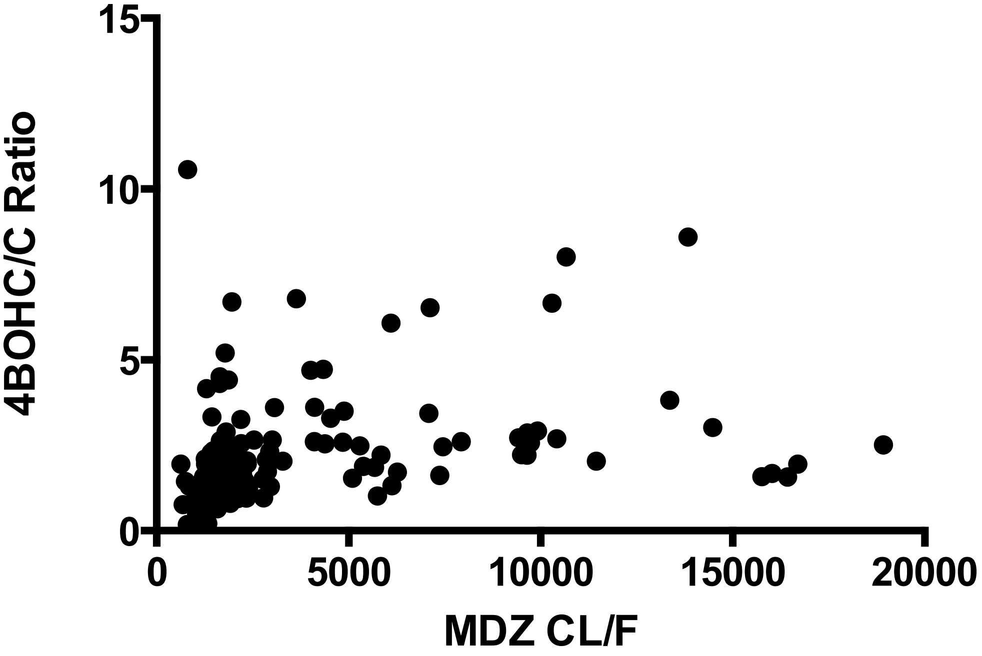


Figure 2.17 The change in midazolam clearance after rifampin treatment was not well predicted by the change in 4BOHC/C ratio after rifampin treatment ($r^2 = 0.2329$, $p = 0.0229$).

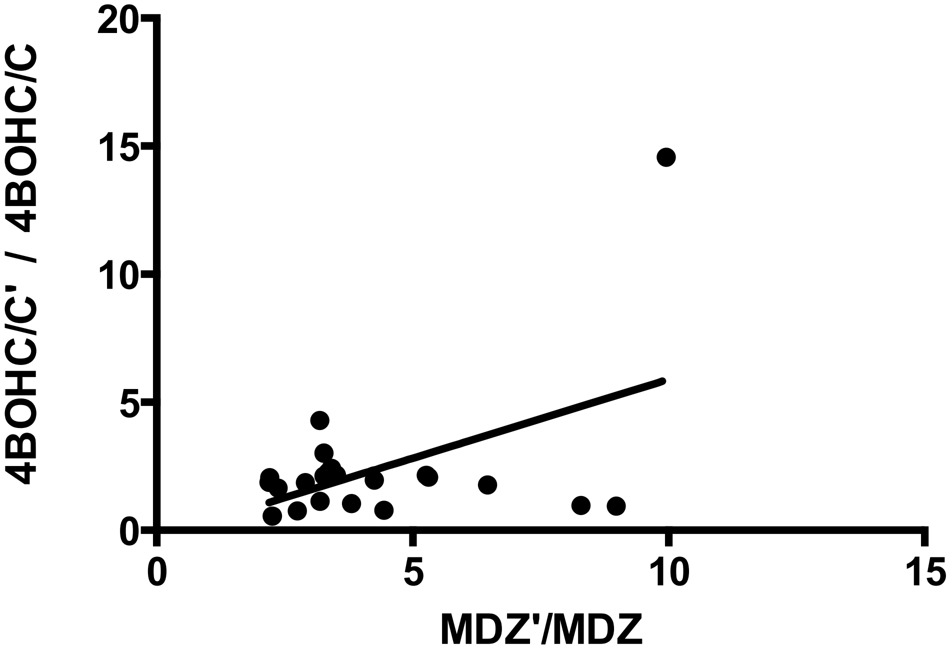


Figure 2.18 6βOHF/F baseline ratios among subjects were distributed normally ($p = 0.67$, $n = 23$).

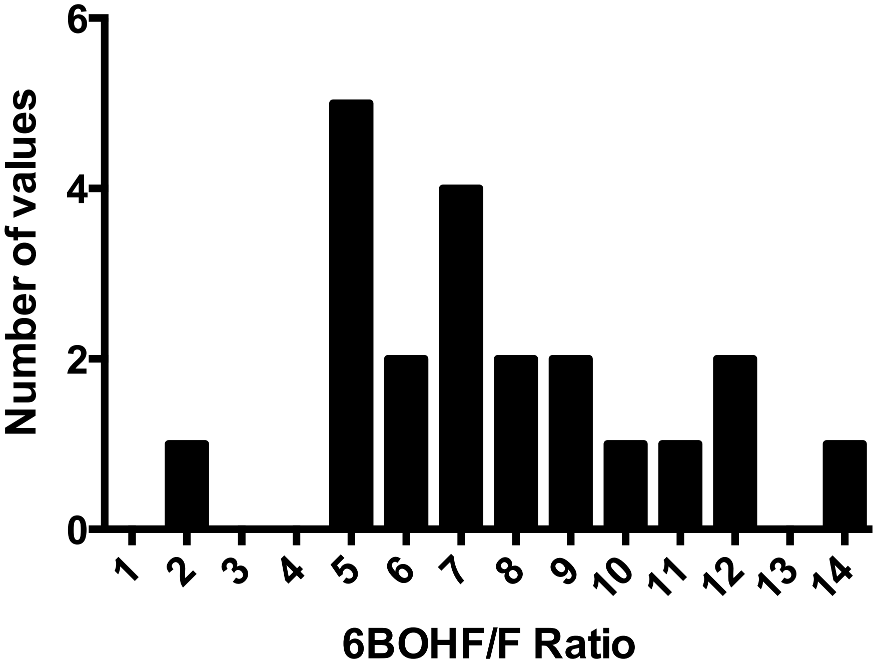


Figure 2.19 6βOHF/F concentration were significantly higher after rifampin treatment (AT) compared to before treatment (BT) (n=19, 28.68 ± 3.3 vs. 7.04 ± 0.88, p < 0.0001).

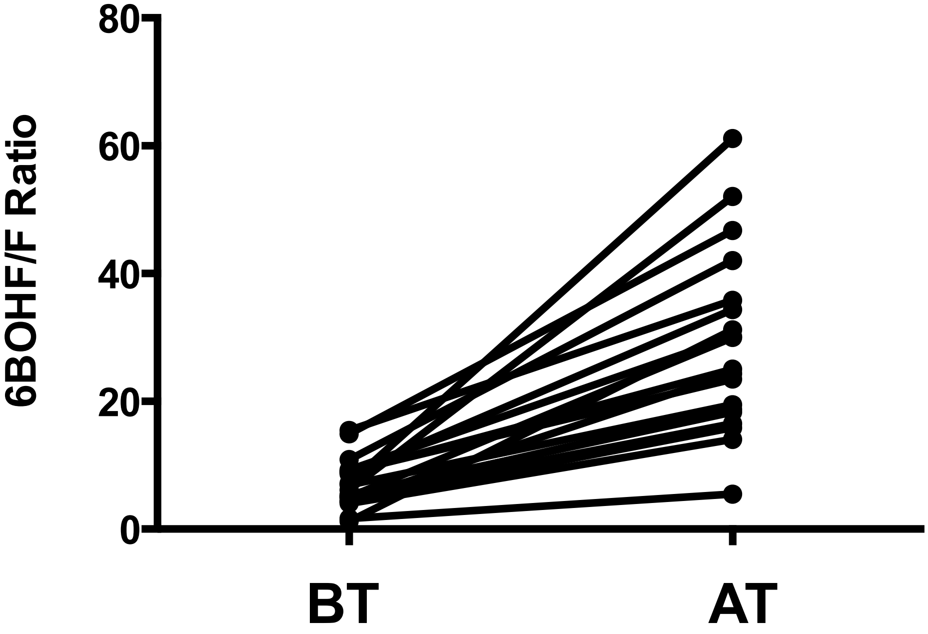


Figure 2.20 The 6β-hydroxycortisol/cortisol ratios (6βOHF/F) were moderately correlated with oral midazolam clearance (ρ = 0.6563, p < 0.0001).

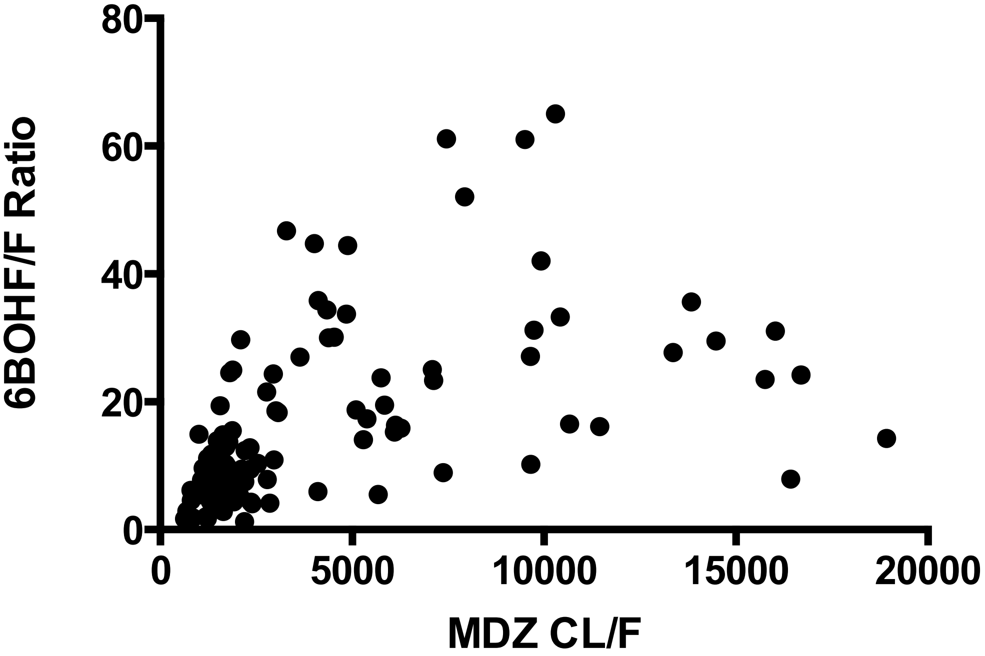


Figure 2.21 The change in 6βOHF/F ratio (6βOHF'/6βOHF) after CYP3A induction by rifampin was not correlated with the change in midazolam clearance (MDZ'/MDZ) ($r^2 = 0.017$).

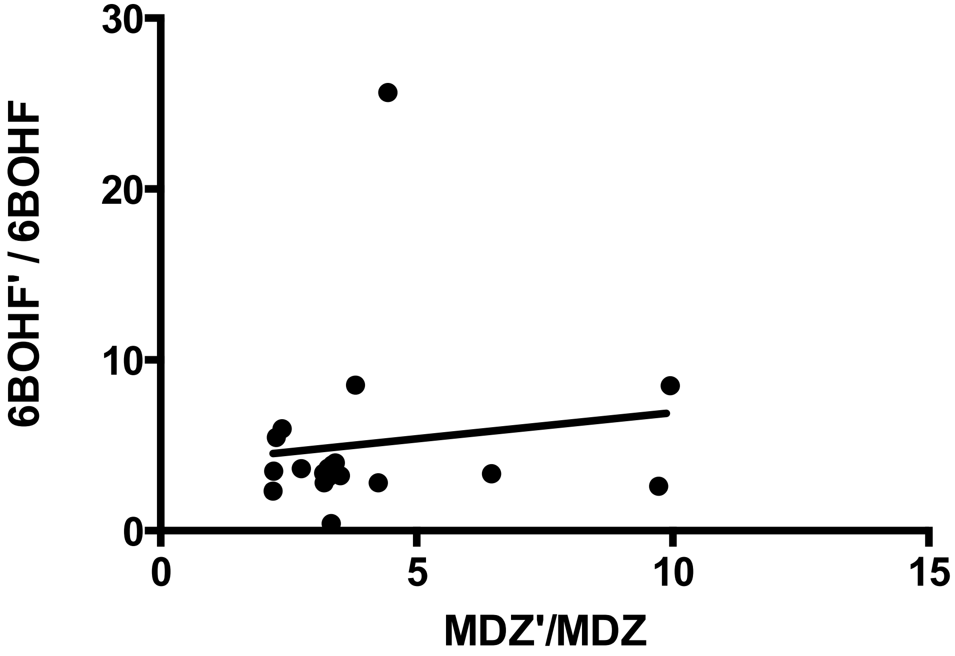


Figure 2.22 6βOHE/E ratios were distributed normally among subjects (p = 0.16, n = 23).

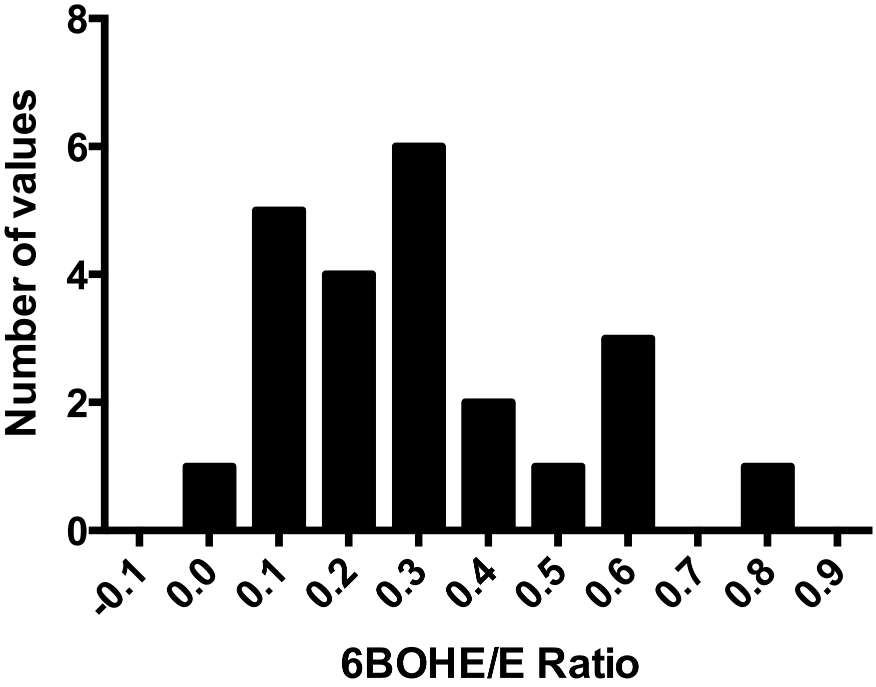


Figure 2.23 6βOHE/E ratio was significantly higher among patients following rifampin treatment (AT) compared to before treatment (BT) (1.27 ± 0.16 vs. 0.32 ± 0.05, $p < 0.0001$).

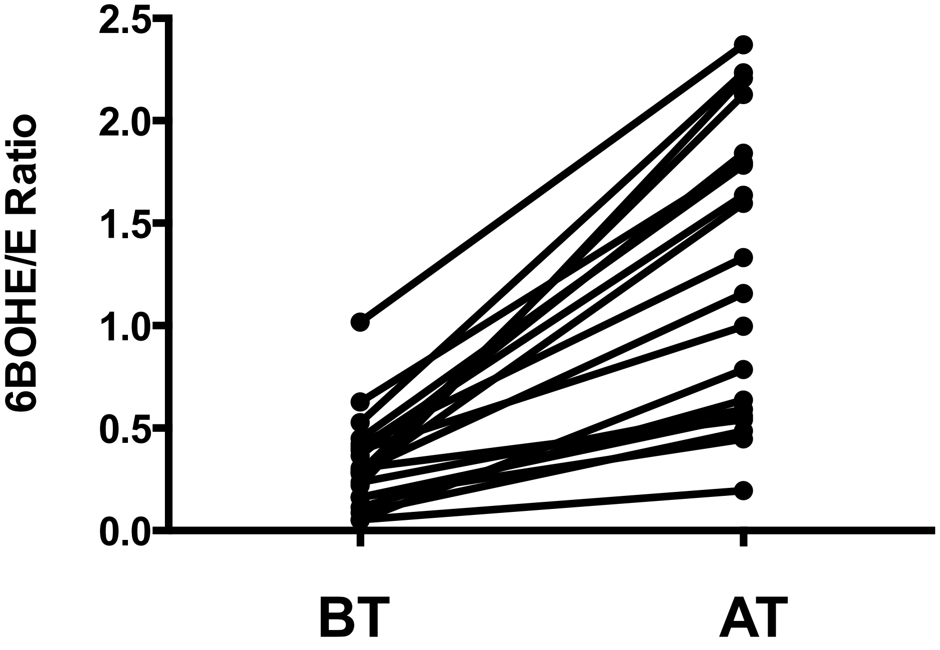


Figure 2.24 There was a moderate correlation between midazolam oral clearance and 6β-hydroxycortisone/cortisone ratios ($\rho = 0.5972$, $p < 0.0001$).

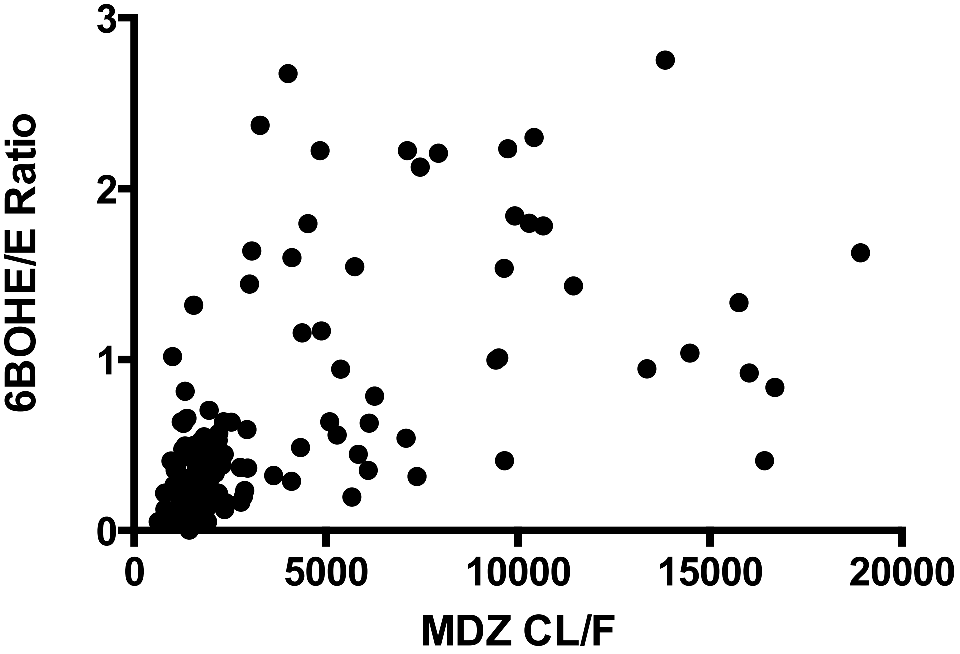


Figure 2.25 The change in midazolam clearance (MDZ'/MDZ) was not associated with a change in 6βOHE/F ratio (6βOHE'/6βOHE) after CYP3A induction by rifampin ($r^2 = 0.022$, $p = 0.7364$).

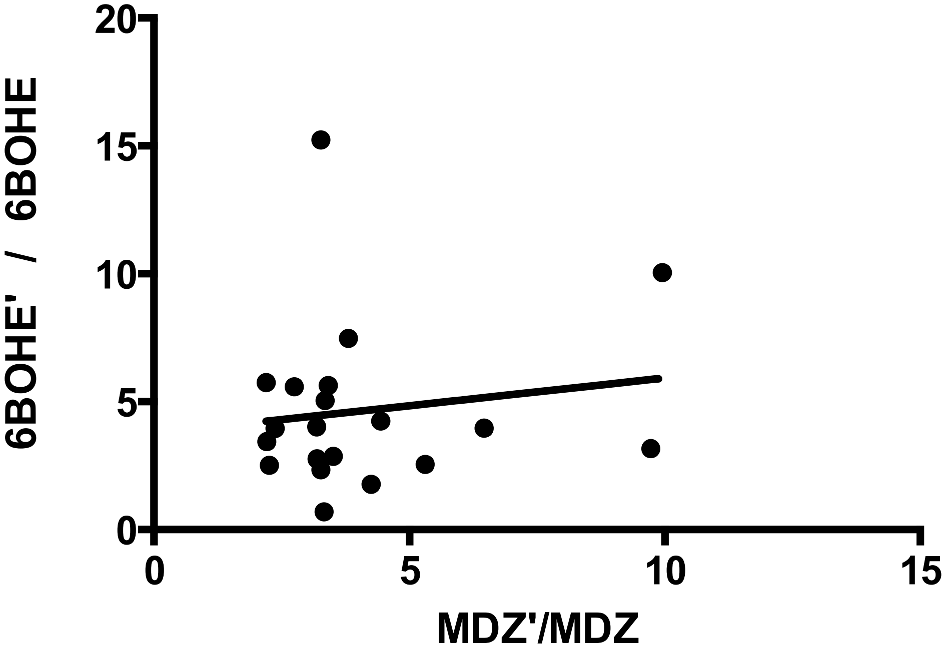


Figure 2.26 The 4β,25-dihydroxyvitamin D_3/25-hydroxyvitamin D_3 (4β,25OHD3/25OHD3) ratio was higher following rifampin treatment (AT) compared to before treatment (BT) (3.8 ± 0.44 vs. 2.12 ± 0.16, p = 0.0002).

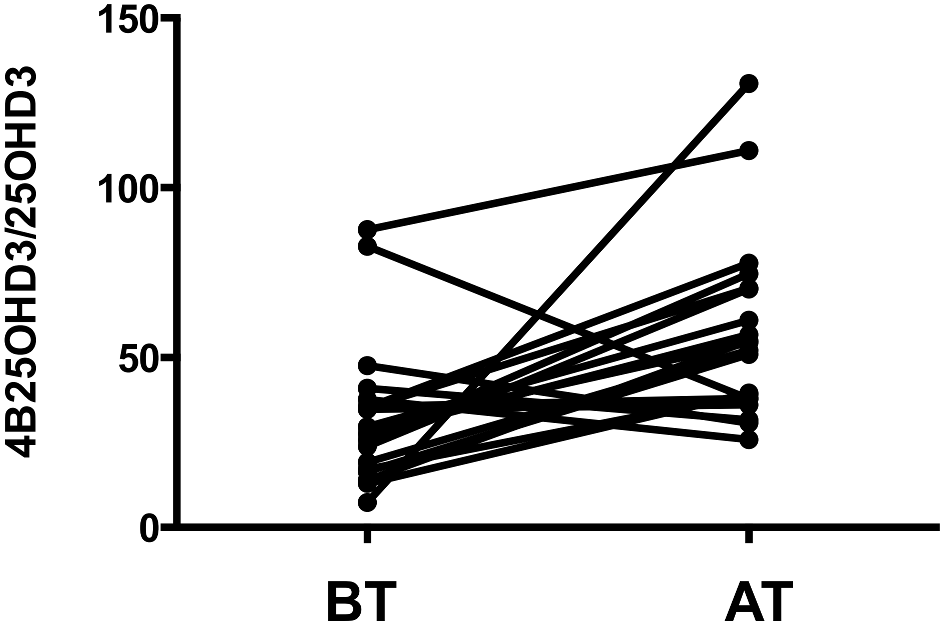


Figure 2.27 Midazolam oral clearance (MDZ CL/F) was poorly correlated with 4β,25-dihydroxyvitamin D_3/25-hydroxyvitamin D_3 ratio (4β,25OHD3/25OHD3) ($\rho = 0.1$, $p = 0.5599$)

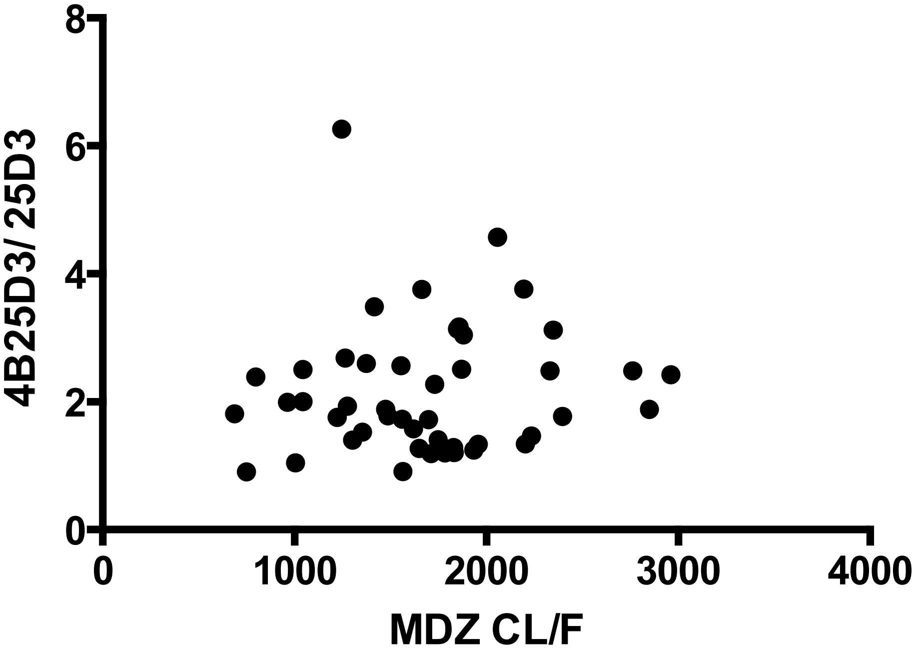


Figure 2.28 Correlation between change in midazolam oral clearance (MDZ'/MDZ) and change in 4β,25-dihydroxyvitamin D_3: 25-hydroxyvitamin D_3 (4BD3R'/4BD3R) ratio following rifampin treatment ($r^2 = 0.0026$, $p = 0.7563$).