Estrogen Regulation of the Cytochrome P450 3A Subfamily in Humans

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**Running Title:**
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**Abbreviations:**
CYP3A – cytochrome P450 3A subfamily
QRT-PCR – quantitative real-time PCR

**Recommended Section:**
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This study examines the possible role of estrogen in regulating the expression of the human CYP3A subfamily; CYP3A4, CYP3A5, CYP3A7, and CYP3A43. To accomplish this goal, mRNA was quantified from human livers and endometrial samples and total CYP3A protein levels evaluated by Western immunoblot analysis of the liver samples. The human endometrial samples were from both pre-menopausal and post-menopausal women. The pre-menopausal endometrium was either in the proliferative or secretory phase, while for the post-menopausal endometrium samples, the women had been treated with either a placebo or estropipate, an estrogen substitute. After analyses, CYP3A4 mRNA was shown to have lower hepatic expression in females than in males. In the endometrium, CYP3A4 and CYP3A43 are down-regulated by estrogen while CYP3A5 is expressed at higher levels during the secretory phase. CYP3A7 was not detected in the endometrium. In addition, the CYP3A subfamily showed increased mRNA expression in the liver as age increased. The expression levels of total CYP3A protein and total CYP3A mRNA showed good correlation. Despite apparent regulation of CYP3A4 mRNA expression by estrogen, the effects of estrogen may be overshadowed by additional regulators of gene expression.
It is known that men and women do not respond equally to certain drugs administered for therapeutic relief (Harris et al., 1995); however, the reasons have yet to be fully identified and appear to be multifaceted. A variety of possibilities may contribute to therapeutic discordance, but a probable explanation is that estrogen directly or indirectly regulates a gene or genes responsible for the differences in response. Of the genes that could cause variations in drug response, a metabolizing enzyme would be a likely candidate. In particular, the cytochrome P450 (CYP450) superfamily is known for metabolizing a diverse set of substrates, with the cytochrome P450 3A (CYP3A) subfamily metabolizing the most diverse range of substrates (Guengerich, 1999). It is possible that an estrogen-regulated CYP450 of the CYP3A subfamily contributes to the gender dimorphism exhibited in the drug response between women and men.

In humans, four CYP3As are known to be expressed; CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 is the most abundant CYP450 (Guengerich, 1995) and contributes to the metabolism of the largest percentage of clinically-used drugs (Evans and Relling, 1999). CYP3A5 is polymorphically expressed; and in some individuals, CYP3A5 expression can equal that of CYP3A4 (Kuehl et al., 2001). CYP3A7 is known for its expression in fetal liver (Komori et al., 1990); however, expression has also been shown in some adult tissues (Burk et al., 2002). The latest human CYP3A to be discovered, CYP3A43, is thought to have the strongest mRNA expression in the liver and testis (Westlind et al., 2001) or the prostate (Gellner et al., 2001), but the expression of CYP3A43 is significantly lower than that of CYP3A4 (Gellner et al., 2001) and CYP3A5 (Westlind et al., 2001). Some of the substrates known to be metabolized by the CYP3As include erythromycin (Brian et al., 1990), cyclosporine (Kronbach et al., 1988), warfarin (Kaminsky and Zhang, 1997), and 17β-estradiol (Lee et al., 2001).

Conducting in vivo research based on humans is extremely difficult, especially when
examining estrogen regulation. Most of the techniques used in animal models are not acceptable for use on humans. Therefore, many researchers have relied on available tissue resources.

Recent studies of gender-based CYP3A expression in the liver include those of Westlind-Johnsson, et al., (2003) and Wolbold, et al. (2003). Westlind-Johnsson, et al., did not uncover a significant difference between men and women for CYP3A4, CYP3A5, or CYP3A43 mRNA expression; however, Wolbold, et al., reported that women had two-fold higher expression of CYP3A4 mRNA and three-fold higher protein expression than men.

The classical tissue for assaying CYP450 is the liver, since it is the major site of detoxification for the body and the principal location of drug metabolism. Comparing male and female livers will not directly identify genes that are regulated in part by estrogen; however, knowing gender differences in mRNA expression may suggest possible candidates for further investigation.

The endometrium may be a better tissue to investigate in terms of responsiveness to estrogen. It is not a primary site for drug metabolism; nevertheless, the CYP3As are present in the endometrium and may play an endogenous role. For pre-menopausal endometrium, studies of CYP3A mRNA expression include studies by Schuetz, et al. (1993), Hukkanen, et al. (1998), and Sarkar, et al. (2003). Schuetz, et al., reported that CYP3A7 was expressed higher in the secretory phase than the proliferative phase. Hukkanen, et al., detected CYP3A4 and CYP3A5 but did not explore differences between phases and CYP3A7 was not detected. Sarkar, et al., found a significant difference between the two phases for CYP3A7, but not CYP3A4.

Similar to the results reported for CYP3A expression in liver, the literature discussing CYP3A expression in pre-menopausal endometrium is equally contradictory. Hukkanen, et al., (1998) did not detect CYP3A7 expression; however, Schuetz, et al., (1993) and Sarkar, et al.,
(2003) did detect CYP3A7 expression, but they contradict each other in terms of the phase in which CYP3A7 is expressed at higher levels. Sarkar, et al., showed that CYP3A7 was expressed at higher levels in the proliferative phase while Schuetz, et al., found higher expression during the secretory phase than during the proliferative phase.

The current study provides a more inclusive exploration into the relationship between estrogen and the human CYP3A subfamily by examining the expression of the CYP3A forms utilizing samples from the liver, pre-menopausal endometrium, and post-menopausal endometrium with and without exogenous estrogen treatment. Additionally, in the current paper, the effect of age on CYP3A expression has also been analyzed.
**Human Liver and Endometrial Samples:**

A total of twenty-seven liver samples, twenty post-menopausal endometrial samples, and thirteen pre-menopausal endometrial samples were obtained from various sources.

Of the human liver samples, twenty (HL1-HL20) were obtained under approved protocols from the Medical College of Wisconsin or the Indiana University School of Medicine, six (HL21-HL26) from Steve Strom at the University of Pittsburgh, and one (HL27) from the International Institute for the Advancement of Medicine (IIAM; Exton, PA). Most of the livers originated from transplant sources, although four originated from biopsy. All liver samples were from persons that died of accidental causes.

Thirteen human pre-menopausal endometrial samples were collected from uteri at the time of hysterectomy. Of the thirteen hysterectomies, seven were performed for cervical cancer, four for leiomyomas, and two for ovarian neoplasms. All thirteen samples were histologically normal. Determination of the phase of the menstrual cycle, whether proliferative or secretory, was made by histological staining.

The twenty human post-menopausal endometrial samples consisted of two groups of ten. One group was obtained from women treated with a placebo while the other was obtained from women treated with estropipate (Wyeth Research; Philadelphia, PA), formerly identified as piperazine estrone sulfate. Each group was randomly selected from a larger group of samples. The estropipate-treated women were administered 0.625 mg of estropipate daily for six months, prior to sample collection or biopsy. Please refer to Table 1 for clinical information regarding the post-menopausal endometrial samples.

**RNA Isolation From Human Samples:**

For each human liver sample, 100 mg of whole liver was added to 1 mL of RNA STAT-
60 Reagent (CS-111; Tel-Test; Friendswood, TX). The samples were homogenized until no
debris was visible and allowed to sit at room temperature for five minutes. After adding 200 μL of
chloroform, the mixtures were shaken for fifteen seconds and allowed to sit at room temperature
for two to three minutes. Then, the samples were centrifuged at 4°C for fifteen minutes. The
clear supernatant fractions were transferred to clean tubes, mixed with 500 μL of isopropanol,
and allowed to sit at room temperature for five to ten minutes. Next, the mixtures were
centrifuged at 4°C for ten minutes and the supernatant fractions were discarded. The remaining
pellets were washed with 1 mL of 75% ethanol and centrifuged at 4°C for five minutes. The
supernatant fractions were discarded, the pellet was dried and resuspended in water treated with
diethyl pyrocarbonate (DEPC).

The endometrial samples were processed as specified by Deng, et al. (Deng et al., 2003).

All samples were DNase I-treated and stored at -80°C until analysis.

**Quantitative Real-Time PCR:**

All liver and endometrial RNA samples were quantified using quantitative real-time PCR
with an ABI Prism 7700 sequence detection system (Applied Biosystems; Foster City, CA).

The primer and probe sets were designed to overlap the exon/exon junctions of mRNA
using the Primer Express Software (Applied Biosystems; Foster City, CA). Regions of high
homology between the human CYP3As were excluded during the design. The probes contain a 5'
6-FAM and a 3' TAMRA. The standards were oligonucleotides spanning the region of the
amplimer, three bases upstream of the forward primer to three bases downstream of the reverse
primer. The primers, probes, and standards utilized in this study are listed in Supplemental
Material Table 1 and were synthesized by SeqWright (Houston, TX), Integrated DNA
Technologies (Coralville, IA), or BioSource (Camarillo, CA).
For each sequence quantitated, four parameters were measured: 5-log dilution series of the oligonucleotide standards, no template controls (NTC), unknown samples, and no amplification controls (NAC) per unknown sample. The NTC omits RNA to ensure contaminant-free reagents, while the NAC omits the reverse transcriptase to ensure the lack of DNA contamination. All standards, NTCs, and unknown samples were conducted in triplicate with one NAC per unknown sample. The assays used reagents provided in the Superscript One-Step RT-PCR with Platinum Taq kit (10928-042; Invitrogen; Carlsbad, CA). A total of 50 μL was aliquoted to each well and consisted of 1X Reaction Mix (a buffer with 0.4 mM of each dNTP and 2.4 mM magnesium sulfate), an additional 1.8 mM magnesium sulfate, 800 nM each of forward and reverse primers, and 200 nM probe. The standards, samples, and NTCs contained 1 μL of RT/Taq mixture, while the NACs contained 1U of Platinum Taq DNA Polymerase (10966-018; Invitrogen; Carlsbad, CA).

The template used for the standards was a serial ten-fold dilution of the oligonucleotide standard ranging from 100 aM to 1 pM (quantity calculation described below). The NTC contained water instead of a template. For the liver samples, a template of 100 ng of RNA was used for each well containing samples and NAC. On the other hand, the endometrial samples had a template of 10 ng of RNA for the β-actin assay and 40 ng of RNA for the CYP3A transcripts quantitated.

Each assay was tested for cross-reactivity using the assay mixture outlined above. The template mixture for the CYP3A4 assay contained the full length cDNA for CYP3A5, CYP3A7, and CYP3A43. As a positive control, 1 ng of the full length CYP3A4 cDNA was used as the template, while the negative control had no template. Likewise, the other CYP3A assays were tested for cross-reactivity. Only in the positive control reactions did amplification occur,
suggesting no cross-reactivity among assays.

The results are reported in quantity of transcripts. By using the molecular weight of the standards, the number of grams to achieve the desired number of standard templates was calculated. ABI Prism's software then used the quantity of the standards to calculate the quantity of the samples.

**Western Immunoblot Analysis:**

From the human liver samples, microsomes were prepared as previously described (Kalsotra et al., 2002). The protein concentration of the microsomes was determined using the “BCA Protein Assay Kit” from Pierce (23225; Rockford, IL). A total of 50 μg of each sample was loaded onto 4-20% gradient gels (161-1105EDU; Bio-Rad Laboratories; Hercules, CA) and electrophoresed at 100V until the dye-front reached the end of the gel. The gels were transferred to pure nitrocellulose membranes using the suggested protocol supplied by the Semi-Dry Electrophoretic Transfer Cell from Bio-Rad (170-3940; Hercules, CA). The membranes were blocked overnight using 5% (w/v) evaporated milk in Tris-buffered saline containing 0.0005% (v/v) Tween 20. The membranes were probed at a 1:1000 dilution using the CYP3A4 polyclonal antibody from Research Diagnostics Inc. (RDI-CYP3A4abr; Flanders, NJ) as the primary antibody for two hours and at 1:1250 using a Goat Anti-Rabbit – Horseradish Peroxidase Conjugate (170-5046; Bio-Rad Laboratories; Hercules, CA) as the secondary antibody for one hour. “SuperSignal West Pico Chemiluminescence” (34080; Pierce; Rockford, IL) was used for detection in the ChemiGenius® (SynGene; Frederick, MD). GeneTools 3.04b by SynGene (Frederick, MD) quantitated the bands.

For separation of the individual CYP3A isoforms, CYP3A4 (456207; GenTest; Bedford, MA), CYP3A5 (456235; GenTest; Bedford, MA), and CYP3A7 (456237; GenTest; Bedford, MA).
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MA) were purchased. CYP3A43 was excluded since it is not commercially available. All enzymes were loaded either individually or mixed.

**Statistical Analyses:**

In the liver samples, gender and age were examined for effects. The data were transformed to normality using the Box-Cox transformation. Power transformations of -0.25, logarithm, -0.25, and logarithm were used in the case of CYP3A4, CYP3A5, CYP3A7, and CYP3A43, respectively.

A previous study using human liver samples was reported by Westlind-Johnsson, *et al.* (Westlind-Johnsson et al., 2003); therefore, these data were also examined for gender and age effects utilizing the same statistical methods used to analyze the new data presented in this study. Power transformations of 0.25, 0.25, 0.25, and 0.50 were used in the case of CYP3A4, CYP3A5, CYP3A43, and PXR, respectively.

For any CYP3A mRNA expression in the human liver that showed both an age and gender effect, a nonparametric test (Kruskal-Wallis) was conducted to confirm the parametric results. This analysis divided the human liver samples into two groups; females 55 years of age and under versus males of all ages and females over the age of 55. The age of 55 years was chosen as a conservative approximation of the median age for menopause.

In the endometrial samples, differences in mRNA expression were tested between proliferative and secretory stages in pre-menopausal individuals, as well as the placebo and estropipate treatments in the post-menopausal individuals. For CYP3A5, only data from pre-menopausal individuals were available due to the lack of available post-menopausal samples. Power transformations of 0.5, logarithm, and -0.25 were used in the case of CYP3A4, CYP3A5, and CYP3A43, respectively. Differences between different stages and treatments were tested
using Scheffe’s test for multiple comparisons.

For all the above analyses, in the SAS proc mixed procedure, compound symmetry covariance structure was used on observations from the same individual. We also standardized each observed level of RNA by the average level of β-actin in each individual.

The mRNA expression levels of all four enzymes collected in this study were combined for each sample and analyzed using a power transformation of -0.25. The Kruskal-Wallis analysis described above was also conducted on the combined data. The total protein expression levels were analyzed using a logarithmic power transformation and analyzed by the Kruskal-Wallis analysis described above. Spearman's correlation was conducted between the total mRNA expression and the total protein expression.

A p-value ($p$) less than 0.05 is considered to be statistically significant.
In this study, RNA samples from two human tissues were analyzed as well as protein expression from the liver. The first tissue analyzed was a comparison of human liver samples from males and females for CYP3A mRNA expression levels. The second tissue analyzed was the human endometrium.

A total of twenty-seven human liver samples were collected. For each sample, the mRNA was quantified by quantitative real-time PCR (QRT-PCR) for β-actin, CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNA expression. Each CYP3A was normalized against β-actin, and the results are listed in Supplemental Material Table 2.

Statistical analyses were conducted on the CYP3A4 mRNA levels of the liver samples for age and gender effects. For CYP3A4 mRNA, age and gender effects were found to be statistically significant covariates, as shown in Table 2, with younger females (Figure 1A) expressing lower mRNA levels than younger males (Figure 1B). For CYP3A7 and CYP3A43, only an age effect was found to be statistically significant. For these three genes, mRNA expression was found to increase with age, as seen in Figures 1A, 1B, 1D, and 1E. The estimated model for each is given in Supplemental Material Table 3 and shown in Figure 1. On the other hand, no significant age or gender effect was found for CYP3A5, as shown in Figure 1C.

As confirmation of the results obtained from the CYP3A4 regression analyses in this study, the Kruskal-Wallis non-parametric test was used on the mRNA expression levels of hepatic CYP3A4. The data divided into two groups, as described in the Methods section, reveals a significant result ($p = 0.0190$), with women 55 years and younger had a much lower expression of CYP3A4 mRNA. More samples, particularly from younger females, would allow for a refinement of the statistical model.
Recently, a similar study was reported by Westlind-Johnsson, et al. (Westlind-Johnsson et al., 2003). In their study, the mRNA levels for CYP3A4, CYP3A5, CYP3A43, and PXR were quantified in the human liver after normalization against human acidic ribosomal phosphoprotein. The results of the Westlind-Johnsson, et al., study were analyzed in a similar manner to the data generated in this study. An age effect was found to be statistically significant for CYP3A4, CYP3A5, and CYP3A43, as shown in Table 2. CYP3A7 was not quantified in the Westlind-Johnsson, et al., study. Similar to the results reported here, the data from Westlind-Johnsson, et al., show that the level of mRNA increases with age. The estimated models are given in Supplemental Material Table 3 and shown in Figure 2. No statistically significant age or gender effect was found for PXR.

A total of twenty-seven human livers were obtained and assayed for the mRNA levels of all four human CYP3A forms (Supplemental Material Table 2) with β-actin as the normalizer. In the study conducted by Westlind-Johnsson, et al., forty-six human livers were assayed for CYP3A4, CYP3A5, CYP3A43, and PXR mRNA with human acidic ribosomal phosphoprotein as the normalizer (Westlind-Johnsson et al., 2003). Since these two studies used different genes for normalization, the results were not combined.

In addition to the human liver samples, CYP3A mRNA levels from human endometrial samples were analyzed. Two sets of samples from human endometria were obtained, pre-menopausal and post-menopausal. The pre-menopausal samples were separated into proliferative and secretory phases. A few samples were found to be inactive and atrophic, suggesting the possibility that the women were undergoing menopause. Other samples were found to have progestational effects, suggesting that this group of women were using oral contraceptives. The endometrial samples that were inactive or showed progestational effects were excluded from
analyses. Supplemental Material Table 4 shows the levels of CYP3A gene expression, normalized to β-actin, for the samples that were included and excluded from statistical analyses.

The post-menopausal samples were divided into two treatment groups, either placebo or estropipate. To determine which human CYP3A genes should be quantified, a pool of ten randomized samples for initial testing was generated for both groups; the results are shown in Supplemental Material Table 5. After quantitating the pool samples, a new group of ten randomized samples were analyzed for both groups and normalized to β-actin; the results are shown in Supplemental Material Table 6. Clinical information regarding each group is compiled in Table 1. For expression levels for CYP3A4 mRNA, the placebo-treated endometrium was higher than the estropipate-treated endometrium and pre-menopausal (both phases) endometrium, as shown in Figure 3A. The multiple comparison adjusted p-values were determined for placebo-treated versus estropipate-treated (p = 0.0213), placebo-treated versus proliferative phase (p < 0.0001), and placebo-treated versus secretory phase (p = 0.0019). Verification of the results was obtained using the Kruskal-Wallis non-parametric test which gave a statistically significant p-value (p = 0.0003). For CYP3A5 (Figure 3B), the proliferative phase was significantly lower than the secretory phase (p = 0.0058). The Kruskal-Wallis non-parametric test that was used as confirmation of CYP3A5 had a statistically significant p-value (p = 0.0087). No statistical differences were found for CYP3A43 (Figure 3C). Table 3 provides the estimated means and standard errors for these determinations.

From the human liver samples gathered in this study, all mRNA expression levels were combined for each sample and analyzed in a fashion similar to the analysis for CYP3A4. Both age (p = 0.0051) and gender (p = 0.0498) effects were found to be significant, but the interaction between the two was not statistically significant. The Kruskal-Wallis analysis for two groups was
The results presented thus far reveal associations between age and/or gender effects and mRNA expression levels for human CYP3A subfamily members as determined by QRT-PCR. In order to extend the results seen with mRNA expression to protein expression, the separation of individual CYP3A isoforms was attempted, as seen in Figure 4. The CYP3A isoforms were not separated by electrophoresis, which is consistent with previous results reported (Domanski et al., 2001) using different conditions. Therefore, the total CYP3A protein expression was examined for associations with age and/or gender effects. Using similar statistical analyses for total protein expression, as had been conducted for total mRNA expression, yielded no statistically significant relationships with age and/or gender. However, the Spearman's correlation coefficient (0.55617), which is a measure of the degree of relatedness between variables, was significant ($p = 0.0026$) for the relationship between total protein and total mRNA expression.

The human endometrium samples were not analyzed due to limited quantity of samples obtainable at collection.
The focus of this study was to determine whether estrogen regulates the expression of the human Cytochrome P450 3A subfamily. In order to accomplish this goal, liver and endometrial samples were assayed for the mRNA levels for CYP3A4, CYP3A5, CYP3A7, and CYP3A43 and normalized against β-actin. The mRNA of the liver was then compared to liver protein expression.

The increase in liver mRNA expression with age is consistent across all human CYP3A isoforms. The reason for this observation is not obvious, but one possibility is a tie to a common function of the CYP3A isoforms, the ability to metabolize exogenous compounds. Some of the exogenous substrates are known to up-regulate the expression of CYP3A4 through activation of the pregnane X receptor (PXR) (Goodwin et al., 1999). The possibility for increased PXR activation with age exists despite the fact that PXR mRNA expression was not found to increase with age, as shown by the data from the Westlind-Johnsson, et al., study (Figure 2D). The increase in PXR activation may be the result of an increase in medications as age increases; thus, no increase in PXR mRNA expression would be observed. This increase in medications taken as one ages would be expected in the general population. However, according to the known medical histories of the liver donors used in the current study, no such difference was evident. Thus, PXR activation causing the increase in CYP3A expression as age increases cannot be confirmed.

A reason the CYP3As increase with age may lie in the source of the liver samples obtained. Most of the livers were obtained from transplantation, others from biopsy, but the source is not known for all samples. Therefore, the liver samples may have been handled differently depending upon the source. Of the samples with known sources, the samples of persons aged 58 and younger were from transplantation, and the samples of persons aged 59 and older were from biopsy. Our observations suggest a difference in mRNA expression quantified
for each CYP3A versus the source of the sample; however, upon analysis, the difference was not statistically significant. Therefore, it is unlikely that the source of the samples had any significant effects on the expression levels of the CYP3As as a function of age.

In liver, CYP3A4 showed both gender and age dependencies. The results show that post-menopausal women express CYP3A4 mRNA at equal levels with men, which supports the notion that estrogen down-regulates CYP3A4 mRNA expression and is consistent with the results of the human endometrial samples in this study. Another study (Wolbold et al., 2003) reports differing findings, but did not examine age. The mean age for their female control group is about fifty (pre-menopausal) while the mean age for females exposed to drugs is about sixty-five (post-menopausal), in their study. Using the results of our study, the findings by Wolbold, et al., would be expected since their female control group is pre-menopausal (suppressed CYP3A4 expression) and their female test group is post-menopausal (unsuppressed CYP3A4 expression) versus men of similar ages (unsuppressed CYP3A4 expression).

Pre-menopausal and post-menopausal endometrial samples were also collected and the mRNA levels of the CYP3A isoforms were quantified. In order to conserve the post-menopausal samples, pools of ten samples each were generated from placebo and estropipate-treated. The results of the pools suggested that CYP3A7 could not be quantified but that the other three CYP3A forms would be good candidates for quantification. CYP3A4 was chosen to be quantified since it is the dominant CYP3A subfamily member expressed in the human liver and has been reported to show gender bias in the liver (Wolbold et al., 2003). Since CYP3A43 is the latest CYP3A discovered in humans, it was also examined. CYP3A5 was not analyzed due to limited sample quantity.
For the pre-menopausal endometrial samples, the results of this study are in agreement with the results of Sarkar, *et al.*, (Sarkar et al., 2003) for CYP3A4. Specifically, CYP3A4 remains constant during the proliferative and secretory phases (Figure 3A). Post-menopausal endometrial samples, treated with a placebo, show a drastic increase in CYP3A4 mRNA expression; however, those treated with an exogenous estrogen express lower levels of CYP3A4, although not as low as pre-menopausal samples. This suggests that CYP3A4 is regulated by estrogen. At least two possibilities exists as to why the pre-menopausal endometrium expresses lower levels of CYP3A4 than the estrogen-treated post-menopausal endometrium. One possibility is that the cycling endometrium has additional regulators of CYP3A4 expression that are inactive in the post-menopausal state. A second possibility refers to a result of this study in the human liver, that is CYP3A4 mRNA expression increases as age increases. The post-menopausal women are on-average older than the pre-menopausal women. Also, in conjunction, these explanations are plausible.

According to this study, CYP3A5 expression is significantly higher in the secretory phase than the proliferative phase of the endometrium (Figure 3B). Another study reported CYP3A5 expression in pre-menopausal endometrium without exploring differences between phases (Hukkanen et al., 1998).

The current study did not detect CYP3A7 mRNA in the vast majority of the endometrial samples, in agreement with a previous study (Hukkanen et al., 1998), but in disagreement with two other studies (Sarkar et al., 2003; Schuetz et al., 1993). The findings of Schuetz, *et al.*, for CYP3A7 are similar to the findings for CYP3A5 in our study. For Schuetz, *et al.*, the possibility exists for cross-reactivity of the CYP3A7 probe with CYP3A5. At the time of the Schuetz, *et al.*, publication, the degree of similarity for a large portion of the probe between CYP3A5 and
CYP3A7 was not known. Therefore, the Schuetz, et al., findings could be reporting CYP3A5 mRNA expression in the endometrium as well as CYP3A7.

CYP3A43 appears to be down-regulated by estrogen (Figure 3C) since the endometrial samples from the placebo-treated, post-menopausal women appear to have higher mRNA expression than those of the estrogen-treated post-menopausal and pre-menopausal women, though the changes are not significant. CYP3A43 could be expressed in higher levels in the proliferative rather than the secretory phase, though additional studies are necessary for a more definitive answer.

The results of the current study are consistent with the notion of estrogen decreasing the mRNA levels of CYP3A4 in the human post-menopausal endometrium, and may do the same to CYP3A43 (based on our data). With this information and setting aside the effects of other possible regulators of transcription, the mRNA levels of CYP3A4 and CYP3A43 would be expected to be lower during the proliferative phase in human pre-menopausal endometrium compared to the secretory phase. This difference is seen with CYP3A5, but not with CYP3A4 and CYP3A43. In fact, the results of CYP3A43 are opposite to the prediction; the proliferative phase is higher than the secretory phase. For both CYP3A4 and CYP3A43, additional regulators of transcription may cause the deviation from the model described above. The addition of estrogen to the post-menopausal endometrium may be causing the atrophic endometrium to mimic the pre-menopausal endometrium (Nilsson et al., 1980). Nilsson, et al., demonstrated that morphologically atrophic endometrium subjected to estrogen resembles pre-menopausal endometrium. If the post-menopausal endometrium does resemble pre-menopausal endometrium as a result of estrogen treatment, then the down-regulation observed for CYP3A4, and possibly CYP3A43, may be a result of other factors found in pre-menopausal endometrium and estrogen.
may or may not be regulating the CYP3A forms. In addition, the CYP3A mRNA levels of the liver samples, except for CYP3A4, showed no difference between genders. Since additional regulators of transcription are most likely involved, it is not surprising to observe a difference between the results obtained and what was predicted.

In contrast to the mRNA levels observed, reports have been published indicating no clinical difference of midazolam (Gorski et al., 2000) and erythromycin (Harris et al., 1996) metabolism between untreated, post-menopausal women and post-menopausal women taking hormone replacement therapy. Therefore, despite CYP3A4 mRNA levels being suppressed by estrogen, this form of regulation appears to play a small role, if any, in regards to clinical significance for the individual possibly due to an increase in the metabolic role played by other CYP forms.

The results of the mRNA expression are consistent with the results of the total protein expression reported in this study. While total protein did not significantly increase with age or gender as total mRNA did, a significant positive relationship exists between the two, based on the Spearman's correlation coefficient. This means that as the expression of total mRNA or total protein increases, the other will also tend to have a higher expression value. However, the relationship is not very strong, indicating that factors other than mRNA expression may influence protein expression level.

On the other hand, CYP3A4 may be regulated by estrogen only in certain tissues, similar to rat CYP3A9 (Anakk et al., 2003). Anakk, et al., showed that CYP3A9 was regulated by estrogen differently in the liver and brain. The liver predominantly expressed estrogen receptor alpha (ERα), while the brain expressed ERβ.
In conclusion, estrogen may be important for tissue-specific expression of the CYP3As. The literature is divided on the issue of gender differences in the liver expression of CYP3A4. Perhaps this reflects, among many other things, the difficulty of doing population studies when in actuality other individual differences among women and among men make strong contributions to overall expression of the drug metabolizing enzymes.
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Legends for Figures

Figure 1 – Data from Supplemental Material Table 2 plotted for females (○) and males (Δ) with the estimated model (solid line) from Supplemental Material Table 3 and 95% confidence interval (dashed lines) for CYP3A4 [A and B], CYP3A7 [D], and CYP3A43 [E]. CYP3A5 [C] only shows data plotted due to the absence of statistically significant effects. p-values are listed in Table 2.

Figure 2 – Data from Westlind-Johnsson, et al. (2003) plotted for females (○) and males (Δ) with the estimated model (solid line) from Supplemental Material Table 3 and 95% confidence interval (dashed lines) for CYP3A4 [A], CYP3A5 [B], and CYP3A43 [C]. PXR [D] only shows data plotted due to the absence of statistically significant effects. p-values are listed in Table 2.

Figure 3 – Comparison of pre-menopausal phases (Supplemental Material Table 4) and post-menopausal treatments (Supplemental Material Table 6) for CYP3A4 [A], CYP3A5 [B], and CYP3A43 [C]. The error bars represent the standard error (Table 3). A single asterisk (*) denotes $p < 0.05$, and a double asterisk (**) denotes $p < 0.01$. Estimated means and standard errors are listed in Table 3.

Figure 4 – Separation of commercially-available human CYP3A proteins. The proteins were loaded onto the gel either individually or mixed. The bands shown are as follows: CYP3A4, CYP3A5, CYP3A7, CYP3A4/CYP3A5, CYP3A5/CYP3A7, CYP3A4/CYP3A7, and CYP3A4/CYP3A5/CYP3A7.
Table 1 – Clinical features of post-menopausal samples

There was not a significant difference in the mean age of the two groups ($p=0.08$) based on a two-sided, unpaired t-test.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Age (mean ± SD)</th>
<th>ethnicity$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>placebo</td>
<td>10</td>
<td>56.6 ± 3.97</td>
<td>4AA, 6C</td>
</tr>
<tr>
<td>ERT$^a$</td>
<td>10</td>
<td>53.1 ± 4.56</td>
<td>3AA, 7C</td>
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</tbody>
</table>

$^a$ERT – estrogen-replacement therapy

$^b$AA – African-American; C – Caucasian
Table 2 – Significant P-values from statistical analyses of human liver CYP3A mRNA expression

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value</th>
<th>Current&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Previous&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>CYP3A4</td>
<td>age</td>
<td>0.0014</td>
<td>0.0028</td>
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<tr>
<td></td>
<td>gender</td>
<td>0.0092</td>
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</tr>
<tr>
<td></td>
<td>age * gender</td>
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</tr>
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<td>age</td>
<td></td>
<td>0.0479</td>
</tr>
<tr>
<td>CYP3A7</td>
<td>age</td>
<td>0.0126</td>
<td>NT&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>CYP3A43</td>
<td>age</td>
<td>0.0250</td>
<td>0.0017</td>
</tr>
</tbody>
</table>

<sup>a</sup>current – this study
<sup>b</sup>previous – Westlind-Johnsson, et al. (2003)
<sup>c</sup>NT – not tested
Table 3 – Estimated means and standard errors of human endometrium mRNA expression

Each cell contains mean minus standard error, mean, and mean plus standard error. Mean ± standard error is not symmetric since the model was fitted to transformed data. The intervals of the data transformed back to the original units reflect the fact that this data is not symmetric.

<table>
<thead>
<tr>
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<th>Pre-Menopause</th>
<th>Post-Menopause</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Proliferative</td>
<td>Secretory</td>
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<tr>
<td>CYP3A4</td>
<td>3.464</td>
<td>3.232</td>
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<tr>
<td></td>
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<td></td>
<td>5.920</td>
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<td>CYP3A5</td>
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<tr>
<td></td>
<td>7.147</td>
<td>4.921</td>
</tr>
</tbody>
</table>

<sup>a</sup>NT – not tested
Figure 1

CYP3A4 (Females)

CYP3A4 (Males)

CYP3A5

CYP3A7

CYP3A43
Figure 2
Figure 3

(A) CYP3A4

(B) CYP3A5

(C) CYP3B43
Figure 4

[Image of a gel electrophoresis pattern with bands of varying intensity.]