Plasma Letrozole Concentrations in Postmenopausal Women With Breast Cancer Are Associated With *CYP2A6* Genetic Variants, Body Mass Index, and Age

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The associations between plasma letrozole concentrations and *CYP2A6* and *CYP3A5* genetic variants were tested in the Exemestane and Letrozole Pharmacogenomics (ELPH) trial. ELPH is a multicenter, open-label prospective clinical trial in women randomly assigned ($n \approx 250$ in each arm) to receive 2 years of treatment with either oral letrozole (2.5 mg/day) or oral exemestane (25 mg/day). CYP2A6 and CYP3A showed effects on letrozole metabolism *in vitro*. DNA samples were genotyped for variants in the *CYP2A6* and *CYP3A5* genes. Plasma letrozole concentrations showed high interpatient variability (>10-fold) and were associated significantly with *CYP2A6* genotypes ($P < 0.0001$), body mass index (BMI) ($P < 0.0001$), and age ($P = 0.0035$). However, *CYP3A5* genotypes showed no association with plasma letrozole concentrations. These data suggest that CYP2A6 is the principal clearance mechanism for letrozole *in vivo*. CYP2A6 metabolic status, along with BMI and age, may serve as a biomarker of the efficacy of letrozole treatment or a predictor of adverse effects.

Estrogens play an important role in the initiation and promotion of estrogen receptor– and/or progesterone-positive breast cancers; patients with hormone receptor–positive breast cancers are candidates for endocrine therapy. Aromatase (cytochrome P450 (CYP) 19) is the rate-limiting enzyme in the biosynthesis of estrogens from androgens.[1] Letrozole is a nonsteroidal triazole derivative, one of the third-generation potent and selective competitive inhibitors of aromatase,[2] which effectively depletes plasma and tissue estrogen.[3,4] Letrozole is now widely used as adjuvant therapy in postmenopausal women with hormone receptor–positive early-stage breast cancer and for extended therapy in patients with early-stage breast cancer who have already received 5 years of adjuvant tamoxifen therapy.[5,6] Despite its proven efficacy, letrozole does not benefit all patients, and a significant proportion of patients are at increased risk for serious long-term adverse effects, notably musculoskeletal symptoms,[7] bone loss, and fractures.[6] The mechanisms underlying interpatient variability in letrozole response remain unknown, and there are therefore no biomarkers that help identify women who will derive benefit from letrozole treatment.

One factor that appears to contribute to variable drug response in breast cancer therapy is variability in drug metabolism, as has been shown with tamoxifen.[8–10] Letrozole is cleared predominantly by metabolism through the CYP enzyme system to pharmacologically inactive carbinol (4,4′-methanol-bisbenzonitrile) and probably to ketone metabolites.[11] The exact pattern of these metabolic pathways is currently unclear. The carbinol metabolite is rapidly excreted, primarily in urine, in the form of glucuronide conjugates.[12] Evidence exists that letrozole pharmacokinetics varies widely among patients;[11] this, in turn, may contribute to differences in biochemical and clinical responses to this drug. It is therefore important to identify mechanisms and factors that are responsible for the interindividual variability in the pharmacokinetics of letrozole.

Given that letrozole is metabolized mainly by the CYP system, the pharmacokinetics of the drug is likely to be controlled by

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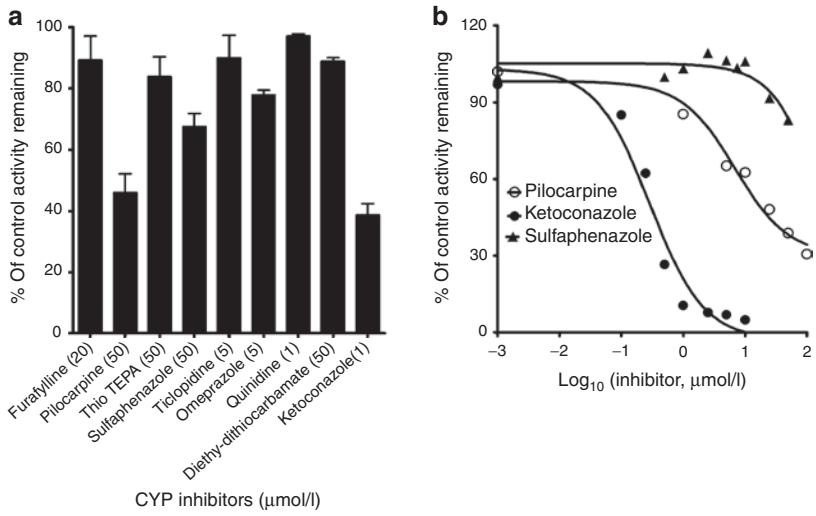

Figure 1 Inhibition of the metabolism of letrozole to 4,4′-methanol-bisbenzonitrile in human liver microsomes (HLMs) by isoform specific inhibitors. (**a**) Letrozole (10 µmol/l) was incubated with HLMs (0.5 mg/ml) and cofactors with and without the following inhibitors: furafylline (20 µmol/l, CYP1A2), pilocarpine (50 µmol/l, CYP2A6), thioTEPA (50 µmol/l, CYP2B6), ticlopidine (5 µmol/l, CYP2B6, and CYP2C19), sulfaphenazole (25 µmol/l, CYP2C9), omeprazole (5 µmol/l, CYP2C19), quinidine (1 µmol/l, CYP2D6), diethyldithiocarbamate (50 µmol/l, CYP2E1), and ketoconazole (1 µmol/l, CYP3A); (**b**) IC_{50} values were determined by incubating letrozole with HLMs and cofactors with and without multiple inhibitor concentrations. The data represent either mean values ± SD in **a** or average of duplicate incubations in **b**. CYP, cytochrome P450.

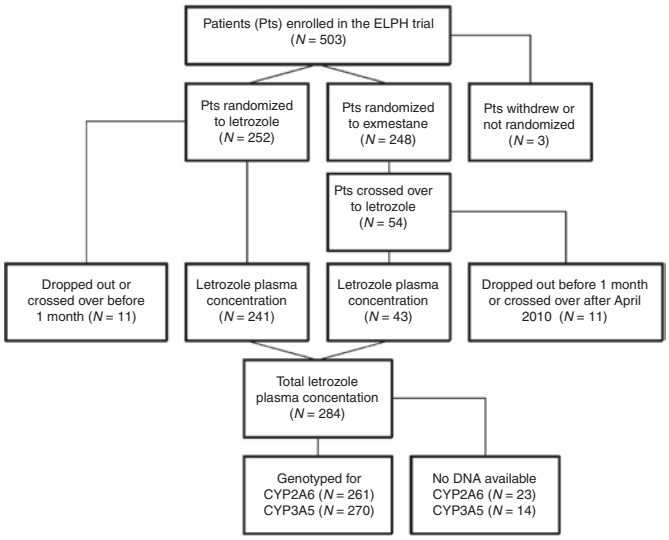

Figure 2 Flow diagram illustrating enrollment of patients (Pts) and analyses of samples. ELPH, Exemestane and Letrozole Pharmacogenomics.

the activity of the CYPs involved. However, the specific CYPs that catalyze letrozole metabolism have not been fully characterized. In an *in vitro* study, letrozole was shown to be a competitive inhibitor of CYP2A6, with marginal effects on other CYPs tested;[13] these findings are consistent with a recent *in vitro* report suggesting that CYP2A6 might mediate the high-affinity enzymatic component for letrozole metabolism.[14] Also, information provided in the product label implicates CYP3A and CYP2A6 in letrozole metabolism.[11] However, there are no published data that systematically address the contribution of these or other enzymes *in vitro*. More importantly, the value of these *in vitro* data in predicting *in vivo* letrozole exposure has not been studied.

The purpose of the present study was therefore to identify the pathways of letrozole metabolism and the CYPs involved in the process, using *in vitro* human liver cellular fractions and expressed CYPs. We next tested the hypothesis that genetic variants involved in letrozole metabolism *in vitro* also predict letrozole concentrations in plasma in patients with breast cancer.

RESULTS

In vitro studies

Incubation of 10 µmol/l letrozole with human liver microsomes (HLMs) and cofactors resulted in the formation of one main metabolite that was identified as 4,4′-methanol-bisbenzonitrile. No other metabolites of letrozole, including

the ketone metabolite mentioned in the product label,[11] were identified after incubation of letrozole with HLMs (or expressed enzymes) and cofactors using high-performance liquid chromatography (HPLC) with ultraviolet or fluorescent detection and by liquid chromatography–tandem mass spectrometry approaches (see **Supplementary Methods** online). Subsequent experiments showed that the formation rate of 4,4′-methanol-bisbenzonitrile (i) was characterized by a Hill equation: K_m = 37.5 ± 1.2 μmol/l, V_{max} = 18.6 ± 14.0 pmol/min/mg protein, and the Hill coefficient = 1.80 ± 0.42; (ii) showed ~26-fold variability among 21 HLMs and was significantly correlated with CYP3A (Spearman r = 0.72; P = 0.0002) and CYP2A6 (r = 0.45; P = 0.04); (iii) was inhibited by the CYP3A inhibitor ketoconazole (IC_{50} = 0.28μmol/l) and by the CYP2A6 inhibitor pilocarpine (IC_{50} = 8.2μmol/l) (**Figure 1**); and (iv) was formed by CYP2A6>CYP3A4 (and, to some extent, CYP2C19 and CYP2D6).

Clinical studies

Distribution of letrozole plasma concentrations. Plasma samples were available from 241 of the patients who had initially been randomized to the letrozole arm (n = 252) (**Figure 2**). Additional plasma samples were obtained from 43 patients who had initially been randomized to the exemestane arm but crossed over to letrozole before the 1-month (n = 7), 3-month (n = 35), or 6-month (n = 1) blood sample draw (**Figure 2**). The distribution of plasma concentrations of letrozole in the total cohort (N = 284) was characterized by large interpatient variability (median, 88.4 ng/ml; range, 0–349.2ng/ml) (**Figure 3**). In four of the patients, plasma letrozole concentrations were not detectable. Compliance with treatment regimens was not formally assessed in this trial, and the information obtained from patients' self-reports in the charts indicates that two of the patients took their last letrozole dose within the 3 h prior to the blood draw, whereas there was no record of the timing with respect to the other two patients. The samples were assayed twice to rule out errors. The plasma letrozole concentration remained highly variable, with a median of 89.7 ng/ml (range: 28.4–349.2ng/ml; 12.3-fold difference) even after the data for the four aforementioned patients were excluded. The inset in **Figure 3** illustrates frequency distribution and probit analysis of letrozole concentrations, which was skewed toward high concentrations.

Associations between plasma letrozole concentrations and demographic variables. The demographic characteristics of the patients are shown in **Supplementary Table S1** online. As shown in **Figure 4** and **Table 1**, plasma letrozole concentrations were positively correlated with age (Pearson r = 0.17; P = 0.0035) and negatively correlated with body mass index (BMI) (r = −0.26; P < 0.0001). Linear regression analysis revealed that age (R^2 = 0.03; P = 0.0035) and BMI (R^2 = 0.068; P = 8.6 × 10^{-6}) were associated with plasma letrozole concentrations (**Figure 4**; and **Table 1**). The sample size was too small (Asian, 6; black, 25) to test for associations with race (**Supplementary Table S1** online).

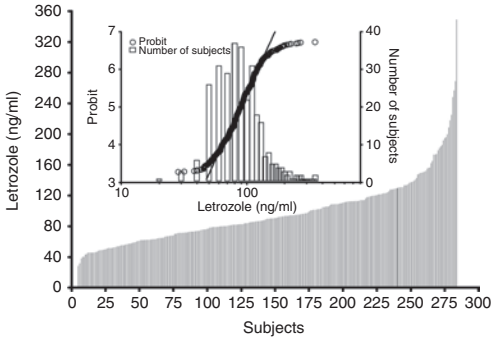

Figure 3 Distribution of plasma letrozole concentrations in all (N = 284) patients with breast cancer. Plasma letrozole concentrations were measured using high-performance liquid chromatography at 1 month (n = 22), 3 months (n = 258), and 6 months (n = 4) after intake of 2.5 mg/day of letrozole. Among these, 43 subjects were initially randomized to exemestane and later crossed over to letrozole. Of the total number of subjects, the majority (88%) were whites (n = 250); 9.9% were blacks (n = 28), and 2.1% were Asians (n = 6). The frequency distribution is shown in the inset and was skewed to the right (high exposure).

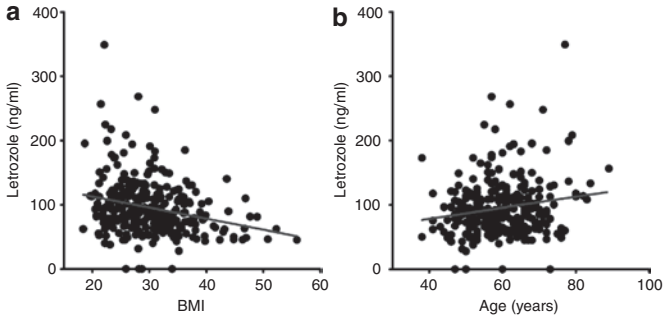

Figure 4 Associations between plasma letrozole concentrations and (**a**) body mass index (BMI) and (**b**) age in patients with breast cancer.

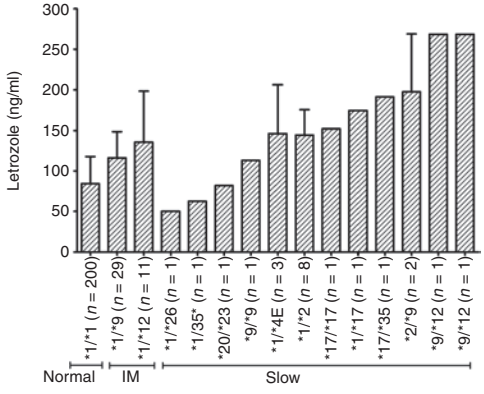

Figure 5 Plasma letrozole concentrations in individual *CYP2A6* genotypes. On the basis of genotype-predicted phenotypes, patients were grouped into normal (n = 200; *1/*1), intermediate (IM) (n = 40; *1/*9 *and* *1/*12*), and slow (n = 21) metabolizer groups.

Associations between CYP2A6 genotype groups and plasma letrozole concentrations. DNA samples from 235 patients who had initially been randomized to receive letrozole, and from 40 other patients who had crossed over from the exemestane arm to the

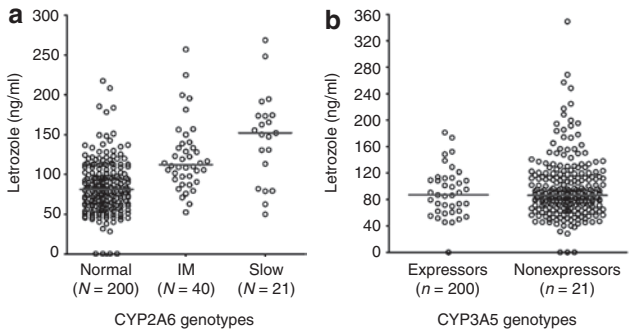

Figure 6 Plasma letrozole concentrations in patients with breast cancer are significantly associated with (**a**) *CYP2A6* genetic variations but not with (**b**) *CYP3A5* genotypes.

Table 1 Associations of letrozole plasma concentrations with *CYP2A6* and *CYP3A5* genotypes and demographic variables

Independent variable	Adjusted r^2	β Coefficient (SE)	*T* value	*P* value
Simple variate (simple linear regression) analysis				
CYP2A6 gene score Normal Intermediate Slow	0.229	−32.963 (3.76)	−8.77	2.49×10^{-16}
CYP3A5 gene score Expressor Nonexpressor	0.002	−5.66 (7.72)	−0.73	0.47
Age	0.0299	0.85 (0.29)	2.95	0.0035
BMI	0.068	−1.7261 (0.38)	−4.53	0.00000861
Multivariate analysis				
CYP2A6 gene score Normal Intermediate Slow		−33.57 (3.55)	−9.45	$<2 \times 10^{-16}$
Age		0.59 (0.246)	2.4	0.0172
BMI		−1.82 (0.327)	−5.57	0.000000064
Age+BMI+CYP2A6 genotype	0.323			$<2.2 \times 10^{-16}$

All *CYP2A6* genotype data were included and analyzed after the genotype-predicted phenotypes were categorized as normal, intermediate, or slow metabolizer according to nicotine metabolism (see Methods section for details). CYP3A5 expressors carry one or two functional alleles, and CYP3A5 nonexpressors carry two nonfunctional alleles.

BMI, body mass index.

letrozole arm, were genotyped for *CYP2A6* variants (total n = 275). In 261 patients, data related to both genotype and plasma letrozole concentrations were available (Figure 2). Most of the subjects in this study were white (**Supplementary Table S1** online). Data from 259 subjects were used in the final analyses. No significant differences in demographic characteristics were observed among the three genotype groups (**Supplementary Table S1** online). The frequencies of the *CYP2A6* genotypes, depicted in Figure 5, were in Hardy–Weinberg equilibrium. The genotype groups were categorized as normal, intermediate, or slow metabolizer groups (see Methods section) on the basis of genotype-predicted phenotype as shown by nicotine metabolism.[15,16] The median (minimum to maximum) plasma letrozole concentrations in normal, intermediate, and slow metabolizers of CYP2A6 were 81.2 (0–217.5), 112.4 (52.7–1256.9), and 152.1 (50.0–268.6) ng/ml, respectively. Statistically significant differences in letrozole concentrations were observed among the three genotype groups ($P < 0.0001$; Kruskal–Wallis test) (Figure 6a). *Post hoc* analysis revealed significantly higher plasma letrozole concentrations in slow and intermediate metabolizers relative to those in normal metabolizers ($P < 0.0001$ for each) (Figure 6a). Large interindividual variability was seen within each genotype (Figure 6a). Simple linear regression analysis (Table 1) revealed that CYP2A6 genetic variation was significantly associated with plasma letrozole concentration (adjusted $R^2 = 0.229$; $P = 2.49 \times 10^{-16}$) (Table 1).

The data shown in Figure 5 reveal that the phenotype predicted by *CYP2A6*1/*26* and *CYP2A6*1/*35* genotypes was consistent with normal metabolizers of letrozole rather than slow metabolizers, whereas *CYP2A6*12* was associated more with slow metabolizers than with intermediate metabolizers. These data are in contrast to the *CYP2A6* genotype–phenotype associations reported on the basis of nicotine metabolism.[15,16] Given the ambiguity of the predictions regarding phenotype, and in order to ensure that no bias was introduced into estimates of the strength of genotype–phenotype associations on this account, additional analyses were performed after the two genotypes (*CYP2A6*1/*26* and *CYP2A6*1/*35*; n = 1 each) were excluded from the analysis, whereas data from *CYP2A6*1/*12* (n = 11) were analyzed on the assumption that they related to slow metabolizers.**Supplementary Table S2** online shows that the data from both analyses were essentially similar to those listed in Table 1, although the strength of the association was slightly stronger when the genotype-predicted phenotype was on the basis of letrozole concentrations ($R^2 = 0.265$) than on the basis of nicotine metabolism ($R^2 = 0.229$).

***CYP3A5* genotypes and their associations with plasma letrozole concentrations.** A total of 288 DNA samples were genotyped for *CYP3A5* variants (**3, *6,* and **7*). Letrozole concentrations and genotypes were available for only 270 of the patient samples. When the data were analyzed using the same approach as with CYP2A6 genotype-predicted phenotype (normal, intermediate, and slow metabolizers), no significant associations were observed (one-way analysis of variance; $P = 0.16$) among the three groups. The r^2 derived from linear regression was 0.0048 ($P = 0.256$). Given that most of the patients studied were white, it is not surprising that the number of patients with the *1/*1 genotype was very small (n = 7) (**Supplementary Table S1** online). We found no statistically significant differences in letrozole concentrations between *CYP3A5* expressors (n = 42: *1/*1, n = 7; *1/*3, n = 31; *1/*6, n = 3; and n = 1, *1/*7) and nonexpressors (n = 237: 3/*3, n = 228; *3/*6, n = 3; *3/*7, n = 4; *6/*6, n = 1; and *6/*7, n = 1) (Figure 6b; Table 1).

Multivariate analyses. In the multivariate analyses, significant associations were noted between plasma letrozole concentrations and *CYP2A6* genotype ($P < 2 \times 10^{-16}$), BMI

(6.4×10^{-8}), and age ($P = 0.017$). The incorporation of age and BMI in the multiple regression analyses significantly improved the overall predictive value of *CYP2A6* genotype; the R^2 value of 0.229 in the model containing *CYP2A6* genetic markers alone rose to an adjusted value of 0.323 in the model containing *CYP2A6* genotypes plus demographic variables. The absolute difference in the variation in letrozole concentrations attributable to age and BMI was ~9.4% (**Table 1**).

DISCUSSION

In the present study, CYP2A6 and CYP3A enzymes were identified as the principal catalysts in the metabolism of letrozole into 4,4′-methanol-bisbenzonitrile *in vitro*. The genes coding for these enzymes were therefore selected for focused study of genotype–phenotype associations in a large-scale clinical trial in postmenopausal women with breast cancer. Letrozole concentrations exhibited considerable interpatient variability in this cohort of patients. This variability can be explained to a large extent by *CYP2A6* genetic variations and to a smaller extent by demographic factors, notably BMI and age; together, all these (*CYP2A6* variations plus demographic factors) explain ~32.3% of the interpatient variability. CYP3A5 genotype had no evident association with plasma letrozole concentrations *in vivo*. These data impart important information that may be applied to individualize letrozole therapy. In addition, they suggest that CYP2A6 may be involved in the principal clearance mechanism of letrozole *in vivo*.

Plasma letrozole concentrations varied more than 10-fold among patients with breast cancer who had quantifiable levels of letrozole. We estimate that ~23% of the letrozole variability is attributable to *CYP2A6* genetic variation. These *in vivo* data broadly concur with our *in vitro* findings showing the participation of CYP2A6 in letrozole metabolism (present data and ref. 13). Other investigators have also reported that CYP2A6 and CYP3A represent the high- and low-affinity components, respectively, in the formation of the carbinol metabolite of letrozole.[14] Although the results of these *in vitro* studies point toward a role for CYP2A6 in the *in vivo* metabolism of letrozole, it remains less clear whether this *in vivo* effect can be fully predicted from *in vitro* studies evaluating formation of 4,4′-methanol-bisbenzonitrile. According to the information provided in the product label, CYP2A6 is the sole catalyst in the metabolism of letrozole to a ketone metabolite, whereas CYP2A6 and CYP3A are involved in the formation of 4,4′-methanol-bisbenzonitrile.[11] As proposed in our previous study, these two metabolites might be sequentially formed from letrozole, with the ketone being formed first, probably by CYP2A6, and subsequently being converted to 4,4′-methanol-bisbenzonitrile by CYP2A6 and CYP3A.[13] If the latter model holds true, CYP2A6 could be the rate-limiting step in letrozole metabolism, as shown in our *in vivo* results; under this condition, assessing 4,4′-methanol-bisbenzonitrile alone would substantially underestimate the quantitative *in vivo* role of CYP2A6, given that there appears to be significant participation from CYP3A in the formation of this metabolite *in vitro* (present data and ref. 14). Unfortunately, this suggestion, although plausible, remains speculative because no ketone metabolite standard was available to us and repeated attempts to identify the ketone metabolite in our *in vitro* and *in vivo* studies did not succeed. Regardless of the specific mechanisms involved, the findings of this study provide *in vivo* evidence that CYP2A6 is involved in the main clearance mechanism of letrozole and that this drug can now be added to the list of the few clinically important substrates of CYP2A6. The data suggest that letrozole may serve as a safe, alternative *in vivo* probe of CYP2A6 activity. Unfortunately, metabolite data were not obtained in this study to enable a proper evaluation of this suggestion. Attempts to measure concentrations of letrozole metabolites in plasma were not successful because these were below the limit of quantification of the assay, even after enzymatic hydrolysis by β-glucuronidase. This is not surprising because the quantity of letrozole metabolite in plasma is expected to be minimal given that letrozole has a very long elimination half-life (90–120 h) with very low oral clearance (1.2 l/h at steady state)[12] and that any metabolites that are slowly produced undergo efficient phase II conjugation and rapid elimination through the kidney. Although a high abundance of conjugated letrozole metabolite is expected in urine,[12] urine samples were not collected in these patients, and therefore these metabolic ratios could not be calculated. This study was not designed to address the utility of letrozole as a probe for CYP2A6. Formal pharmacokinetic studies, including determination of metabolite(s) in urine and comparison with established probes, are needed to validate the suggestion that letrozole can serve as an alternative probe for CYP2A6 *in vivo*.

Our data show that BMI and age are independent predictors of plasma letrozole concentrations. The inclusion of age and BMI in the multivariate model significantly improved the overall predictive value from 22.9% in a model containing only *CYP2A6* genetic markers to 32.3% in the model containing *CYP2A6* genetic markers plus demographic factors, yielding an absolute difference of ~9.4% of the variation in letrozole concentration as being attributable to age and BMI. The relatively lower plasma concentrations of letrozole in the peripheral compartment (i.e., plasma) may reflect the fact that letrozole is a highly lipophilic drug with a large volume of distribution (183 l)[12] and that the volume of distribution increases with increasing BMI. The study finding that letrozole concentrations increase with increasing age concurs with previous reports showing an effect of age on CYP2A6 activity.[17,18] One study reported no effect of age on letrozole exposure[12]; however, this result was possibly due to the small size of the study cohort.

Interestingly, ~68% of the variability in letrozole concentrations remains unaccounted for. First, although a relatively comprehensive genotyping was performed, it is possible that other (rare) *CYP2A6* variants that were not considered in this study, including gain-in-function alleles,[19] may contribute to this variability. Second, drug interactions may influence CYP2A6 activity, given that patients with breast cancer often take other drugs concomitantly.[9] Medication data were carefully collected in this study population, but their impact on letrozole concentrations was not assessed because the inhibitors and inducers of CYP2A6 are less characterized than those that influence other CYPs

(http://medicine.iupui.edu/clinpharm/ddis). Third, although our data show little effect of *CYP3A5* genotypes on letrozole concentrations, the possibility of the involvement of CYP3A4 in letrozole metabolism *in vivo* cannot be excluded. Finally, the possibility that other unidentified metabolic pathways may contribute cannot be fully ruled out, although this is unlikely given that metabolites other than the ketone and carbinole metabolites account for <5% of the dose in mass-balance studies.[11,12] Although female sex is an independent predictor of increased CYP2A6 activity,[20] this was not an issue in this study because all the patients were postmenopausal women.

We realize that genotype–phenotype associations may be prone to be false positives in the absence of replication, particularly when the analysis deals with one of many secondary end points of the study, as in this instance. Consequently, the lack of external replication is a limitation of this study. In addition, our suggestion that CYP2A6 might be involved in the main clearing mechanism for letrozole in *vivo* cannot be confirmed in the absence of metabolite data; such data could have provided further information on whether there is an inverse relationship between letrozole and metabolite levels and whether the metabolic ratios are related to the CYP2A6 status. Despite these limitations, there are findings that constitute compelling evidence implicating CYP2A6 as the dominant clearance mechanism of letrozole *in vivo*. These findings include: the robust association between CYP2A6 genetic variations and plasma letrozole concentrations (the present study); the available *in vitro* data supporting the hypothesis that CYP2A6 is involved in letrozole metabolism (the present study as well as refs. 11,13,14); and the fact that the functional consequences of CYP2A6 variants, assessed from plasma letrozole concentrations, cosegregate for the most part with those assessed from nicotine metabolic status,[15,16,21] supporting the proposition that nicotine and letrozole are metabolized by the same enzyme, i.e., CYP2A6. Therefore, we believe that the findings of our study have important implications. First, if letrozole plasma concentrations and/or *CYP2A6* genetic variants can predict letrozole efficacy and adverse effects, they may eventually be utilized to individualize breast cancer therapy using letrozole. In the Exemestane and Letrozole Pharmacogenomics (ELPH) trial, several biomarkers of efficacy and side effects of letrozole have been studied. Analyses of the trial data are ongoing to test the associations between letrozole concentrations and/or *CYP2A6* genetic variations with regard to a large number of phenotypic outcomes. The results of these analyses may also have relevance to other settings in which letrozole is widely used, especially as an inducer of ovulation in women with infertility.[22] Second, given that CYP2A6 appears to be involved in the dominant clearance mechanism of letrozole *in vivo*, letrozole may serve as an alternative *in vivo* marker of CYP2A6 activity in postmenopausal women and in men. Third, because the letrozole metabolic pattern appears to diverge from nicotine metabolism with respect to a few *CYP2A6* variants (*12, *26, and *35), consistent with a previous report for variants such as *CYP2A6*17* between coumarin and nicotine,[23] letrozole may be a valuable novel tool to resolve the issue of potential substrate-dependent effects of *CYPA6* genetic variants, particularly those that lead to structural changes. In conclusion, steady-state plasma concentrations of letrozole exhibit high interpatient variability in postmenopausal women with breast cancer, and this variability is partially explained by CYP2A6 metabolic status.

METHODS

In vitro studies

Kinetic, correlation,[24] and inhibition[13,24,25] analyses in HLMs and experiments in expressed CYPs were performed to identify letrozole metabolites and the specific CYPs involved (for details see **Supplementary Methods** online).

Clinical studies: the ELPH trial

Study patients. Postmenopausal women (18 years and older) with stage 0–III hormone receptor–positive breast cancer who were being started on aromatase inhibitor therapy, either "up-front" or after 1–5 years of tamoxifen treatment, were eligible. Eligible patients were recruited from August 2005 through July 2009 at the following COBRA member institutions: Indiana University Cancer Center, Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University, and the University of Michigan Comprehensive Cancer Center. Inclusion and exclusion criteria for participating in this study are detailed elsewhere.[26] All indicated treatments (surgery, radiation therapy, and chemotherapy) for breast cancer were completed prior to study enrollment. The protocol was approved by the institutional review boards of all three participating study sites, and all the enrolled patients provided written informed consent. The clinical trial was reviewed on a biannual basis by an independent data and safety monitoring committee. The trial is registered at http://www.clinicaltrials.gov (trial identifier NCT00228956).

Study design. The ELPH trial is a multicenter, open-label, randomized, prospective clinical trial. Patients were randomly assigned to receive either exemestane 25 mg orally daily or letrozole 2.5 mg orally daily for 2 years. DNA samples, plasma samples, and multiple phenotypic outcomes were prospectively collected at baseline and at multiple time points (up to 24 months) after drug administration for pharmacogenetic correlative analyses. The primary objective of the study was to correlate changes in breast density with aromatase (CYP19) genetic variation. The secondary objectives included testing for associations between various phenotypes (e.g., bone density and bone turnover metabolites, serum lipids, estrogen concentrations, hot flashes, quality-of-life measures, and drug concentrations) and variants in candidate genes. This report addresses a secondary hypothesis that genetic variants in drug-metabolizing enzymes predict letrozole concentrations. Briefly, blood samples (10 ml) were obtained at baseline and at 1, 3, 6, and 12 months after letrozole initiation. Plasma was separated from blood samples by centrifugation at 2,060*g* and stored at −80 °C until analysis. Plasma letrozole concentrations were determined from samples collected 1, 3, or 6 months after initiating letrozole. Plasma samples from patients who were randomized to the letrozole arm, and who stayed in this arm for at least 1 month, were analyzed. No plasma samples were available for those who discontinued the study or crossed over to the exemestane group before the 1-month blood draw. In addition, plasma samples from patients who were initially randomized to exemestane and were switched later to letrozole were also assayed for letrozole concentrations.

DNA samples. Genotyping was performed using genomic DNA extracted from whole blood using the QIAamp DNA Blood Maxi Kit–Spin Protocol in accordance with the manufacturer's instructions (Qiagen, Valencia, CA). DNA samples from subjects who had initially been randomized to the letrozole arm, and from those who had crossed over from exemestane to letrozole, were analyzed.

CYP2A6 genotyping. All DNA samples were genotyped for *CYP2A6 *2*, **4E* (detects **4A/D*, *C*, and *E*), **9*, and **12*, and African Americans were additionally genotyped for **17*, **20*, **23*, **24*, **25*, **26*, **27*, and **35* and Asians for **7*, **8*, and **10*, using methods previously described.[15,21,27]

Subjects were then divided into three categories on the basis of previously published associations between *CYP2A6* genotypes and nicotine metabolism.[21] Individuals with one copy of the decrease-of-function alleles (**9* and **12*) were categorized as intermediate metabolizers; those with two copies of the decrease-of-function alleles or one or two copies of loss-of-function alleles (*CYP2A6*2*, **4*, **7*, **10*, **17*, **20*, **23*, **24*, **25*,**26*, **27*, and **35*) or one decrease-of-function allele together with one loss-of-function allele as slow metabolizers; and those without these genetic variants as normal metabolizers (**1/*1*).

Despite the fact that individuals with alleles **26* and **35* have previously been categorized as slow metabolizers of nicotine,[15,16] our study showed these alleles to be associated with relatively lower letrozole concentrations (**Figure 5**). The **1/*12* genotype, which codes for an intermediate nicotine metabolizer,[21] was consistent with other loss-of-function alleles when assessed from the viewpoint of letrozole concentrations (**Figure 5**). Therefore, in addition to the analyses according to categorizations based on the effects of the genotypes on nicotine metabolism, secondary analyses were performed after recategorization of the **1/*12* ($n = 11$) genotype as slow metabolizer. The **1/*26* ($n = 1$) and **1/*35* ($n = 1$) genotypes were excluded from these analyses because of uncertainty regarding genotype-predicted phenotype and small sample sizes. In effect, regarding the genotype-predicted phenotypes for the alleles **12*, **26*, and **35*, there were differences depending on whether nicotine[21] or letrozole (the present study) was used as a substrate. If these results could be further confirmed, a novel categorization of substrate-dependent allele functionality and genotypic effect could be proposed on the basis of the results of our study.

CYP3A5 genotyping. DNA samples were genotyped for the alleles **3*, **6*, and **7* using allelic-discrimination TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA).

HPLC assay. A new HPLC assay was developed to measure letrozole concentrations in plasma. To 100 µl of plasma, 50 µl of 1µg/ml internal standard and 25 µl of methanol were added; this was vortex-mixed and centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkmann Instruments, Westbury, NY). The supernatant was extracted with 0.5 ml of 1mol/l NaOH/glycine buffer (pH = 11.3) and 6 ml ethyl acetate. The organic phase was evaporated to dryness. The residue was reconstituted with 150 µl of mobile phase, and 50 µl was injected into the HPLC system. The separation system included a Zorbax SB-C18 column (3.5 µm particle size, 150 × 4.6 mm; Phenomenex, Torrance, CA), a Nova-Pak C18 guard column (Waters, Milford, MA)(4-µm particle size), and a mobile phase consisting of 30% acetonitrile and 70% 10mmol/l KH_2PO_4 (adjusted to pH = 6.5), with a flow rate of 1 ml/min. The column elute was monitored by fluorescent detector (excitation, 230 nm; emission, 295 nm) (letrozole) or by ultraviolet detection set at 234 nm (internal standard). Letrozole was quantified using the ratio of peak area of letrozole to the peak area of internal standard, and calibration curves were constructed using known letrozole concentrations spiked to blank plasma. The limit of quantification of this assay was 7 ng/ml, with interday and intraday coefficients of variation of <6.5% and <10%, respectively.

Data and statistical analyses. *In vitro* data are provided in **Supplementary Methods** online.

Continuous variables were summarized groupwise using descriptive statistics. The observed and expected frequencies of alleles and genotypes within populations were tested for deviation from Hardy–Weinberg equilibrium, using the χ^2-test. Differences in letrozole concentrations among the three different genotype groups of *CYP2A6* were analyzed using nonparametric analysis of variance (Kruskal–Wallis test), with Dunnett's multiple-comparison post-test. Simple linear regression analysis was used to analyze the correlations between letrozole concentrations and genetic (*CYP2A6*, *CYP3A5*) as well as demographic variables (age, race, BMI). The contributions of these variables to the overall intersubject variability of plasma letrozole concentrations were subsequently tested using multiple linear regression analyses. In particular, *CYP2A6* was coded in the categories normal, intermediate, and slow metabolizers. CYP3A5 was coded as expressors (1) and nonexpressors (0). Statistical analyses were performed using the statistics software packages SPSS, version 16.0 (SPSS, Chicago, IL) and GraphPad Prism (version 5.02, San Diego, CA). All tests were two sided. $P < 0.05$ was considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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CONFLICT OF INTEREST

R.F.T. owns shares in and participates in Nicogen Research Inc., a company focused on novel approaches to smoking-cessation treatment. No Nicogen funds were used in this work, and no other Nicogen participants reviewed the manuscript. R.F.T. has also consulted for Novartis. N.L.H. received research funding from AstraZeneca. Although R.F.T. is an Associate Editor of *Clinical Pharmacology & Therapeutics*, she had no involvement in the review or decision process regarding publication of this article. The other authors declared no conflict of interest.

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