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CLINICAL TRIAL

CYP3A4 and seasonal variation in vitamin D status in addition to CYP2D6 contribute to therapeutic endoxifen level during tamoxifen therapy

Wendy A. Teft · Inna Y. Gong · Brian Dingle · Kylea Potvin · Jawaid Younus · Theodore A. Vandenberg · Muriel Brackstone · Francisco E. Perera · Yun-Hee Choi · Guangyong Zou · Robin M. Legan · Rommel G. Tirona · Richard B. Kim

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Abstract Tamoxifen is a widely utilized adjuvant anti-estrogen agent for hormone receptor-positive breast cancer, known to undergo CYP2D6-mediated bioactivation to endoxifen. However, little is known regarding additional genetic and non-genetic determinants of optimal endoxifen plasma concentration. Therefore, 196 breast cancer patients on tamoxifen were enrolled in this prospective study over a 24-month period. Blood samples were collected for pharmacogenetic and drug-level analysis of tamoxifen and metabolites. Regression analysis indicated that besides CYP2D6, the recently described *CYP3A4**22 genotype, seasonal variation, and concomitant use of CYP2D6-inhibiting antidepressants were significant predictors of endoxifen concentration. Of note, genetic variation explained 33 % of the variability while non-genetic variables accounted for 13 %. Given the proposed notion of a sub-therapeutic endoxifen concentration for predicting breast cancer recurrence, we set the therapeutic threshold at 18 nM, the 20th percentile for endoxifen level among enrolled patients in this cohort. Nearly 70 % of CYP2D6 poor metabolizers as well as extensive metabolizers on potent CYP2D6-inhibiting antidepressants exhibited endoxifen levels below 18 nM, while carriers of *CYP3A4**22 were twofold less likely to be in sub-therapeutic range. Unexpectedly, endoxifen levels were 20 % lower during winter months than mean levels across seasons, which was also associated with lower vitamin D levels. *CYP3A4**22 genotype along with sunshine exposure and vitamin D status may be unappreciated contributors of tamoxifen efficacy. The identified covariates along with demographic variables were integrated to create an endoxifen concentration prediction algorithm to pre-emptively evaluate the likelihood of individual patients falling below the optimal endoxifen concentration.

Keywords Endoxifen · CYP3A4 · Vitamin D · CYP2D6 · Tamoxifen efficacy · Therapeutic threshold

Introduction

Tamoxifen remains the most commonly prescribed adjuvant anti-estrogen therapy for estrogen receptor (ER)-

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W. A. Teft · I. Y. Gong · R. M. Legan · R. G. Tirona · R. B. Kim
Division of Clinical Pharmacology, Department of Medicine, University of Western Ontario, London, ON, Canada

I. Y. Gong · R. G. Tirona · R. B. Kim
Department of Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

B. Dingle · K. Potvin · J. Younus · T. A. Vandenberg · M. Brackstone · F. E. Perera · R. B. Kim
Department of Oncology, University of Western Ontario, London, ON, Canada

M. Brackstone
Department of Surgery, University of Western Ontario, London, ON, Canada

Y.-H. Choi · G. Zou
Department of Epidemiology and Biostatistics, University of Western Ontario, London, ON, Canada

R. B. Kim (✉)
Department of Medicine, LHSC-University Hospital, Room A-LL-152, 339 Windermere Road, London, ON N6A 5A5, Canada
e-mail: richard.kim@lhsc.on.ca

positive breast cancers [1]. Although the clinical benefit of tamoxifen therapy has been evidently demonstrated, significant interindividual variability in the benefit and efficacy has been observed. In fact, up to 50 % of patients acquire tamoxifen resistance and eventually relapse [2, 3]. Accordingly, considerable interest has been given to evaluating the clinical relevance of pharmacogenetics in tamoxifen efficacy.

Tamoxifen is considered a prodrug which undergoes hepatic bioactivation by cytochrome P450 (CYP) enzymes to produce primary metabolites *N*-desmethyl-tamoxifen (NDM-tamoxifen) and 4-OH-tamoxifen and the active metabolite endoxifen, all of which show variable potencies of ER inhibition [1]. Endoxifen and 4-OH-tamoxifen bind the ER with approximately 50–100 fold higher affinity than tamoxifen, but endoxifen has 6 times higher plasma exposure than 4-OH-tamoxifen originally thought to be the active form [4].

CYP2D6 is considered to be the rate-limiting enzyme for tamoxifen metabolism and contributes to the near tenfold interpatient variability in endoxifen concentration [5, 6]. *CYP2D6* harbors numerous functional single nucleotide polymorphisms (SNPs), allowing for classification of individuals into phenotypic categories including ultrarapid (UM), extensive (EM), intermediate (IM), and poor (PM) metabolizers, which has been well documented to correlate with plasma endoxifen levels [5, 7]. There are many medications known to interact with CYP2D6 including several selective-serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) which are often co-prescribed with tamoxifen for depression and control of hot flashes [8]. Concomitant use of these medications can lead to reduced tamoxifen metabolism resulting in lower endoxifen levels [5, 6]. The association of *CYP2D6* genotype to overall survival and recurrence rates has been highly controversial with inconsistent results likely due in part to the many confounding factors inherent within retrospective studies including source and quality of DNA, unknown CYP2D6 inhibitor coadministration, and differential tamoxifen combination therapy [9–13]. To date, there have been few prospective studies correlating *CYP2D6* genotype, endoxifen levels and outcomes [1, 14].

Importantly, it is evident that additional factors must contribute to the formation of endoxifen as PMs lacking any CYP2D6 enzyme activity are still able to generate this active metabolite [5, 7]. Other CYP enzymes including CYP3A4/5 and CYP2C9 have been shown to be important in tamoxifen metabolism, and SNPs in these enzymes may become more prominent in individuals lacking CYP2D6 activity [7, 12]. Additionally, we have previously demonstrated that endoxifen is a substrate of multidrug resistance protein 1 (MDR1, encoded by *ABCB1*), suggesting that polymorphisms in drug transporters may play a role in endoxifen exposure [15]. However, beyond CYP2D6, little is known regarding clinically relevant genetic and non-genetic contributors of tamoxifen metabolism and response.

It has been recently demonstrated that tamoxifen efficacy is dependent on attaining a threshold level of endoxifen [16]. Madlensky et al. [16] performed a large retrospective analysis to compare tamoxifen metabolite levels and outcomes. They found that women in the upper quintiles of endoxifen concentration had a 26 % lower recurrence rate compared to those in the lowest quintile (<15 nM), indicating the significance of a defined therapeutic threshold. Additionally, tamoxifen and other metabolite levels were not associated with recurrence, highlighting the importance of endoxifen to tamoxifen efficacy. A similar endoxifen level (13 nM) was noted to occupy 90 % of the ER in vitro and 93 % of *CYP2D6* PMs fell below this concentration [7]. Furthermore, we performed PK-PD modeling on xenograft efficacy data obtained from breast cancer tumor bearing mice dosed with a range of clinically relevant endoxifen concentrations to define the relationship between exposure and percent tumor growth inhibition (TGI) [17]. Indeed, PK-PD simulations using steady-state concentrations observed in patients of different CYP2D6 genotypes demonstrate that endoxifen levels within this threshold range have suboptimal TGI.

Accordingly, we performed a detailed analysis to determine predictors of endoxifen plasma level to better understand the observed interpatient variation. Furthermore, a predictive algorithm was developed for pre-emptive identification of patients at risk for sub-therapeutic response to tamoxifen based on prediction of endoxifen level which may be easily integrated into clinical practice to maximize efficacy.

Methods

Study population

Patients ($n = 196$) on adjuvant tamoxifen therapy were recruited between March 2010 and March 2012. All study participants provided written informed consent. The study was approved by the Research Ethics Board at the University of Western Ontario.

Genotyping

DNA was extracted from whole blood using the Gentra Puregene Blood Kit (Qiagen, Toronto, Ontario, Canada).

The following TaqMan allelic discrimination assays (Applied Biosystems, Carlsbad, CA) were used for genotyping: *CYP3A4**22 (intron 6 C > T), *POR*28*, *CYP 2C9*2*, *CYP2C9*3*, *CYP2B6*4*, *CYP2B6*5*, *CYP2B6*6*, *MDR1 c.3435C > T*, *BCRP c.421C > A*, *BCRP c.34G > A*, *CYP3A5**3, *CYP2D6* *3, *4, *9, *10, and *41 and *CYP2D6* TaqMan Gene Copy Number Assay (intron 6) for gene deletion (*5) and duplication. We considered CYP2D6 intermediate alleles as *9, *10, *41 and CYP2D6 poor alleles as *3, *4, *5. Hardy–Weinberg equilibrium was assessed for all genotypes using the Chi square goodness-of-fit test.

Metabolite measurements

Steady-state levels of tamoxifen and its metabolites (NDM-tamoxifen, 4-OH-tamoxifen, Z-endoxifen, Z-3-OH-tamoxifen, and Z-α-OH-tamoxifen), normalized 4-β-OH-cholesterol to total free cholesterol (4-β-HC/Total-C), and 25-OH-vitamin D levels were measured in patient plasma as described in Supplementary Methods.

Statistical analysis

Multiple linear regression analysis was performed to elucidate parameters affecting interindividual variability in endoxifen level (natural log-transformed). The variables considered were age, body mass index (BMI), SSRI/SNRI use, season of blood draw, 25-OH-vitamin D level, 4-β-HC/Total-C, and *CYP2D6*, *CYP3A4*, *CYP3A5*, *POR*, *CYP2C9*, *CYP2B6*, *BCRP*, and *MDR1* genotype. These variables were added to the model according to the stepwise forward procedure to determine significant predictors (P value < 0.05; SPSS v. 18.0, Chicago, IL). Significant covariates were entered into a multiple linear regression model to determine the contribution of each parameter, following adjustment for age and BMI. Classification of CYP2D6 inhibitory medications, *CYP2D6* genotype, and season can be found in Supplementary Methods. Stepwise regression analysis was also used to determine significant variables affecting tamoxifen, NDM-tamoxifen, and 4-OH-tamoxifen plasma exposure.

To make predictions for risk of sub-therapeutic endoxifen concentration, logistic regression analysis was performed with variables age, BMI, *CYP2D6* and *CYP3A4* genotype, SSRI/SNRI use and season (R Development Core Team). The accuracy of identifying patients falling below the therapeutic range was assessed by Receiver Operating Characteristics curve. A cut-off value of 0.8 was used for optimal identification of sub-therapeutic patients. A tenfold cross-validation of the logistic model was performed to validate the model for predictive accuracy.

Results

Patient characteristics

Patient demographics and clinical characteristics are reported in Table 1. Seven patients were excluded from data analysis: two due to low tamoxifen levels assumed to be a result of non-compliance, and five male patients.

CYP2D6 contributes to but cannot fully explain endoxifen formation

Initial plasma concentrations were measured from patients at steady state on tamoxifen therapy (median time on tamoxifen 27.9 weeks, range: 4.1–231.1 weeks). Endoxifen concentrations were not significantly different between initial and follow-up blood samples at 6 and 12 months (Supplementary Fig. S1). Mean plasma levels (17 ± 4.8 h post-dose) of tamoxifen, NDM-tamoxifen, (Z)-4-OH-tamoxifen, (Z)-endoxifen, as well as two other metabolites 3-OH-tamoxifen and α-OH-tamoxifen can be found in Supplementary Table S1. *CYP2D6* genotype did not appear to significantly affect tamoxifen and 4-OH-tamoxifen concentrations (Supplementary Fig. S2). Conversely, NDM-tamoxifen, 3-OH-tamoxifen, α-OH-tamoxifen, and endoxifen levels were significantly affected by the presence of reduced or loss-of-function *CYP2D6* alleles in a gene-dose dependent manner (Supplementary Fig. S2, Fig. 1). Metabolic ratios of endoxifen to tamoxifen and endoxifen to both primary metabolites showed a significant correlation with *CYP2D6* genotype (Supplementary Fig. S3), supporting the notion that CYP2D6 is an important contributor to endoxifen bioactivation.

Since little is known regarding the in vivo endoxifen concentrations required for sufficient tumor suppression, we examined the relationship between endoxifen concentration and tumor inhibition in a xenograft efficacy model [17]. The KC_{50} (describing the endoxifen tumor-inhibitory effect) of endoxifen efficacy was 14 nM. A recent study by Madlensky et al. [16] indicated that patients in the lowest quintile of endoxifen concentration were at the highest risk of recurrence and proposed the 20th percentile as a therapeutic threshold. Our xenograft efficacy model supports these findings as these concentrations would result in suboptimal TGI. As such, we defined the therapeutic threshold as the 20th percentile (18 nM) of the current cohort.

We observed an increasing percentage of patients within sub-therapeutic range with greater reduction in CYP2D6 function (Fig. 1a). Interestingly, PMs with no CYP2D6 enzymatic activity are still able to generate endoxifen (15.7 ± 6.7 nM), although the majority of these PMs fall below the therapeutic threshold (Fig. 1a). Of note, the formation of endoxifen is complex with <10 % formed via

Table 1 Patient demographics and clinical characteristics grouped by CYP2D6 phenotype ($n = 189$)

Characteristic	EM ($n = 71$)	IM ($n = 108$)	PM ($n = 10$)
Median age, years	50	49	45
Range	26–82	28–82	36–87
>50 years (%)	34 (47.9)	40 (37.0)	4 (40.0)
Race/ethnicity			
Caucasian	68	100	10
Middle Eastern	1	1	0
Asian	0	5	0
Other	2	2	0
Height, cm			
Median	164.3	164.0	163.9
Range	144.8–182.9	152.4–185.4	152.4–172.7
Weight, kg			
Median	73.1	69.8	70.7
Range	51.2–124.7	46.7–126.1	49.5–99.3
BMI, kg/m^2			
Median	27.0	25.6	26.1
Range	19.0–43.7	18.0–49.3	18.2–33.3
SSRI/SNRI use			
Total (%)	14 (19.7)	21 (19.3)	1 (10)
Strong inhibitor	3	2	1
Moderate inhibitor	3	1	0
Mild inhibitor	8	18	0
CYP3A4*22, *n* (%)	WT = 61 (85.9)	WT = 91 (84.4)	WT = 8 (80)
	Carrier = 10 (14.1)	Carrier = 17 (15.6)	Carrier = 2 (20)
CYP2D6 phenotype, *n* (%)	UM/EM = 3 (1.6)	EM/IM = 37 (20.0)	PM/PM = 10 (5.2)
	EM/EM = 68 (31.4)	EM/PM = 54 (28.3)	
		IM/IM = 6 (3.1)	
		IM/PM = 11 (5.8)	

BMI body mass index, *EM* extensive metabolizer, *IM* intermediate metabolizer, *PM* poor metabolizer, *SSRI* selective-serotonin reuptake inhibitor, *SNRI* serotonin-norepinephrine reuptake inhibitor, *UM* ultrarapid metabolizer, *WT* wildtype

the conversion of tamoxifen to 4-OH-tamoxifen while the majority is generated through the demethylation of tamoxifen to NDM-tamoxifen and its subsequent oxidation to endoxifen [18]. In CYP2D6 EMs, there was a strong correlation between endoxifen and 4-OH-tamoxifen ($r^2 = 0.74$, Supplementary Fig. S4A) plasma levels. However, only a weak association between endoxifen and NDM-tamoxifen ($r^2 = 0.22$, Supplementary Fig. S4B) plasma levels was observed. Collectively, the large

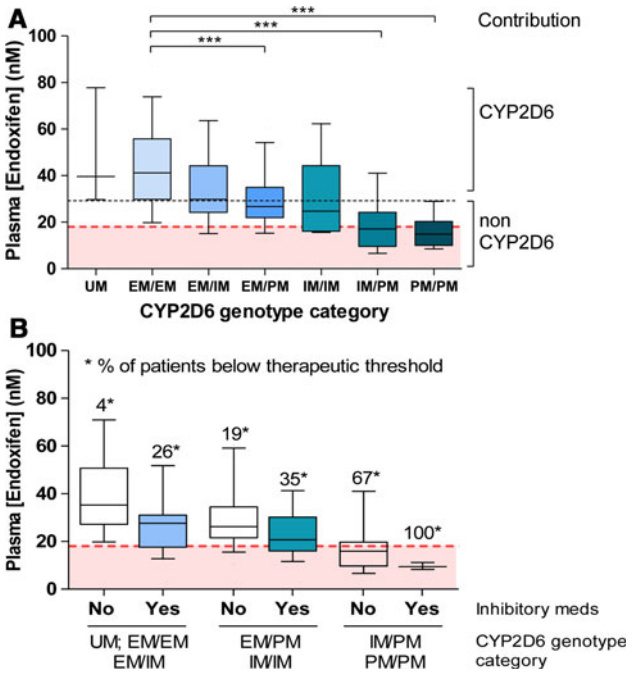


Fig. 1 Influence of *CYP2D6* genotype and inhibitory medications on endoxifen concentrations with respect to the therapeutic threshold. Trough plasma levels of endoxifen **a** in patients not on any known CYP2D6 inhibitors were significantly associated with CYP2D6 genotype. CYP2D6 contribution is denoted by the *dotted line*. **b** Plasma levels of endoxifen in CYP2D6 genotype defined groups not on (No) or concurrently taking CYP2D6 inhibitory medications (Yes). *Numbers* represent percentage of patients below the therapeutic threshold. The *top* and *bottom* of the *box*-and-*whisker* plots represents 25th and 75th percentile, respectively; median is represented by the *middle line*, *whiskers* are the 95 % confidence interval (CI). Therapeutic threshold is represented by the *dashed line* with the *shaded region* indicating the sub-therapeutic range. ***$P < 0.0001$

variability in endoxifen levels observed within CYP2D6 EMs along with the ability of PMs to generate endoxifen suggests that factors in addition to CYP2D6 contribute to the observed variation and importantly, attaining therapeutic endoxifen levels.

Influence of CYP2D6 inhibitory medications on endoxifen concentrations

We examined the effect of concomitant medications on endoxifen levels. In CYP2D6 EM/EM and EM/IM patients a trend toward lower endoxifen levels with increasing SSRI/SNRI inhibitory activity was observed, where patients on strong inhibitors have statistically lower levels compared to patients not on inhibitors (Table 2). In fact, patients concurrently on strong SSRI/SNRI medications had similar endoxifen concentrations as CYP2D6 PMs. Furthermore, taking CYP2D6 inhibitory medications concurrently with tamoxifen lowered endoxifen concentrations in all CYP2D6 defined genotype groups (UM, EM/EM,

Table 2 Effect of CYP2D6 inhibitory medications on endoxifen concentrations in EM/EM and EM/IM patients ($n = 100$) compared to PM patients ($n = 10$)

Covariate (n)	Mean endoxifen nM (SD)	% in sub-therapeutic range	P value
CYP2D6 EM/EM; EM/IM			
No inhibitor (79)	39.6 (16.2)	3.8	
SSRI/SNRI: strong (3)	17.6 (6.8)	66.7	0.0219
SSRI/SNRI: moderate (4)	27.1 (14.3)	25.0	0.1319
SSRI/SNRI: mild (14)	26.0 (8.2)	28.6	0.0028
CYP2D6 PM	15.2 (6.6)	70.0	<0.0001

Table 3 Multiple linear regression model for effect on Ln-transformed endoxifen concentration (adjusted R squared: 0.46)

Predictor variable	Estimate	Standard error	P	Adjusted r^2
Intercept	1.838	0.502	<0.001	
Age	0.005	0.002	0.05	
Ln (BMI)	0.185	0.150	0.220	
CYP2D6 UM	1.155	0.248	<0.001	
CYP2D6 EM/EM	0.960	0.128	<0.001	
CYP2D6 EM/IM	0.808	0.136	<0.001	
CYP2D6 EM/PM	0.658	0.130	<0.001	
CYP2D6 IM/IM	0.534	0.195	0.006	
CYP2D6 IM/PM	0.040	0.167	0.807	0.301
CYP3A4*22 (C/C)	−0.241	0.079	<0.001	0.329
SSRI/SNRI: strong	−0.740	0.159	<0.001	
SSRI/SNRI: moderate	−0.516	0.194	0.008	
SSRI/SNRI: mild	−0.366	0.114	0.002	
Venlafaxine Use	−0.214	0.110	0.052	0.433
Season: summer	0.242	0.073	0.001	
Season: spring	0.225	0.090	0.013	
Season: fall	0.123	0.081	0.132	0.460

BMI body mass index, *EM* extensive metabolizer, *IM* intermediate metabolizer, *Ln* natural log, *PM* poor metabolizer, *UM* ultrarapid metabolizer

EM/IM; EM/PM, IM/IM and IM/PM, PM/PM) resulting in an increased percentage of patients falling below the therapeutic threshold (Fig. 1b; Table 2).

Seven patients on CYP2D6 SSRI/SNRI inhibitors were informed of the potential inhibitory effect on endoxifen and voluntarily discontinued or lowered the inhibitor dose. Endoxifen concentrations were increased by approximately twofold following SSRI/SNRI discontinuation or dosing change compared to a 1.3-fold change observed in patients who remained on a stable SSRI dose at 6-month follow-up ($p = 0.041$) (data not shown).

CYP3A5, P450 oxidoreductase, CYP2C9, CYP2B6 and transporters BCRP and MDR1 do not affect endoxifen levels

Additional genetic factors that may contribute to the variability of endoxifen levels were assessed in genes involved in tamoxifen metabolism as well as drug transporters that may aid in determining tamoxifen metabolite exposure. Endoxifen concentrations, as well as all other metabolites measured (data not shown), were not associated with CYP3A5*3 genotype or the *POR*28 SNP in the gene encoding P450 oxidoreductase that has been associated with increased CYP3A4 activity in vivo (Supplementary Fig. S5) [19]. Carriers of *CYP2C9*2 or *3 or *CYP2B6* variant alleles (*1/*4, *1/*5, *5/*5, *4/*6, *5/*6, *1/*6, or *6/*6) did not have significantly different endoxifen levels, compared to wildtype patients (Supplementary Fig. S5). Polymorphisms in BCRP (*ABCG2*) at positions 34 (G > A) and 421 (C > A) or in MDR1 (*ABCB1*) at position 3435 (C > T) did not associate with differences in endoxifen level compared to wildtype (Supplementary Fig. S5).

Influence of CYP3A4 on endoxifen and metabolite concentrations

We examined the effect of the recently identified SNP *CYP3A4*22* [20] on tamoxifen and metabolite levels. *CYP3A4*22* CT/TT patients (allele frequency 0.079) had significantly higher levels of tamoxifen, endoxifen and all other metabolites measured (Fig. 2a, b and Supplementary Fig. S6 A–D). Among the *CYP3A4*22* CC versus CT/TT carriers, 23 and 10 % of patients had endoxifen levels below 18 nM, respectively, indicating that carriers were twofold less likely to be in sub-therapeutic range (Fig. 2b). Since the *CYP3A4*22* SNP was only recently described, we assessed its utility as a marker of CYP3A4 activity by evaluating its relationship with the endogenous CYP3A4 biomarker 4-β-HC/Total-C [21]. We found that *CYP3A4*22* CT/TT carriers, with reduced expression of CYP3A4, had significantly lower 4-β-HC/Total-C compared to wildtype patients (Fig. 2c). Thus, we postulate that this SNP is likely predictive of CYP3A4 activity. We observed a significant correlation between the metabolic ratios of NDM-tamoxifen/tamoxifen ($r^2 = 0.13$) and 4-OH-tamoxifen/tamoxifen ($r^2 = 0.19$) to 4-β-HC/Total-C suggesting that CYP3A4 activity is associated with the conversion of tamoxifen to both primary metabolites (Supplementary Fig. S6E, F). While no association with *CYP3A4*22* genotype was found for metabolic ratios of endoxifen to tamoxifen and endoxifen to both primary metabolites, the total metabolite (NDM-tamoxifen + 4-OH-tamoxifen + endoxifen) to tamoxifen ratio was significantly lower in *CYP3A4*22* CT/TT carriers

(Fig. 2d). Interestingly, we observed increased endoxifen levels in *CYP3A4**22 CT/TT carriers within all CYP2D6 genotype defined groups (Fig. 2e), suggesting that the presence of this SNP may be clinically important for tamoxifen efficacy. This may be especially pertinent in CYP2D6 PMs as the majority of PMs who carry the *CYP3A4**22 allele had endoxifen levels above the therapeutic threshold compared to PMs wildtype for this SNP who fell mostly within sub-therapeutic range.

Seasonal variation of endoxifen levels

There is extensive evidence indicating that vitamin D3 can regulate intestinal CYP3A4 expression through the binding of VD3/vitamin D receptor (VDR) complexes to proximal promoter elements [22, 23]. Additionally, VDR polymorphisms are associated with a seasonally dependent intestinal CYP3A4 expression [24]. Therefore, we hypothesized that tamoxifen metabolism may be affected seasonally by vitamin D levels. Interestingly, endoxifen levels measured from patients in winter months (Jan–Mar) were ~20 % lower than the mean endoxifen concentration while drug levels obtained from patients in the summer (July–Sept) months were more than 8 % above average (Fig. 3a). A similar trend was observed for tamoxifen and other metabolites (data not shown). Patients taking vitamin D supplements tended to have a greater increase in endoxifen levels compared to those not taking vitamin D supplements in each season (Fig. 3b), suggesting that dietary supplementation in addition to sunlight exposure may increase endoxifen levels. Furthermore, a significant association was found between endoxifen and 25-OH-vitamin D level (Supplementary Fig. S7). Finally, we note that there was a twofold increase for patients to be in the sub-therapeutic range during fall and winter seasons compared to spring and summer (Fig. 3c).

Predictors of endoxifen level

Multi-step linear regression analysis was performed to determine significant covariates affecting endoxifen concentration. Endoxifen levels were found to be dependent on *CYP2D6* genotype, concomitant use of SSRI/SSNI, season of blood draw, and *CYP3A4**22 genotype, in order of entry into the stepwise regression. Parameter estimates of the final endoxifen concentration model are given in Table 3.

Predictors of endoxifen therapeutic concentration

Key determinants of sub-therapeutic endoxifen concentration were *CYP2D6* genotype, SSRI/SNRI use, season, and *CYP3A4**22 genotype (Fig. 4a, Supplementary Table S2).

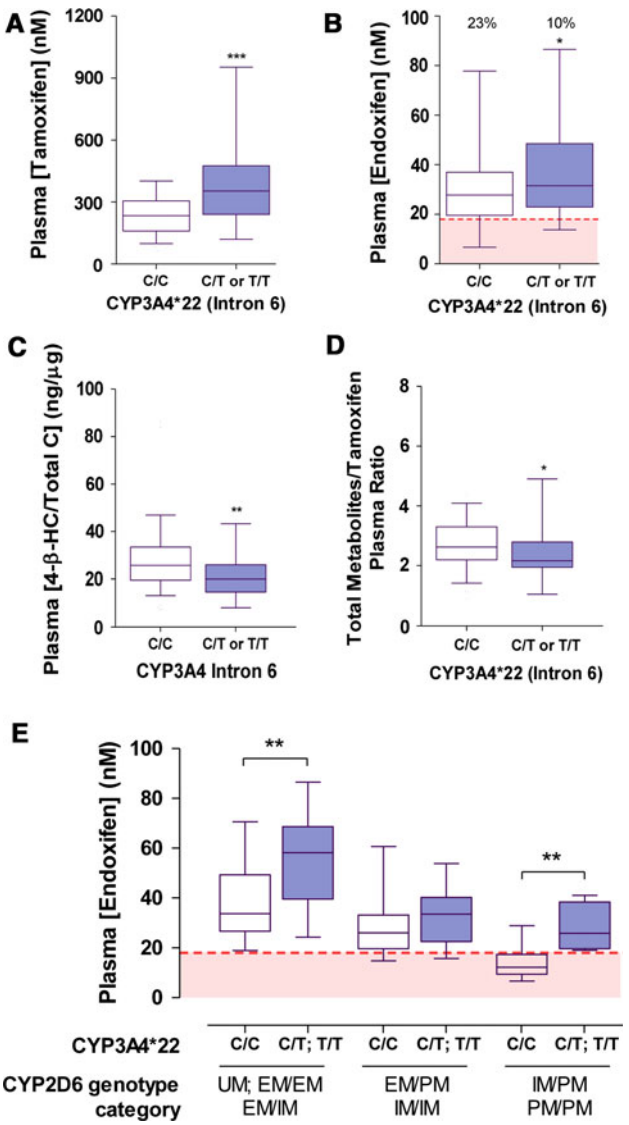


Fig. 2 Influence of *CYP3A4* genotype and activity on tamoxifen metabolite concentrations. Association of *CYP3A4**22 genotype with trough plasma concentrations of tamoxifen (**a**) and endoxifen (**b**). Percentage of patients within sub-therapeutic range is shown (**b**). **c** Association of *CYP3A4**22 genotype with the endogenous CYP3A4 biomarker, 4-β-OH-cholesterol/total cholesterol. **d** Association of *CYP3A4**22 genotype with the metabolic ratio of total metabolite (NDM-tamoxifen + 4-OH-tamoxifen + endoxifen) and tamoxifen. **e** Plasma levels of endoxifen in CYP2D6 genotype defined groups in the absence (C/C) or presence (C/T; T/T) of *CYP3A4**22 SNP. The *top* and *bottom* of the *box*-and-*whisker* plots represents 25th and 75th percentile, respectively; median is represented by the *middle line*, *whiskers* are the 95 % CI. Therapeutic threshold is represented by the *dashed line* with the *shaded region* indicating the sub-therapeutic range. *$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$

The predictive accuracy of the logistic model was 89 %. At an optimal cut-off probability of 0.8, sensitivity and specificity of the model was 81 and 77 %, respectively, with a false positive rate of 23 % and a false negative rate of 19 %

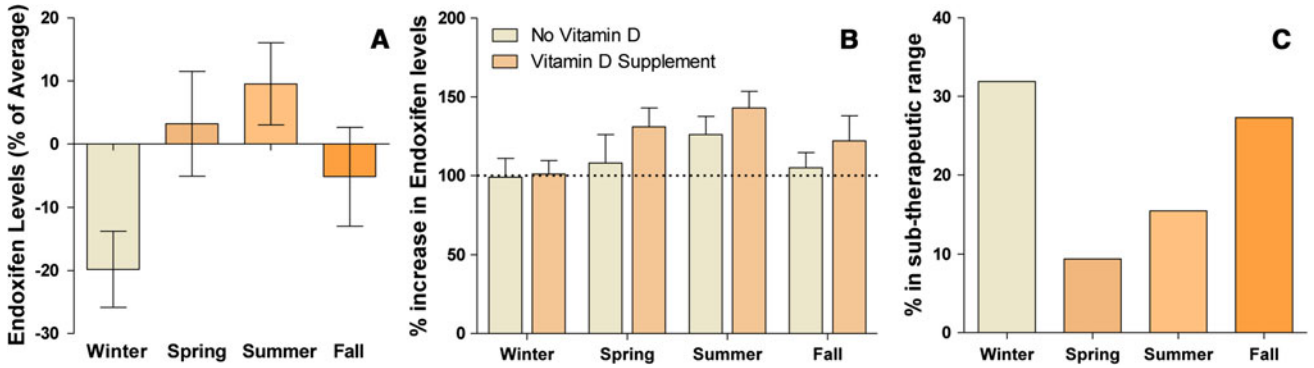


Fig. 3 Effect of seasonal variation and vitamin D levels on endoxifen concentrations. **a** Percent of the mean endoxifen concentration stratified by season in which the blood sample was collected. Winter: January–March, Spring: April–June, Summer: July–September, Fall: October–December. **b** Percent increase in endoxifen levels in patients concomitantly taking or not taking vitamin D supplements compared to patients in winter (not taking vitamin D) stratified by season. **c** Percent of patients in sub-therapeutic range stratified by season

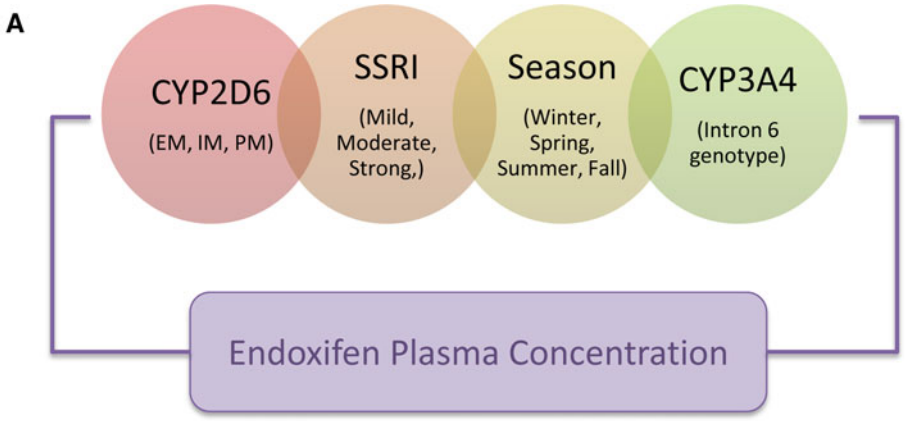
(Supplemental Table S3). The cross-validation estimate of accuracy was 85 %. Together, these factors along with patient demographics can be used in an algorithm to predict endoxifen concentration (Fig. 4b, supplementary excel file).

Discussion

Considerable attention has been given to the role of *CYP2D6* genetic variation in predicting tamoxifen therapeutic benefit. Studies of various designs have shown that carriers of reduced or non-functional *CYP2D6* alleles exhibit worse breast cancer outcomes [9, 25] while two recently published trials refuted the link between CYP2D6 and tamoxifen [10, 26]. However, the interpretation of those trials is complicated by the use of tumor-derived DNA. Overall, *CYP2D6* genotype has been consistently linked to endoxifen exposure and recent results of the WHEL study indicated that patients falling below a threshold level of endoxifen concentration are at an increased risk of therapeutic failure [16, 27]. However, significant variability in endoxifen levels still exists within *CYP2D6* genotype groups, suggesting that additional variables remain to be elucidated.

The results of this study confirm that *CYP2D6* genotype accounts for a large proportion (30 %) of the variability in endoxifen concentration. Given the high level of variance explained by a single gene, CYP2D6 is evidently the central enzyme involved in modulating tamoxifen metabolism and efficacy. Furthermore, CYP2D6 inhibitory medications place patients at significantly increased risk of attaining sub-therapeutic endoxifen levels demonstrated by the observation that increasing severity of CYP2D6 inhibition by SSRI/SNRIs results in exacerbated risk of falling below the threshold level. Overall, our data suggests that use of CYP2D6 inhibitory medications should be avoided to control for depressive and vasomotor symptoms.

As endoxifen formation is not solely reliant on CYP2D6 activity, other genetic and environmental factors must contribute to plasma endoxifen exposure. CYP3A4 is the most abundant CYP enzyme in the liver and metabolizes 50 % of all drugs [28], but its activity and expression can vary 10–100 fold and genetic markers to explain this variability are lacking [29]. Recently, a newly identified SNP in intron 6 was associated with decreased mRNA expression [20]. We demonstrate for the first time that *CYP3A4**22 associates with lower 4-β-HC/Total-C, an in vivo marker of CYP3A4 activity. Unexpectedly, lower CYP3A4 activity resulted in higher levels of all metabolites measured including endoxifen while retaining the predicted higher tamoxifen levels. This correlates with recent data showing that CYP3A4 induction by rifampicin resulted in decreased levels of both tamoxifen and endoxifen [30]. A reduction in intestinal CYP3A4 activity in *CYP3A4* *22 carriers may reduce first pass metabolism resulting in enhanced tamoxifen bioavailability as was seen recently with the administration of curcumin, a CYP3A4 inhibitor, in rats [31]. Additionally, the increased levels of endoxifen and other metabolites in *CYP3A4**22 carriers may be due to the higher level of tamoxifen in these patients as tamoxifen concentration was the best predictor of metabolite formation in our regression analysis. Although individual metabolite to parent ratios did not associate with *CYP3A4**22, total metabolite to tamoxifen ratio was significantly lower in T carriers indicating modulation of CYP3A4-mediated metabolite formation. As tamoxifen metabolism is extremely complex, it is difficult to determine the effect of CYP enzymes in isolation. It is likely that the role of additional CYP enzymes becomes more important in the absence of CYP2D6. Indeed, we observed higher endoxifen levels (within therapeutic



B

Prediction of Therapeutic Endoxifen Level

Patient ID	PGTM1	
Clinical Data		
Age	50	Years
Weight	70	Kg
Height	1.7	Meters
Season	Spring	
SSRI Use	None	
Genetic Markers		
CYP2D6		
CYP2D6*3	A/del	(2549A>del; A/A, A/del, del/del)
CYP2D6*4	G/G	(1846G>A; G/G, G/A, A/A)
CYP2D6*9	AGA/AGA	(2613_2615delAGA; AGA/AGA, AGA/del, del/del)
CYP2D6*10	C/C	(100C>T; C/C, C/T, T/T)
CYP2D6*41	G/G	(2988G>A; G/G, G/A, A/A)
CYP2D6 CNV	None	(CNV)
CYP2D6 Phenotype	EM/PM	
CYP3A4		
CYP3A4*22	C/C	(Intron6C>T; C/C, C/T, T/T)
Calculated Concentration (nM)		28.22
Percentile Rank		49%

Fig. 4 Clinical guideline model of factors associated with prediction of therapeutic endoxifen concentrations in individual patients. **a** Significant factors contributing to endoxifen plasma concentration. **b** Endoxifen plasma level predictor and percentile ranking based on genotypes and clinical variable is available for download (see Supplemental file: endoxifen concentration calculator)

range) in PMs carrying the *CYP3A4**22 allele compared to the sub-therapeutic levels seen in PMs wildtype for this SNP. Taken together, our data suggests that the role of CYP3A4 in tamoxifen metabolism and efficacy may have been previously underestimated, likely due to the lack of a reliable marker of its activity. Retrospective analysis of *CYP3A4**22 association with tamoxifen outcomes in clinical trials is needed to determine its clinical relevance in predicting tamoxifen efficacy.

As we have previously shown that endoxifen is a substrate of MDR1 [15], transport proteins may play a role in the overall exposure of endoxifen. Here, we show that functional polymorphisms in *ABCB1* (MDR1) and *ABCG2* (BCRP) do not associate with endoxifen levels. However, Kiyotani et al. [32], demonstrated that polymorphisms in *ABCC2* (MRP2) were significant predictors of recurrence rates without affecting endoxifen plasma concentrations. Therefore, the effect of polymorphisms in transporters may modulate the overall tumor exposure to endoxifen resulting in enhanced risk of recurrence.

Our study indicates for the first time that environmental factors such as sunlight exposure and vitamin D status may

impact tamoxifen metabolism. While insufficient vitamin D levels and low amounts of sunlight exposure have been suggested to increase the risk of breast cancer [33], the association of vitamin D levels and recurrence rates has been inconsistent [34]. There is considerable evidence indicating that elevated vitamin D levels can induce CYP3A4 expression through activation of the VDR [23, 35]. However, we found no correlation between vitamin D level or season and 4-β-HC/Total-C, and thus, the effect of vitamin D exposure due to seasonal variation likely affects endoxifen formation through an alternate mechanism. Nevertheless, vitamin D levels can be influenced by diet and more importantly by sunlight exposure [36]. In many geographical locations the exposure to sunlight can vary dramatically throughout the year, especially in northern climates [36]. Evidence suggests there is little sunshine associated vitamin D production during the winter months among those residing in latitudes above 45° including much of the USA, all of Canada, and most of Europe [37]. Therefore, patients in more northern climates may have lower or more variable endoxifen concentrations throughout the year. Our observations provide rationale for a larger clinical trial designed to investigate the association of vitamin D levels and endoxifen concentrations with repeat measures over time.

Published analytical methods to measure endoxifen have varied resulting in a wide range of endoxifen concentrations. Our measured endoxifen levels are similar to recent reports by Madlesky et al., and Murdter et al., however, are much lower than older studies representing an important caveat to determining endoxifen concentrations [5–7, 16]. Analytical capabilities for therapeutic drug monitoring of endoxifen may not be feasible in many clinical settings. Probe drugs including dextromethorphan have been suggested as a method to predict endoxifen concentration [38], however, this strategy requires administration of an additional drug with timed sampling for analysis that may be impractical for many patients. As such, use of covariates including only demographic and genotype information that are predictive of endoxifen concentration may be a more feasible and cost effective approach to identify patients at risk for reduced tamoxifen benefit. This strategy can be easily implemented prior to or during initiation of therapy with a single blood sample.

Although this study is limited by the lack of clinical outcomes, recent evidence suggests that the 20th percentile of endoxifen concentration represents the relevant cut-off for determining breast cancer recurrence during tamoxifen therapy [16]. We show that clinically observed endoxifen levels, particularly those of CYP2D6 EMs, are associated with optimal tumor response in our endoxifen xenograft model [17]. Taken together, this provided rationale for defining the therapeutic threshold in this study at 18 nM (the 20th percentile of our study). As tamoxifen remains a necessary treatment for many pre-menopausal women, attaining sufficient endoxifen level is likely essential to prevent recurrence. This may require a method to predict the appropriate tamoxifen dose to reach therapeutic endoxifen levels. A recent dose-escalation study showed similar endoxifen concentration between CYP2D6 IMs and PMs on 40 mg of tamoxifen compared to EMs on the standard 20 mg, indicating that dose adjustment can compensate for metabolic deficiency [14].

Accordingly, the algorithm formed here incorporates genetic and environmental covariates that aid in predicting sub-therapeutic endoxifen concentration. The identification of patients at risk for sub-optimal treatment should improve tamoxifen efficacy by tailoring the therapy for these individuals. Additionally, this strategy may be particularly useful in the post-menopausal population when considering tamoxifen therapy for those who are not ideal candidates for aromatase inhibitors [11]. Further validation of our algorithm, particularly in the setting of a large randomized trial is needed to determine its utility in predicting breast cancer-associated outcomes.

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Conflict of interest None.

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