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ORIGINAL ARTICLE

Tamoxifen metabolism predicts drug concentrations and outcome in premenopausal patients with early breast cancer

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Tamoxifen is the standard-of-care treatment for estrogen receptor-positive premenopausal breast cancer. We examined tamoxifen metabolism via blood metabolite concentrations and germline variations of CYP3A5, CYP2C9, CYP2C19 and CYP2D6 in 587 premenopausal patients (Asians, Middle Eastern Arabs, Caucasian-UK; median age 39 years) and clinical outcome in 306 patients. *N*-desmethyltamoxifen (DM-Tam)/*(Z)*-endoxifen and CYP2D6 phenotype significantly correlated across ethnicities (R^2: 53%, $P<10^{-77}$). *CYP2C19* and *CYP2C9* correlated with norendoxifen and (Z)-4-hydroxytamoxifen concentrations, respectively ($P<0.001$). DM-Tam was influenced by body mass index ($P<0.001$). Improved distant relapse-free survival (DRFS) was associated with decreasing DM-Tam/*(Z)*-endoxifen ($P=0.036$) and increasing CYP2D6 activity score (hazard ratio (HR) = 0.62; 95% confidence interval (CI), 0.43–0.91; $P=0.013$). Low (<14 nM) compared with high (>35 nM) endoxifen concentrations were associated with shorter DRFS (univariate $P=0.03$; multivariate HR = 1.94; 95% CI, 1.04–4.14; $P=0.064$). Our data indicate that endoxifen formation in premenopausal women depends on CYP2D6 irrespective of ethnicity. Low endoxifen concentration/formation and decreased CYP2D6 activity predict shorter DRFS.

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INTRODUCTION

The standard-of-care for estrogen receptor (ER)-positive breast cancer patients who are functionally premenopausal is a 5-year treatment with the selective ER modulator tamoxifen[1] as aromatase inhibitors are of limited use due to the strong hypothalamic–pituitary control of estrogen levels.[2] Tamoxifen for 5 years substantially lowers the yearly relapse rates and mortality in primary breast cancer.[3] Data from the Adjuvant Tamoxifen, Longer Against Shorter trial and the Adjuvant Tamoxifen Treatment offers more trial indicate further benefit by maintaining tamoxifen treatment for 10 years with 25% mortality reduction relative to 5 years.[4,5] Despite this success, about 50% of patients do not benefit from tamoxifen and frequent adverse drug reactions (ADR) including hot flashes, vasomotor and gynecologic symptoms as well as depression and diminished sexual functioning prevent particularly young women from staying on the drug.[6,7] Toward a personalized strategy of premenopausal breast cancer treatment, the possible association between serum concentration of the active tamoxifen metabolite endoxifen and the occurrence of side effects has been addressed;[7,8] yet, the potential of such an approach regarding treatment outcome in young women is unclear.

Tamoxifen, which acts at the ER, requires conversion into active metabolites (*Z*)-4-hydroxytamoxifen and (*Z*)-endoxifen that have up to 100-fold higher ER affinity than the parent drug.[9–11] The cytochrome P450 enzyme CYP2D6 has a major role in the formation of endoxifen[12] in postmenopausal women.[13–15] *CYP2D6* is highly polymorphic with more than 100 genetic variants (http://www.imm.ki.se/cypalleles/cyp2d6.htm) contributing to the high interindividual variability of enzyme activity. Impaired metabolism by CYP2D6 can be accurately predicted by loss- and reduced-function alleles resulting in the poor metabolizer (PM) and intermediate metabolizer (IM) phenotypes. Likewise, functional and duplicated *CYP2D6* alleles correlate with extensive (EM) and ultra rapid (UM) metabolizer phenotypes, respectively.[16] There are significant interethnic differences of *CYP2D6* allele frequencies across geographic regions and populations leading to a shift of metabolizer phenotype prevalence with higher frequencies of IMs in Asians and UMs in Arabic/North African countries as compared with populations of European descent.[17]

Clinical outcome of adjuvant tamoxifen in postmenopausal patients is influenced by their CYP2D6 metabolizer phenotype which can be predicted by genetic testing[18–20] using germline

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rather than tumor DNA.[21,22] However, the current debate on the validity of the postmenopausal data and the lack of CYP2D6 association with clinical outcome in some of these studies[21–28] point to the need of combined pharmacokinetic and pharmacogenetic analyses particularly in the case of testing the hypothesis in another patient group, i.e. premenopausal patients. Recently, lower endoxifen concentrations were shown to be associated with poor clinical outcome in a mixed cohort of pre- and postmenopausal patients.[14] Notably, the *in vitro* pharmacological modeling of endoxifen concentrations for the treatment of ER positive breast cancer showed the essential requirement of endoxifen to block breast cancer cell growth in the presence of high estrogen concentrations equivalent to premenopausal patients.[29,30] Therefore, it is reasonable to hypothesize that variable endoxifen formation contributes to tamoxifen efficacy in premenopausal patients. Here, we present combined pharmacokinetic and pharmacogenetic analyses in purely premenopausal breast cancer patient cohorts of different ethnic origin to evaluate (i) the factors that influence active tamoxifen metabolite concentrations with a particular emphasis on CYP2D6 and (ii) whether tamoxifen metabolite concentrations and/or genetic variants of drug-metabolizing enzymes (DME) are suitable biomarkers for the prediction of clinical outcome.

PATIENTS AND METHODS

Patients and study design

Three ethnic groups of prospectively recruited hormone receptor-positive premenopausal breast cancer patients with adjuvant tamoxifen treatment were investigated (Figure 1). Of these, 164 Asian patients in part previously described[31] (136 Chinese, 14 Malays and 14 Indians) have been provided by the Division of Medical Sciences, Humphrey Oei Institute of Cancer Research, Singapore. Another 78 consecutively recruited patients (2009–2011) in part previously described[32] have been provided by the Hematology-Oncology Division, Internal Medicine, American University of Beirut, Lebanon (Leb). Furthermore, 345 patients were selected from the Prospective study of Outcomes in Sporadic versus Hereditary breast cancer (POSH) cohort, an observational cohort study comprised mainly of Caucasian patients (2956 patients recruited between 2001 and 2007) by the Cancer Sciences Academic Unit and University of Southampton Clinical Trials Unit, University of Southampton, UK.[33,34] Selection of patients for our current study was based on the availability of tamoxifen steady-state serum and germline DNA. Patients taking CYP2D6 inhibitors were excluded from the Singapore cohort and comprised only seven patients who received weak CYP2D6 inhibitors, venlafaxine, escitalopram or clomipramine in the Lebanon cohort. For the POSH cohort, data on co-medication was not available. In total, 587 premenopausal patients were investigated for the quantitation of tamoxifen metabolites and genotyping. Figure 1 shows the patient inclusion scheme to explain the underlying rationale for the survival analyses, which was performed in the POSH cohort but not in the Singapore and Lebanon cohorts due to incomplete follow-up and delayed study entry of their patients, respectively (Figure 1).

Steady-state blood samples of patients treated with tamoxifen (20 mg per day) were collected on-site within the first year of treatment and immediately stored at −20 °C. Study approvals were obtained from the National Cancer Centre Ethics Review Committee (Singapore), American University of Beirut Institutional Review Board (Lebanon) and South and West MultiCentre Research Ethics Committee (MREC 00/6/69; POSH). All patients provided informed consent.

Measurement of tamoxifen and metabolites

Tamoxifen and its metabolites DM-Tam, (*Z*)-4-hydroxytamoxifen ((Z)-4-OH-Tam), (*Z*)-endoxifen, *N*-desmethyltamoxifen (DM-Tam), *N*,*N*-Didesmethyltamoxifen

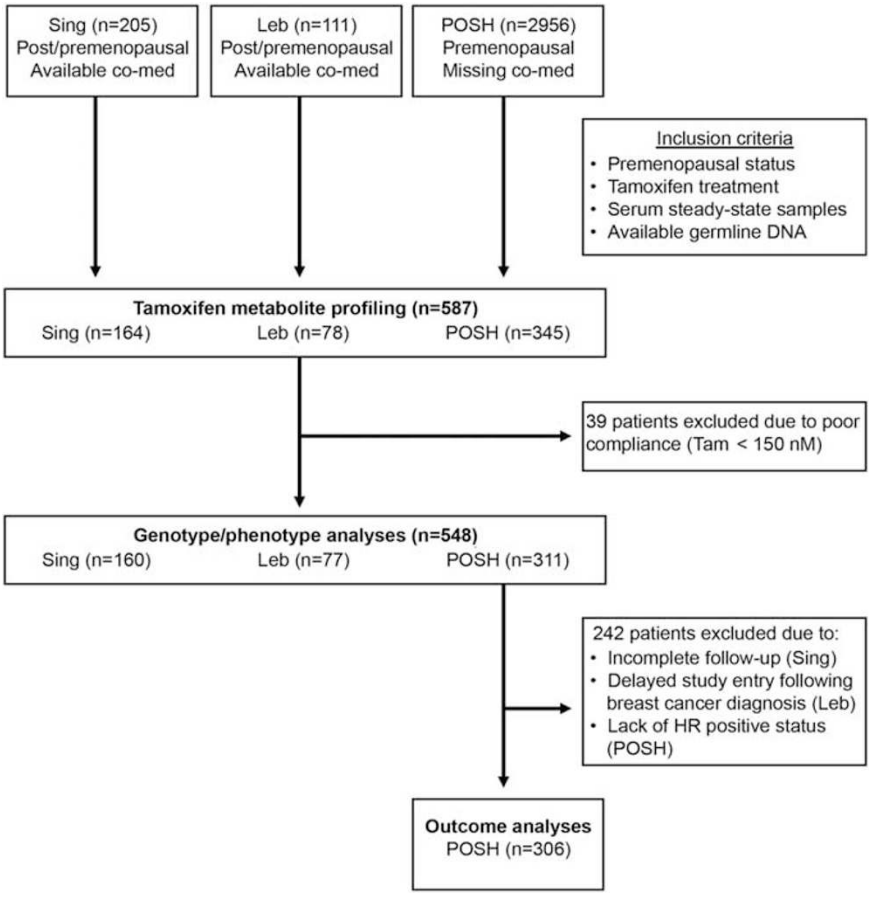

Figure 1. Study flow diagram of premenopausal study. co-med, co-medication; HR, hormone receptor; Leb, Lebanon; POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer; Sing, Singapore; Tam, tamoxifen.

(DDM-Tam) and (*Z*)-norendoxifen ((Z)-4-OH-DDM-Tam; Supplementary Figure 1) were measured by liquid chromatography tandem mass spectrometry in the multiple reaction monitoring mode on a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) as described previously.[13] All analyses refer to the active (*Z*)-isomers of the tamoxifen metabolites which were separated from their respective inactive (*E*)-isomers.[35]

DNA isolation and genotyping

DNA samples were genotyped for CYP2D6 alleles associated with null (PM: *3, *4, *5 and *6) and reduced (IM: *9, *10 and *41) CYP2D6 function by matrix-assisted, laser desorption/ionization, mass spectrometry.[18,31] CYP2D6 gene duplication (UM) was determined via TaqMan Copy Number Assay (Applied Biosystems, Foster City, CA, USA). Absence of variant alleles or gene duplications was assigned to normal CYP2D6 function (EM). Individuals with ambiguous genotypes were verified by AmpliChip P450 assay (Roche Molecular Diagnostics, Mannheim, Germany), which revealed additional CYP2D6 alleles *14, *15 and *17. For genotype interpretation, we applied a CYP2D6 activity score[36] to predict PM, IM, EM and UM phenotypes: PM/PM (0), PM/IM (0.5), IM/IM (0.75), PM/EM (1), IM/EM (1.5), EM/EM (2) and EM/UM (3). In survival analyses, CYP2D6 activity scores were classified into three phenotype classes: PM (0), hetEM/IM (0.5–1.5) and EM and UM (2 to 3). Genetic testing was also performed for CYP2C9 (*2, *3), CYP2C19 (*2,*3,*17) and CYP3A5*3, known to be involved in tamoxifen metabolism using TaqMan or Sequenom mass array genotyping.[13]

Study aims, power calculation and statistical analyses

Primary aim of this study was to determine factors that influence active tamoxifen metabolite concentrations with special emphasis on CYP2D6-catalyzed metabolite endoxifen. Thus, we performed a power calculation assuming a 50% decrease or increase of square root transformed endoxifen or log-transformed metabolic ratio (MR) DM-Tam/endoxifen per one unit of CYP2D6 activity score as a relevant gene-dose effect.[13,37] On the basis of the s.d. of the plasma concentrations in the three cohorts (excluding poorly/non-compliant patients), we computed that the sample size in the smallest cohort ($n = 77$, Leb) would already provide 92.9% power in the analyses. Secondary aim was to test whether tamoxifen metabolite concentrations and/or genetic variants of DME can reliably predict clinical outcome in premenopausal patients. As there is no such data published in a purely premenopausal clinical setting, we consider this an exploratory investigation.

SPSS (version 20, Chicago, IL) and R-3.01 including libraries coin-1.0–22, MASS-7.3–27, mfp-1.4.9, party-1.0–8 and survival-2.37–4 (www.r.project.org) were used for statistical analyses. Clinical characteristics and DME genotypes were compared between study cohorts by Kruskal–Wallis and χ^2-tests. Tamoxifen concentrations across all patients displayed two splits operationally used to define non-compliance ($\leqslant 40$ nM) and poor compliance (40–150 nM). Frequencies of CYP2D6 phenotypes between these groups were compared by χ^2-tests. All following analyses excluded poorly/non-compliant patients.

Correlations of square root transformed endoxifen concentrations or log-transformed MR DM-Tam/endoxifen and CYP2D6 activity score were examined by linear models with stratification for ethnicity.

Survival analyses were strictly confined to the POSH cohort because of delayed study entry following breast cancer diagnosis and incomplete follow-up in the Lebanon and Singapore cohorts, respectively. The endpoint was distant relapse-free survival (DRFS) defined as the time from diagnosis to the earliest occurrence of distant metastasis or death from any cause. First, we tested which clinical characteristics, tamoxifen metabolites or CYP genotypes were associated with DRFS using univariate Cox regression and general asymptotic independence tests. Step-wise model selection (R-library mfp) revealed nodal status and chemotherapy use to be included as covariates in subsequent multivariate Cox regression. Conditional inference trees (*P*-value cutoff 0.15) and receiver operating characteristic analyses on DRFS were applied to identify cutoffs for MR DM-Tam/endoxifen and endoxifen concentration. For endoxifen, there was neither a split nor a significant association in multivariate Cox regression, hence we explored non-monotonic effects by dividing the POSH population into quarters. Associations between DRFS and classified endoxifen concentrations, MR DM-Tam/endoxifen or CYP2D6 phenotypes were investigated by Kaplan–Meier analyses and multivariate Cox regression adjusted for nodal status and chemotherapy use. All Cox models were stratified for ethnicity. All statistical tests were two-sided and statistical significance was defined as $P < 0.05$.

RESULTS

Patient characteristics and cytochrome P450 genotypes

The median age of diagnosis was 39.1 years (range 22–59 years) (Table 1). Follow-up, age, body mass index (BMI), ethnicity, tumor size and proportion of patients treated with chemotherapy differed among cohorts. Genotypes were successfully obtained from 583 patients for *CYP2D6* (99%) and in 97–99% for *CYP3A5*, *CYP2C9* and *CYP2C19*. Genotypes met Hardy–Weinberg Equilibrium with few exceptions of minor deviations (Table 2). Notably, UM frequency in the POSH cohort was lower than expected[38] likely due to sample size.

Tamoxifen metabolite profiling, compliance and CYP2D6 activity

There was a strong interindividual variability for tamoxifen and the five metabolites in each ethnic group (Figure 2). According to two tamoxifen concentration splits, we defined non-compliant ($\leqslant 40$ nM), poorly compliant (40–150 nM) and compliant (> 150 nM) patients (Figure 2, dashed lines). Twenty-four POSH patients were non-compliant (7%) in their first-year serum sample, 10 patients were poorly compliant (2.9%). Of the other two cohorts, two patients were below 40 nM, whereas three patients were below 150 nM. There were no differences in CYP2D6 phenotype frequencies between poorly/non-compliant patients and the remaining patients, therefore subsequent genotype-phenotype correlations were not biased by excluding poorly/non-compliant patients (data not shown).

The compliant patients from each cohort are shown according to genotype-predicted CYP2D6 activity scores (Table 3). There was a strong gene-dose effect for an association between the CYP2D6 activity score and endoxifen concentrations ($P < 10^{-40}$) in all ethnic cohorts (Figure 3a). The contribution of CYP2D6 to the interindividual variability of endoxifen formation via DM-Tam, as deduced from MR DM-Tam/(*Z*)-endoxifen, was 46% (Singapore, $P < 10^{-17}$), 55% (Lebanon, $P < 10^{-9}$) and 55% (POSH, $P < 10^{-46}$), respectively (Figure 3b). Altogether, 53% of the interindividual variability of endoxifen formation from DM-Tam was attributed to CYP2D6 (Figure 3c; $R^2 = 0.53$; $P < 10^{-77}$).

Other factors influencing tamoxifen metabolism

The formation of norendoxifen from DDM-Tam precursor and *(Z)*-4-OH-Tam converted from tamoxifen was decreased in patient carriers of *CYP2C19*2* and/or **3* (Figure 4a, $P < 0.001$) and *CYP2C9*2* and/or**3* alleles (Figure 4b), $P < 0.001$, respectively. Lower levels of DM-Tam were observed in patients with a BMI higher than 30, both in Caucasians and non-Caucasians (Figure 4c, $P < 0.001$). Notably, DM-Tam formation did not depend on the *CYP3A5*3* reduced-function allele. Multivariate regression analyses across all cohorts showed that the combined genetic (*CYP2C9, CYP2C19, CYP3A5*) and non-genetic factors (age, BMI) contributed to only 2.8% of DM-Tam/endoxifen ratio as compared with 53% by CYP2D6.

Tamoxifen metabolites and clinical outcome

POSH outcome analysis ($N = 306$) was performed excluding hormone receptor-negative and poorly/non-compliant patients. There was no association between endoxifen concentrations and DRFS given the lack of a significant trend (Table 4, Figure 5a). However, following Madlensky *et al.*,[14] when we classified patients into percentiles, we observed that patients with low endoxifen concentrations (< 14.15 nM) had higher risk for distant relapse or death compared with those with high concentrations (> 35 nM, $P = 0.03$, Figure 5a), and showed a trend by multivariate Cox regression analysis of an increased hazard ratio (HR) of 1.94; 95% confidence interval (CI) 0.96–3.93; $P = 0.064$ (Table 4). We next explored the MR DM-Tam/endoxifen and observed an increased HR with increasing MR (decreasing endoxifen formation rate)

Table 1. Clinical characteristics of premenopausal patients

	Singapore (n = *164*)		*Lebanon* (n = *78*)		*POSH* (n = *345*)		*Overall* (n = *587*)		P-*value*[a]
	No. of patients	*%*	*No. of patients*	*%*	*No. of patients*	*%*	*No. of patients*	*%*	
Follow-up, years									<0.001
Median	4.0		4.1		6.4		5.5		
Range	1.3–14.2		1.9–8.5		1.2–11.6		1.2–14.2		
Age at diagnosis, years									<0.001
Median	47.0		43.0		37.5		39.1		
Range	30.0–59.0		24.0–51.0		22.0–41.0		22.0–59.0		
BMI									<0.001
⩽30	153	93.3	62	79.5	272	78.8	487	82.9	
>30	10	6.1	16	20.5	65	18.8	91	15.5	
Unknown	1	0.6			8	2.3	9	1.5	
Ethnicity									<0.001
Caucasian					320	92.8	320	54.5	
Asian	164	100			5	1.4	169	28.8	
African					15	4.3	15	2.6	
Middle Eastern			78	100			78	13.3	
Unknown					5	1.4	5	0.9	
Tumor size (cm)									0.004
⩽2	95	57.9	30	38.5	174	50.4	299	50.9	
2–5	58	35.4	35	44.9	142	41.2	235	40.0	
>5	11	6.7	13	16.7	20	5.8	44	7.5	
Unknown					9	2.6	9	1.5	
Nodal status									0.08
Negative	92	56.1	40	51.3	159	46.1	291	49.6	
Positive	69	42.1	38	48.7	184	53.3	291	49.6	
Unknown	3	1.8			2	0.6	5	0.9	
Grading									0.21
G1			14	17.9	38	11.0	52	8.9	
G2			35	44.9	178	51.6	213	36.3	
G3			27	34.6	125	36.2	152	25.9	
Unknown	164	100	2	2.6	4	1.2	170	29.0	
Hormone receptor status									0.472
(ER or PR) Positive	164	100	78	100	340	98.6	582	99.1	
(ER and PR) Negative					3	0.9	3	0.5	
Unknown					2	0.6	2	0.3	
HER2 status									0.252
Positive	43	26.2	18	23.1	66	19.1	127	21.6	
Negative	112	68.3	60	76.9	158	45.8	330	56.2	
Unknown	9	5.5			121	35.1	130	22.1	
Chemotherapy									0.001
Yes	143	87.2	69	88.5	261	75.7	473	80.6	
No	21	12.8	9	11.5	84	24.3	114	19.4	
CYP2D6 phenotypes									<0.001
PM/PM			4	5.1	30	8.7	34	5.8	
hetEM/IM	129	78.7	37	47.4	205	59.4	371	63.2	
EM/UM	33	20.1	37	47.4	110	31.9	180	30.7	
Unknown	2	1.2					2	0.3	

Abbreviations: BMI, body mass index; EM, extensive metabolizer; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; het, heterozygous; PM, poor metabolizer; POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer; PR, progesterone receptor; IM, intermediate metabolizer; UM, ultra rapid metabolizer. [a]Refers to test for differences between the three cohorts.

Table 2. Observed frequencies of DME gene variants

					Genotype frequency				*Minor allele frequency*			
Allele	*Variant*	*rs number*	*Function*	*Genotypes*	*Sing (n = 164)*	*Leb (n = 78)*	*POSH (n = 345)*	*Total (n = 587)*	*Sing*	*Leb*	*POSH*	*Total*
CYP2D6												
*3	2549delA	rs35742686	Abrogated	A/A	1.00	1.00	0.97	0.98	0	0	0.02[†]	0.01
				A/del	0	0	0.03	0.02				
				del/del	0	0	0.003	0.002				
*4	g1846 G>A	rs3892097	Abrogated	G/G	0.99	0.76	0.63	0.75	0.003	0.141	0.21	0.15
				G/A	0.01	0.21	0.31	0.21				
				A/A	0	0.04	0.06	0.04				
*5	Chromosomal deletion		Abrogated	wt/wt	0.87	0.94	0.93	0.91	0.067	0.032	0.035	0.04
				wt/del	0.13	0.06	0.07	0.09				
				del/del	0	0	0	0				
*6	1707delT	rs5030655	Abrogated	T/T	1.00	0.97	0.97	0.98	0	0.013	0.02	0.01
				T/del	0.00	0.03	0.03	0.02				
				del/del	0	0	0	0				
*9	2615_2617delAAG	rs5030656	Reduced	AAG/AAG	1.00	1.00	0.94	0.97	0	0	0.03[†]	0.02
				AAG/del	0	0	0.05	0.03				
				del/del	0	0	0.01	0.003				
*10	g100 C>T	rs1065852	Reduced	C/C	0.31	0.99	0.97	0.79	0.488[π]	0.006	0.02	0.15
				C/T	0.40	0.01	0.03	0.13				
				T/T	0.29	0	0	0.08				
*41	g2988 G>A	rs28371725	Reduced	G/G	0.93	0.72	0.79	0.82	0.037	0.154	0.11	0.1
				G/A	0.06	0.26	0.19	0.16				
				A/A	0.01	0.03	0.01	0.01				
**XN*	Duplication		Increased	2 copies	0.99	0.87	0.99	0.98	0.007[‡]	0.128[‡]	0.003[‡]	0.02[‡]
				>2 copies	0.01	0.13	0.002	0.02				
CYP2C19												
*2	681 G>A	rs4244285	Abrogated	G/G	0.48	0.79	0.75	0.67	0.326	0.116	0.145	0.19
				G/A	0.40	0.18	0.23	0.27				
				A/A	0.13	0.03	0.02	0.05				
*3	636 G>A	rs4986893	Abrogated	G/G	0.90			0.90	0.052	nd	nd	0.05
				G/A	0.09			0.09				
				A/A	0.01			0.01				
*17	−806 C>T	rs12248560	Increased	C/C	0.92	0.62	0.67	0.74	0.04	0.216	0.2[#]	0.15
				C/T	0.08	0.32	0.26	0.22				
				T/T	0.00	0.05	0.07	0.04				
CYP2C9												
*2	c430 C>T	rs1799853	Reduced	C/C	1.00	0.83	0.75	0.83	0	0.087	0.14	0.09
				C/T	0.00	0.17	0.23	0.15				
				T/T	0.00	0.00	0.02	0.01				
*3	c1075 A>C	rs1057910	Reduced	A/A	0.91	0.88	0.85	0.87	0.046	0.06	0.08	0.07
				A/C	0.08	0.12	0.14	0.13				
				C/C	0.01	0.00	0.003	0.002				
CYP3A5												
*3	6986 A>G	rs776746	Markedly reduced	A/A	0.10	0.01	0.02	0.05	0.72	0.91	0.91[Φ]	0.85
				A/G	0.36	0.15	0.14	0.21				
				G/G	0.54	0.83	0.84	0.75				

Abbreviations: c, cDNA; del, deletion; DME, drug-metabolizing enzymes; g, gDNA; Leb, Lebanon; nd, not determined; POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer; Sing, Singapore; w, wildtype; wt, wildtype referring to major, that is, functional alleles. Note-Nucleotide positions refer to numbering according to the ATG start codon. CYP2D6*14, *15 and *17 alleles were detected by AmpliChip quality control only and were not included in the table. **XN*, refers to duplication of EM alleles resulting in UM phenotype. [‡]Refers to prevalence of UM phenotype based on multiple copies of functional alleles. Genotype frequencies that deviate from HWE have the following *P* values: [†]$P = 0.02$; [π]$P = 0.045$; [#]$P = 0.006$; [Φ]$P = 0.003$.

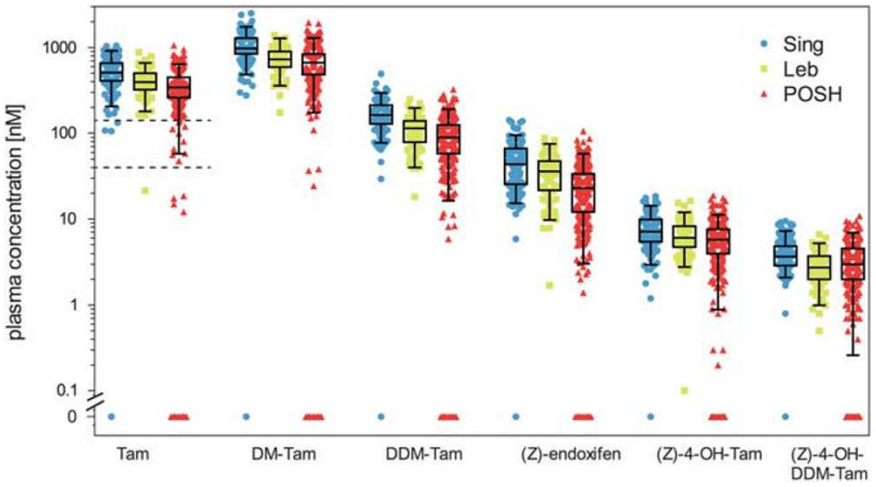

Figure 2. Metabolic profiling for tamoxifen (Tam) and five measured metabolites, *N*-desmethyltamoxifen (DM-Tam), *N*,*N*-didesmethyltamoxifen (DDM-Tam), (Z)-endoxifen, 4-hydroxytamoxifen [*(Z)*-4-OH-DDM-Tam] and norendoxifen [*(Z)*-4-OH-DDMT-Tam] in study cohorts from Singapore (Sing, $N = 164$), Lebanon (Leb, $N = 78$) and Prospective study of Outcomes in Sporadic versus Hereditary breast cancer (POSH, $N = 345$). Metabolite concentrations are presented as boxplots with whiskers defined by the 5th and 95th percentiles and extreme values outside the whiskers. The two dashed lines for Tam delineate putative non-compliant ($\leqslant 40$ nM) and poorly compliant (40–150 nM) patients as defined from Tam plasma concentrations. Patients with Tam concentrations < 150 nM were excluded from further analyses.

Table 3. CYP2D6 activity scores of compliant patients

	Singapore (n = *160*)		*Lebanon* (n = *77*)		*POSH* (n = *311*)		*Overall* (n = *548*)	
CYP2D6 activity scores	*No. of patients*	*%*	*No. of patients*	*%*	*No. of patients*	*%*	*No. of patients*	*%*
0.0	0		4	5.2	25	8.0	29	5.3
0.5	12	7.5	4	5.2	28	9.0	44	8.0
0.75	52	32.5	2	2.6	7	2.25	61	11.1
1.0	13	8.1	15	19.5	95	30.5	123	22.4
1.5	48	30.0	15	19.5	53	17.0	116	21.2
2.0	32	20.0	27	35.1	102	32.8	161	29.4
3.0	1	0.6	10	13.0	1	0.3	12	2.2
Unknown	2	1.2					2	0.4

Abbreviation: POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer.

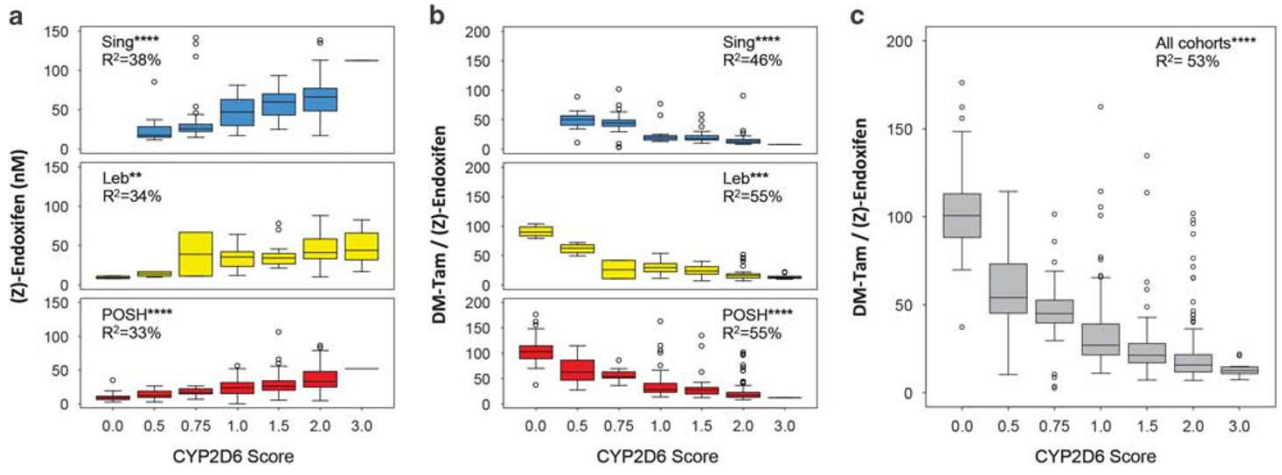

Figure 3. Steady-state plasma concentrations of endoxifen and metabolic ratio DM-Tam/*(Z)*-endoxifen in premenopausal breast cancer patients according to genotype-based CYP2D6 activity score: (**a**) Endoxifen concentrations in Singapore (upper panel, $N = 160$, $R^2 = 0.38$; $P < 10^{-12}$), Lebanon (middle panel, $N = 77$, $R^2 = 0.34$; $P < 10^{-4}$) and POSH (lower panel, $N = 306$, $R^2 = 0.33$; $P < 10^{-21}$) cohorts. (**b**) Metabolic ratios of DMT/*(Z)*-endoxifen in Singapore (upper panel, $R^2 = 0.46$; $P < 10^{-17}$), Lebanon (middle panel, $R^2 = 0.55$; $P < 10^{-9}$) and POSH (lower panel, $R^2 = 0.55$; $P < 10^{-46}$) cohorts. (**c**) Metabolic ratio DM-Tam/*(Z)*-endoxifen across all cohorts ($N = 548$, $R^2 = 0.53$; $P < 10^{-77}$). Data are presented as boxplots with whiskers defined by 5th and 95th percentiles and extreme values outside the whiskers. DM-Tam, *N*-desmethyltamoxifen. $^{**}P < 10^{-3}$; $^{***}P < 10^{-5}$; $^{****}P < 10^{-10}$. POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer.

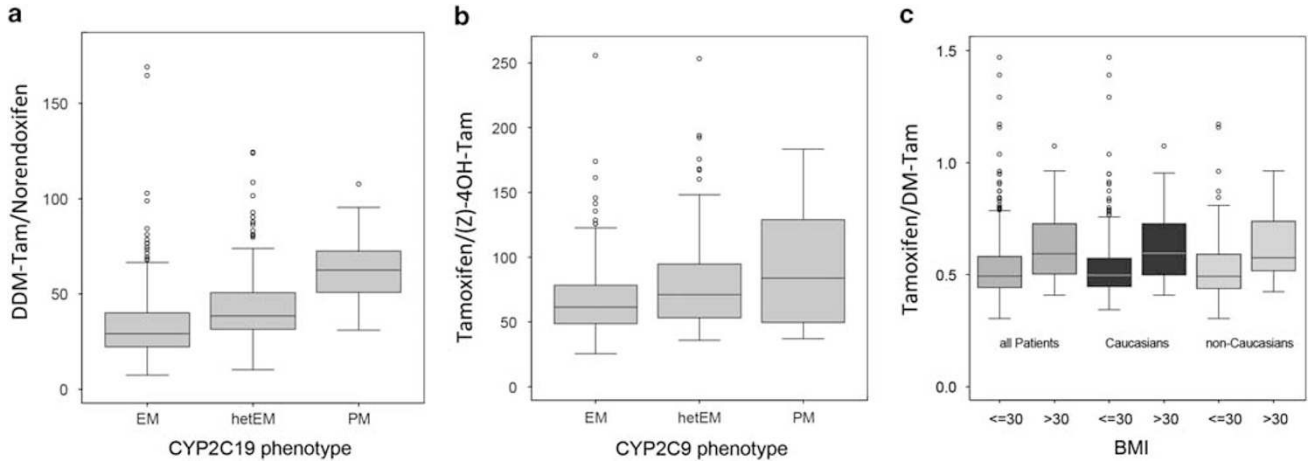

Figure 4. Impact of CYP2C19, CYP2C9 and body mass index on tamoxifen metabolite ratios: (**a**) Metabolic ratio (MR) DDM-Tam/norendoxifen according to the loss-of-function alleles *CYP2C19*2/*3* predicting EM, hetEM (heterozygous **2* or **3*) and PM (homozygous **2* or **3*). (**b**) MR tamoxifen/*(Z)*-4-OH-TAM according to *CYP2C9 *2/*3* reduced activity alleles defining hetEM (heterozygous **2* or **3*) and PM (homozygous **2* or **3*) versus EM with normal activity (absence of **2* or **3*). (**c**) MR tamoxifen/DM-Tam stratified by BMI ($\leqslant 30$ or >30) in all patients, Caucasians and non-Caucasians. Data are presented as boxplots with whiskers defined by 5th and 95th percentiles and extreme values outside the whiskers. BMI, body mass index; DDM-Tam, DiDesmethyltamoxifen; DM-Tam, Desmethyltamoxifen; EM, extensive metabolizer; hetEM, heterozygous EM; PM, poor metabolizer; *(Z)*-4-OH-TAM, *(Z)*-4-hydroxytamoxifen.

Table 4. Cox proportional hazard models of DRFS for (*Z*)-endoxifen plasma levels, MR DM-Tam/(Z)-endoxifen or CYP2D6 activity score/phenotype

Quantitative analyses				*Categorical analyses*				
	HR (per unit)[a]	*95% CI*	*Wald P*		*Factor*	*HR*	*95% CI*	*Wald P*
(Z)-Endoxifen (nM)				*(Z)-Endoxifen (nM)*	>35	1 (Reference)		
Unadjusted	0.989	0.973–1.004	0.142		24.7–35	1.61	0.76–3.38	0.210
Adjusted	0.992	0.977–1.007	0.291		14.15–24.7	1.45	0.69–3.06	0.329
					<14.15	1.94	0.96–3.93	0.064
				Nodal status	Negative	1 (Reference)		
					Positive	2.09	1.11–3.92	0.022
				Chemotherapy	No	1 (Reference)		0.243
					Yes	1.65	0.71–3.83	
MR DM-Tam/(Z)-Endoxifen				*MR DM-Tam/(Z)-Endoxifen*	<31	1 (Reference)		
Unadjusted	1.008	1.001–1.015	0.025		31–115	1.43	0.86–2.37	0.167
Adjusted	1.007	1.000–1.014	0.036		>115	3.82	1.47–9.89	0.006
				Nodal status	Negative	1 (Reference)		
					Positive	2.09	1.12–3.89	0.021
				Chemotherapy	No	1 (Reference)		
					Yes	1.62	0.70–3.74	0.260
CYP2D6 activity score				*CYP2D6 phenotype class*	EM/UM	1 (Reference)		
Unadjusted	0.611	0.425–0.88	0.008		hetEM/IM	1.55	0.88–2.72	0.132
Adjusted	0.623	0.429–0.905	0.013		PM	1.98	0.82–4.79	0.128
				Nodal status	Negative	1 (Reference)		
					Positive	2.15	1.14–4.04	0.018
				Chemotherapy	No	1 (Reference)		
					Yes	1.60	0.69–3.73	0.277

Abbreviations: CI, confidence interval; DRFS, distant recurrence-free survival; HR, hazard ratio; MR DM-Tam/(*Z*)-endoxifen, metabolic ratio *N*-desmethyltamoxifen/(*Z*)-endoxifen; TAM, tamoxifen. Node status was binary coded with factor levels negative vs positive; chemotherapy was binary coded with levels no (no chemotherapy given) and yes (chemotherapy given); CYP2D6 and tamoxifen metabolite predictor variables were coded categorically. Models were corrected for nodal status and chemotherapy and stratified for ethnicity. [a]For univariate analysis, HR refers to an increase by one unit (1 nM) of steady-state endoxifen plasma concentration, increase of the dimensionless MR DM-Tam/*(Z)*-endoxifen ranging between 8 (highest) and 176 (lowest) endoxifen formation rates and an increase by one unit of CYP2D6 phenotype score ranging from 0 (PM) to 3 (UM).

in multivariate Cox regression: $HR_{per\ 1\ unit} = 1.007$; 95% CI 1.000–1.014; $P = 0.036$ (Table 4). Because the per unit effect of this HR is rather small on a linear scale, we applied two cutoff values demonstrating worse (MR > 115), moderate (MR 31–115) and better DRFS (MR < 31) by Kaplan–Meier analysis (Log-rank $P = 0.001$; Figure 5b). This was confirmed in multivariate Cox regression with an increased HR for patients with MR > 115 (low endoxifen formation rate) compared with patients with MR < 31

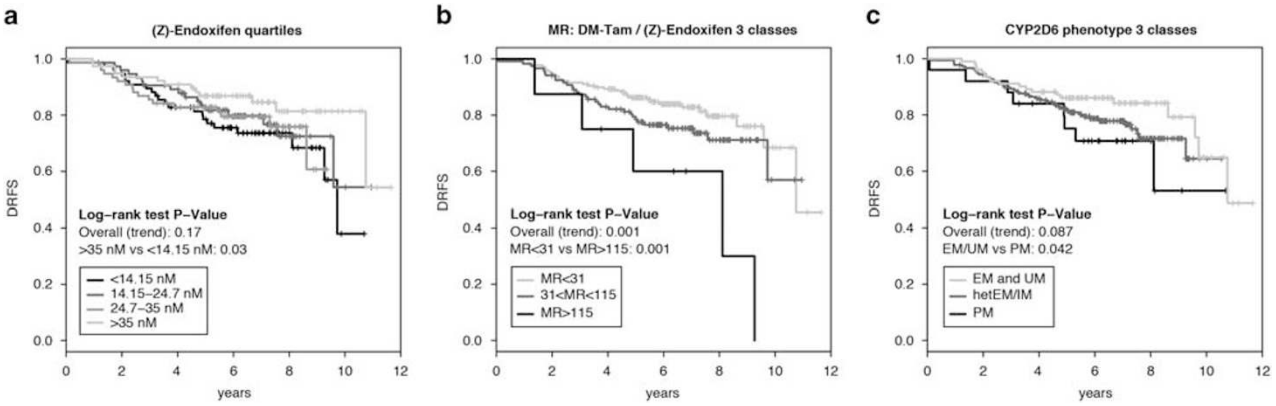

Figure 5. Kaplan–Meier analyses for an association between *(Z)*-endoxifen concentrations, metabolic ratio (MR) Desmethyltamoxifen (DM-Tam)/*(Z)*-endoxifen or CYP2D6 phenotype score and distant relapse-free survival (DRFS) in the POSH cohort. Kaplan–Meier analyses for DRFS and the three predictor variables classified into groups. (**a**) Steady-state endoxifen concentrations split into four equally-sized patient groups (<14.1, 14.1–24.7, 24.7–35 and >35 nM), (**b**) MR DM-Tam/*(Z)*-endoxifen classified by conditional inference trees into three splits (<31, 31–115 and >115), (**c**) CYP2D6 phenotypes grouped into EM (plus UM), hetEM and PM. Corresponding Mantel–Cox log-rank tests are stratified for ethnicity. EM, extensive metabolizer; hetEM/IM, heterozygous EM/IM; IM, intermediate metabolizer; PM, poor metabolizer; POSH, Prospective study of Outcomes in Sporadic versus Hereditary breast cancer; UM, ultra rapid metabolizer.

(high endoxifen formation rate): HR = 3.82; 95% CI 1.47–9.89; $P = 0.006$ (Table 4).

Importantly, none of the remaining metabolites including tamoxifen and DM-Tam showed an association between plasma concentrations and clinical outcome (Supplementary Figure 2).

CYP2D6 genotype and clinical outcome

Cox modeling revealed a significant linear association between CYP2D6 activity score and outcome (DRFS), both in univariate ($P = 0.008$) and multivariate models ($HR_{per\ 1\ CYP2D6\ score\ unit} = 0.62$; 95% CI 0.43–0.91; $P = 0.013$; Table 4). When grouping activity scores into the phenotype classes EM/UM, hetEM/IM and PM, Kaplan–Meier analysis indicated that PM patients have worse DRFS compared to EM subjects (Figure 5c; log-rank test $P = 0.042$). When adjusted for nodal status and chemotherapy use, the association was no longer significant ($HR_{PM\ vs\ EM} = 1.98$; 95% CI 0.82–4.79; $P = 0.13$; Table 4).

DISCUSSION

This is the first study that investigated a purely premenopausal breast cancer population for interindividual variability of tamoxifen metabolism and its influence on clinical outcome. In contrast to postmenopausal patients,[13,18–20,37] little is known regarding the clinical relevance of variable tamoxifen metabolism for drug response in premenopausal patients. Although postmenopausal patients may receive aromatase inhibitor treatment as an alternative to tamoxifen, a personalized approach in the premenopausal setting must confront the lack of available alternatives and address the dilemma of young women stopping tamoxifen prematurely due to ADRs. Emerging data report a link between steady-state endoxifen concentrations and ADRs;[7,8] however, the two retrospective studies reporting an association between endoxifen blood concentrations and clinical outcome do not reflect the premenopausal setting. Notably, Madlensky *et al.*[14] provided the first clinical outcome data for endoxifen; however, their study lacked premenopausal subgroup analysis. Similarly, a small exploratory study performed with 48 oophorectomized women is not reminiscent of the premenopausal setting.[39]

Our comprehensive approach with premenopausal women accounts for potential determinants of tamoxifen response, including tamoxifen metabolites, CYP2D6 phenotypes and drug compliance. Notably, compliance is a serious issue as non-compliance pertains to up to 50% of patients by year five of the recommended tamoxifen treatment and has been associated with poor survival.[40–43] In our study, tamoxifen compliance assessment via blood concentrations was limited to the first year of treatment and the poor/non-compliance rate of 10% (POSH) was lower than expected.[41,44] Although first-year compliance may not be predictive for compliance for the full 5-year treatment duration, this has been accounted for by excluding all poorly/non-compliant patients from subsequent analyses. Regardless of the true compliance rate known to us we suggest, that outcome stratification based on CYP2D6 metabolism would be perhaps even better in a fully compliant population. We observed a clear *CYP2D6* gene-dose effect for plasma endoxifen and endoxifen formation from DM-Tam. This strong effect was independent of age, BMI or non-*CYP2D6* DME polymorphisms, which together contributed less than 3% of the observed variability in endoxifen formation. The CYP2D6 effect is similar to that observed in postmenopausal patients and holds true across ethnic groups (Asians, Europeans and Middle Eastern Arabs) independent of the differences in *CYP2D6* allele frequencies.[45] Our data clearly show that endoxifen formation follows the same principle in pre- and postmenopausal women driven by CYP2D6. Yet, its contribution to formation is estimated at approximately 53% suggesting that other factors contribute to the bioavailability of endoxifen. Additional findings of a correlation between impaired CYP2C9 activity and decreased 4-OH-Tam formation, as well as CYP2C19 loss-of-function and decreased formation of the anti-estrogen norendoxifen, corroborate previous *in vitro* observations and point to additional relevant metabolic pathways involved in the formation of antiestrogens.[46,47] Moreover, a potential non-genetic influence on tamoxifen metabolism by adipose tissue acting as sequestering 'compartment' for lipophilic tamoxifen metabolites is supported by the observed association between BMI and decreased DM-Tam concentrations.

To understand the clinical relevance of tamoxifen metabolism in premenopausal patients, we conducted an outcome analysis (POSH) after excluding all patients with incomplete follow-up and/or delayed study entry (Singapore, Lebanon). The association of endoxifen concentrations with clinical outcome was inconclusive; yet, when we grouped endoxifen levels into distinct classes, patients with low concentrations (<14.1 nM) were at increased risk of distant relapse or death compared with patients with high endoxifen (>35 nM; Figure 5a). Importantly, the low endoxifen class contained 88% of all PMs (24 of 27 patients) thereby support-

ing the notion of a link between deficient CYP2D6 phenotype, reduced endoxifen concentration, and impaired clinical efficacy of tamoxifen. Our data are in line with those of Madlensky *et al.*[14] who reported an distant recurrence rate for patients with low endoxifen concentrations (5.9 ng ml^{-1}, 15.8 nM) in a patient cohort comprising pre- and postmenopausal patients. In contrast to endoxifen alone, using MR DM-Tam/endoxifen as a surrogate for CYP2D6 endoxifen formation demonstrated a strong association between high MR (low endoxifen concentrations) and an increased risk for recurrence or death, thereby substantiating the clinical relevance of the CYP2D6-mediated pathway for tamoxifen response. Together these data support the antagonistic potency of active metabolites at the ER, rather than the inhibition of ER by abundant tamoxifen and major metabolites through receptor saturation[48,49] in premenopausal patients. This is further substantiated by the lack of an outcome association with variable concentrations of tamoxifen and DM-Tam (Supplementary Figure 2). Notably, our *in vivo* data confirm the *in vitro* prediction by Maximov *et al.*[29,30] who demonstrated that endoxifen, rather than ER low-affinity tamoxifen metabolites, matters in blocking breast cancer cell replication in the presence of estrogen concentrations equivalent to premenopausal patients.

Our observation that CYP2D6 PM or low activity score patients had less favorable outcome compared with EM or high activity score patients supports the relevance of the *CYP2D6* genotype for the prediction of tamoxifen outcome in premenopausal women, a hypothesis that has been originally postulated for the postmenopausal setting.[18–20,50] This association was considerably stronger when using activity scores as compared with classifying into three CYP2D6 phenotype groups (Table 4) supporting a gene-dose effect. Unlike in postmenopausal breast cancer where chemotherapy has been suggested to abrogate the CYP2D6 tamoxifen outcome effect,[51] our data do not support this notion despite the large majority of POSH patients having received chemotherapy as it is the standard in the premenopausal setting. Combination chemotherapy produces chemical oophorectomy in the majority of premenopausal women over the age of 40[52] resulting in lower circulating estradiol levels compared with premenopausal patients taking tamoxifen alone;[53] however, the ovarian function of most POSH patients may have remained intact as inferred from their median age of 37.5 years. Another confounding factor in pharmacogenetic studies is the deviation from Hardy–Weinberg Equilibrium, which has been observed in the POSH cohort for *CYP2D6*3* and **9,* likely due to rare frequency or population admixture. Borderline deviation from Hardy–Weinberg Equilibrium was observed for *CYP2D6*10* in the Singapore cohort, which could be attributed to the **36-*10* genotype (not analyzed)[54] and/or population admixture (17% Malays/Indians vs 83% Chinese). Because genotyping was done from germline DNA, Hardy–Weinberg Equilibrium deviation is not considered a confounder in our outcome analysis.

A limitation of this study is its moderate sample size, given that more than 800 patients would be required to detect a CYP2D6-related clinical effect in postmenopausal women with sufficient power.[22] This low power might explain the lack of significance with CYP2D6 phenotype in multivariate Cox analysis ($P = 0.13$). However, breast cancer in young women is less frequent than in postmenopausal women, resulting in smaller available cohorts with simultaneous collection of serum, genomic DNA and detailed follow-up per patient. We therefore restricted our outcome analyses to the 306 compliant patients for whom, both pharmacogenetic and pharmacokinetic data were available. This design differs from a previous preliminary dataset with more patients but without control for compliance.[55] It may be argued that this design may give rise to a selection bias; however, we believe that strict inclusion criteria including first-year compliance, serum availability for pharmacokinetic measurements and a reasonable follow-up time are integral to CYP2D6-related outcome analysis. Notwithstanding, our study presents the largest analyses of purely premenopausal patients for which pharmacokinetic and pharmacogenetic outcome associations have been described. No information on co-medication was available for the POSH cohort, a reason why we cannot comment on their possible influence on the results. In the Singapore and Lebanon cohorts, pharmacokinetic analyses were likely not influenced because no patient received moderate/strong CYP2D6 inhibitors. Collectively, our data for the association of tamoxifen outcome with endoxifen formation/concentrations in premenopausal patients must be considered exploratory therefore, its potential clinical relevance awaits confirmation by independent studies.

Our data support the notion that tamoxifen efficacy in premenopausal breast cancer patients is influenced by CYP2D6-mediated metabolism. If replicated, therapeutic drug level monitoring at steady-state could identify patients with high endoxifen levels or low DM-Tam/endoxifen ratio expected to have a lower risk of recurrence and who should therefore be encouraged to adhere to tamoxifen. On the basis of current research and developments toward improving therapeutic levels of endoxifen, patients with impaired/deficient CYP2D6 and suboptimal therapeutic endoxifen concentrations could be considered for the following: (i) increase of the standard tamoxifen dose as previously suggested for postmenopausal women,[15,56,57] (ii) standard tamoxifen dose supplemented with endoxifen, a possibility currently explored by *in silico* modeling of CYP2D6 phenotype guided dosing schemes,[58] (iii) treatment with (*Z*)-endoxifen hydrochloride of which first-in-man studies have already been published[59] and further clinical trials are underway (www.clinicaltrials.gov: NCT01327781, NCT01273168) and (iv) aromatase inhibitor combined with ovarian suppression,[60] as currently investigated in the Tamoxifen and EXemestane (TEXT) and Suppression of Ovarian Function Trial (SOFT) trials.[61]

CONFLICT OF INTEREST

HB and MS report scientific collaborations with Roche Molecular Diagnostics and Siemens Healthcare Diagnostics Products in the previous 3 years. The remaining authors declared no conflict of interest.

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AUTHOR CONTRIBUTIONS

HB, TM, PS, WS and MS designed the research. BC, RD, BE, DE, BG, SG, MZH, JSLL, TM, RCHN, PS, ES, WS, AT, NSW, YSY and NKZ performed the research. HB, BC, BE, DE, ME, TM, PS, WS, MS, SW and NKZ analyzed the data. BC, DE and AT contributed study materials or patients. WS, MS and HB led the study. qDE established the POSH study cohort. BC established the Singapore study cohort. NZK and AT established the Lebanon study cohort as co-principle investigators. BC, RD, BE, DE, SG, MZH, JSLL, RCHN, AT, NSW, YSY and NKZ collected samples and data. PS and WS performed the genotyping. BG and TM performed the drug metabolite analytics. PS, WS, TM and SW performed the statistical analysis. HB, BC, DE, TM, PS, WS, MS, SW and NKZ wrote the manuscript. All other authors contributed primarily to the patient ascertainment and/or data, and sample collection and preparation. All authors reviewed the final manuscript.

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Supplementary Information accompanies the paper on the The Pharmacogenomics Journal website (http://www.nature.com/tpj)