Factors Affecting Tamoxifen Metabolism in Patients With Breast Cancer: Preliminary Results of the French PHACS Study

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In addition to the effect of cytochrome P450 (*CYP*) *2D6* genetic polymorphisms, the metabolism of tamoxifen may be impacted by other factors with possible consequences on therapeutic outcome (efficacy and toxicity). This analysis focused on the pharmacokinetic (PK)-pharmacogenetic evaluation of tamoxifen in 730 patients with adjuvant breast cancer included in a prospective multicenter study. Plasma concentrations of tamoxifen and six major metabolites, the genotype for 63 single-nucleotide polymorphisms, and comedications were obtained 6 months after treatment initiation. Plasma concentrations of endoxifen were significantly associated with *CYP2D6* diplotype ($P < 0.0001$), *CYP3A4*22* genotype ($P = 0.0003$), and concomitant intake of potent CYP2D6 inhibitors ($P < 0.001$). Comparison of endoxifen levels showed that the CYP2D6 phenotype classification could be improved by grouping intermediate metabolizer (IM)/IM and IM/poor metabolizer diplotype into IM phenotype for future use in tamoxifen therapy optimization. Finally, the multivariable regression analysis showed that formation of tamoxifen metabolites was independently impacted by *CYP2D6* diplotype and *CYP3A4*22*, *CYP2C19*2*, and *CYP2B6*6* genetic polymorphisms.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ The clinical utility of pretherapeutic cytochrome P450 (*CYP*) *2D6* genotyping or therapeutic drug monitoring of ENDO, the major active metabolite of tamoxifen (TAM), remains uncertain.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ What is the impact of genetic polymorphisms and comedications on plasma concentrations of endoxifen (ENDO) and could the current *CYP2D6* genotype to phenotype classification system be improved for TAM therapy?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ Based on data from 730 patients with adjuvant breast cancer, ENDO concentrations were strongly correlated with *CYP2D6* diplotype, *CYP3A4*22* genotype, and concomitant intake of potent CYP2D6 inhibitors. CYP2D6 phenotype classification could be improved by grouping intermediate metabolizer (IM)/IM and IM/poor metabolizer diplotypes into IM phenotype for future use in TAM therapy optimization.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ The results of this study provide information on factors affecting TAM metabolism, which might be helpful for further evaluations of its relationship with clinical outcome (efficacy and toxicity).

High interindividual variability in pharmacokinetics (PK) of tamoxifen (TAM) has been attributed to genetic polymorphisms of cytochrome P450 (CYP) 2D6 encoding for the most important enzyme involved in TAM bioactivation to endoxifen (ENDO), its

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major active metabolite. However, individualization of TAM therapy based on *CYP2D6* genotype could not meet consensus due to conflicting results about the association between *CYP2D6* genetic polymorphisms and recurrence in patients with breast cancer.[1–3] The achievement of response to TAM therapy may be related to a combination of *CYP2D6* genotype and other factors influencing TAM metabolism, such as genetic polymorphisms (notably *CYP3A4/5*, *CYP2C9*, and *CYP2C19*) and comedications with CYP inhibitors.[4–8] Indeed, it has been shown that plasma concentrations of ENDO exhibit 20–40-fold variation among patients treated with TAM 20 mg/day[9] and only 39–58% of this interindividual variability can be explained by *CYP2D6* diplotype.[8,10] *CYP3A4*22*, *CYP2C19*2*, *CYP2C9*2*, and **3* genetic polymorphisms have also been shown to impact plasma concentrations of ENDO, 4-hydroxy tamoxifen (4-OHTAM) and other TAM metabolites.[4,6,7,9,11]

Some studies have reported a positive association between plasma ENDO concentrations and TAM efficacy and proposed a therapeutic threshold of 16 nmol/L for ENDO.[7,12] However, these results have not been reproduced in later studies[13,14] questioning the utility of therapeutic drug monitoring of ENDO concentrations in optimization of TAM therapy. More prospective studies are needed to confirm or denounce these findings.

Because over 100 single-nucleotide polymorphisms (SNPs) of the *CYP2D6* gene have been reported, a validated genotype to phenotype translation system is required to correctly investigate the impact of *CYP2D6* genotype on the metabolism of TAM and other drugs primary metabolized by CYP2D6. Previous Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines[15,16] recommended classification of patients into CYP2D6 phenotypes based on the sum of activity scores of the two alleles.[17] However, these classifications have been proposed based on dextromethorphan[17] or codeine[15,16] data and recent studies have shown that they are not adequate to predict ENDO levels.[8,18] In consequence, recent CPIC guidelines[19] discussed the need for re-evaluation of the existing CYP2D6 phenotype classification to guide TAM therapy.

Finally, comedication with CYP2D6 inhibitors may decrease plasma ENDO levels in TAM-treated patients.[20] However, data regarding the impact of CYP3A4 inhibitors on TAM metabolism are limited. Therefore, evaluation of the impact of CYP2D6 and CYP3A4 inhibitors based on data from an observational clinical trial could provide additional information helpful for better management of TAM-treated patients.

In light of the above considerations, the aims of this study were to evaluate the impact of genetic polymorphisms and comedications on ENDO concentrations and to compare ENDO levels and metabolic ratio N-desmethyl tamoxifen/endoxifen ($MR_{NDT/ENDO}$ as a marker of CYP2D6 activity) between *CYP2D6* genotypes and diplotypes in order to improve the existing genotype to phenotype classification system for TAM therapy. The third objective was to evaluate the PK/pharmacogenetic (PG) relationship of TAM and its major metabolites based on metabolic ratios. Data come from a large prospective multicenter 3-year follow-up study aiming to investigate the relationship among PK, PG, and toxicity of TAM and aromatase inhibitors in adjuvant setting (PHACS, ClinicalTrials.gov registration number NCT01127295).

RESULTS

Patients and data

Eight hundred seventy-nine patients starting treatment with TAM at 20 mg/day were included in the PHACS study; 864 patients performed the first follow-up visit, and PK samples were available for 789 patients. Of those, 59 patients were excluded from the statistical analysis of the first follow-up visit due to TAM or ENDO plasma concentrations below the limit of quantification suggesting noncompliance ($n = 12$), missing genotype data or missing/uncertain *CYP2D6* copy number ($n = 47$). Finally, 730 patients were included in the analysis, and the distributions of their baseline characteristics (**Table 1**) were not statistically different from those of the 134 excluded patients. The first follow-up visit was performed after a median time of 6.2 months after treatment initiation (interquartile range: 6.0–6.6). Because concomitant treatment with CYP2D6 and CYP3A4 inhibitors or inducers may significantly impact formation of TAM metabolites, patients taking concomitant CYP2D6 inhibitors (weak/moderate CYP2D6 inhibitors: celecoxib, citalopram, duloxetine, escitalopram, and sertraline; potent CYP2D6 inhibitors: fusidic acid, amiodarone, clomipramine, flecainide, fluoxetine, paroxetine, and propafenone); moderate or potent CYP3A4 inhibitors (amiodarone, ciprofloxacin, clarithromycin, diltiazem, fluconazole, fusidic acid, and verapamil); or CYP3A4 inducers (dexamethasone and phenobarbital) at the time of PK sampling were excluded from the PK/PG analysis ($n = 53$) unless otherwise stated. The 63 SNPs analyzed in the study (**Supplementary Material S1**) were in Hardy–Weinberg Equilibrium (HWE).

Plasma concentrations of TAM and metabolites

Steady-state plasma concentrations of TAM, N-desmethyl tamoxifen (NDT), tamoxifen N-oxide (NOX), 4-OHTAM, Z-4′-hydroxy tamoxifen (4′-OHTAM), Z-ENDO, and Z′-endoxifen (Z′-ENDO), as well as metabolic ratios (MRs) and antiestrogenic activity score (AAS) are presented in **Table S2**. Plasma concentrations of NDT, 4-OHTAM, ENDO, and Z′-ENDO, as well as $MR_{TAM/4\text{-}OHTAM}$, $MR_{TAM/NDT}$, $MR_{NDT/ENDO}$, $MR_{4\text{-}OHTAM/ENDO}$, and AAS varied significantly among CYP2D6 diplotypes. ENDO levels, $MR_{NDT/ENDO}$, and $MR_{4\text{-}OHTAM/ENDO}$ showed the most significant gene-dose effect. ENDO concentrations increased with increasing CYP2D6 activity, whereas an inverse correlation was observed with $MR_{NDT/ENDO}$. High interindividual variability in ENDO concentrations was observed within CYP2D6 diplotypes (coefficient of variation: 42–105%), which supports the hypothesis that factors other than CYP2D6 contribute to these interindividual differences. Because CYP2D6 is the only enzyme involved in the formation of ENDO from NDT, further analyses focused on both $MR_{NDT/ENDO}$ (as a marker of CYP2D6 activity) and plasma ENDO concentrations (as a reflection of exposure to active metabolite).

Comparison of $MR_{NDT/ENDO}$ between CYP2D6 genotypes and diplotypes

$MR_{NDT/ENDO}$ was compared among *CYP2D6* genotypes classified into the same diplotype. Significant differences in

Table 1 Patients' baseline characteristics (*n* = 730)

Characteristic	Median [range] or number (%)
Age at inclusion	47 [25–74]
Lymph node status	
pN0	453 (62.2)
pN+	275 (37.8)
Missing	2
Hormonal receptor status	
ER–/PR+	6 (0.8)
ER+/PR–	78 (10.7)
ER+/PR+	643 (88.4)
Missing	3
Hormonal status	
Nonmenopausal	601 (84.5)
Menopausal	108 (15.2)
Menopausal under hormone replacement therapy	2 (0.3)
Missing	19
Neo-adjuvant chemotherapy	
Yes	63 (8.7)
No	665 (91.3)
Missing	2
Adjuvant chemotherapy	
Yes	415 (57.0)
No	313 (43.0)
Missing	2
Radiotherapy	
Yes	698 (95.6)
No	32 (4.4)
Trastuzumab treatment	
Yes	79 (10.9)
No	649 (89.1)
Missing	2

ER, estrogen receptor; pN0, no regional lymph node metastasis; pN+, regional lymph node metastasis; PR, progesterone receptor.

$MR_{NDT/ENDO}$ among **9/*4*, **10/*4*, and **41/*4* genotypes were identified ($P = 0.003$), whereas they all belong to intermediate metabolizer (IM)/poor metabolizer (PM) diplotype (**Figure 1a**). $MR_{NDT/ENDO}$ was significantly higher in **41/*4* patients than in **9/*4* patients ($P = 0.0009$); however, there was no significant difference between **9/*4* and **10/*4* patients and between **10/*4* and **41/*4* patients ($P = 0.2$ and $P = 0.08$, respectively). $MR_{NDT/ENDO}$ was similar between **41/*4* and PM/PM patients ($P = 0.3$) but not between **10/*4* and PM/PM patients ($P = 0.01$). No statistically significant differences in $MR_{NDT/ENDO}$ between genotypes of the remaining diplotypes were detected.

Comparisons of $MR_{NDT/ENDO}$ between *CYP2D6* diplotypes are presented in **Figure 1b**. Ultrarapid metabolizer (UM) had similar $MR_{NDT/ENDO}$ to UM/PM and normal metabolizer (NM)/NM patients ($P = 0.7$ and $P = 0.2$, respectively). $MR_{NDT/ENDO}$ was significantly lower in NM/IM than in NM/PM patients ($P = 0.02$) suggesting higher CYP2D6 activity of NM/IM diplotype (**Figure 1b**). NM/PM patients had significantly lower $MR_{NDT/ENDO}$ than IM/IM patients ($P = 0.0002$), despite the same sum of allele activity score (AS). Finally, $MR_{NDT/ENDO}$ was significantly lower in IM/IM patients than in IM/PM patients ($P = 0.02$).

Comparison of ENDO concentrations between CYP2D6 genotypes and diplotypes

Significant differences in ENDO levels among **9/*4*, **10/*4*, and **41/*4* genotypes were identified ($P = 0.006$), whereas they all belong to IM/PM diplotypes (**Figure 2a**). ENDO concentrations in **9/*4* patients (28.1 ± 13.5 nmol/L) were significantly higher than in **10/*4* (13.1 ± 7.1 nmol/L) and **41/*4* patients (15.0 ± 19.0 nmol/L; $P = 0.02$ and $P = 0.0017$, respectively) whereas **10/*4* and **41/*4* genotypes had similar ENDO levels ($P = 0.72$). Plasma ENDO levels were then compared among **10/*4*, **41/*4*, and PM/PM patients. Plasma ENDO levels were similar between **10/*4* and PM/PM and between **41/*4* and PM/PM patients ($P = 0.2$ and $P = 0.3$, respectively). No statistically significant differences in ENDO levels between genotypes of the remaining diplotypes were detected.

Next, plasma ENDO levels were compared across *CYP2D6* diplotypes. Similarly to $MR_{NDT/ENDO}$, plasma ENDO levels were not statistically different between UM and UM/PM ($P = 0.6$) or NM/NM patients ($P = 0.3$). Concerning NM/NM, NM/IM, NM/PM, and IM/IM diplotypes that are collapsed into NM phenotype according to the previous CPIC classification,[15] NM/NM patients had significantly higher plasma ENDO concentrations than NM/IM ($P < 0.0001$), NM/PM ($P < 0.0001$), and IM/IM patients ($P < 0.0001$; **Figure 2b**). Plasma ENDO levels were not significantly different between NM/IM and NM/PM patients ($P = 0.09$). NM/PM patients had significantly higher plasma ENDO levels than IM/IM patients ($P = 0.002$), despite the same sum of allele AS. Finally, plasma ENDO levels in IM/IM and IM/PM patients were similar ($P = 0.4$).

Impact of genetic polymorphisms and comedications on ENDO concentrations

In the univariate analysis of all 63 SNPs with ENDO concentrations, only SNPs related to the *CYP2D6* gene: rs1135840, rs3892097 (**4*), and rs1065852 (**10*), and to the *CYP3A4*: rs35599367 (**22*) were found significant after correction for multiple testing. In order to account for the *CYP2D6* copy number variation (CNV) and the presence of different gene variants, we combined all the information regarding patients' *CYP2D6* genotype into diplotype for the multivariable regression analysis. In this analysis, plasma ENDO concentrations were independently associated with *CYP2D6* diplotype and *CYP3A4*22* genotype ($P < 0.0001$ and $P = 0.0001$, respectively). NM/PM and IM/IM patients carrying *CYP3A4*22* allele had significantly higher plasma ENDO levels than patients with the same *CYP2D6* diplotype and absence of *CYP3A4*22* allele (**Figure 3**).

Table 2 summarizes plasma ENDO levels according to CYP2D6 phenotype (in which IM/IM and IM/PM diplotypes

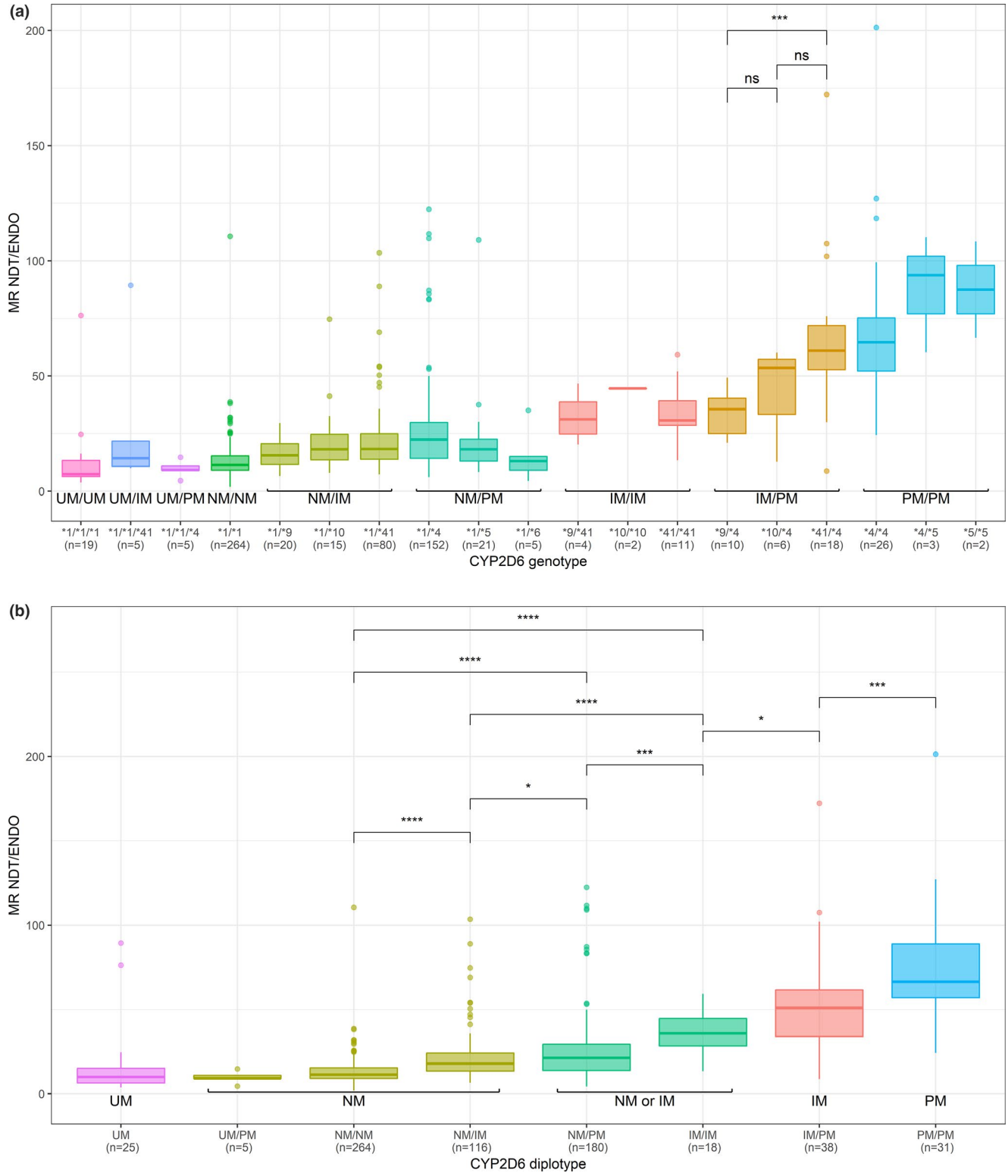

Figure 1 (**a**) Metabolic ratio N-desmethyl tamoxifen/endoxifen ($MR_{NDT/ENDO}$) according to cytochrome P450 (*CYP*) *2D6* genotype. Patients taking concomitant CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors, or CYP3A4 inducers (n = 53), and genotypes represented by only one patient (>**1x4*, **1/*4/*4*, **1/*17*, **1/*7*, **10/*41*, **10/*5*, **17/*4*, **41/*5*, and **41/*7*; n = 9) were excluded. (**b**) $MR_{NDT/ENDO}$ according to *CYP2D6* diplotype. The second *x*-axis represents the classification into CYP2D6 phenotype according to recent Clinical Pharmacogenetics Implementation Consortium guidelines[19] where ultrarapid metabolizer (UM)/UM and UM/intermediate metabolizer (IM) diplotypes were regrouped into UM. Patients taking concomitant CYP2D6 inhibitors, CYP3A4 moderate or potent inhibitors, or CYP3A4 inducers were excluded (n = 53). Not significant (ns) $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. NM, normal metabolizer; PM, poor metabolizer. [Colour figure can be viewed at wileyonlinelibrary.com]

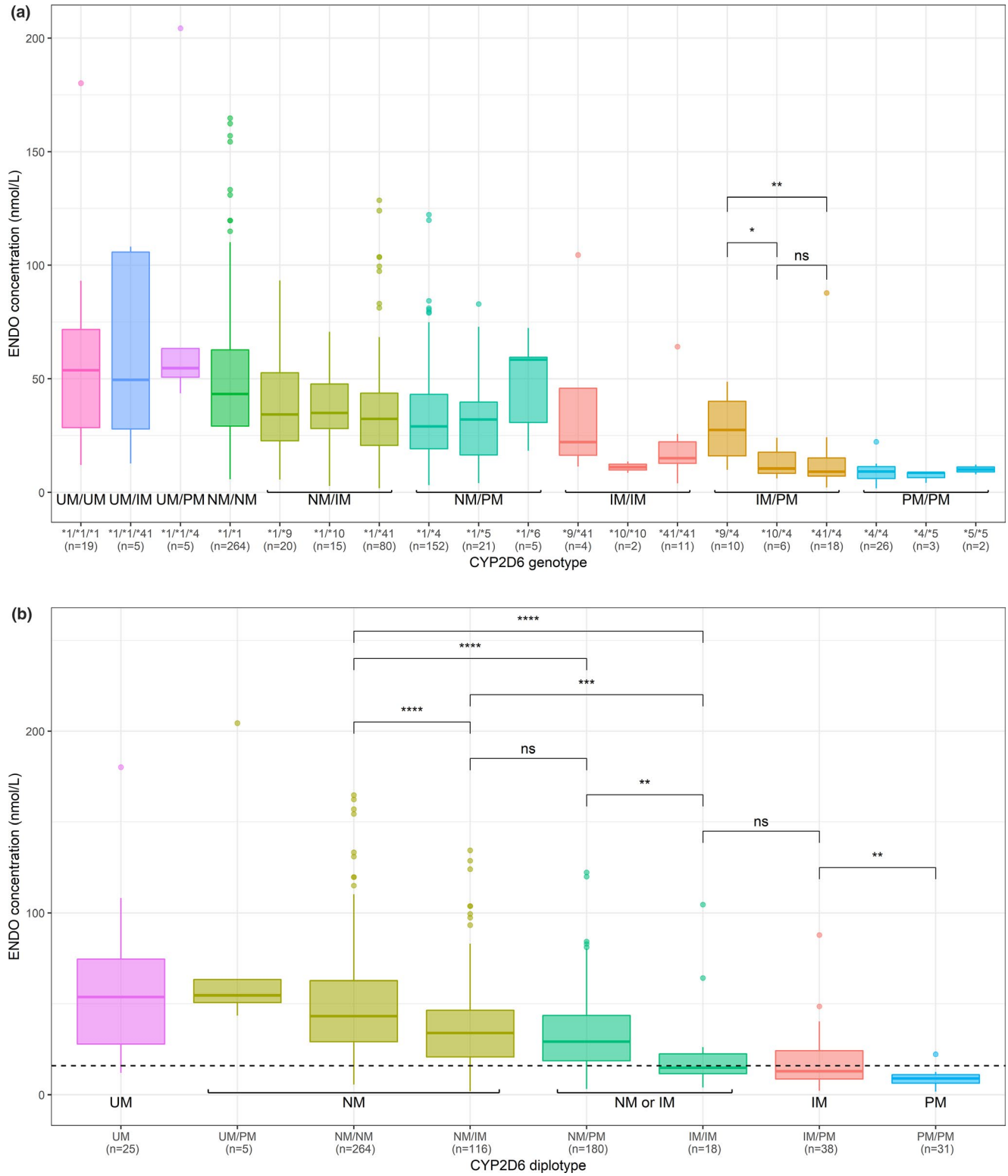

Figure 2 (**a**) Plasma endoxifen (ENDO) concentration according to cytochrome P450 (*CYP*) *2D6* genotype. Patients taking concomitant CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors, or CYP3A4 inducers (n = 53), and genotypes represented by only one patient (**1/*17*, **1/*7*, **10/*41*, **4/*17*, **7/*41*, **5/*10*, **5/*41*, **1/*4/*4*, and >**1x4*; n = 9) were excluded. (**b**) Plasma ENDO concentration according to *CYP2D6* diplotype. The second *x*-axis represents the classification into CYP2D6 phenotype according to recent Clinical Pharmacogenetics Implementation Consortium guidelines[19] where ultrarapid metabolizer (UM)/UM and UM/intermediate metabolizer (IM) diplotypes were regrouped into UM. The dashed line represents the threshold for plasma ENDO concentration (16 nmol/L) associated with lower breast cancer recurrence proposed by Madlensky *et al.*[12] Patients taking concomitant CYP2D6 inhibitors, CYP3A4 moderate or potent inhibitors, or CYP3A4 inducers were excluded (n = 53). Not significant (ns) $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. NM, normal metabolizer; PM, poor metabolizer. [Colour figure can be viewed at wileyonlinelibrary.com]

were classified into IM phenotype) and concomitant intake of CYP2D6 and CYP3A4 inhibitors. Both weak/moderate and potent CYP2D6 inhibitors significantly decreased plasma ENDO levels in NM patients, whereas no significant difference was observed in IM patients probably due to the small number of patients. The number of UM and PM patients taking concomitant CYP2D6 inhibitors was too low to perform statistical analysis. Intake of weak or moderate CYP2D6 inhibitor decreased ENDO concentrations by 35% in NM patients ($P = 0.005$), in particular, escitalopram, a weak CYP2D6 inhibitor often prescribed to patients with breast cancer treated with TAM to avoid the use of paroxetine, decreased ENDO levels by 38% in NM patients ($n = 14$) compared with NM patients not taking comedications (from 42.3 to 25.9 nmol/L, respectively). Potent CYP2D6 inhibitors decreased plasma ENDO concentrations by 54% in NM patients ($P = 0.0006$) to the level observed in IM patients not taking a CYP2D6 inhibitor (no significant difference between ENDO levels under potent inhibitors and IM patients not taking a CYP2D6 inhibitor, $P = 0.5$). In the final multivariable regression analysis, intake of potent CYP2D6 inhibitors remained significantly associated with plasma ENDO concentrations when accounting for CYP2D6 diplotype and *CYP3A4*22* genotype (**Table 3**). *CYP2D6* diplotype alone explained 16.8% of the variation in ENDO levels, further inclusion of *CYP3A4*22* genotype and of concomitant CYP2D6 inhibitors increased the explained variability to 17.6% and 19.4% (final model), respectively.

Weak or moderate/potent CYP3A4 inhibitors did not have a significant impact on plasma ENDO levels in the univariate analysis ($P = 0.8$ and $P = 0.2$, respectively). However, when patients were classified according to CYP2D6 phenotype (**Table 2**), NM patients treated with a moderate or potent CYP3A4 inhibitor had significantly lower plasma ENDO levels than NM patients without concomitant treatment ($P = 0.048$; **Table 2**), whereas weak CYP3A4 inhibitors did not have an impact on ENDO concentrations ($P = 0.3$). Due to an insufficient number of patients with other CYP2D6 phenotypes taking CYP3A4 inhibitors, their impact on plasma ENDO could not be evaluated. The impact of CYP3A4 inducers on plasma ENDO levels could not be evaluated because only two patients were concomitantly treated with a CYP3A4 inducer.

Impact of genetic polymorphisms on MRs

CYP2D6 diplotype was the only factor significantly associated with $MR_{NDT/ENDO}$ ($P < 0.0001$; **Table 4**). The multivariable analyses

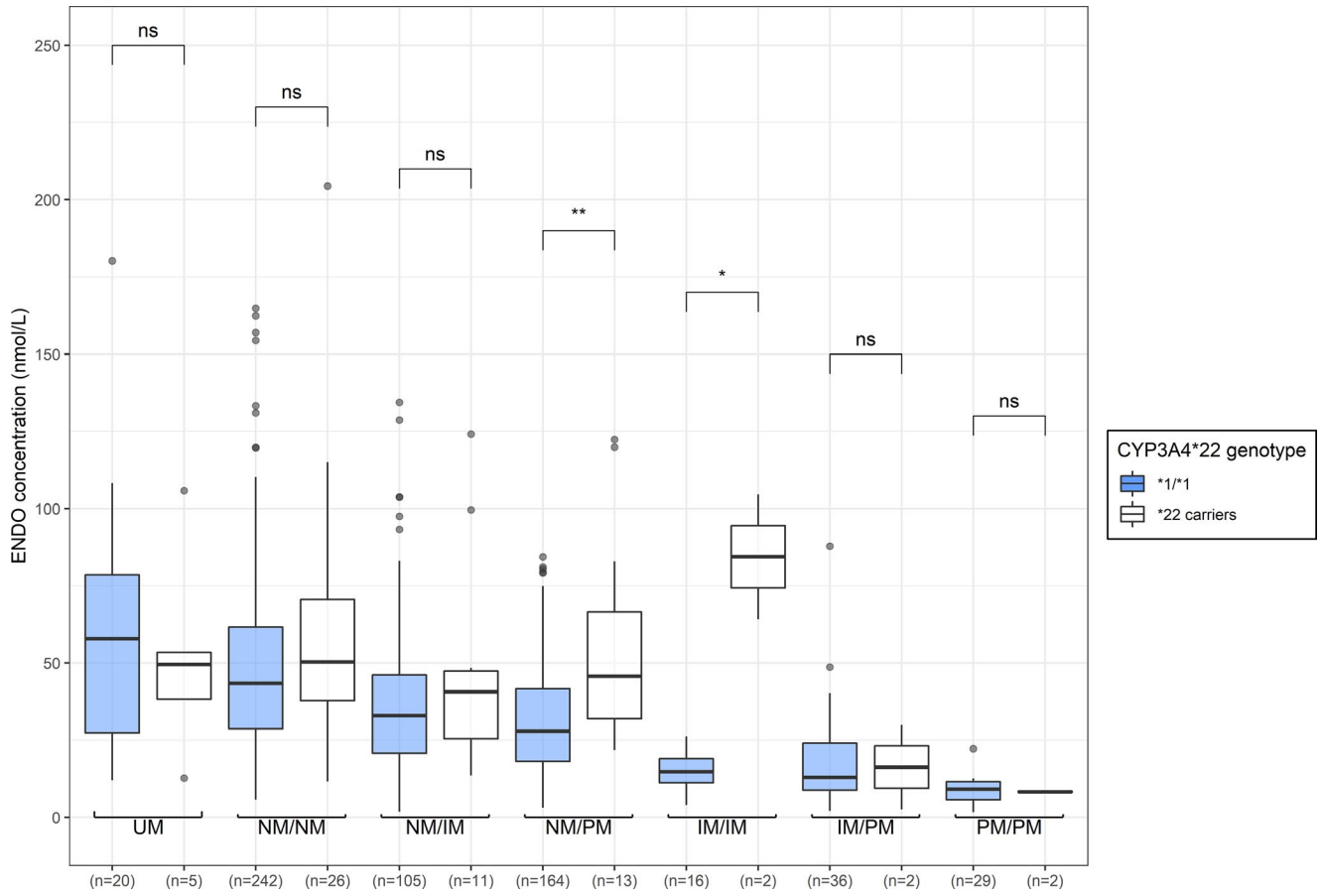

Figure 3 Plasma endoxifen (ENDO) concentration according to cytochrome P450 (*CYP*) *2D6* diplotype and *CYP3A4*22* genotype. Patients with missing *CYP3A4*22* genotype ($n = 4$) or concomitantly treated with CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors, or CYP3A4 inducers were excluded ($n = 53$). Not significant (ns) $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 2 Plasma ENDO concentrations according to CYP2D6 phenotype and concomitant intake of CYP2D6 inhibitors

	CYP2D6 phenotype									
	UM		NM[a]			IM[b]			PM	
	ENDO (nmol/L)	*n*	ENDO (nmol/L)	*n*	*P* value[c]	ENDO (nmol/L)	*n*	*P* value[c]	ENDO (nmol/L)	*n*
CYP2D6 inhibitor (*n* = 721)										
No CYP2D6 inhibitor	59.07 ± 39.17	25	42.28 ± 27.15	565	–	19.87 ± 19.11	56	–	8.87 ± 3.80	31
Weak or moderate[d]	71.97	1	27.25 ± 15.25	23	0.005	16.78 ± 10.07	5	0.9	10.10	1
Potent[e]	20.90	1	19.31 ± 11.77	11	0.0006	5.98 ± 4.81	2	0.1	NA	0
CYP3A4 inhibitor[e] (*n* = 682)										
No CYP3A4 inhibitor	NA	0	42.21 ± 27.13	557	–	20.07 ± 19.22	55	–	8.77 ± 3.83	30
Weak[f]	NA	0	49.11 ± 28.46	8	0.3	8.60	1	NA	11.76	1
Moderate or potent[g]	NA	0	23.11 ± 10.37	5	0.048	NA	0	NA	NA	0

Patients concomitantly treated with moderate or strong CYP3A4 inhibitors or CYP3A4 inducers were excluded (*n* = 9) for CYP2D6 inhibitor effect analysis, and patients concomitantly treated with CYP2D6 inhibitors (*n* = 46) or CYP3A4 inducers (*n* = 2) were excluded for CYP3A4 inhibitory effect analysis. Data are presented as mean ± SD.

CYP, cytochrome P450; ENDO, endoxifen; IM, intermediate metabolizer; NA, not applicable; NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer.

[a]UM/PM, NM/NM, NM/IM, and NM/PM diplotypes. [b]IM/IM and IM/PM diplotypes. [c]Wilcoxon unpaired test for comparison between patients concomitantly treated with the respective inhibitor and without concomitant inhibitor. [d]Weak/moderate CYP2D6 inhibitors: escitalopram (*n* = 22), citalopram (*n* = 3), duloxetine (*n* = 3), celecoxib (*n* = 1), and sertraline (*n* = 1). [e]Potent CYP2D6 inhibitors: paroxetine (*n* = 7), flecainide (*n* = 3), amiodarone (*n* = 1), clomipramine (*n* = 2), fluoxetine (*n* = 1), fusidic acid (*n* = 1), and propafenon (*n* = 1). [f]Weak CYP3A4 inhibitors: esomeprazole (*n* = 13). [g]Moderate or potent CYP3A4 inhibitors: amiodarone (*n* = 1), ciprofloxacin (*n* = 1), clarithromycin (*n* = 1), diltiazem (*n* = 1), fluconazole (*n* = 1), fusidic acid (*n* = 1), and verapamil (*n* = 1).

Table 3 Multivariable regression analysis for plasma ENDO concentrations

	Coefficient[a]	95% CI	*P* value (*t*-test of coefficient)	*P* value (likelihood ratio test of global significance of variable)
Reference category: NM/NM diplotype, *CYP3A4*1/*1* genotype, and no concomitant CYP2D6 inhibitors	47.99	44.99; 50.99	< 0.001	
CYP2D6 diplotype				< 0.0001
UM (*n* = 27)	8.62	–1.29; 18.53	0.088	
NM/NM (*n* = 291)				
NM/IM (*n* = 123)	–10.43	–15.72; –5.14	< 0.001	
NM/PM (*n* = 187)	–15.26	–19.88; –10.65	< 0.001	
IM/IM (*n* = 19)	–25.90	–37.53; –14.28	< 0.001	
IM/PM (*n* = 44)	–29.11	–37.11; –21.10	< 0.001	
PM/PM (*n* = 33)	–38.60	–47.63; –29.58	< 0.001	
*CYP3A4*22* genotype				0.0003
**1/*1* (*n* = 659)				
**1/*22* or **22/*22* (*n* = 65)	11.90	5.48; 18.32	< 0.001	
CYP2D6 inhibitors				0.0001
No concomitant inhibitors (*n* = 678)				
Weak or moderate (*n* = 22)	–9.75	–20.47; 0.98	0.075	
Potent (*n* = 24)	–20.67	–30.90; –10.43	< 0.001	

Patients treated with CYP3A4 inducers (*n* = 2) and with missing *CYP3A4*22* genotype (*n* = 4) were excluded from the analysis.

CI, confidence interval; CYP, cytochrome P450; ENDO, endoxifen; IM, intermediate metabolizer; NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer.

[a]As all the variables listed are indicator variables, the interpretation of each coefficient is as follows: a positive (or negative) value of the coefficient (*a*) indicates that the response variable (ENDO) is increased by *a* (respectively decreased by *a*) compared to the reference category. For example, for a given CYP3A4 genotype and use of CYP2D6 inhibitors, the mean ENDO value of NM/IM patients is 10.43 nmol/L lower than that of NM/NM patients.

showed that $MR_{TAM/4\text{-}OHTAM}$ was significantly and independently associated with *CYP2D6* diplotype ($P < 0.0001$), *CYP3A4*22* ($P < 0.0001$), and *CYP2C19*2* ($P = 0.0001$) genotype (**Table S3**). $MR_{TAM/4\text{-}OHTAM}$ was decreased in *CYP3A4*1/*1* and **1/*22* patients compared with **22/*22* patients suggesting that in patients with the same *CYP2D6* diplotype, higher TAM and lower

Table 4 Results of the linear regression analysis of CYP2D6 diplotype on $MR_{NDT/ENDO}$

	Coefficient[a]	95% CI	*P* value (*t*-test of coefficient)
$MR_{NDT/ENDO}$ (*n* = 677)			
CYP2D6 diplotype			
UM (*n* = 25)	3.49	−3.44; 10.42	0.323
NM/NM (reference) (*n* = 269)	12.89	10.87; 14.91	< 0.001
NM/IM (*n* = 116)	8.59	4.90; 12.27	< 0.001
NM/PM (*n* = 180)	12.44	9.25; 15.63	< 0.001
IM/IM (*n* = 18)	22.48	14.40; 30.55	< 0.001
IM/PM (*n* = 38)	38.44	32.70; 44.18	< 0.001
PM/PM (*n* = 31)	62.16	55.87; 68.45	< 0.001

Patients concomitantly treated with CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors, or CYP3A4 inducers were excluded (*n* = 53).
CI, confidence interval; CYP, cytochrome P450; IM, intermediate metabolizer; $MR_{NDT/ENDO}$, N-desmethyl tamoxifen to endoxifen metabolic ratio; NM, normal metabolizer; PM, poor metabolizer; UM, ultrarapid metabolizer.
[a]As all the variables listed are indicator variables, the interpretation of each coefficient is as follows: a positive (or negative) value of the coefficient (*a*) indicates that the response variable ($MR_{NDT/ENDO}$) is increased by *a* (respectively decreased by *a*) compared to the reference category. For example, the mean $MR_{NDT/ENDO}$ value of NM/IM patients is 8.59 higher than that of NM/NM patients.

4-OHTAM concentrations are observed in the presence of two *CYP3A4*22* alleles, possibly because of a decreased conversion of TAM to 4-OHTAM. Increased $MR_{TAM/4\text{-}OHTAM}$ was also observed in *CYP2C19*2* carriers.

$MR_{TAM/NDT}$ and $MR_{4\text{-}OHTAM/ENDO}$ were associated with *CYP2D6* diplotype ($P < 0.0001$) and phenotype ($P < 0.0001$), respectively (**Table S3**). *CYP3A5*3* was significantly associated with $MR_{TAM/NDT}$ and $MR_{4\text{-}OHTAM/ENDO}$ when coded as **1/*1* vs. **1/*3* or **3/*3* ($P < 0.0001$ and $P < 0.0001$, respectively). However, this SNP was no longer significant ($P = 0.7$ and $P = 0.3$, respectively) when coded as CYP3A5 expressor (**1/*1* and **1/*3*) vs. nonexpressor (**3/*3*) genotypes as suggested by Sanchez Spitman *et al.*[10] Because of the small number of patients with *CYP3A5*1/*1* genotype ($n = 5$) and the difficulty in interpreting the clinical meaning of the first classification compared to the expressor vs. nonexpressor classification, this SNP was not included in the multivariable analyses. *CYP3A4*1B* genotype (coded as **1/*1* and **1/*1B* vs. **1B/*1B*) was also found to be significantly associated with $MR_{TAM/NDT}$ in the univariate analysis ($P < 0.001$). However, the functional significance of this SNP has not been demonstrated, and there is a linkage disequilibrium with the wild-type allele (A) of rs776746 (SNP encoding the nonfunctional *CYP3A5*3* allele).[21] It may be speculated that *CYP3A5*3*, rather than *CYP3A4*1B* genotype, has contributed to significant associations with $MR_{TAM/NDT}$, especially because when *CYP3A5*3* (coded as **1/*1* vs. **1/*3* or **3/*3*) and *CYP3A4*1B* (coded as **1/*1* and **1/*1B* vs. **1B/*1B*) were entered in the multivariable analysis together with *CYP2D6* diplotype, the second one was no longer significant ($P = 0.11$). The presence of two *CYP2B6*6* (rs3745274) alleles was associated with increased $MR_{TAM/NOX}$ ($P < 0.001$; **Table S3**). Finally, no statistically significant SNPs were found on $MR_{TAM/4'OHTAM}$ and $MR_{NDT/Z'ENDO}$.

DISCUSSION

This work is, to our knowledge, the largest prospective study simultaneously evaluating factors that might influence the concentrations of TAM and six of its metabolites, including genetic polymorphisms in all the major enzymes involved in its metabolism. *CYP2D6* diplotype and *CYP3A4*22* genotype as well as concomitant intake of potent CYP2D6 inhibitors were significant predictors of ENDO concentrations. In addition, the comparison of plasma ENDO concentrations and $MR_{NDT/ENDO}$ among *CYP2D6* diplotypes showed that IM/IM patients had lower ENDO levels than NM/PM patients despite the same AS for both diplotypes. These results support the need to improve the *CYP2D6* genotype to phenotype classification system for TAM therapy.

Recent CPIC recommendations for TAM therapy discussed uncertainties in the translation of *CYP2D6* genotype into diplotypes and phenotypes.[19] We performed a comparison of plasma ENDO levels and $MR_{NDT/ENDO}$ between *CYP2D6* genotypes classified into the same diplotype. We showed that the presence of **10* and **41* alleles among IM/PM diplotype did not result in the same plasma ENDO levels as in **9* allele carriers, whereas all of these alleles are considered to be associated with reduced enzyme activity (AS = 0.5). This is consistent with previous studies showing that the activities of **10* and **41* alleles were lower than that of **9* allele.[18] Moreover, in our analysis, plasma ENDO levels were similar for **10/*4* or **41/*4* and PM/PM patients, confirming lower CYP2D6 activity of **10* and **41* alleles compared to **9* allele. Further studies including more patients with these genotypes are needed to evaluate if patients carrying **10/*4* and **41/*4* genotypes should be assigned into PM/PM instead of IM/PM diplotype (as suggested by our results) for prediction of ENDO concentration.

Furthermore, we found that NM/NM (AS = 2) and UM patients (AS > 2; UM/UM and UM/IM diplotypes) had similar ENDO levels and $MR_{NDT/ENDO}$. This is consistent with Hicks *et al.*,[16] who suggested that patients with AS = 2.5 or 3 should be attributed NM phenotype. Regarding patients with CYP2D6 AS = 1 (NM/PM and IM/IM), plasma ENDO levels were significantly lower in IM/IM patients than in NM/NM, NM/IM, and NM/PM patients but similar to IM/PM patients. Similar results were observed by Hertz *et al.*,[18] which altogether indicates that classification of IM/IM patients into NM phenotype as proposed by previous CPIC recommendations[15] needs to be re-evaluated. Based on ENDO levels in our study, IM/IM could be classified together with IM/PM (AS = 0.5) patients into the IM phenotype. Moreover, NM/PM had similar ENDO levels to NM/IM but lower than NM/NM patients, which might suggest classification of these diplotypes separately from NM/NM.

In our analysis, higher plasma ENDO levels were observed in *CYP3A4*22* carriers, a genotype associated with reduced mRNA expression and enzyme activity.[22] In particular, NM/

PM and IM/IM patients carrying *CYP3A4*22* allele had significantly higher ENDO levels than patients with the same CYP2D6 diplotype and *CYP3A4*22* noncarriers. Although unexpected, this result is consistent with previous studies showing that the presence of *CYP3A4*22* allele is associated with higher ENDO concentrations, particularly in patients with low CYP2D6 activity.[4,6,10]

One of the possible hypotheses for the increased concentrations of ENDO in *CYP3A4*22* carriers could be its impaired metabolism through CYP3A4. Although there is not much evidence in the literature that ENDO could be metabolized by CYP3A4, it has been suggested that norendoxifen, another metabolite of TAM, could be formed via N-demethylation of ENDO, which is a phase I reaction involving CYP isoenzymes.[23,24] In addition, a recent *in vitro* study showed that an estrogen-like metabolite of TAM (bisphenol tamoxifen) can be formed from ENDO in presence of CYP3A4.[25] More studies are needed to investigate the relative implication of CYP3A4 in ENDO metabolism compared with other enzymes.

In our study, NM patients cotreated with potent CYP2D6 inhibitors had 54% lower ENDO concentrations than NM patients without comedication with CYP2D6 inhibitors and, therefore, reached similar concentrations to IM patients, consistently with previous reports.[20,26] Concerning CYP3A4 inhibitors, lower ENDO levels in NM patients treated with moderate or strong CYP3A4 inhibitors were observed, but the low number of patients may have limited statistical significance. The final multivariable analysis, including *CYP2D6* diplotype, *CYP3A4*22* genotype, and CYP2D6 inhibitors explained 19.4% of the variability in ENDO levels. *CYP2D6* diplotype had the most substantial effect on ENDO as it explained 16.8% of the variability. This result is lower than that recently reported by Sanchez-Spitman *et al.*[10] ($R^2 = 0.42$), but their analysis was performed on ln-transformed concentration data, which partly explains our different result. However, our value is consistent with Hertz *et al.*[18] who reported that only 11% of the variability in ENDO levels was explained by *CYP2D6* phenotype.

Although the consideration of these factors will help to predict ENDO concentration, there is still uncertainty concerning the correlation of ENDO or other metabolites with clinical outcomes. Two retrospective analyses in patients with adjuvant breast cancer[7,12] have shown that ENDO concentration > 16 nmol/L and AAS > 1,798[27] are associated with a better therapeutic outcome. However a recent prospective study did not observe any significant association between clinical benefit of TAM and plasma ENDO exposure in 247 patients with neo-adjuvant and metastatic breast cancer,[13] corroborated by another recent study in 667 patients.[14] On the contrary, Helland *et al.*[28] reported a correlation between ENDO and 4-OHTAM concentrations and long-term survival in 99 retrospectively analyzed patients with adjuvant breast cancer. Therefore, the clinical utility of therapeutic drug monitoring of ENDO concentrations remains uncertain. This issue requires further elucidation based on data from prospective studies. Meanwhile, if we consider the 16 nmol/L threshold for ENDO,[7,12] our study shows that 97% of PM, 59% of IM, and 12% of NM + UM patients were below this value.

Finally, the analysis of genetic polymorphisms on MRs showed that *CYP3A4*22*, *CYP2C19*2*, and *CYP2B6*6* contribute to the variability in the metabolism of TAM. However, the results on MRs should be interpreted with caution as they are dependent not only on enzymes involved in a given metabolic pathway but also on the elimination of the metabolite.

The compliance to treatment is an important factor affecting clinical efficacy of TAM.[29] In this study, we excluded clearly noncompliant patients based on their plasma TAM or ENDO concentrations below the limit of quantification so that this should not be a confounding factor. Nevertheless, future analyses will further investigate the combined impact of genetic and nongenetic factors, such as age, body weight, and compliance, on TAM metabolism.

In conclusion, based on a large dataset of 730 patients with adjuvant breast cancer treated with TAM 20 mg/day, we provide results supporting re-evaluation of the existing *CYP2D6* genotype to phenotype classification system for TAM therapy. Moreover, this study refines the previous findings about the impact of genetic polymorphisms on TAM metabolism. Although personalization of TAM therapy based on *CYP2D6* genotype or ENDO concentrations is not currently recommended due to inconsistent results concerning their relationship with TAM efficacy or toxicity, the results of this study are of importance for future evaluations of factors impacting TAM outcome and to standardize the genotype to phenotype translation systems for TAM and other drugs with similar metabolic pathways.

METHODS

Patients and data collection

Eligible patients started treatment with TAM at 20 mg/day and were followed up every 6 months over 3 years. Inclusion criteria were histologically proven primary breast cancer, no metastatic disease at diagnosis, and estrogen receptor–positive and/or progesterone receptor–positive tumor assessed by locally performed immunohistochemistry. Each visit consisted in a clinical examination looking for side effects and a PK sampling; the comedications at each follow-up visit were recorded. The present report focuses on the evaluation of PK, PG, and comedication at first follow-up visit. All patients provided written informed consent in compliance with the ethical principles of the revised Declaration of Helsinki and with European regulations.

Plasma concentrations of TAM and its metabolites

Blood samples (10 mL) were collected in Vacutainer Lithium Heparin tubes from each patient during first the follow-up visit when steady-state concentrations of TAM and its metabolites were achieved. Patients were instructed not to take a TAM dose on the day of the PK sampling to assure trough plasma levels. Samples were immediately centrifuged at 1,400 *g* at ambient temperature, and plasma was stored at −20°C until analysis. Plasma concentrations of TAM, its two active metabolites 4-OHTAM and ENDO, as well as NDT, NOX, 4′-OHTAM, and Z′-ENDO were measured by a validated ultra-performance liquid chromatography-tandem mass spectrometry, as described previously.[30] MRs were calculated for TAM/4-OHTAM, TAM/NDT, NDT/ENDO, 4-OHTAM/ENDO, NDT/Z′-ENDO, TAM/NOX, and TAM/4′-OHTAM. AAS was calculated according to a recently proposed algorithm based on *in vitro* proliferation assays on Michigan Cancer Foundation-7 breast adenocarcinoma cells expressing estrogen receptors:

$$\begin{aligned} AAS = {} & 1 \times [C]_{TAM} + 0.38 \times [C]_{NDT} \\ & + 21.8 \times [C]_{4\text{–}OHTAM} + 74.4 \times [C]_{ENDO} \end{aligned}$$

where [C] is the plasma concentration of the respective compound.[27]

Genotyping

Blood samples for PG analysis were collected in Vacutainer EDTA tubes for each patient at inclusion. Description of the methods used for DNA extraction, genotyping of 63 selected SNPs, and CNV determination is provided in **Supplementary Material S1**.

CYP2D6 phenotype and AS

Patients were genotyped for the presence of decreased (**9*, **10*, **17*, and **41*) and nonfunctional (**4*, **6*, and **7*) alleles and gene copy number (**5* or duplication). The absence of one of these alleles led to **1* allele assignment. A phenotype and a score were assigned to each *CYP2D6* allele according to its activity: extensive NM (AS = 1) for wild-type (**1*), IM (AS = 0.5) for decreased activity, and PM (AS = 0) for nonfunctional alleles.[17] Based on the combination of alleles and the number of *CYP2D6* copies, patients were assigned a diplotype and an AS, which is the sum of the scores assigned to each allele: PM/PM (AS = 0), IM/PM (AS = 0.5), IM/IM (AS = 1), NM/PM (AS = 1), NM/IM (AS = 1.5), NM/NM and NM/NM/PM (UM/PM; AS = 2), and NMxn (xn represents the number of *CYP2D6* gene copies; UM/UM) and NM/NM/IM (UM/IM), which were collapsed into UM (AS > 2). Finally, patients were classified into CYP2D6 phenotypes: PM (AS = 0), IM (AS = 0.5), NM or IM (AS = 1), NM (AS = 1.5–2), and UM (AS > 2)[19]; the corresponding frequencies in our study were: 4.7%, 6.0%, 28.6%, 57.0%, and 3.7%, respectively.

Statistical analysis

HWE was assessed for all 63 SNPs using the Benjamini–Hochberg correction for multiple testing. For *CYP2D6*, *CYP2A6*, and *UGT2B17* SNPs, the analysis was performed in patients with two copies of the corresponding gene, as the HWE is a widely used model to describe the distribution of genotypes without CNV in a population. The associations of plasma ENDO levels and MRs with SNPs were examined using the qtlsnp Stata command and adjusted for multiple testing (Benjamini–Hochberg correction). After identification of SNPs significantly associated with ENDO concentrations or the different MRs, univariate and multivariable linear regression analyses with the respective SNPs were carried out in order to determine which SNPs were independently associated with ENDO levels and MRs (in these analyses, *CYP2D6* SNPs were replaced by *CYP2D6* diplotypes combining all the genetic polymorphisms and number of gene copies). The global significance of each variable was tested using likelihood ratio tests on the nested models. Kruskal–Wallis or Wilcoxon tests were used for comparisons of plasma concentrations or MRs between *CYP2D6* diplotypes and genotypes or patients with and without comedications. Data are presented as mean ± SD. All tests were two-sided, and a P value < 0.05 was considered statistically significant unless otherwise stated. Statistical analyses were performed in R version 3.4.2 coupled with RStudio and in Stata version 13.0 (StataCorp, College Station, TX).

SUPPORTING INFORMATION

Supplementary information accompanies this paper on the *Clinical Pharmacology & Therapeutics* website (www.cpt-journal.com).

Supplementary Material S1. Methods for genotyping and CNV analysis.
Table S1. List of single-nucleotide polymorphisms (n = 63) analyzed in the PHACS study with the corresponding gene.
Table S2. Mean ± SD plasma concentrations of TAM, major TAM metabolites, and MRs in patients with breast cancer from PHACS study at 6 months after treatment start according to *CYP2D6* diplotype. Patients concomitantly treated with CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors or CYP3A4 inducers were excluded (n = 53).
Table S3. Results of the linear regression analyses of genetic polymorphisms on the MRs. Patients concomitantly treated with CYP2D6 inhibitors, moderate or potent CYP3A4 inhibitors, or CYP3A4 inducers were excluded (n = 53). Patients without variant allele for the investigated single-nucleotide polymorphism were considered **1/*1* genotype.

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CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

A.P., M.W.-K., and F.T. wrote the manuscript. H.R., A.E., J.R., M.D., W.J., T.F., E.C., M.W.-K., and F.T. designed the research. A.P., C.A., C.V., A.E., V.L.M., J.C.B., J.R., C.D., F.D., M.D., L.V.-B., W.J., E.S., I.S.B., H.R., M.W.-K., and F.T. performed the research. A.P., C.A., A.E., J.C.B., J.R., T.F., M.W.-K., and F.T. analyzed the data.

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