Original Research

*CYP2C19*17* association with higher plasma 4-hydroxy tamoxifen in Pakistani (estrogen-positive) breast cancer patients

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Impact Statement

This study contributes to the field of pharmacogenomics. Adverse drug reactions as well as non-responsiveness to the tamoxifen chemotherapy are observed in many patients. Our findings will help the clinicians to cope up with the problem of inappropriate drug prescription for South Asian and Pakistani patients.

Abstract

Breast cancer (BC) continues to be the most common cancer in the women worldwide. Since estrogen receptor (ER)-positive BC accounts for the majority of newly diagnosed cases, endocrine therapy is advised to utilize either tamoxifen (Tam) or aromatase inhibitors. The use of Tam as a monotherapy or in conjunction with an aromatase inhibitor following two or three years of endocrine therapy has long been recommended. When used adjuvantly, Tam medication reduces BC mortality and relapses, while it extends survival times in metastatic BC when used in conjunction with other treatments. Unfortunately, the efficiency of Tam varies considerably. This study was conducted to explore the influence of genetic polymorphisms in *CYP2C19* gene on Tam's pharmacogenetics and pharmacokinetics in estrogen-positive BC patients. Data from healthy, unrelated individuals ($n=410$; control group) and ER-positive BC patients ($n=430$) receiving 20 mg of Tam per day were recruited. Steady-state plasma concentrations of Tam and its three metabolites were quantified using the high-performance liquid chromatography in the patients. The CYP2C19 polymorphisms were genotyped using an Amplification Refractory Mutation System-Polymerase Chain Reaction (ARMS-PCR) approach. More than 65% of healthy individuals were extensive metabolizers (*1/*1) for CYP2C19, whereas more than 70% of ER-positive BC patients were rapid and ultrarapid metabolizers (*1/17*, *17/17*). The polymorphism CYP2C19*17 is significantly associated with higher 4-hydroxytamoxifen (4-OH-Tam). Patients with the *17/*17 genotype exhibited 1- to 1.5-fold higher 4-OH-Tam, which was also high in patients with the *1/*2 and *2/*2 genotypes.

Keywords: Tamoxifen, CYP2C19, ER-positive breast cancer, personalized medicine, pharmacogenomics, cytochrome p450

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Introduction

Breast cancer (BC) is a significant health issue worldwide, and Asia bears most of this burden due to greater disease linked morbidity and mortality.[1] According to a recent analysis, BC incidence remained higher in Pakistan as compared to its neighboring and similar socioeconomic countries like China, India, and Thailand,[2] possibly due to higher rates of consanguineous marriages, inaccessibility to basic healthcare facilities, social stigma to talk about the disease, and a lack of awareness programs. One of the meta-analyses exhibited an increased prevalence of BC (31%) among Pakistani women at an early age with the progressive stage of the disease.[3]

Tamoxifen (Tam) is an antiestrogenic drug used to treat estrogen receptor (ER)-positive BC for more than three decades, leading to strikingly reduced disease recurrence and mortality rate.[4] Tam is a prodrug which must be metabolized into its active components: 4-hydroxytamoxifen (4-OH-Tam), *N*-desmethyl-tamoxifen (*N*-DesM-Tam), and 4-hydroxy-*N*-desmethyl-tamoxifen (Endoxifen). In contrast to their parent moiety – Tam, 4-OH-Tam, and Endoxifen possess more significant activity toward ER receptors and are also responsible for the decreased proliferation of BC cells.[5,6] Tam plays a potent role in activating the transforming growth factor (TGF) signal transduction pathway by inhibiting tumor cell growth. Endoxifen and 4-OH-Tam have profound effects on tumor cell growth

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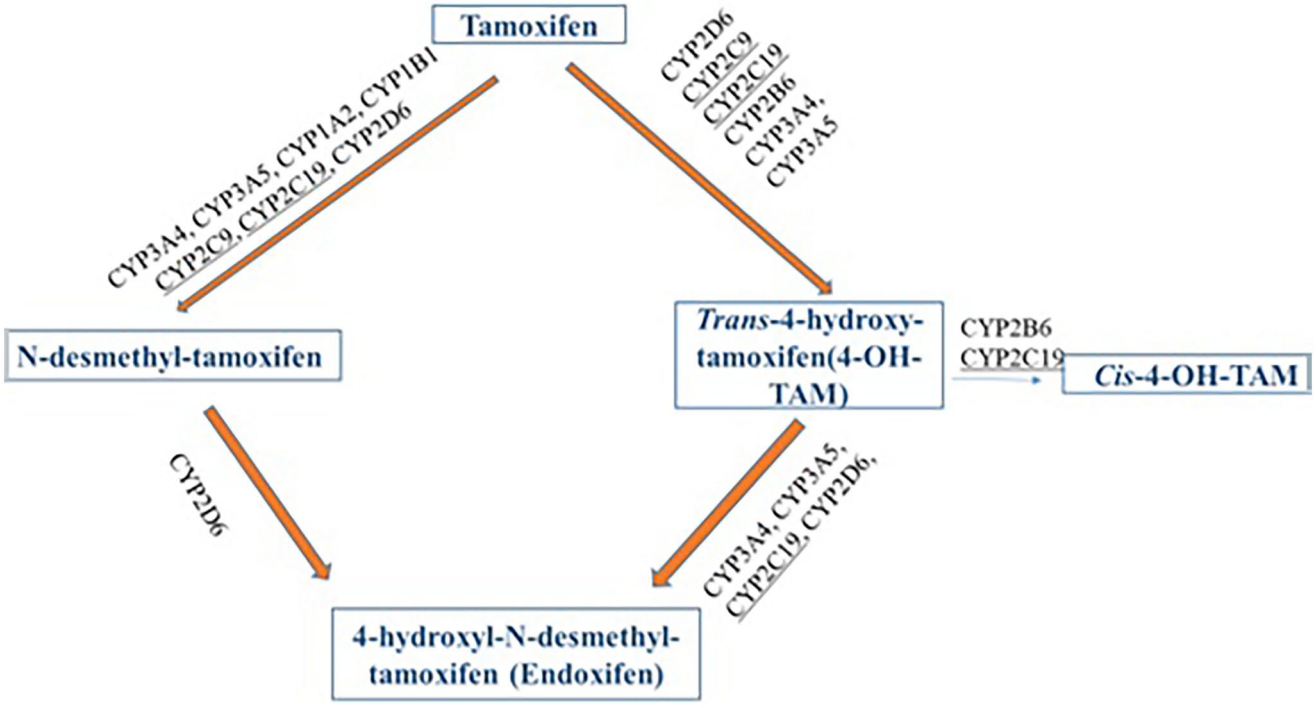

Figure 1. The role of CYP2C19 in the hepatic metabolism of tamoxifen.

via TGFβ2 and TGFβR2 expression compared to other metabolites.[7]

Tam is converted into Endoxifen and 4-OH-Tam, which exhibit 30–100 times greater potency in ER-binding ability via cytochrome P450 isoenzymes.[8] Tam has a very complex metabolism (Figure 1), encompassing a wide range of P450 enzymes.[9] *CYP2C19* is one of the xenobiotic-metabolizing enzymes along with CYP2D6 and CYP2B6, that is polymorphically expressed and involved in Tam metabolism,[10] but the underlying mechanisms are still unknown.[11] It plays a key role in transforming Tam into its primary metabolites, 4-OH-Tam and Endoxifen.[12] Based on data from over 1000 cases and controls, Justenhoven *et al.*[13] discovered that having more CYP2C19*17 alleles was associated with a lower risk of BC. The *CYP2C19* phenotype is measured by comparing the drug metabolites via urine or plasma samples. Involvement of genetic polymorphism in the metabolism of a drug categorized the patients into four classes: normal or extensive metabolizers (EMs), poor metabolizers (PMs), intermediate metabolizers (IMs), and ultrarapid metabolizers (UMs) for a certain drug.[14]

*A CYP2C19*2* genetic variant arises because of a single-base alteration, 681G > A, rs4244285 located in exon 5. This mutation is responsible for the premature stop codon, leading to a truncated, malfunctioning l protein.[15] The *CYP2C19*3* variant results from 636G > A mutation, rs4986893 present in exon 4, leading to a premature stop codon and truncated protein product.[16] *The CYP2C19*17* variant has two linked mutations in total non-disjunction equilibrium at −806C > T and −3402C > T in the 5′ regulatory region. This mutation is responsible for the increased expression and activity of enzyme.[17]

*CYP2C19*2* is associated with prognosis in Tam-treated BC patients.[18] One of the studies reported that individuals who carry the "A" allele of *CYP2C19*3* have a higher risk of BC than those carrying the "G" allele in the Chinese population.[19] Polymorphism of *CYP2C19*2* was linked with prolonged survival in BC patients taking Tam,[18] while *CYP2C19*17* (but not *3 and *2) is linked with decreased BC risk.[13] These *CYP2C19* genetic polymorphisms in BC patients have been investigated in European populations.[18] There is a need to explore the phenomenon in the Pakistani population as the ethnic diversity makes it distinct from other Asian populations. Therefore, this study was designed to determine the role of *CYP2C19*2*, *3, and *17* variants among Tam-treated BC cases by particularly categorizing them into ultrarapid (UM), extensive (EM), intermediate (IM), or poor (PM) metabolism groups.

Materials and methods

This study was approved by the Bio-Ethical Committee (BEC) of Quaid-i-Azam University Islamabad vide protocol # BEC-FBS-QAU-40. Samples were collected from Sir Ganga Ram Hospital (SGRH), Lahore, Pakistan; Institute of Radiotherapy and Nuclear Medicine, Peshawar; Centre for Nuclear Medicine and Radiotherapy (CENAR), Quetta; and Nuclear Oncology Research Institute (NORI), Islamabad, Pakistan. The sample size was calculated with the formula provided $\{n = Z\alpha_{/2}^2 pq/(\text{MOE})^2\}$ using the frequency of BC from the literature as 12–15%. Therefore, applying an estimated population size of 15% with a margin of error of 5%, the calculated sample size was 195,[20] where $Z\alpha_{/2}$ is a statistical constant, p is the prevalence, and MOE is the margin of

Table 1. Primers for *CYP2C19* allele.

Primers	Sequence (5'-3')	Length	Melting temperature	Fragment size (bp)	PCR product
2C19*2F	CAGAGCTTGGCAATATTGTATC	22	57.1°C	291	Control
2C19*2R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
2C19*2A	GTAATTTGTTATGGGTTCCT	20	52.3°C	169	A-allele fragment
2C19*2F	CAGAGCTTGGCAATATTGTATC	22	57.1°C		
2C19*2G	ACTATCATTGATTATTTCCCG	21	55.6°C	202	G-allele fragment
2C19*2R	ATACGCAAGCAGTCACATAAC	21	57.4°C		
*2C19**3F2	TATTATTATCTGTTAACAAATATGA	25	52.7°C	253	Control
*2C19**3R	AACTTGGCCTTACCTGGATC	20	58.4°C		
*2C19**3F1	TATTATTATCTGTTAACAAATATG	24	51.6°C	253	Allele fragment
*2C19**3R(T)	AACTTGGCCTTACCTGGATT	20	56.4°C		
2C19*17F	AAGAAGCCTTAGTTTCTCAAG	21	55.5	507	Control
2C19*17R	AAACACCTTTACCATTTAACCC	22	56.6		
2C19*17C	ATTATCTCTTACATCAGAGATG	22	54.7	330	C-allele fragment
2C19*17F	AAGAAGCCTTAGTTTCTCAAG	21	55.5		
2C19*17T	TGTCTTCTGTTCTCAAAGTA	20	52.3	218	T-allele fragment
2C19*17R	AAACACCTTTACCATTTAACCC	22	56.6		

PCR: polymerase chain reaction.

error or relative precision. Sampling was carried out after taking written informed consent from both patients and controls. For this study, 430 subjects (425 female and 5 male patients) were enrolled. Clinically diagnosed ER-positive BC patients at any stage of the disease and taking Tam as adjuvant therapy with a day-to-day dosage of 20 mg for at least three months prior to sampling were included in the study.

Demographic characteristics – such as age, race, weight, marital status, family history, and smoking – were collected using a specially designed questionnaire (Supplementary Table 1a). In addition, we recruited patients' reports and hospital records or clinical information such as surgery, chemotherapy, radiotherapy, tumor size, and cancer stage. Patients with liver, kidney, heart or neurological disorders or those diagnosed with diabetes mellitus or on any other medication for the last seven days except Tam were excluded from the study. To compare the genotyping results with the control population, 410 age- and gender-matched healthy individuals from the same cities and socioeconomic backgrounds were also enrolled (Supplementary Table 1b).

Genomic DNA extraction and genotyping of CYP2C19 variants

Blood and plasma samples of ER-positive BC patients taking Tam monotherapy as well as that of healthy controls were collected in vacutainers. Plasma samples were stored at −20°C and high-performance liquid chromatography (HPLC) was performed to analyze Tam and its metabolites.

The phenol–chloroform extraction method was employed for the genomic DNA extraction from peripheral blood samples.[20] Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) was utilized for the genotyping of *CYP2C19*2*, *CYP2C19*3*, and *CYP2C19*17* by allele-specific polymerase chain reaction (AS-PCR). The sequences of primers are given in Table 1.

Tam and its metabolites quantification in plasma

HPLC was performed on the plasma samples of ER-positive BC samples to quantify Tam and metabolites. To determine the association of CYP2C19*2, CYP2C19*3, and CYP2C19*17 in the formation of 4-OH-Tam from Tam, and Endoxifen from 4-OH-Tam, we employed HPLC Agilent 1100 UV system (Santa Clara, CA, USA) for the plasma analysis of 430 ER-positive BC patients at a 80-nm wavelength. The separation was performed on a CNW Athena C18 column (China) (150 mm × 4.6 mm, particle diameter 5.0 µm). The flow rate was set at 1.0 mL/min until the end of the analysis. The column temperature was set at 30°C. The total run time was 16 min. The retention time was 2.05 min for verapamil (internal standard [IS]), 4.14 min for Endoxifen, 4.5 min for 4-OH-Tam, 11.13 min for *N*-DesM-Tam, and 13.7 min for Tam.

Preparation of solutions and standards for HPLC

Separate methanolic stock solutions for Tam, 4-OH-Tam, *N*-DesM-Tam, and Endoxifen were prepared. The stock concentrations included 1 mg/mL for Tam, 0.1 mg/mL for 4-OH-Tam, 1 mg/mL for *N*-DesM-Tam, and 1 mg/mL for Endoxifen. Verapamil (1 µg/mL) was used as an IS. Working solutions of Tam (40 ng/mL), 4-OH-Tam (20 ng/mL), *N*-DesM-Tam (10 ng/mL), and Endoxifen (5 ng/mL) were prepared in methanol. Mobile phase buffer was prepared by diluting 1 M triethylammonium phosphate buffer with 1000 mL ultrapure water, and it was filtered afterwards using a Millex Syringe-driven unit (Merck Millipore, Germany). Tris buffer (0.2 M) was prepared and its pH was adjusted to 10.0 using 0.1 M NaOH. The solvent for extraction was set up by combining 95 mL hexane with 5 mL of *n*-propanol.[21]

Sample preparation for HPLC

A volume of 2 mL of the patient's plasma sample was mixed in 0.7 mL Tris buffer (pH 10.0) and 5.2 mL extraction solvent in a 15-mL falcon tube. The samples were gently mixed for 10 min and centrifuged at 2000*g* for 10 min. The organic layer was transferred into another falcon tube, and 200 µL of phosphoric acid 0.1% (v/v) was added in it. After homogenization, it was again centrifuged at 4000 r/min for 15 min. The aqueous layer was collected in a new tube, and 20 µL of the aqueous layer was injected into Agilent HPLC 1100 system.[20]

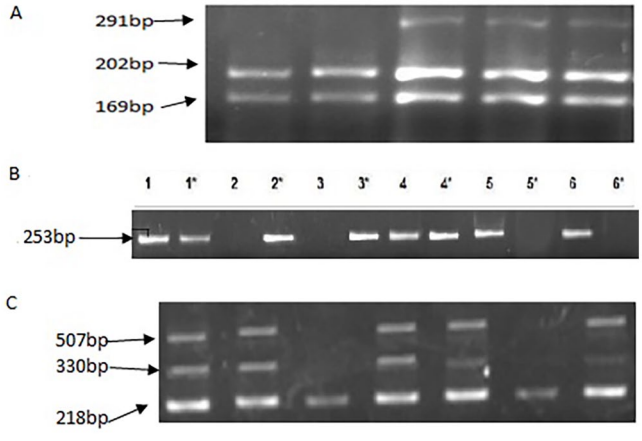

Figure 2. Genotyping of CYP2C19 SNPs. (A) Electropherogram of ARMS PCR for CYP2C19*2 allele. The control band is 291 bp, the wild-type G-allele band is 202 bp, and the mutated A-allele band is 169 bp. (B) Electropherogram of allele-specific PCR for CYP2C19*3 allele. Asterisk (*) represents the mutant allele. Wild-type C-allele band = 253 bp, mutant type T-allele = 253 bp. 1 and 1* lane showing heterozygous major C and minor T-allele, 2* and 3* showing homozygous minor T-allele, while 5 and 6 showing homozygous major C-allele. (C) Electropherogram of ARMS PCR for CYP2C19*17. Control band of 507 bp, wild-type C-allele band of 330 bp, and mutated T-allele band of 218 bp. 100 bp ladder are used for comparison.

Data analyses

The calculation of calibration curves (weighted $1/\times$) and quantification were performed using the MassHunter Quantitative Analysis (Agilent). Metabolic ratios were estimated as substrate concentration/metabolite concentration. Statistical analyses were performed using SPSS 21. Conditional logistic regression was applied to determine the association between *CYP2C19* variants and the risk of BC, represented by odds ratio (OR) and 95% confidence interval (CI).

Results

Genotyping frequency for CYP2C19*2 SNP in exon 5 at 681 G > A

AS-PCR at this locus produced three sizes of bands: 291 bp (positive control for the locus), 202 bp (wild type), and 169 bp (mutant). The subject was considered heterozygous (*CYP2C19*1/*2*) in case three bands of size 291 bp, 202 bp, and 169 bp were detected; homozygous wild type (*CYP2C19*1/*1*), if two bands of size 291 bp and 202 bp were detected; and homozygous mutant (*CYP2C19*2/*2*), if two bands of size 291 bp and 169 bp were observed. *CYP2C19*2* heterozygous condition was a BC risk factor (OR: 0.6; 95% CI: 0.43–0.84; $P = 0.003$) in our population, whereas no association was found for the homozygous mutant condition (Figure 2(A) and Table 2).

Genotyping frequency for CYP2C19*3 SNP in exon 4 at 636 G > A

AS-PCR amplified *CYP2C19*3* gene. Normal "C" allele band of 253 bp and mutant "T" allele band of 253 bp were amplified to determine genotype. The allele-specific band marked the presence of respective alleles in the *CYP2C19* gene. Homozygous with major allele "C" and homozygous with minor allele "T" have shown only a single band, whereas heterozygous has shown bands with both primers. Statistical analysis revealed a significant contribution of *CYP2C19*3* heterozygous variant (OR: 0.34; 95% CI: 0.24–0.48; $P < 0.001$) toward BC development in this study population (Figure 2(B) and Table 2).

Genotyping frequency for CYP2C19*17 (–806C > T; rs12248560)

Three bands produced in this case were of 507 bp (control), 330 bp (wild type), and 218 bp (mutant). In the case of detecting all three bands, the subject was considered as heterozygous (*CYP2C19*1/*17*). The subject was designated as homozygous wild type (*CYP2C19*1/*1*), if two bands of the sizes 507 bp and 330 bp were detected, and homozygous mutant (*CYP2C19*17/*17*), if two bands of the sizes 507 bp and 218 bp were observed. Heterozygous individuals (OR: 7.17; 95% CI: 5.08–10.1; $P < 0.001$) and homozygous mutant individuals (OR: 5.36; 95% CI: 3.18–9.01; $P < 0.001$) were at significant risk of BC development (Figure 2(C) and Table 2).

Analysis of tamoxifen and its metabolites Tam is metabolized by enzymes such as *CYP2D6, CYP2C19, CYP2C9, CYP2B6*, and *CYP3A* into 4-OH-Tam. *CYP2C19* is a polymorphic gene. *CYP2C19*2* and *CYP2C19*3* mutants have low enzyme action, whereas *CYP2C19*17* heterozygotes and mutants have enhanced enzyme activity.

CYP2C19 polymorphisms impact the plasma concentrations and total metabolic ratio of Tam and its metabolites

Median plasma concentrations of Tam and the three metabolites measured in patients of each genotypic group are shown in Table 3. Figure 3 displays the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*17* (heterozygous), and *CYP2C19*17/*17* (mutant) genotypes. No significant difference was observed for the median plasma Tam and Endoxifen concentrations among the three different genotypes of patients (Table 3 and Figure 3). However, this locus showed a strong association with the median plasma concentrations of 4-OH-Tam, which was recorded to be 288.59 ng mL/L in the wild-type genotypes, 354.53 ng mL/L ($P < 0.001$) in the heterozygous genotypes (62% increased than the wild-type patients), and 378.25 ng mL/L ($P < 0.001$) in the mutant genotypes (75% increased than the wild-type patients). A slight reduction ($P = 0.362$) in the median concentration of *N*-DesM-Tam was observed in heterozygous and mutant patients (Table 3 and Figure 3).

Figure 4 shows the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*3* (heterozygous), and *CYP2C19*3/*3* (mutant) genotypes. No significant association was observed for Tam or *N*-DesM-Tam among the wild-type, heterozygous, and mutant genotypes for *CYP2C19*3* locus. However, an insignificant decrease was observed in the median plasma concentrations of 4-OH-Tam in the heterozygous (240.63 ng mL/L) ($P = 0.420$) or mutant

Table 2. Association of CYP2C19 genotypes with ER-positive breast cancer.

Genotype			Controls (410)		Cases (430)		OR (95% CI)	*P* value
			n	%	*n*	%		
*CYP2C19*2*								
GG	EM	*1/*1	210	51.2	214	49.8	1.0 (reference)	
GA	IM	*1/*2	180	43.9	190	44.2	0.6 (0.43–0.84)	0.003
AA	PM	*2/*2	20	4.9	26	6	0.81 (0.39–1.71)	0.594
*CYP2C19*3*								
GG	EM	*1/*1	152	37.1	159	37.0	1.0 (reference)	
AG	IM	*1/*3	214	52.2	205	47.7	0.34 (0.24–0.48)	>0.001
AA	PM	*3/*3	44	10.7	66	15.3	0.68 (0.39–1.16)	0.165
*CYP2C19*17*								
CC	EM	*1/*1	265	64.6	80	18.6	1.0 (reference)	
CT	RM	*1/*17	107	26.1	281	65.3	7.17 (5.08–10.1)	>0.001
TT	UM	*17/*17	38	9.3	69	16.1	5.36 (3.18–9.01)	>0.001

ER: estrogen receptor; OR: odds ratio; CI: confidence interval; EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; RM: rapid metabolizer; UM: ultrarapid metabolizer.

Table 3. Effects of *CYP2C19* polymorphisms on median, mean, and SEM plasma concentrations (ng/mL) of tamoxifen and its metabolites.

Parameters *CYP2C19*2* (681G>A; rs4244285)	Genotype *1/*1 (EM)[a]		Genotype *1/*2 (IM)[a]		Genotype *2/*2 (PM)[a]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	116.96	122.94 (1.30)	119.68	126.12 (1.47)	118.49	128.15 (2.1)
4-OH-Tam	255.75	301.43 (10.51)	272.65	307.09 (8.01)	305.25	387.8 (18.09)
N-DesM-Tam	3.96	5.8 (0.44)	4.10	6.49 (0.39)	4.91	10.89 (1.30)
Endoxifen	19.34	27.62 (1.80)	27.15	35.10 (1.73)	32.50	55.88 (5.1)
Parameters *CYP2C19*3* (636G>A; rs4986893)	Genotype *1/*1 (EM)[b]		Genotype *1/*3 (IM)[b]		Genotype *3/*3 (PM)[b]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	116.87	119.20 (0.68)	116.99	119.65 (0.58)	117.26	124.27 (1.08)
4-OH-Tam	288.43	306.37 (3.90)	240.63	266.71 (5.67)	276.29	303.2 (5.67)
N-DesM-Tam	3.02	5.50 (0.58)	3.13	4.52 (0.36)	1.68	3.60 (0.63)
Endoxifen	19.34	34.36 (3.05)	24.73	37.43 (2.48)	35.40	44.32 (5.38)
Parameters *CYP2C19*17* (–806C>T; s12248560)	Genotype *1/*1 (EM)[c]		Genotype *1/*17 (RM)[c]		Genotype *17/*17 (UM)[c]	
	Median	Mean (SEM)	Median	Mean (SEM)	Median	Mean (SEM)
Tamoxifen	118.97	129.48 (2.37)	120.15	129.22 (1.31)	121.88	131.40 (2.51)
4-OH-Tam	288.59	296.48 (1.80)	354.53	358.13 (0.73)	378.25	392.16 (3.20)
N-DesM-Tam	4.78	5.92 (0.52)	3.47	5.01 (0.29)	3.20	4.74 (0.56)
Endoxifen	29.49	34.96 (1.64)	27.04	36.34 (1.47)	31.40	39.55 (2.57)

EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; RM: rapid metabolizer; UM: ultrarapid metabolizer; 4-OH-Tam: 4-hydroxytamoxifen; *N*-DesM-Tam: *N*-desmethyl-tamoxifen; Endoxifen: 4-hydroxy-*N*-desmethyl-tamoxifen.
[a]For RM $n=214$, IM $n=190$, and PM $n=26$.
[b]For RM $n=159$, IM $n=205$, and PM $n=66$.
[c]For RM $n=69$, UM $n=299$, and UM $n=62$.

(276.29 ng mL/L) ($P=0.879$) genotypes, compared to the wild-type (288.43 ng mL/L) genotypes at this locus (Table 3 and Figure 4(B)). Conversely, an increase in the median plasma concentrations was recorded for Endoxifen in the heterozygous (24.73 ng mL/L) and mutant (35.40 ng mL/L) genotypes, compared to the wild-type (19.73 ng mL/L) genotypes (Table 3 and Figure 4(D)).

Figure 5 shows the associations of Tam and its derivatives with *CYP2C19*1/*1* (wild-type), *CYP2C19*1/*2* (heterozygous), and *CYP2C19*2/*2* (mutant) genotypes. Weak associations of the three types of genotypes were recorded for the median plasma concentrations of Tam and *N*-DesM-Tam (Figure 5(A) and (C)). However, an increase in the median plasma concentrations of 4-OH-Tam was observed in the heterozygous (272.65 ng mL/L) ($P<0.021$) and mutant (305.25 ng mL/L) ($P<0.004$) genotypes, compared with the concentrations in the wild-type (255.75 ng mL/L) genotypes (Table 3 and Figure 5(B)). Similarly, a stepwise increase was observed in the median plasma concentrations of Endoxifen: 19.43 ng mL/L in the wild type, 27.15 ng mL/L in the heterozygous, and 32.50 ng mL/L in the mutant genotypes (Table 3 and Figure 5(D)).

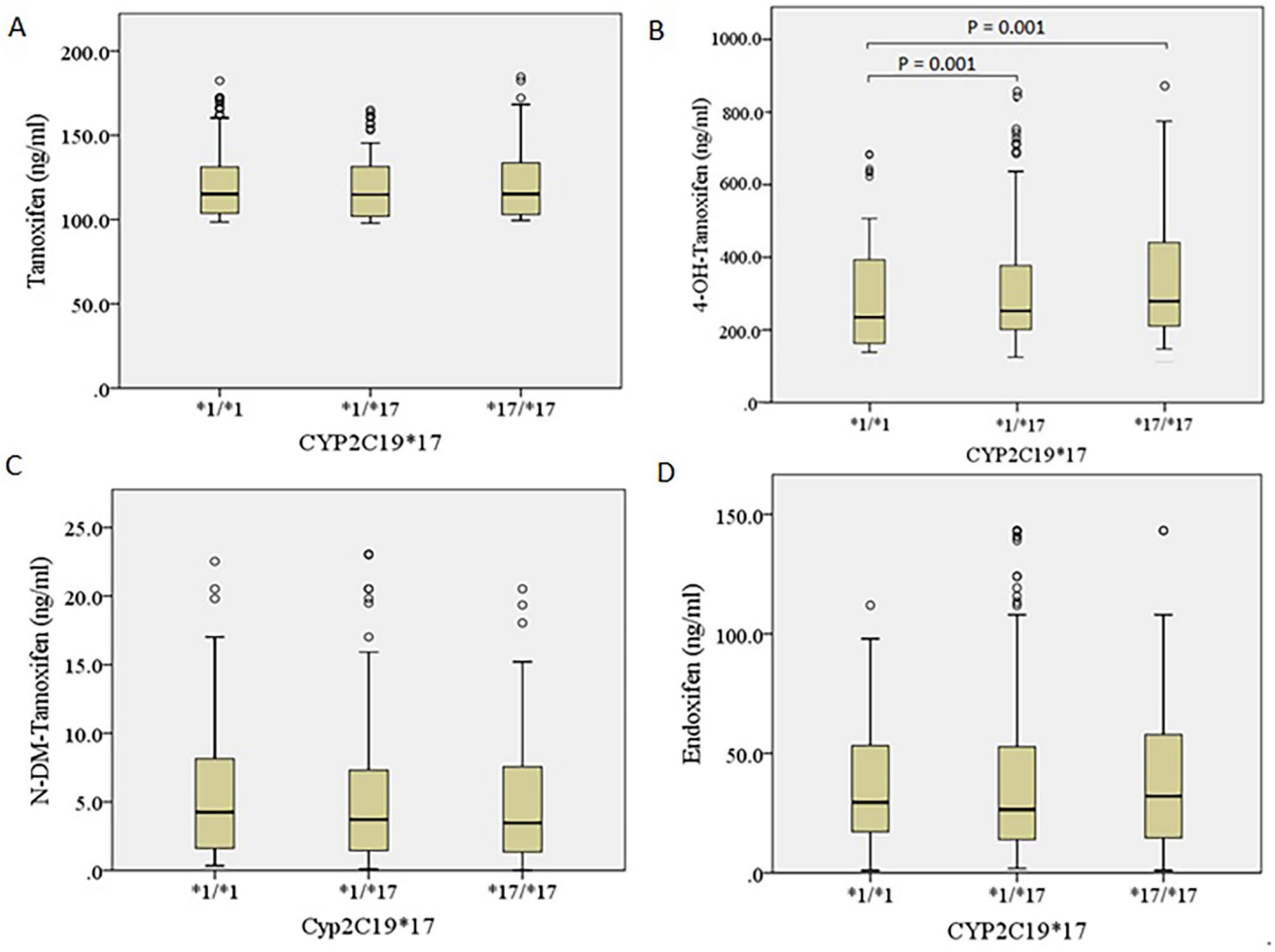

Figure 3. Association between CYP2C19*17 (*1/*1, *1/*17, and *17/*17) genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-Tam, and (D) Endoxifen.
No significant difference in the median plasma concentration of tamoxifen and Endoxifen between the three genotypes. Significantly higher quantity of 4-OH-Tam concentration in *17 genotypes as compared to the wild type ($P = 0.001$). Insignificant reduction in median plasma concentration of *N*-DesM-tamoxifen ($P = 0.362$) in *17 genotypes as compared to the wild type.

For the *CYP2C19*17* locus, the median total metabolic ratio of 4-OH-Tam (TMR-4-OH-Tam) was significantly higher in the heterozygous (187.91) ($P < 0.001$) and mutant (210.90) ($P < 0.001$) genotypes, compared to the wild-type (122.46) genotypes (Table 4 and Figure 6(A)). Similarly, TMR-*N*-DesM-tamoxifen median values also show an increasing trend: 7.68 in the wild-type, 8.90 in heterozygous, and 9.23 in the mutant genotypes (Table 4 and Figure 6(B)). Another significant association was observed between the *CYP2C19*2* polymorphism and the TMR-4-OH-Tam (Table 4 and Figure 6(C)), patients harboring the **1/*2* heterozygous ($P < 0.001$) and **2/*2* homozygous mutant genotypes ($P = 0.227$) displaying higher median TMR-4-OH-Tam. In contrast, TMR-*N*-DesM-tamoxifen were similar among the patients carrying **1/*1*, **1/*2*, and **2/*2* (Table 4 and Figure 6(D)). No significant association was observed for the median TMR-4-OH-Tam and TMR-*N*-DesM-tamoxifen in the patients bearing **1/*1*, **1/*3*, and **3/*3* genotypes (Table 4 and Figure 6(E) and (F)).

Discussion

CYP2C19 gene is involved in the metabolism of most of the proton-pump inhibitors (PPIs) and antiepileptic drugs. The metabolic pathways have not been fully described yet. Cytochrome P450 enzymes are key players in the metabolism of xenobiotic drugs. BC is the most commonly diagnosed cancer among women across the globe.[22] All women, irrespective of their ethnic or racial origin or family history, are vulnerable to BC, while males are at a reduced risk of developing BC.[23] Key factors involved in breast carcinoma progression are hormones (endogenous and exogenous) in females, genetic factors, environmental factors, etc.

We previously reported the frequency of *CYP2C19*2* and *CYP2C19*17* alleles in different indigenous groups of Pakistan and also reported that the overall ratio of expected PM (*2/*2) allele was 29.0% in contrast to UM allele (*17/*17) 23.70%.[24] A German study could not find any correlation

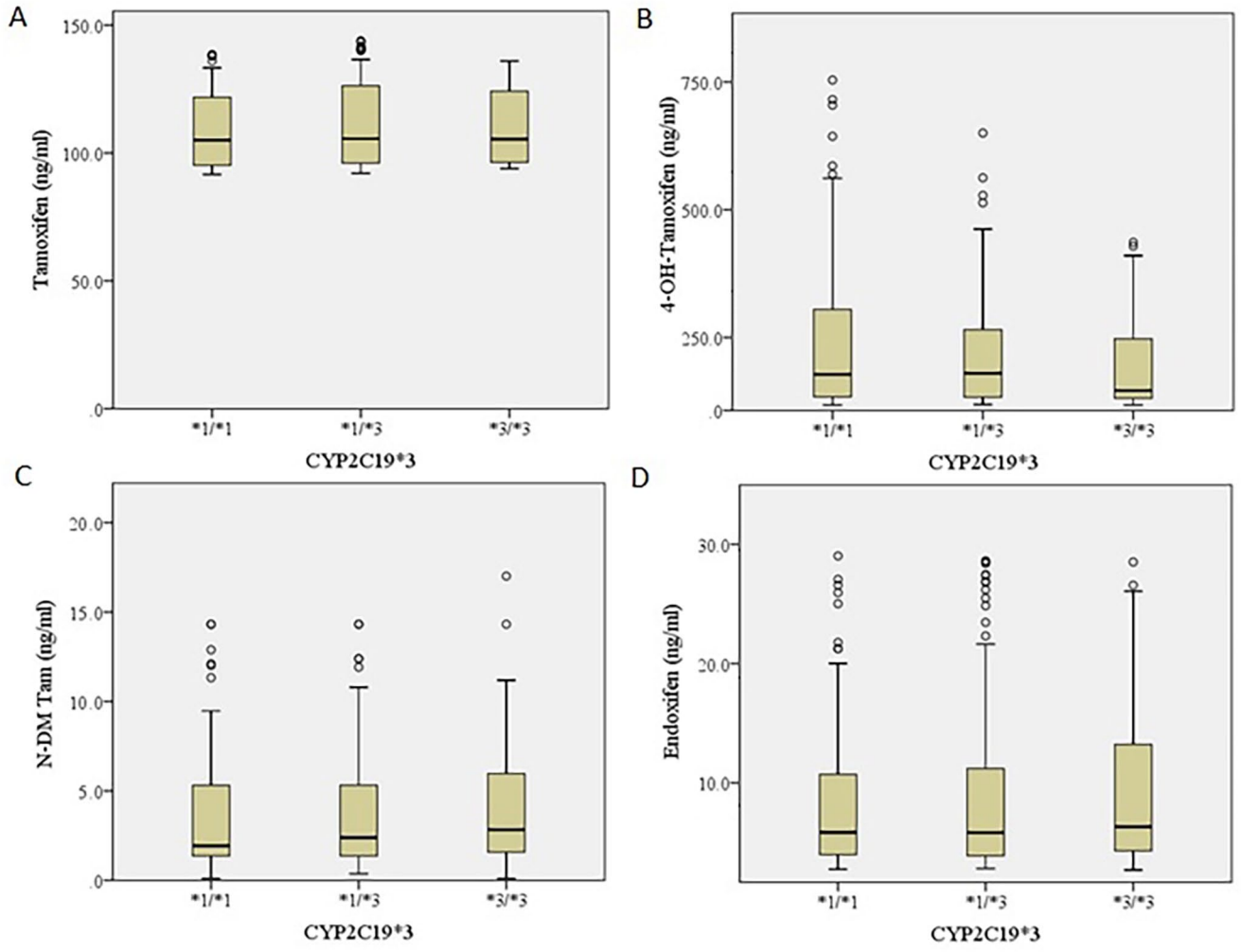

Figure 4. Association between CYP2C19*3 (*1/*1, *1/*3, and *3/*3) genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-tamoxifen, and (D) Endoxifen.
No significant change observed in median plasma concentration of tamoxifen and *N*-DesM-Tam between the three genotypes. Insignificant decrease in concentrations 4-OH-Tam in *3 genotypes as compared to wild type ($P = 0.420$), whereas slight increase in the median plasma concentration of Endoxifen in *3 genotypes compared to *1/*1.

between *CYP2C19*2* polymorphism and BC risk.[13] Iraqi population reported *CYP2C19*2* polymorphism with the proliferation of BC.[25] An Indian study elucidated the heterozygous *CYP2C19*2* as a cause of BC. *CYP2C19*2* heterozygous mutant BC patients receiving Tam have higher chances of survival.[16] Furthermore, *CYP2C19*3* allele was found to be associated with increased BC. As per Chinese studies, arachidonic acid metabolism can be influenced by *CYP2C19*'s antiapoptotic effect, subsequently causing BC.[19] Another study conducted by A.B. Sanchez-Spitman reported that *CYP2C19* has no significant impact on Tam metabolism or BC relapse.[9] *CYP2C19*17* allele has been related to a decline in BC in a German population, proposing that *CYP2C19* increases the estrogen catabolism, reducing the risk of BC.[13]

In this study, the total sample was 410 unrelated healthy individuals and 430 ER-positive BC patients. The genotype frequencies for *CYP2C19*2*, *CYP2C19*3*, and *CYP2C19*17* were calculated between the two groups and allele frequencies of the *CYP2C19* variants in control and ER-positive BC patients were expressed, as shown in Table 2.

Our findings (Table 2) demonstrate the genotyping results for unrelated healthy individuals and estrogen-positive BC patients in Pakistani population. No noteworthy difference was detected between the allele frequencies of *CYP2C19*2*, but conditional logistic regression shows that *CYP2C19*2* heterozygous condition was a risk factor for BC (OR: 0.6; 95% CI: 0.43–0.84; $P = 0.003$). This study strongly implicates the statistical contribution of *CYP2C19*3* heterozygous variant in the development of BC (OR: 0.34; 95% CI: 0.24–0.48; $P < 0.001$). Majority of the samples belonged to EM group in unrelated healthy individuals and ER-positive BC patients. Table 2 illustrates the allele frequencies of *CYP2C19* variants, but there is a significant difference between unrelated healthy individuals and ER-positive BC patients for *CYP2C19*17* allele frequency. More than 65% of unrelated healthy individuals were EMs (**1/*1*) for *CYP2C19*. In

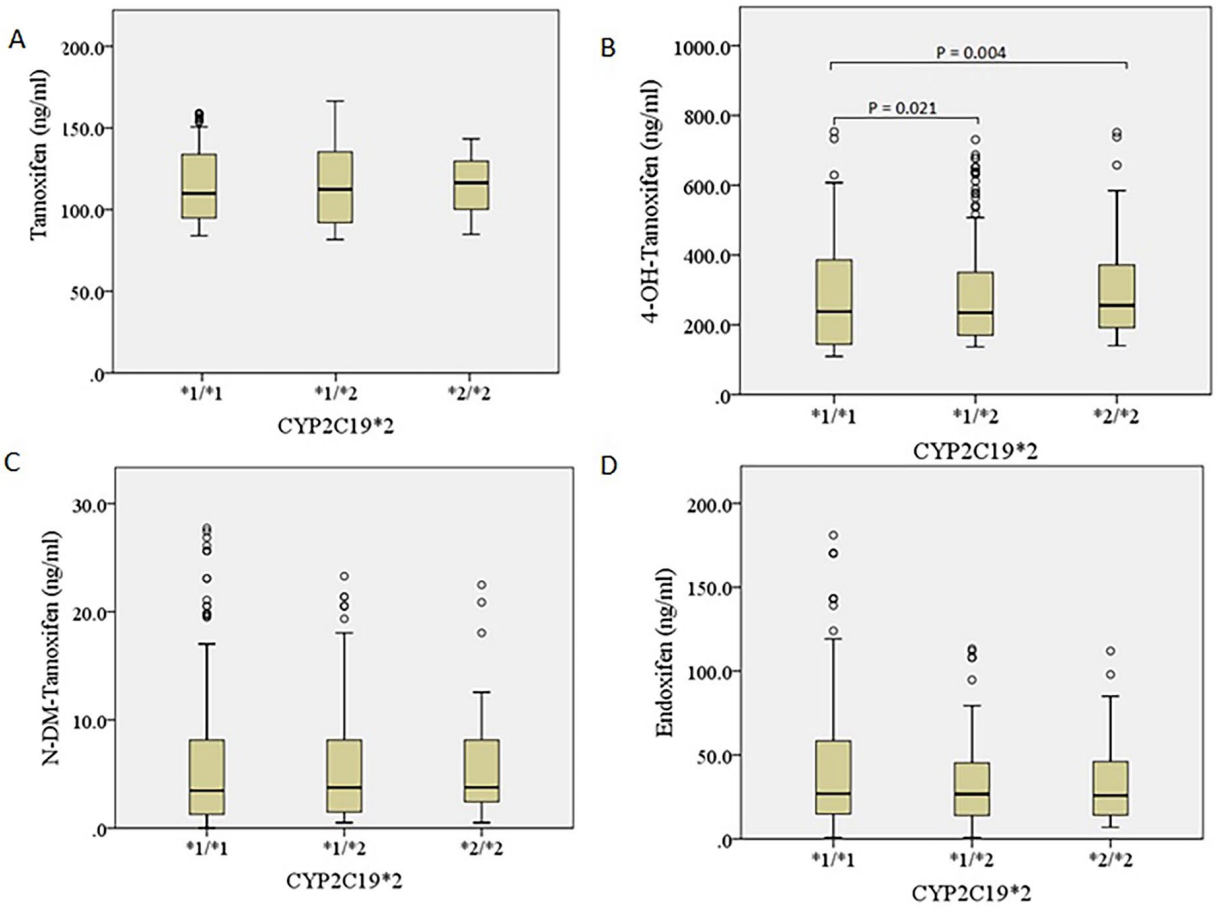

Figure 5. Association between CYP2C19*2 genotypes and steady-state median plasma concentrations of (A) tamoxifen, (B) 4-OH-Tam, (C) *N*-DesM-Tam, and (D) Endoxifen. No significant difference in tamoxifen and *N*-DesM-Tam concentration between the three genotypes, significant increase in the median plasma concentration of 4-OH-Tam ($P = 0.021$) for *1/*2 and ($P = 0.004$) for *2/*2 compared to wild type. Endoxifen concentration shows insignificant increase in *1/*2 and *2/*2 as compared to *1/*1.

Table 4. Effects of *CYP2C19* polymorphisms on median metabolic ratios of tamoxifen and its analytes.

Parameters *CYP2C19*2* (681G > A; rs4244285)	Genotype *1/*1 (EM)[a]	Genotype *1/*2 (IM)[a]	Genotype *2/*2 (PM)[a]
Plasma metabolic ratios (MRs)			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.21	0.26	0.37
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	7.87	7.46	12.31
Total metabolic ratios (TMRs)			
$TMR_{N\text{-DesM-Tam}}$	6.64	7.30	8.76
$TMR_{4\text{-OH-Tam}}$	143.78	183.79	213.7
Parameters *CYP2C19*3* (636G > A; rs4986893)	**Genotype *1/*1 (EM)[b]**	**Genotype *1/*3 (IM)[b]**	**Genotype *3/*3 (PM)[b]**
Plasma MRs			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.32	0.32	0.27
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	13.72	12.67	12.01
TMRs			
$TMR_{N\text{-DesM-Tam}}$	7.88	7.81	8.34
$TMR_{4\text{-OH-Tam}}$	93.81	82.04	87.27
Parameters *CYP2C19*17* (–806C > T; rs12248560)	**Genotypes *1/*1 (EM)[c]**	**Genotype *1/*17 (RM)[c]**	**Genotype *17/*17 (UM)[c]**
Plasma MRs			
$MR_{(N\text{-DesM-Tam})-(Tam)}$	0.28	0.28	0.26
$MR_{(Endoxifen)-(4\text{-OH-Tam})}$	0.26	0.16	0.19
TMRs			
$TMR_{N\text{-DesM-Tam}}$	6.68	8.98	9.23
$TMR_{4\text{-OH-Tam}}$	122.48	187.91	210.90

EM: extensive metabolizer; IM: intermediate metabolizer; PM: poor metabolizer; UM: ultrarapid metabolizer; 4-OH-Tam: 4-hydroxytamoxifen; *N*-DesM-Tam: *N*-desmethyl-tamoxifen; Endoxifen: 4-hydroxy-*N*-desmethyl-tamoxifen.
[a]For EM $n = 214$, IM $n = 190$, and PM $n = 26$.
[b]For EM $n = 159$, IM $n = 205$, and PM $n = 66$.
[c]For EM $n = 69$, RM $n = 299$, and UM $n = 62$.

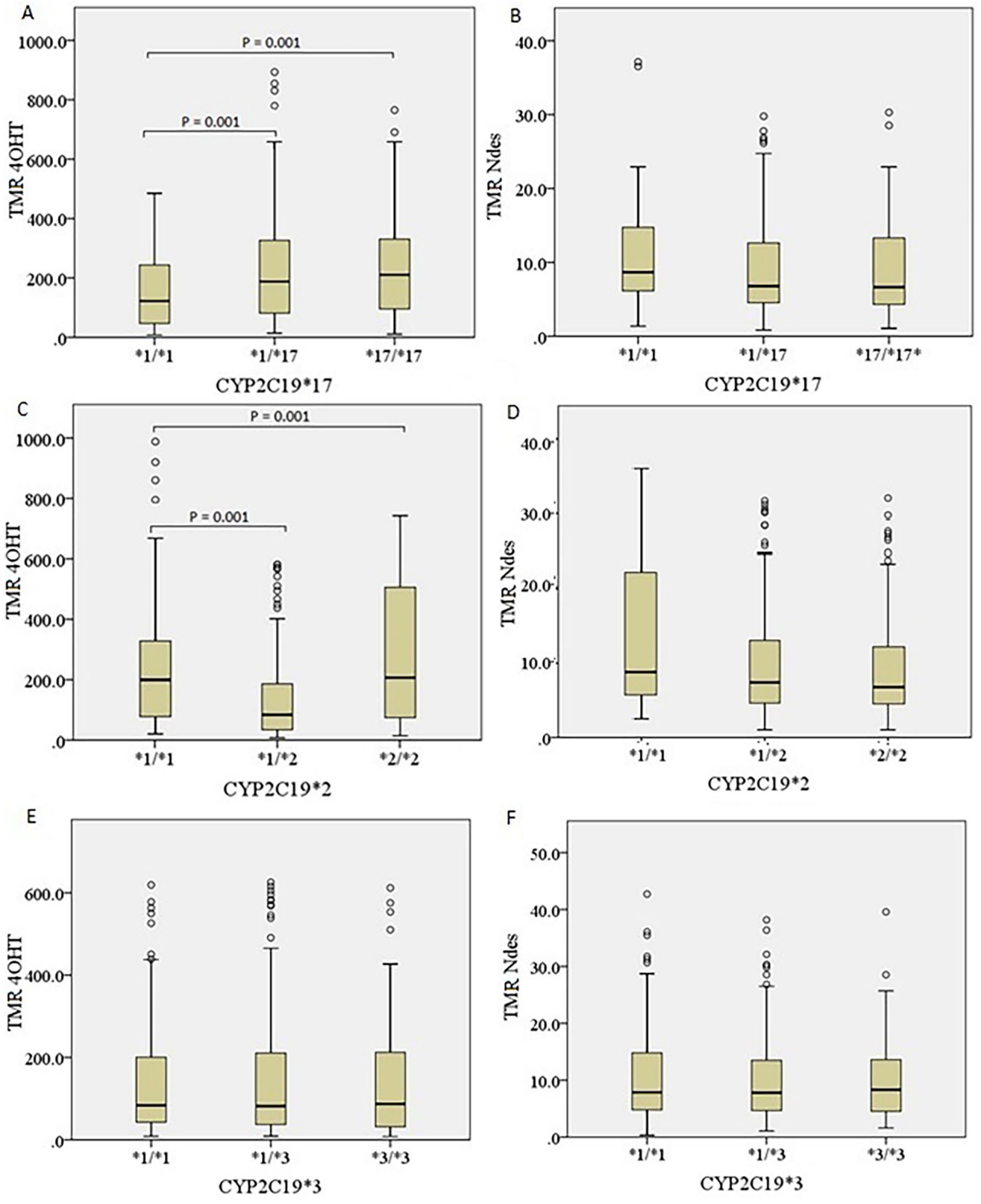

Figure 6. CYP2C19 genotypes and metabolic ratios of *N*-DesM-tamoxifen and 4-hydroxytamoxifen. (A) The median total metabolic ratio of 4-OH-tamoxifen (TMR-4-OH-Tam) was significantly higher in **1/*17* ($P < 0.001$) and **17/*17* ($P < 0.001$) genotypes than **1/*1* genotype. (B) An insignificant increase in the median total metabolic ratio of *N*-DesM-tamoxifen (TMR-*N*-Des-Tam) median values with genotype **1/*17* and **17/*17* as compared to **1/*1*. (C) Significantly higher median total metabolic ratio of 4-OH-Tam (TMR-4-OH-Tam) between the CYP2C19*2 genotypes **1/*2* ($P < 0.001$) and **2/*2* genotypes ($P = 0.227$). (D) The median TMR-*N*-DesM-Tam was similar among **1/*1, *1/*2*, and **2/*2* genotypes. (E) No significant association was observed for the median TMR-4-OH-Tam between **1/*1, *1/*3*, and **3/*3* genotypes. (F) No significant association was observed for the median TMR-*N*-DesM-Tam between **1/*1, *1/*3*, and **3/*3* genotypes.

contrast, the findings were quite different for ER-positive BC patients, revealing more than 70% of them to be UMs (**1/17**, **17/17*) (OR: 7.17; 95% CI: 5.08–10.1).

Numerous cytochrome P450 enzymes and polymorphism in the genes that produce these enzymes facilitate the multifaceted metabolism of Tam, imparting a great impact on the plasma concentration of Tam and its various metabolites. In this study, we have tried to investigate the impact of previously reported polymorphisms in genes encoding the enzymes that are accountable for Tam's metabolism and three metabolites of Tam in the Pakistani patients of BC.

In this study, the *CYP2C19*17* (−806C > T; rs12248560) allele was found to be strongly associated with higher plasma metabolic ratios of 4-OH-Tam in the plasma, while the association with *N*-DesM-Tam was not found to be significant. This suggests an accumulation of 4-OH-Tam and Tam in plasma when the metabolic alteration of 4-OH-Tam to Endoxifen is reduced. Total metabolic ratios also suggest an impaired conversation of 4-OH-Tam to Endoxifen. A significant increase has also been observed in the plasma concentration of 4-OH-Tam in patients carrying CYP2C19 **1/*2* and **2/*2 genotypes.

The plasma concentration of *N*-DesM-Tam was comparable between patients with **1/*2, *1/*17* and **2/*2, *17/*17* genotypes. There was minor escalation in the Endoxifen plasma concentration in patients with genotypes *CYP2C19*1/*1, *1/2, *2/*2, *1/*17*, and **17/*17*. A decrease was observed in the plasma concentration of 4-OH-Tam in patients with **1/*3* and **3/*3* genotypes compared with those with **1/*1* genotype.

An amplified gene expression of the *CYP2C19*17* alleles results in a putative UM phenotype.[17] *CYP2C19* is responsible for Tam metabolism to antiestrogenic metabolite 4-OH-Tam, exhibiting *in vitro* activities similar to *CYP2D6*.[26,27] Our data suggest that *CYP2C19*17* has a significant role in the plasma concentration of 4-OH-Tam. An active form of *CYP2C19*17* can cause significant benefits toward the reduction of BC recurrence, as reported earlier by Schroth and his co-workers.[28] However, our studies contradict the study of Joanne S.L. Lim *et al.*,[29] who reported no correlation between *CYP2C19* polymorphism and the pharmacokinetics of Tam.

Our findings are predominantly significant in light of the efficacy of Tam for women having hormone receptor-positive BC. In conclusion, this study indicates that *CYP2C19*17* is an essential factor that influences the plasma concentrations of Tam, its three metabolites, and metabolic ratios of Tam in a BC population in Pakistan. The influence of other *CYP2C19* polymorphic variants needs evaluation with a much larger pool.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and, analysis of the data and review of the manuscript. MUT, SR, SZ, AK, FT, FA, and MNM conducted the experiments. MUT and AS wrote the manuscript. SSM, RAA, MA, and IM contributed to the concept and design of the study and analysis and interpretation of the data.

DECLARATION OF CONFLICTING INTERESTS

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ETHICAL APPROVAL

Bio-Ethical Committee of QAU, Protocol # BEC-FBS-QAU-40.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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