Boucenna *et al.*
Egyptian Journal of Medical Human Genetics (2022) 23:122
https://doi.org/10.1186/s43042-022-00332-7

Egyptian Journal of Medical Human Genetics

RESEARCH Open Access

Influence of CYP2D6, CYP2C19 and CYP3A5 polymorphisms on plasma levels of tamoxifen metabolites in Algerian women with ER$^+$ breast cancer

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Abstract

Background: Tamoxifen, a selective estrogen receptor modulator, is indicated for breast cancer developed in response to estrogen.

Findings: In the current study we explored the relationship between the different variants of *CYP2D6*, *CYP2C19*, *CYP3A5* and plasma Endoxifen levels in Algerian patients with ER + breast cancer. We further conducted the relationship between the candidate genes and the recurrences rate. Endoxifen levels differed significantly ($p < .005$) between carriers of two functional alleles and patients genotyped as *CYP2D6*10*, *CYP2D6*17*, *CYP2D6*41* or *CYP2D6*5/*5*. Patients with elevated Endoxifen concentrations were significantly more likely to not report recurrences than patients with reduced or nul alleles. Such nul/nul, red/red, and red/nul diplotypes have been associated with a higher rate of recurrences than other genotypes during treatment.

Conclusion: Our findings suggest that the *CYP2D6* genotype should be considered in tamoxifen-treated women. While quantitatively, *CYP2D6* represents only a minor fraction of the total drug metabolizing capacity of the liver, it is polymorphic and, therefore, may alter the balance of metabolism of tamoxifen toward the activation pathways. Breast cancer patients with the *CYP2D6* nul/nul or red/nul diplotype may benefit less from Tamoxifen treatment and are more likely to develop recurrences. Comprehensive *CYP2D6* genotyping has a good predictive value for *CYP2D6* activity. Common variants in *CYP2C19* and *CYP3A5* did not have a significant impact on the recurrences in this cohort of patients with ER + breast cancer.

Keywords: Breast cancer, Tamoxifen, Endoxifen, CYP2D6, Pharmacogenetics

Introduction

Breast cancer is a multidisciplinary disease that is the leading cause of death in women globally in 2018. Breast cancer will be diagnosed in over 3 million women by 2040, according to projections [1–5]. In Algeria, the incidence rate was 12,536 new cases in 2020 [5].

Tamoxifen, a Selective Estrogen Receptor Modulator (SERM), is recommended for breast cancer patients that are classified as Estrogen Receptor Positive Breast Cancer (ER +) [6–9], as it greatly reduces the risk of recurrence up to 15 years with 12% and the mortality risk by 9% [10, 11]. It is known as one of the drugs that revealed germline pharmacogenomics (PGx) level association of interest [12–14]. A person who inherited PGx variants

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associated with decreased enzyme activity may require nonstandard dosing or may benefit from avoiding certain drugs [15]. The Clinical Pharmacogenetics Implementation Consortium (CPIC) has recently published a level of evidence for PGx drug–gene combinations' clinical actionability, where the highest levels are A and B, displaying that nonstandard doses or alternative therapies are crucial in the administration of drugs (https://cpicpgx.org/genes-drugs/) (accessed on May 1, 2022). However, levels C and D are not linked with any genetically based prescribing changes. Moreover, three degrees of extent have been published by the US Food and Drug Administration (FDA's) recently, reporting that degree 1 is most clinically actionable (https://www.fda.gov/medicaldevices/precision-medicine/tablepharmacogenetic-associations) (accessed on May 1, 2022). More than 87 drugs metabolized involve *CYP2C9, CYP2C19,* and/or *CYP2D6*. Their significance is the consequence of the high genetic variations that impact drug metabolism. The overall prevalence of inter-variability of *CYP* genes leads to 5 different categories of metabolic phenotype, ranging from no enzyme activity to increased enzyme activity: Poor Metabolizer (PM), Intermediate Metabolizer (IM), Normal Metabolizer (NM), Rapid Metabolizer (RM), and Ultra Rapid Metabolizer (URM) [16, 17].

Tamoxifen metabolism occurs via two pathways: 4-hydroxylation and N-demethylation (Fig. 1). The 4-hydroxylation pathway leads to the production of 4-hydroxytamoxifen, which is approximately 30–100 fold more potent in the suppression of tumor cells than tamoxifen itself [16]. It is catalyzed by multiple *CYPs,* including *CYP2D6.* However, this pathway accounts for about 7% of tamoxifen metabolism. On the other hand, the N-demethylation pathway leads to the formation of N-desmethyltamoxifen, considered the most potent metabolite since its concentration in plasma is 6 to 12 times higher if compared with 4-hydroxytamoxifen. Furthermore, it has the lowest IC50 (inhibitor concentration) at the ERs (estrogen receptor) [18]. It is essentially catalyzed by *CYP3A4* and *CYP3A5*; and contributes for nearly 92% of tamoxifen's metabolism [16].

CYP2D6/tamoxifen has been rated as the most important gene/drug-type pair as surveyed by the American Society for Clinical Pharmacology and Therapeutics members in 2010 [19]. Currently, approximately 150 single nucleotide polymorphisms (SNPs) and 100 allelic variants are described [13], resulting in increased to nonfunctional alleles. *The most important intermediate metabolizer (IM) alleles are CYP2D6*10 (100C > T), CYP2D6*17 (1022C > A), (1022C > T), CYP2D6*35 (31G > A), CYP2D6*39 (4181G > C), and CYP2D6*41 (1662G >); (2851C > T); (2989G > A); (4181G > C).* The allele *CYP2D6*5*, consisting of a gene deletion, is representative of a missing enzymatic

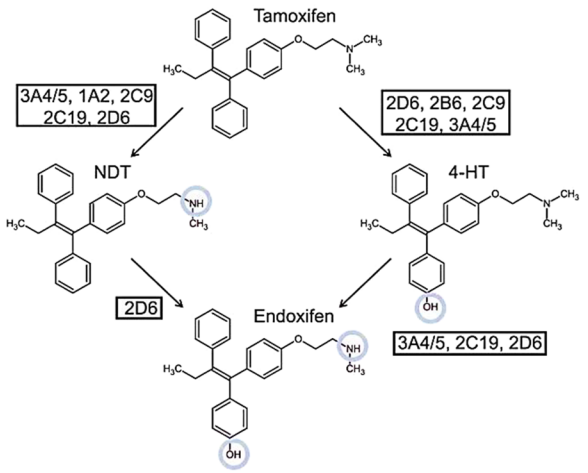

Fig. 1 Tamoxifen metabolism pathway in humans [16]. *CYP 2D6* is the major isoform responsible for the formation of 4-hydroxytamoxifen and *CYP3A4/5* is the major isoform responsible for the formation of N-desmethyltamoxifen, whereas the generation of Endoxifen is predominantly catalyzed by *CYP2D6*. Other *CYP* isoforms, including *CYP2C19*, *CYP2C9*, and *CYP2B6*, appear to play less important roles in Tamoxifen metabolism in vitro at therapeutically relevant concentrations

activity [20]. Following the Activity Scores (AS) results, in which NM are assigned a value of 2, with decreased activity alleles ranging from 0.25 to 1.5, no enzymatic activity assigned a score of 0.0 [16], in 2019, it was internationally agreed to harmonize the *CYP2D6*1/*4* interpretation from an extensive/normal metabolizer phenotype (CPIC definition until 2017, mostly used in the US) to an intermediate metabolizer phenotype (the definition used by the Dutch Pharmacogenetics Working Group (DPWG), mostly used in Europe). The second important change is concerning the *CYP2D6*10* allele, which was downgraded from AS = 0.5, comparable to other decreased activity alleles such as **9* and **41*, to AS = 0.25 [18, 21].

Among the other CYP enzymes involved in the biochemical pathway, the influence of CYP2C19 activity on the disposition of tamoxifen and its metabolites has generated considerable interest [22–25]. Cytochrome P450 2C19 (*CYP2C19*) is located within a cluster of cytochrome P450 genes on chromosome 10 (10q24.1-q24.3) and encodes a 490-amino-acid protein. CYP2C19 is involved in metabolizing several important therapeutic drugs. Common variants of the *CYP2C19* gene are associated with impaired drug metabolism. Therefore, *CYP2C19*2 (681G > A)* and *CYP2C19*3 (636G > A)* are the most common alleles, encoding enzymes with decreased activity. However, *CYP2C19*17 alleles (806C > T) and (340C > T)* result in increased gene transcription and high enzyme activity [16].

A comprehensive kinetic characterization of tamoxifen sequential metabolism in vitro demonstrated that CYP3A is the major CYP isoform responsible for the formation of N-desmethyltamoxifen [26]. *Cytochrome* P450 family 3 subfamily A member 5 is localized on chromosome 7: q22.q22.1. The protein expression is largely attributed to four alleles: *CYP3A5*1, CYP3A5*3 (6981A > G), CYP3A5*6 (624G > A)* and *CYP3A5*7 (27126_27127insT),* of which only *CYP3A5*1* is associated with CYP3A5 expression; the other three are non-expressed [16].

Although given the lack of clinical trials where patients are receiving Tamoxifen as a part of their adjuvant therapy for hormone receptor-positive breast cancer, according to Algerians population the current data is the first reference to serve for future large-scale PGx studies that improve prescribing decisions before the administration of Tamoxifen, resulting in reducing drug-related adverse events and achieving optimal treatment response. Patients with ER + breast cancer are analyzed for *CYP2D6, CYP2C19,* and *CYP3A5* to test the association with plasma concentrations of tamoxifen and its principal metabolites. Likewise, here we report preliminary data addressing associations of disease recurrence with *CYP2D6, CYP2C19, CYP3A5* genotypes and Endoxifen plasma concentrations in Algerian subjects we have studied in the trial.

Methodology

Study design and patients recruitment

A total of 97 Algerian females with ER^+ Breast Cancer (mean age 44, 65 ± 6, 38) were included in the present study between February 2014 and December 2017. All of these patients underwent mastectomy and lumpectomy at the University Hospital Center of Constantine department of Oncology and Radiotherapy and were treated with Tamoxifen (20 mg per day), were recruited between February 2014 and December 2017. Tamoxifen was considered for an average of 30 months (range: 12–77 months), with a median follow-up of 46 months. Our study has been approved by the local Ethics Committee. The use of human blood samples and protocols in this research strictly adheres to the principles expressed in the Declaration of Helsinki, and informed consent was obtained from all participants or from their family members. The age of the patients ranged between 30 and 60 years old**.** Patients treated concomitantly with drugs that could act as *CYP2D6* inhibitors were excluded. All patients complete a full course of chemotherapy either during primary surgical treatment or as an adjunct (adjuvant). The clinical characteristics of ER + breast cancer patients are displayed in Table 1.

Table 1 Relevant Clinico-pathological features of the assessed ER + Breast cancer patients ($n = 97$)

Characteristic	Value
Age at breast cancer diagnosis (y), median (range)	44.65 ± 6.38
Age at menarche	12.80 ± 1.82
Family status	
Single	17 (17.5%)
Married	80 (82.5%)
Surgery	
Breast conserving, n (%)	87 (89.7%)
Mastectomy, n (%)	10 (10.3%)
Chemotherapy, n (%)	
Adjuvant	77 (79.3%)
Neo adjuvant	20 (20.7%)
Tumor size	
≤ 2 cm	29 (29.9%)
2 < size ≤ 5 cm	58 (59.8%)
> 5 cm	10 (10.3%)
Grade	
I	8 (8.3%)
II	62 (63.9%)
III	27 (27.8%)
Node status	
pN + , n (%)	90 (92.78%)
pN0, n (%)	7 (7.22%)
HER2 status	
HER2 + , n (%)	31 (32%)
HER2 − , n (%)	66 (68%)
Histologic type of tumor	
Ductal	2.8%
Lobular	6.9%
Other types	90.3%
Distant metastatic site	
No recurrence	80 (82.5%)
Locally	11 (11.3%)
Metastatic	6 (6.2%)

Tumor size extracted from pathological report, or for neoadjuvant treated patients, the largest size recorded including clinical measurement. Tumors considered ER^+ if ≥ 10% of the cells stained positive for the receptor by immunohistochemistry, Grade classified according to the Nottingham histologic grade N^+ = regional lymph node metastasis; N0 = no regional lymph node metastasis. RLR relapse loco-region

Sample collection and preparation

Blood samples of each participant were collected into a tube containing ethylenediamine-tetra acetic acid (EDTA). Genomic DNA was extracted from 6-8 mL of peripheral venous blood using the salting out method according to the protocol suggested by Miller and co-workers [27]. Samples had been suspended in 15 ml polypropelen centrifugation tubes with 3 ml of nucleic lysis buffer (10 mM Tris-HClt, 400 mM NaCl, and 2 mM Na^2

EDTA, pH 8.2). The cell lysates were digested overnight at 37 °C with 0.2 ml of 10Z SDS and 0.5 ml of a protease K solution (1 mg protease K in 1Z SDS and 2 mM Na^2 EDTA). After digestion, 1 ml of saturated NaCl (approximately 6 M) was added to each tube and shaken vigorously for 15 s, followed by centrifugation at 2500 rpm for 15 min. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 ml polypropylene tube. Two volumes of room temperature absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The obtained DNA strands were transferred to a 1.5 ml microcentrifuge tube containing 100–200pi TE buffer (10 mM Tris–HCl, 0.2 mM Na2EDTA, pH 7.5). The DNA was allowed to dissolve for 2 h at 37 °C before being quantified. The concentration and purity of DNA samples were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

SNP selection and genotype

Because the majority of the subjects enrolled in this study were white and given the lack of data on the *CYPs* in our population, we analyzed the alleles that are common in this population, taking into consideration their positive correlation with plasma concentrations of Tamoxifen and its metabolites in hormone receptor–positive women who were taking Tamoxifen as adjuvant treatment for newly diagnosed breast cancer. SNPs and *indels* were analyzed for the *CYP2D6, CYP2C19,* and *CYP3A5* genes. We screened the following alleles for the *CYP2D6* gene: rs35742686 (*3), rs1065852 (*4), rs3892097 (*5), rs5030655 (*6), rs5030656 (*9), rs1065852 (*10), rs28371706 (*17), rs59421388 (*29), rs769258, rs1080985, rs16947, rs1135840 (*35), and rs28371725 (*41) alleles. Variation in the number of copies (CNV) for this gene was also analyzed. According to CNVs for *CYP2D6*, three different regions were interrogated: intron 2, intron 6, and exon 9, together with an internal 2-copy control (RNAse P). Reference (*1), rs10264272 (*6), rs41303343 (*7), and rs776746 (*3) variant alleles for CYP3A5; and reference (*1), rs4244285 (*2), rs4986893 (*3), and rs12248560 (*17) alleles for *CYP2C19*. Single Nucleotide Variants (SNVs) were chosen because they are representative of crucial haplotypes associated with altered enzyme activity. The genotyping of SNV was performed by allelic discrimination using TaqMan OpenArray Genotyping with a customized panel on the Quant Studio TM 12 K Flex Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, USA) according to the protocol recommended. To ensure the genotyping results were analyzed in the Thermofisher Cloud application. Samples were clustered according to alleles identified with a threshold of 95% confidence. Copy number variation for *CYP2D6* was analyzed by using TaqMan commercial probes according to the TaqMan Copy Number assay protocol recommended by Applied Biosystems. The data was analyzed using CopyCaller® software v.2 and a two-copy as a positive control. The predicted copy number was assessed for the three probes, and the average and standard deviation were also calculated. In order to transform the SNP and CNV results into a concrete genotype, Allele TyperTM Software was employed with predesigned tables for every gene. Information about the different alleles of different genes was selected from the PharmGKB website [17]. All genotyped analysis were carried out in collaboration with the pharmacogenetics laboratory Research Center in Molecular Medicine and Chronic Disease Cimus (Santiago, Spain).

HPLC measurement

We used the method described in the article of Bobin et al. [28] to separate and quantify Tamoxifen and its metabolites in plasma. Blood samples were collected in heparin tube, centrifuged at 2500 g for 10 min, and the separated plasma was stored at −80 °C until analysis. Tamoxifen and its major metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen, and Endoxifen were quantified in Molecular Medicine and Chronic Diseases center (Cimus), Santiago de Compostela, Spain, Department of Pharmacology, by ultra-high-performance liquid chromatography (UHPLC) followed by electrospray tandem mass spectrometry (LC–MS/MS). Briefly standard solutions of analysis and internal standards were prepared at 1 mg/ml of Z-isomer, in methanol. To solubilize the analyst, the stock solution was diluted from 10 to 5000 ng/ml for Tamoxifen and N-desmethyltamoxifen, from 2 to 1000 ng/ml for Endoxifen and from 1 to 500 ng/ml for 4-hydroxytamoxifen in water/Methanol (30/70) mixed with formic acid 0.1% in order to solubilize the analyst. The diluted solution were diluted in blank plasma to generate the calibrate solutions from 1.5, 20, 100, 250 and 500 ng/ml for both Tamoxifen and N-desmethyltamoxifen; from 0.2, 1, 4, 20, 50 and 100 ng/ml and from 0.1, 0.5, 2, 10, 25 and 50 ng/ml for Endoxifen and 4-hydroxytamoxifen. Internal standard solutions were diluted in Acetonotril: formic acide 0.1%, for final concentration 5 and 20 ng/ml for Endoxifen, 4-hydroxytamoxifen, for Tamoxifen and N-desmethyltamoxifen, respectively. For the Quality Control (QC) samples, stock solution was prepared to produce the following concentrations in plasma: 1, 2.5, 40 and 400 ng/ml; 0.2, 0.5, 8 and 80 ng/ml and 0.1, 0.25, 4 and 40 ng/ml, for both Tamoxifen, N-desmethyltamoxifen, Endoxifen and 4-hydroxytamoxifen, respectively. All stock solutions stored at −80 °C until analysis, a total of 100 µl of water: formic acid 100:1 (v:v) was added to 100 µl of plasma samples in 1.5 ml

micro centrifuge tubes, and vigorously vortexed during 30 s in order to remove protein interaction with plasma. Methanol (100 µl) was added and the aliquots were transversely agitated during 10 min at room temperature. The samples (300 µl) were again vortexed after the addition of 400 µl of internal standard solution and then centrifuged at 18000 g for 10 min at 4C°. Finally, 300 µl of supernatant was mixed with 300 µl of water: formic acid (100:0.2, v: v) ammonium format 2 mM directly in the vials.

Statistical analysis

Tamoxifen and its major metabolite concentrations were described as median with an interquartile range (IQR). ANOVA test was used to determine the relationship between Tamoxifen and its metabolites and candidate genes. The Pearson correlation analysis was performed to assess the magnitude of the association between Tamoxifen and its active metabolites. The Kruskal–Wallis test was used to compare differences in Endoxifen levels between homozygous for *CYP2D6* carries of nonfunctional allele and patients homozygous for *CYP2D6* carries of reduced functional allele *red/red* or *CYP2D6 red/null nul* genotypes. Finally, the Fisher's exact test was used to determine the proportion of patients who developed recurrence disease. All the data were analyzed by IBM SPSS Statistics 22 for Windows.

Results

Patient characteristics

Table 2 shows the allele frequency distribution of the Tamoxifen metabolizing enzyme and 15 different polymorphisms in the genes encoding CYP2D6, CYP2C19, and CYP3A5. Complete genotypes for *CYP2D6* and *CYP3A5* were obtained for 97 samples. For *CYP2C19, 5* samples were discarded from the analysis because of the impossibility of obtaining genotypes for the different haplotypes analyzed, mainly for two reasons: low Nanodrop concentration and/or low purity, since they interfered in the final results. All genotype frequencies were in Hardy–Weinberg Equilibrium ($p > 0.05$). A total of 33.03% of patients had 3 or more *CYP2D6* CNVs. The allele frequencies of *CYP2D6*5* (gene deletion), **1* and **2* were 1.03%, 14.40%, and 33.03%, respectively. Following CPIC phenotype assignment, 49.49% and 33.03% of the patients are predicted to be NM (*CYP2D6*1, CYP2D6*2, CYP2D9*35,* and *CYP2D6*39*) and URM (*CYP2D6*1, CYP2D6*2* > 2 copies), respectively. PM *(CYP2D6*5)* and IM *(CYP2D6*10, CYP2D6*17*, and *CYP2D9*41)* were responsible for 2.06% and 8.25% of the cases, respectively. For *CYP2C19,* phenotype frequencies were 1.03% and 93.40% for the *CYP2C19*17 and CYP2C19*1 alleles,* respectively, and 5.52% for the *CYP2C19*2* allele. However, for *CYP3A5* frequencies and distribution in the population, the most frequent allele was *CYP3A5**3, followed by *CYP3A5**1 and *CYP3A5**6, consisting of 76.29%, 22.68%, and 1.03%, respectively.

Table 2 Alleles frequency distribution of Tamoxifen metabolizing enzyme

CYP2D6	Frequency	Value (%)
*CYP2D6*1, CYP2D6*2* > 2 copies	32	33.03
*CYP2D6*1*	14	14.40
*CYP2D6*2*	32	33.03
*CYP2D6*5*	1	1.03
*CYP2D6*10*	2	2.06
*CYP2D6*17*	6	6.18
*CYP2D6*35*	1	1.03
*CYP2D6*39*	1	1.03
*CYP2D6*41*	8	8.25
CYP2C192C19 CYP2C19		Value
*CYP2C19*1*	86	93.40
*CYP2C19*2*	5	5.52
*CYP2C19*17*	1	1.08
CYP3A53A5 CYP3A5		Value
*CYP3A5*1*	22	22.68
*CYP3A5*3*	74	76.29
*CYP3A5*6*	1	1.03

Clinical characteristics of patients in association with different CYPs

Significant differences were shown in clinical characteristics between different *CYP2D6* phenotype groups in grade, tumor size ($p < 0.05$), and recurrence ($p < 0.05$) (Table 3). However, there were no statistically significant differences in demographic characteristics between different *CYP2C19 and CYP3A5* phenotype groups or tumor size grade and recurrence (Table 4).

Association between plasma endoxifen concentration and *CYP2D6, CYP2C19, CYP3A5* phenotypes

According to HPLC results, the limit of detection (LOD) was 0.1, 0.2, and 0.5 ng/ml for 4-hydroxytamoxifen, Endoxifen, Tamoxifen, and N-desmethyltamoxifen. (Table 5) demonstrates the plasma concentrations of Tamoxifen and its metabolites. Endoxifen has a higher median (34.4 ng/mL) compared to 4-hydroxytamoxifen (4.6 ng/mL). Significant variations in Endoxifen concentrations between individuals (coefficient of variation: 145.79%). A Pearson correlation analysis between Tamoxifen and its metabolites revealed a strong positive association between Tamoxifen and the formation of N-desmethyltamoxifen and between Tamoxifen and 4-hydroxytamoxifen ($R = 0.97$) (Fig. 2). Thus, an increased concentration of Tamoxifen is associated with

Table 3 Clinical characteristics of patients in association with *CYP2D6*

Characteristics	CYP2D6 Phenotype			
N (97)	**NM (48)**	**URM (32)**	**IM (16)**	**PM (1)**
Tumor seize				
≤ 2 cm	20 (20.61%)	3 (3.09%)	9 (8.57%)	0 (00%)
2 < size ≤ 5 cm	25 (25.77%)	25 (25.77%)	7 (6.66%)	0 (00%)
> 5 cm	3 (3.09%)	4 (4.23%)	0 (00%)	1 (1.03%)
p-value	Ref	0.02	0.02	0.04
Grade				
I	5 (4.76%)	1 (1.03%)	2 (2.06%)	0 (00%)
II	26 (26.80%)	30 (30.92%)	6 (6.18%)	0 (00%)
III	17 (17.52%)	1 (1.03%)	8 (7.61%)	1 (1.03%)
p-value	Ref	0.02	0.02	0.03
Recurrence				
No recurrence	43 (44.32%)	31 (31.95%)	6 (6.18%)	0 (00%)
Locally	3 (2.85%)	0 (00%)	7 (6.66%)	1 (1.03%)
Metastatic	2 (1.90%)	1 (1.03%)	3 (2.85%)	0 (0%)
p-value	Ref	0.00	0.00	0.00

CYP. _Cytochrome P450; URM _ ultra rapid metabolizer (CYP2D6); NM _ normal metabolizer (CYP2D6); IM _ intermediate metabolizer (CYP2D6) ; PM _ poor metabolizer (CYP2D6); Ref_reference gene

Table 4 Clinical characteristics of patients in association with *CYP2C19*

Characteristics	*CYP2C19* Phenotype				
N (92)	**NM (49)**	**URM (1)**	**RM (30)**	**IM (10)**	**PM (2)**
Tumor seize					
≤ 2 cm	17 (17.52%)	0 (00%)	10 (10.30%)	2 (2.06%)	1 (1.03%)
2 < size ≤ 5 cm	26 (26.80%)	1 (1.03%)	17 (17.52%)	7 (7.21%)	1 (1.03%)
> 5 cm	6 (6.18%)	0 (00%)	3 (3.09%)	1 (1.03%)	0 (00%)
p-value	Ref	0.68	0.90	0.25	0.82
Grade					
I	0 (00%)	0 (00%)	6 (6.18%)	0 (00%)	0 (00%)
II	36 (37.11%)	0 (00%)	18 (18.55%)	7 (7.21%)	1 (1.03%)
III	13 (13.40%)	1 (1.03%)	6 (6.18%)	3 (3.09%)	1 (1.03%)
p-value	Ref	0.15	0.87	0.44	0.35
Recurrence					
No recurrence	46 (47.42%)	0 (00%)	24 (24.74%)	7 (7.21%)	2 (2.06%)
Locally	2 (2.06%)	1 (1.03%)	3 (3.09%)	2 (2.06%)	0 (00%)
Metastatic	1 (1.03%)	0 (00%)	3 (3.09%)	1 (1.03%)	0 (00%)
p-value	Ref	0.27	0.22	0.17	0.46

*CYP*_ Cytochrome P450; URM _ ultrarapid metabolizer (*CYP2C19*); RM _ rapid metabolizer allele (*CYP2C19*); NM _ normal metabolizer (*CYP2C19*); IM intermediate metabolizer(*CYP2C19*); PM poor metabolizer (*CYP2C19*); Ref_ reference gene

Table 5 Plasma concentrations of Tamoxifen and its metabolites

Plasma concentration	Values	CV (%)
Tamoxifen (median + IQR)	226.98 (108.8, 273.9)	139.24
N-Desmethyl Tamoxifen (median + IQR)	597.44 (282.5, 658.6)	138.61
4-HydroxyTamoxifen (median + IQR)	9.16 (4.6, 11.3)	151.66
Endoxifen (median + IQR)	70.00 (34.4, 81.9)	145.79

CV coefficient of variation; IQR interquartile range

a corresponding increase in N-desmethyltamoxifen or 4-hydroxytamoxifen levels, as well as Tamoxifen or Endoxifen ($R = 0.96$).

The relationship between major groups of *CYP2D6* diplotypes and plasma Endoxifen levels is predicted in Fig. 3. Here, it is evident that subjects carrying the *CYP2D6*1* allele showed plasma Endoxifen levels higher (Table 6) than the suggested threshold by 12 times [29]. However, 1.03% of all patients in the study had low Endoxifen levels under the proposed threshold. Patients with two null alleles (CYP2D6*5/*5) had lower Endoxifen levels than those with URM and NM phenotypes, as did those with two reduced functional alleles *(CYP2D6*10/*10, CYP2D6*17/*17, CYP2D6*4/*10, CYP2D6*4/*41*, and *CYP2D6*5/*41).* Endoxifen levels differed statistically significantly ($p < 0.05$) between carriers of the *CYP2D6*1* allele (median 69.13 ng/mL) and patients genotyped as *CYP2D6*10, CYP2D6*17, CYP2D6*41,* or *CYP2D6*5/*5* (median 29.40 ng/mL and 4.40 ng/mL, respectively). For *CYP3A5* and *CYP2C19* phenotypes, lower plasma concentration in patients categorized as PM, but the results didn't reach significant differences (Fig. 4).

Patients' recurrence rate in relation to endoxifen plasma levels

Data on patients who experienced recurrences was obtained retrospectively from medical records. In our cohort, 8 patients were in stage I, 62 were in stage II, and 27 were in stage III. There were 90 nodes that were positive. 17 of the population had disease relapse. One patient was nul/nul *(CYP2D6*5/*5)*, 16 patients were red/red or red/nul, and 8 of 16 were *CYP2D6*41/*41*. For these analyses, we use NM carriers of the functional allele *CYP2D6*1* as a reference group. We realized that the combination genotype red/nul with nul/nul was more strongly associated with disease recurrence than NM carries the *CYP2D6*1* allele ($p < 0.05$).

Discussion

Tamoxifen is widely used in the treatment of all stages of ER^+ breast cancer. *CYP2D6* is a key enzyme involved in the metabolism of tamoxifen into its relevant metabolites

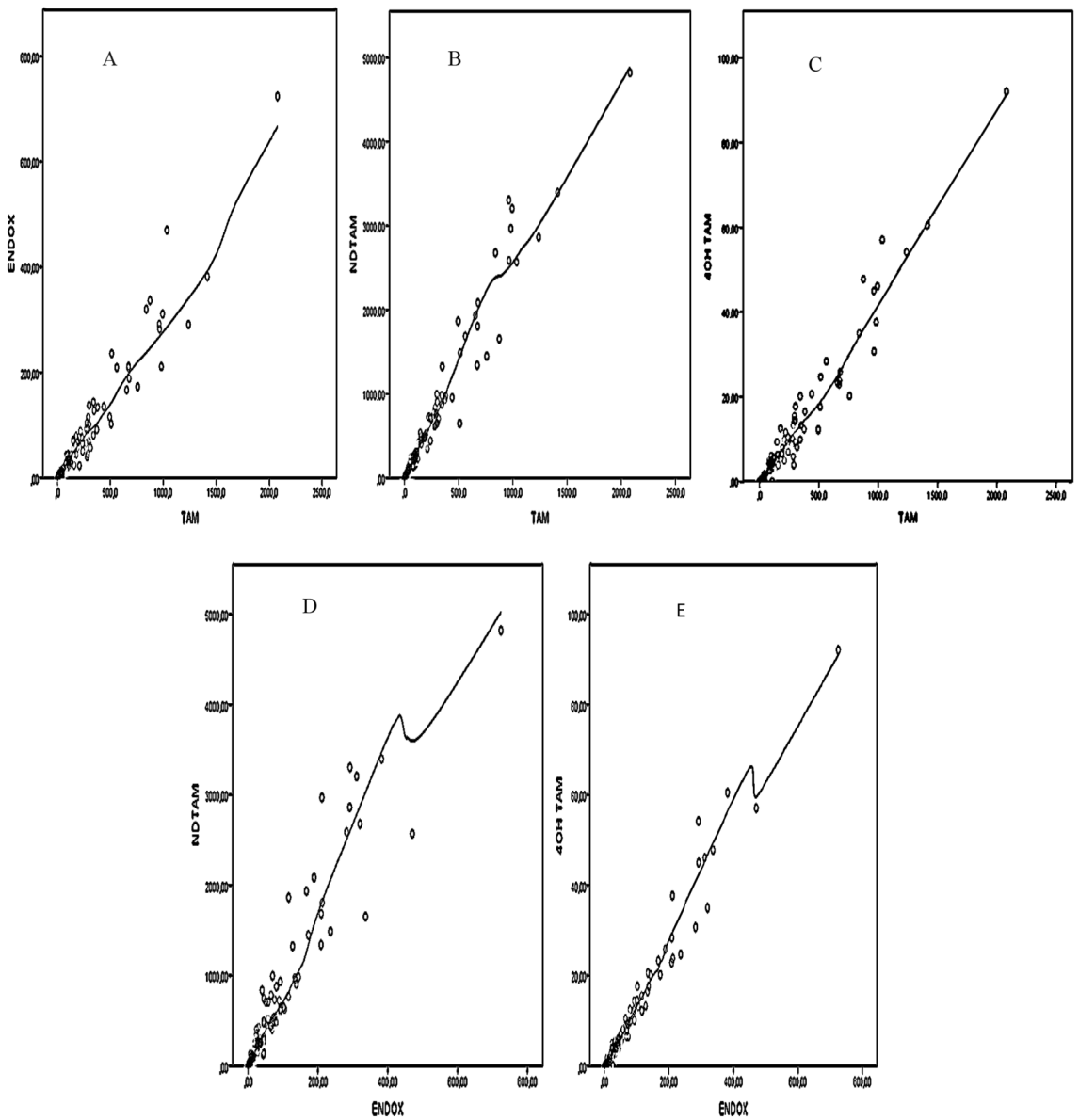

Fig. 2 Scatter plot showing the Pearson's correlation between Endoxifen versus tamoxifen (**A**), N-desmethyltamoxifen versus Tamoxifen (**B**) and 4-hydroxytamoxifen versus Tamoxifen (**C**), Endoxifen versus N-desmethyltamoxifen (**D**) and Endoxifen versus 4-hydroxytamoxifen (**E**)

[30]. In the current study, we explored the relationship between the different variants of *CYP2D6*, *CYP2C19,* and *CYP3A5* and ER positive breast cancer patients. To our knowledge, our study is the first in Algeria looking at the impact of pharmacogenetics of *CYP450* in patients with ER^+ breast cancer.

It is well known that variation in *CYP2D6* is higher in different populations and individuals in the same population. Hence, the prevalence of PM in our population is 1.03%, with a predominance of *CYP2D6*5*, which is comparable to Sistonen J et al. and Fuselli S et al., who found a prevalence of 0.98% in Syrians and 3.3% in Algerians [31, 32]. The functional *CYP2D6*2* allele had the highest frequency of 33.03%, which is similar to a study by Alali, M et al. that found a frequency of 28.3% [33]. Moreover, our population has a high frequency distribution of duplication and multiplication of the functional *CYP2D6*2xN* allele being the most prevalent with a frequency of 33.03% URM, which is in agreement with the predictions of Alali, M et al., who noticed that the frequency of duplications ranged between 7.6% and 31.3% among Arabs [33–35]. This inter-variability in different

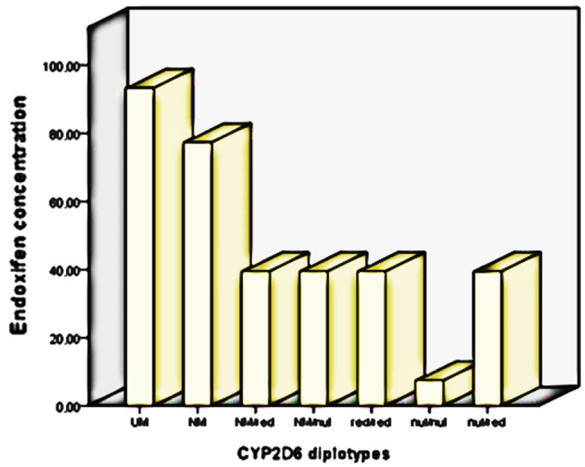

Fig. 3 Plasma Endoxifen concentrations in women with estrogen receptor-positive breast cancer in relation to major groups of *CYP2D6* diplotypes with regards to the combination of fully functional NM, reduced activity (red) and nul allele

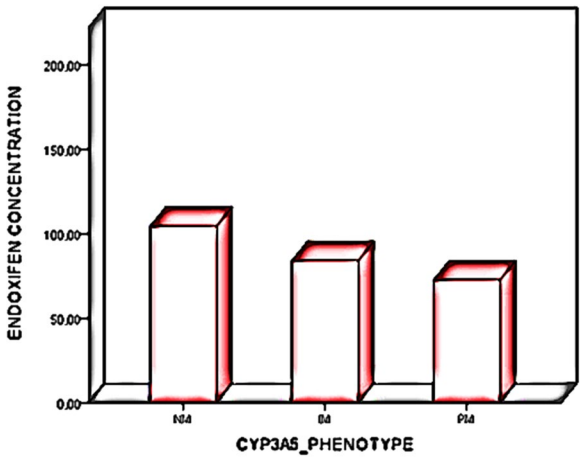

Fig. 4 Plasma Endoxifen concentrations in women with estrogen receptor-positive breast cancer in relation to major groups of *CYP3A5* phenotype

Table 6 Plasma concentrations of Endoxifen in subjects with *CYP2D6*, *CYP2C19* and *CYP3A5* genotype

CYP2D6 **CPIC phenotype**	**(*N* = 97)**	**Endoxifen (ng/mL) median**
URM	32	94.19
NM	48	69.13
IM	16	29.40
PM	1	4.40
CYP3A5 CPIC phenotype	(*N* = 97)	Endoxifen (ng/mL) median
NM	3	114.31
IM	24	90.92
PM	70	70.88
CYP2C19 CPIC phenotype	(*N* = 97)	Endoxifen (ng/mL) median
URM	1	88.70
RM	30	60.53
NM	49	71.77
IM	10	47.34
PM	2	45.60

phenotype groups could be explained by the higher heterogeneity in the Algerian population, which may explain the lower frequency of PM too.

Key findings include a strong relationship between *CYP2D6* and plasma Endoxifen levels; patients with the nul/nul, red/red, and nul/red diplotypes have Endoxifen concentrations below the proposed threshold, but not in the other candidate genes we tested. Accordingly, our results highlight the role of *CYP2D6* in Tamoxifen metabolism, we recognized that low plasma Endoxifen levels in this cohort were observed in patients with two nul alleles of *CYP2D6* diplotypes but also in patients with two reduced functional alleles or patients with one reduced functional allele in combination with a nul allele, implying that the absence of enzyme activity is closely linked to potentially sub therapeutic Endoxifen levels. More specifically, the findings show that breast cancer patients with the *CYP2D6* nul/nul, red/red, or red/nul genotypes might indeed benefit less from Tamoxifen treatment. We could show, in concordance with previous studies [36–38], that the efficacy of Tamoxifen is related to normal and increased *CYP2D6* activity, leading to a favorable treatment outcome. Borges et al. [39] investigated this quantitative relationship between *CYP2D6* variants and Endoxifen plasma concentrations in an updated analysis of 158 patients after 4 months of treatment with 20 mg of daily Tamoxifen. This included variants known to cause a loss of protein (e.g., *4) or exert decreased function (e.g., *10), i.e., PM and IM genotypes, as well as variants known to increase enzyme function, i.e., URM genotypes. The mean Endoxifen/N-Desmethyltamoxifen ratio was low (0.04 + /− 0.02) in patients lacking any functional alleles, intermediate (0.08 + /− 0.04) in patients with 1 active allele, and high (0.15 + /− 0.09) in patients with 2 or more functional alleles. Hence, *CYP2D6* genotypes are highly associated with Endoxifen plasma concentrations and, moreover, account for their variability. In contrast, the American Society of Clinical Oncology (ASCO) reported that mutated *CYP2D6* was associated with increased plasma levels. The investigators concerned significantly higher plasma levels of N-DesmethylTamoxifen in mutation carriers compared to heterozygous or wildtype genotype carriers after 1 year of Tamoxifen ($n = 118$; $p = 0.001$), indicating that conversion into the clinically relevant Endoxifen may be impaired [40].

Therefore, in this pilot we aimed to investigate the relationship between Endoxifen plasma levels and developing recurrences. We realized that subjects with IM and PM phenotype for *CYP2D6* were highly correlated with developing recurrences. Patients with increased plasma Endoxifen concentrations were considerably more likely than patients with reduced or nul alleles to not report recurrences ($p < 0.05$); such nul/nul, red/red, and red/nul diplotypes have been associated with increased rates of recurrences than other genotypes throughout treatment. These findings confirmed that Tamoxifen efficacy is related to attaining a certain level of Endoxifen concentration in plasma patients. The large-scale production of the potent metabolites Endoxifen can explain this trend toward lower recurrences rates as we determined that the mean concentration of Endoxifen was higher than 4-Hydroxytamoxifen, this partly clarify the major role of Endoxifen in the suppression of tumor cells. Our results are in agreement with a study of Chinese women treated with Tamoxifen implying that, patients with an IM phenotype had reduced disease-free survival [37]. Similarly, Madlensky et al. suggests that women in higher quintiles of Endoxifen levels had lower recurrence and side effect in comparison with those with lower quintiles of Endoxifen [29]. Thus, a case control study including 46 women with breast cancer and 136 controls, the frequency of *CYP2D6*4* was higher in patients developing breast cancer than in controls 9vs1% ($p = 0.015$) within Tamoxifen treatment [41]. Contradictory results from Japanese study of patients with primary breast cancer who had ER + or PR + tumors and were treated with Tamoxifen as adjuvant showing that subjects with IM/IM genotype were not predictive of recurrence free survival [42]. In the other hand; Swedish study of post-menopausal women with ER + and ER- breast cancer revealed no significant differences in distance recurrence free survival between patients with PM/PM and EM/EM [43]. It should be noted that all data indicated that *CYP2D6* is not involved in Tamoxifen activation, yet none of them analyses the relationship between *CYP2D6*/Endoxifen/clinical outcomes at the same time.

We also confirmed in this prospective case study that Tamoxifen metabolite stable states took longer to reach than prodrug stable states. Because no systematic pharmacokinetics trials after the administration of these metabolites have been published, the validity of this report is not well established [44]. However, these differences could mainly be explained by the half-life elimination of the tamoxifen metabolites. Likewise, the Pearson correlation coefficient between Endoxifen and Tamoxifen ($R = 0.96$), Endoxifen and N-Desmethyltamoxifen ($R = 0.93$) revealed that Endoxifen levels may be predicted from Tamoxifen. This might be explained by the magnitude of absorption of Tamoxifen, genetic variation between subjects, and also the quality and food association, which could potentially be factors impacting Tamoxifen bioavailability, hence influencing Endoxifene plasma levels.

Regarding non-*CYP2D6* genes, subjects who carried at least one functional allele for *CYP3A5* and *CYP2C19* had higher plasma Endoxifen levels than those who did not carry any functional allele, but the differences were not statistically significant. However, no significant differences in *CYP3A5*, *CYP2C19*, and clinical factors were determined, regardless of the fact that many studies agree with our findings [45–50]. Nevertheless, the different genotypes of *CYP2C19* or *CYP3A5* did not enhance the risk of recurrences in breast cancer while on Tamoxifen treatment, emphasizing the stronger impact of *CYP2D6* on Tamoxifen pharmacokinetics. Our research suggests that pre-treatment *CYP2D6* genotyping from blood samples could predict Tamoxifen clinical outcomes and aid oncologists in treatment decision-making. However, our study has some limitations. First, our sample size was effective to generate significant differences, but the results needed to be confirmed on a large scale to avoid some bias in the results, leading to significant findings interpreted with vigilance. Secondly, N-Desmethyltamoxifen is converted into two isomers, Z-endoxifen and Z'-endoxifen, with different levels of anti-estrogenic activity [51]. It would be worthwhile to explore the relationship between *CYP2D6* genotype and the isomer levels as they are major metabolites of Tamoxifen. Additionally, the alteration of phase II of metabolism may also explain the differences in the response variability. We also obtained evidence of medication use via patients' reports. However, this seems to be an imperfect approach, but it was the only practical way for us to obtain this information. We believe this is preliminary research and encourage replication; our group is currently conducting additional research to develop drug dosage guidelines based on major *CYP450* genotypes.

Conclusion

Our findings suggest that the *CYP2D6* genotype should be considered in Tamoxifen-treated women. While quantitatively, *CYP2D6* represents only a minor fraction of the total drug metabolizing capacity of the liver, it is polymorphic and, therefore, may alter the balance of metabolism of tamoxifen toward the activation pathways. Breast cancer patients with the *CYP2D6* nul/nul or red/nul diplotype may benefit less from Tamoxifen treatment and are more likely to develop recurrences. Comprehensive *CYP2D6* genotyping has a good predictive value for *CYP2D6* activity.

Common variants in *CYP2C19* and *CYP3A5* did not have a significant impact on the recurrences in this cohort of patients with ER + breast cancer.

Abbreviations

CYP: Cytochrome P450; URM: Ultra; RM: Rapid; NM: Normal; IM: Intermediate; PM: Poor metabolizer; SNP: Single-nucleotide polymorphism; CNV: Copy number variation; LC–MS/MS: Liquid chromatography-mass spectrometry; SERM: Selective estrogen receptor modulator; AS: Activity score.

Acknowledgements

We would like to extend our sincere appreciations to all the staff of Molecular Medicine and Chronic Diseases center (CiMUS), Santiago de Compostela, Spain, especially Olalla Maroñas and Almudena Gil for their help and assistance and hardwork in this study and to Medical Oncology and Radiotherapy Services, University Hospital Center, Benbadis, Constantine, Algeria, for their help.

Author contributions

All of the authors mentioned in the article have contributed to this research work. AB protocol/project development, data collection or management, data analysis, and manuscript writing/editing; KB gave idea, helped in protocol/project development and final correction of manuscript;AH helped in data analysis; RML helped in editing manuscript; NA helped in protocol/project development; TF helped in collection data and DS in protocol/project development and final correction of the manuscript. All authors read and approved the final manuscript.

Funding

This study had no funding from any resource.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All procedures performed were in accordance with the ethical standards of the ethics committee of Dr BENBADIS-Constantine University Hospital Centre in accordance with the recommendations of the Algerian national council for ethics in health sciences and with the Declaration of Helsinki (1964). The study was approved by the local ethical committee of Dr BENBADIS-Constantine University Hospital Centre.

Informed consent

Informed consent (written) was obtained from all participants or their family members.

Consent for publication

Not applicable.

Competing interests

All authors declare that there is no conflict of interests. The manuscript has not been published, or submitted for publication elsewhere.

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Received: 10 May 2022 Accepted: 5 August 2022
Published online: 14 August 2022

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