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# Impact of *CYP2D6* Polymorphisms on Predicting the Adverse Effects of Tamoxifen and Recurrence in ER+ Breast Cancer Patients

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ABSTRACT

**Background:** Breast cancer is a major public health concern in Algeria. Tamoxifen has been approved for the treatment of ER+ breast cancer. Some of the negative side effects of tamoxifen are considered as the reason for discontinuation of the treatment, which would otherwise be potentially lifesaving. In the current study, we assessed the association between *CYP2D6* polymorphisms and tamoxifen efficacy in the Algerian population receiving tamoxifen as adjuvant therapy in ER+ breast cancer.

**Methods:** a total of 76 Algerian women recruited using a convenience sampling approach with a histologically confirmed diagnosis of ER+ breast cancer treated with tamoxifen as an adjuvant therapy were investigated. DNA genotyping was performed by TaqMan Open Array technology. Tamoxifen and its metabolite levels were measured by ultra-high-performance liquid chromatography (UHPLC), followed by electro-spray tandem mass spectrometry (LC-MS/MS).

**Results:** A significant association was observed between the presence of a deficit copy of enzyme activity and the development of adverse effects after the commencement of tamoxifen therapy. Low plasma endoxifen was observed in patients categorized as NM/PM, IM/ IM, IM/PM and PM/PM. Patients with increased plasma endoxifen concentrations were significantly more likely not to report recurrences ($P<0.05$) than patients with reduced or null activity. We realized that the combination genotypes NM/PM, IM/IM, IM/PM, and PM/PM were more strongly associated with disease recurrence and adverse effects than NM carriers of CYP2D6**1* allele ($P<0.05$).

**Conclusion:** Our results show that *CYP2D6* polymorphism should be considered in predicting the occurrence of adverse effects of fatty liver in women treated with tamoxifen. Thus, alternative treatment can be considered and lifestyle modifications can be implemented.

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## INTRODUCTION

Breast cancer is a multidisciplinary disease.[1] Over 2.3 million incidences in both sexes combined and 685.000 deaths from breast cancer occurred across ethnicities in 2022.[2] It is a major public health in Algeria, with an incidence rate of about 11847 new cases in 2018. Approximately 75% of breast cancer patients are estrogen receptor - positive (ER+)[3], making this hormone a target of endocrine therapies (Ets). Tamoxifen has been approved by the food and drug administration (FDA) for the treatment of ER+ breast cancer and several studies are currently investigating its therapeutic potential. Tamoxifen undergoes metabolism via cytochrome p450 enzymes

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in the liver, with the major metabolites formed being endoxifen, and 4-hydroxytamoxifen which are believed to be more potent anti-estrogens than tamoxifen itself.[4,5] The main pathway for tamoxifen biotransformation is via its hydroxylation to form 4-hydroxytamoxifen, and then to endoxifen that is catalyzed primarily by Cytochrome P450 2D6 (CYP2D6). To standardize genotype-to-phenotype translations of the various *CYP2D6* variant alleles, the mechanisms for assigning enzyme activity scores were established by Gaedigk and colleagues.[6-7]. In this system, *CYP2D6* variant alleles were assigned enzyme activity scores between 0 and 1, referring to no (‘*null*’) and fully functional enzyme activities, respectively. Based on the assigned activity score of the alleles, individual diplotypes scores are calculated ranging from ‘0’ for *null/null* carriers to ‘≥3’ for carriers of multiplications of fully functional alleles.[8-11] The metabolism of tamoxifen is altered by *CYP2D6* polymorphisms, which can change a drug’s side effects profile and treatment efficacy depending on the CYP2D6 phenotype. Poor Metabolizers (PMs) are at an elevated risk for failure to reach therapeutic levels.[12] Some of the negative side effects of tamoxifen can be attributed to its agonist or antagonist actions depending on the target tissue and the presence or absence of co-activators or co-repressors.[13] Therefore, they are often the reason for discontinuation of the treatment, which would otherwise be potentially lifesaving.[14] Potentially through the agonist action on the uterus, tamoxifen is known to cause uterine fibroids, post-menopausal vaginal bleeding, and menstrual changes. Otherwise, fatty liver is a common side effect of this drug that affects about 43% of patients[15] within the first two years of treatment.[16-17] *CYP2D6* polymorphisms and the risk of side effects during tamoxifen treatment have been the subject of prior research.[18-21] In the current study, we assessed the association between *CYP2D6* polymorphisms and tamoxifen efficacy in the Algerian population receiving tamoxifen as adjuvant therapy in ER+ breast cancer.

## METHODS

*Study design and patient recruitment*

From September to December 2015, in a cross-sectional study, a total of 76 Algerian women recruited using a convenience sampling approach with a histologically confirmed diagnosis of ER+ breast cancer treated with tamoxifen as an adjuvant therapy were investigated during a routine clinical visit at Benbadis Hospital, Constantine Anti-Cancer Center (CAC), Department of Oncology and Radiotherapy. Steady-state blood samples of the patients treated with tamoxifen (20 mg per day) were collected on-site within the first year of treatment. The patients consulted the hospital once a month, three or six times a year, depending on the progress of their treatment. The use of human blood samples and protocols in our study strictly followed the principles expressed in the Declaration of Helsinki. We asked the participants if they would be willing to donate a blood sample for genetic research purposes. All the participants included in our study completed a questionnaire, which allowed us to obtain the necessary information for our study, and a 5ml venous blood sample was obtained from each. Medical and histopathology reports of the patients were reviewed at the hospital. The age of the patients ranged from 30 to 60 years with a median age of 45 years (Table 1). The patients taking CYP2D6 inhibitors were excluded; those who had conditions before starting the tamoxifen therapy that could interfere with tamoxifen’s side effects were also excluded from the study, which was confirmed by interviewing the patients prior to recruitment. Medical reports prepared by doctors treating the patients for the initial assessment of breast cancer and in preparation for surgery after the commencement of tamoxifen therapy were evaluated. Clinical files indicating the adverse effects of tamoxifen such as fatty liver, uterine fibroids and an ovarian cyst were reevaluated. For liver function tests, blood reports were checked.

**Table 1**. Demographic and clinical parameters of the patients

| Characteristic | Value n (%) |
|---|---|
| Age (y), (IQR) | 45.36, (8) |
| Family Status | |
| Single | 11 (14.4%) |
| Married | 64 (84.1%) |
| Divorced | 1 (1.3%) |
| Number of children at diagnosis | |
| 0 | 23 (30.3%) |
| 1 | 2 (2.6%) |
| 2 | 6 (7.9%) |
| 3 | 17 (22.4%) |
| ≥ 4 | 28 (36.8%) |
| Tumor Size | |
| ≤ 2cm | 48 (63.2%) |
| 2 <size≤ 4cm | 19 (25%) |
| > 5cm | 9 (11.8%) |
| Grade | |
| I | 4 (5.3%) |
| II | 50 (65.8%) |
| III | 22 (28.9%) |
| Node Status | |
| PN0 | 13 (17.1%) |
| $PN^+$ | 51 (82.9%) |
| Histologic type of tumor | |
| Ductal | 69 (90.8% ) |
| Lobular | 5 (6.7%) |
| Other Types | 2 (2.5%) |

IQR: interquartile range $N^+$=regional lymph node; N0= no regional lymph node

*SNP selection and genotyping*

Genomic DNA was isolated from the leucocytes of venous blood by proteinase K digestion using the NaCl method of extraction following the protocol suggested by Miller and co-workers.[22] The quality and quantity of DNA were determined by Nanodrop. We examined the common alleles of *CYP2D6* in our sample based on their positive correlation with plasma concentrations of tamoxifen and its metabolites in ER+ breast cancer patients receiving tamoxifen as adjuvant treatment (Table 2). The analysis of *CYP2D6* polymorphisms was performed by the TaqMan polymerase chain reaction. Copy number variation (CNV) for *CYP2D6* was analysed using TaqMan commercial probes according to the TaqMan Copy Number assay protocol recommended by Applied Biosystems. Data were analysed by Copy Caller® software v.2 using a two-copy as a positive control. The predicted copy number was assessed for the three probes, and the mean and standard deviation were also calculated. To transform the Single Nucleotide Polymorphisms (SNP) and CNV results into a concrete genotype, Allele Typer™ Software was employed with predesigned tables for every gene. Information about the different alleles of different genes was selected from the PharmGKB website.[8] The genotype analysis was carried out in collaboration with the pharmacogenetics laboratory placed in the Center for Research in Molecular Medicine and Chronic Disease CiMUS (Santiago, Spain).

*Chromatography and sample preparation*

It is not possible to predict the efficacy of treatment without measuring the different rates of endoxifen levels in plasma patients. We used the method described by Bobin *et al*[23] to separate and quantify tamoxifen and its metabolites in plasma. Blood samples were centrifuged at 3000rpm within 1 hour of collection; the plasma was extracted and stored at -80ºC until analysis. Tamoxifen and its major metabolites N-desmethyltamoxifen, 4-hydroxytamoxifen and endoxifen were quantified by 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, stock solutions of the studied analytes and internal standards were prepared, at 1mg/ml of Z-isomer, in methanol. These stock solutions were diluted from 10 to 5000ng/ml for tamoxifen and N-desmethyltamoxifen, from 2 to 1000ng/ml for endoxifen and from 1 to 500 for 4-hydroxytamoxifen in water/methanol (30/70), with formic acid 0.1%, to solubilize the analytes. These diluted solutions were extemporaneously further diluted in blank plasma to yield the following calibrator concentrations: from 1, 5, 20, 100, 250 and 500 ng/ml; from 0.2, 1, 4, 20, 50 and 100 ng/ml and from 0.1, 0.5, 2, 10, 25 and 50ng/ml for both tamoxifen and N-desmethyltamoxifen, endoxifen and 4-hydroxytamoxifen, respectively. Internal standard solutions were extemporaneously diluted in acetonitrile: formic acid 0.1%, for final concentration 5 and 20ng/ml for endoxifen, 4-hydroxytamoxifen, tamoxifen and N-desmethyltamoxifen, respectively. For the preparation of quality control (QC) samples, independent stock solutions were prepared as above, to yield the following concentrations in plasma: 1, 2.5, 40 and 400ng/ml; 0.2, 0.5, 8 and 80ng/ml and 0.1, 0.25, 4 and 40ng/ml, for both tamoxifen and N-desmethyltamoxifen, endoxifen and 4-hydroxytamoxifen, respectively. All the stock solutions and intermediary solutions were aliquoted and stored at -80◦C. A total of 100 µl of water: formic acid 100:1 (v:v) was added to 100µl of plasma samples in 1.5 ml micro centrifuge tubes, and vigorously vortexed for 30 seconds to remove protein interaction with plasma. Methanol (100µl) was added and the aliquots were transversely agitated for 10 min at room temperature. The samples (300µl) were again vortexed after the addition of 400µl of internal standard solution and then centrifuged at 18,000×g for 10 min at 4◦C. Finally, 300µl of supernatant was mixed with 300µl of water: formic acid (100:0.2, v:v) ammonium formate 2mM directly in the vials.

*Statistical analysis*

IBM SPSS Statistics 22 for Windows was used to statistically analyze the data. The Kruskal–Wallis test was used to compare possible differences in endoxifen levels between homozygous for *CYP2D6* null allele (PM) and patients homozygous for *CYP2D6* reduced functional allele *IM/IM* or heterozygous with deficit allele *CYP2D6 IM/PM*. Two-sided Fisher's exact test was used to study the genotypes and to predict the recurrence of the disease.

**RESULTS**

Table 3 shows the frequency distribution of CYP2D6 phenotype among breast cancer patients. In total, 9 different alleles within 25 genotypes were identified. A total of 29 patients (38.1%) had a multiplication of the *CYP2D6* allele. The most frequent *CYP2D6* gene polymorphism for ultra-rapid metabolizer (URM) was **2/*2XN*, which occurred in 14 patients (18.4%). Also, 40 patients (52.6%) showed a normal metabolizer (NM) phenotype (*CYP2D6*1*, *CYP2D6*2*, *CYP2D9*35* and *CYP2D6*39*) with the most comment allele being **1/*2* with a frequency of 13 (17.1%). PM

(*CYP2D6*5)* and intermediate metabolizer (IM) (*CYP2D6*10, CYP2D6*17,* and *CYP2D6*41*) were seen in 1 (1.3%) and 6 (7.9%) patients, respectively.

Two-sided Fisher's exact test was used to determine the association between the Clinical parameters in and *CYP2D6* diplotype. Significant differences between tamoxifen's adverse effects and different groups of *CYP2D6* diplotype were observed (P<0.00).

**Table 2.** The SNPs selected to be analyzed in our study

| Gene | Haplotype | rs | Alteration | Reference Allele | Taqman probe used |
|---|---|---|---|---|---|
| *CYP2D6* | *CYP2D6*-* | rs1080985 | 1584C>G | G/G | C 32407252_30 |
| | *CYP2D6*4, *10* | rs1080985 | 100C>T | G/G | C 11484460_40 |
| | *CYP2D6*11* | rs1080985 | 883G>C | C/C | C 30634118_A0 |
| | *CYP2D6*17* | rs1080985 | 1023C>T | G/G | C 2222771_A0 |
| | *CYP2D6*6* | rs1080985 | 1707delT | A/A | C 32407243_20 |
| | *CYP2D6*4* | rs1080985 | 1846G>A | C/C | C 27102431_D0 |
| | *CYP2D6*3* | rs1080985 | 2549delA | T/T | C 32407232_50 |
| | *CYP2D6*9* | rs1080985 | 2615_2617delAAG | TTC/TTC | C 32407229_60 |
| | *CYP2D6*-* | rs1080985 | 2850C>T | G/G | C 27102425_10 |
| | *CYP2D6*41* | rs1080985 | 2988G>A | C/C | C 34816116_20 |
| | *CYP2D6*29* | rs1080985 | 3183G>A | C/C | C 34816113_20 |
| | *CYP2D6*-* | rs1080985 | 4180G>C | C/C | C 27102414_10 |
| | *CYP2D6*35* | rs1080985 | 31G>A | C/C | C 27102444_F0 |

*CYP:* Cytochrome p450; SNPs: Single Nucleotide Polymorphisms

**Table 3**. Frequency of *CYP2D6* genotype among study participants

| CYP2D6 phenotype | frequency | Value % |
|---|---|---|
| NM | 40 | 52.6% |
| URM | 29 | 38.1% |
| IM | 6 | 7.9% |
| PM | 1 | 1.3% |

URM= Ultra-Rapid Metabolizer; NM= Normal Metabolizer; IM= Intermediate Metabolizer; PM= Poor Metabolize

Tamoxifen's adverse effects were evaluated in all patients. Table 4 indicates the clinical parameters in association with diplotype. Significant differences between tamoxifen adverse effects and different groups of *CYP2D6* diplotype were observed (P<0.00). No adverse effect was found in 47 (61.8%) of the patients and a positive adverse effect was found in 29 (38.1%) of the study group. Diabetes mellitus was detected in n=8 (10.5%) of patients, those in groups categorized as NM/IM n=6 (7.8%), URM/NM/ n=1 (1.3%) and NM/PM n=1(1.3%) (P<0.00). However, hyperlipidemia was found in n = 8 (10.5%) of patients, those in groups categorized as NM/IM n=3 (3.9%), URM/NM n=1 (1.3%), NM/NM n=1 (1.3%), NM/PM n=1 (1.3%), IM/IM n=1 (1.3%) and PM/PM n=1 (1.3%) (P<0.00). Regarding hypertension, 11 patients (14.8%) developed hypertension after the commencement of tamoxifen treatment. Specifically, 4 patients had NM/IM diplotype (5.2%), 2 patients showed NM/NM (2.6%), 2 patients had PM/IM (2.6%), 1 patient showed URM/URM (1.3%), 1 patient had URM/NM (1.3%), and 1 patient showed PM/PM (1.3%) (P<0.00). Abdominal ultrasound identified hepatosteatosis in 20 (26.3%) patients, while no hepatosteatosis was detected in 56 (73.6%) patients. Also, 13 patients (17.1%) had fatty liver following the commencement of tamoxifen therapy diagnosed as NM/IM, 2 (2.6%) were URM/NM, 2 (2.6%) were IM/IM, 2 (2.6%) were IM/PM and 1 (1.3%) was PM/PM. We observed a sequential association between the initiation of tamoxifen therapy and the occurrence of fatty liver in patients performing at least one copy of deficit enzyme activity (Figure 1). Of patients who developed fatty liver, 8 (40%) had Diabetes mellitus, 6 (30%) had hyperlipidemia and 8 (40%) had hypertension.

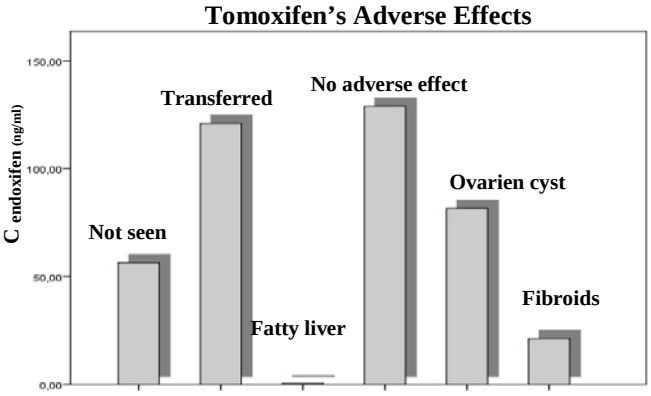


**Figure 1.** Plasma endoxifen concentration in women with ER+ breast cancer was associated with the risk of tamoxifen adverse effects. Patients with high plasma endoxifen concentration are more likely not to experience tamoxifen compared to patients with one deficit or fully deficit *CYP2D6* allele.

**Table 4**. Clinical parameters in association with diplotype

| CYP2D6 Phenotype | | URM | NM | IM | PM |
|---|---|---|---|---|---|
| Tamoxifen_adverse | Positive adverse | 6 (7,0%) | 14 (18.4%) | 6 (7.9%) | 1(1.3%) |
| | No seen | 0 (0.0%) | 1 (1,3%) | 0 (0.0%) | 0 (0.0%) |
| | Transferred | 1 (1,3%) | 1 (1,3%) | 0 (0.0%) | 0 (0.0%) |
| | Negative adverse | 22 (28,9%) | 24 (31,6%) | 0 (0.0%) | 0 (0.0%) |
| P-value | | 0.002* | Ref | p<0.001 | 0.03* |

Ref= reference gene

Data on patients who experienced recurrences was obtained retrospectively from medical records. Table 5 illustrates the association between *CYP2D6* diplotype and recurrence in combination with plasma levels of tamoxifen metabolites. In our cohort, 7 (9.2%) of the population had disease relapse, 1 patient was PM/PM (1.3%), 1 patient was NM/PM (1.3%), 2 patients (2.6%) were IM/IM, and3 (3.9%) patients were IM/PM. The functional allele *CYP2D6*1* was used as a reference group.

**Table 5.** The association between *CYP2D6* diplotype and the likelihood of recurrence in combination with plasma levels of tamoxifen metabolites

| Recurrence | Diplotype | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | URM/URM | URM/NM | NM/NM | NM/IM | NM/PM | IM/IM | IM/PM | PM/PM |
| N (76) | N(3) | N(25) | N(20) | N(21) | N(1) | N(2) | N(3) | N(1) |
| No recurrence | 3 | 25 | 20 | 21 | 0 | 0 | 0 | 0 |
| Locally | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 |
| Metastatic | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 |
| *P-value* | 0.02 | 0.02 | Ref | 0.03 | 0.03 | 0.02 | P<0.001 | P<0.001 |

The relationship between plasma endoxifen concentration and experiencing recurrence is presented in Figure 2. It was found that the combination genotype NM/PM (P=0.03), IM/IM (P=0.02), IM/PM (P<0.001), and PM/PM (P<0.001) was more strongly related to disease recurrence than NM carries of function allele.

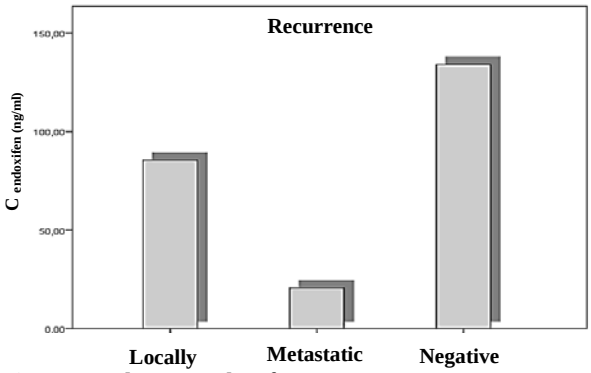


**Figure 2.** Plasma endoxifen concentration in women with ER+ breast cancer was associated with the risk of experiencing recurrences. Patients with high plasma endoxifen concentration are more likely not to experience a recurrence compared to patients with one deficit or fully deficit *CYP2D6* allele.

## DISCUSSION

Tamoxifen is a prodrug indicted in the treatment of all stages of ER+ breast cancer. Pharmacogenetic differences are considered an important factor in oncology because of the potential consequences of toxicities due to the high frequency of genetic intervariability. Numerous studies have been conducted to investigate the relationship between *CYP2D6* polymorphisms and tamoxifen metabolism, and the role of tamoxifen in increasing the risk of breast cancer adverse events and recurrence. *CYP2D6* mutations have a major impact on the appearance of side effects in patients treated with tamoxifen.[24-29] In our study, we investigated the impact of *CYP2D6* polymorphisms on developing side effects and recurrences in pre-menopausal women with ER+ breast cancer treated with tamoxifen as the adjuvant therapy.

Our study showed a significant association between the presence of deficit copy of enzyme activity and the development of tamoxifen adverse effects after the commencement of tamoxifen therapy. During the examination of clinical records, the patients were found to have normal hepatic status before starting tamoxifen. Our study showed a sequential association between the initiation of tamoxifen therapy and the occurrence of fatty liver in patients with at least one copy of deficit enzyme activity. Additionally, our results revealed that a low plasma endoxifen was observed in patients categorized as NM/PM but also in patients with two reduced functional alleles (IM/ IM), patients with one reduced functional allele in combination with one null allele (IM/PM) or patients with two null alleles (PM/PM), implying that the absence of enzyme activity or a decrease in its level is closely related to potentially sub-therapeutic endoxifen levels as well as developing adverse effect of tamoxifen.

As is well-documented, CYP2D6 is involved in the conversion of tamoxifen to 4-hydroxytamoxifen,

which is subsequently oxidized to endoxifen.[26,30] However, tamoxifen has a potential impact on mitochondria; it causes mitochondrial dysfunction [17,19-20] accordingly, the inhibition of mitochondrial β-oxidation, and the accumulation of fatty acids which are converted to triglycerides, resulting in hepatic steatosis. [31] Nevertheless, endoxifen has less impact on mitochondria when compared to tamoxifen. [31] Consequently, patients with lower levels of endoxifen, and those with decreased enzyme activity, are more likely to experience mitochondrial dysfunction and develop fatty liver. These findings may elucidate the considerable frequency of higher levels of fatty liver detected in our population among patients categorized as *CYP2D6* NM/PM, IM/IM, IM/PM or PM/PM diplotypes.

Moreover, we also aimed to investigate the relationship between endoxifen plasma levels and the occurrence of recurrences particularly among patients developing adverse effects. The results indicated that patients with increased plasma endoxifen concentrations were significantly more likely than patients with reduced or null activity not to report recurrences ($P<0.05$); such patients were categorized as PM/PM, IM/IM, IM/PM, NM/PM. Thus, patients with at least one adverse effect of tamoxifen were positively associated with increased rates of recurrences than other genotypes during treatment. These findings revealed that tamoxifen efficacy and minor rates of adverse effects are correlated with a certain level of endoxifen concentration in plasma patients. The large-scale production of the potent metabolites endoxifen can explain this trend toward lower recurrence rates; this partly clarifies the main role of endoxifen in the repression of tumor cells. We could show in concordance with previous studies[32-33] that genotypes associated with normal or increased *CYP2D6* activity lead to a favorable treatment outcome under tamoxifen. Our results are in agreement with a case-control study involving 46 women with breast cancer and 136 controls, where it was found that the frequency of *CYP2D6*4* was higher in patients (9%) than in controls (1%) ($P=0.01$). However, contradictory results have been reported from two studies in the US and Sweden by Owen *et al.*[34] , finding no association between *CYP2D6*4* and the response to tamoxifen or recurrence of breast cancer.

## CONCLUSION

Our results show that *CYP2D6* polymorphism should be considered in predicting the occurrence of adverse effects of fatty liver in women treated with tamoxifen. Alternative treatment can be considered and lifestyle modifications can be implemented. Although *CYP2D6* has a moderate capacity for drug-metabolizing in liver, it is highly polymorphic and, therefore, may alter the metabolism of tamoxifen toward the activation pathways. Breast cancer patients with *CYP2D6* NM/PM, IM/IM, PM/PM and IM/PM diplotypes may benefit less from tamoxifen treatment. Consequently, they are more likely to experience tamoxifen's adverse effects and disease recurrence.

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## CONFLICT OF INTEREST

The authors have declared that no competing interest exists.

## ETHICAL CONSIDERATIONS

This study was approved by the ethics committee of Dr BENBADIS - Constantine University Hospital Centre and complied with the guidelines laid down in Declaration of Helsinki (1964). All procedures involving human subjects were approved by the local ethical committee of Dr. BENBADIS – Constantine University Hospital Centre. Signed informant consent was obtained from individual study participants or their families before being recruited for the study.

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## DATA AVAILABILITY

The data used in the current study are available from the corresponding author upon reasonable request.

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