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CYP2D6 genotype in relation to hot flashes as tamoxifen side effect in a Dutch cohort of the tamoxifen exemestane adjuvant multinational (TEAM) trial

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ABSTRACT

In tamoxifen treated breast cancer patients the occurrence of hot flashes may be associated with effective estrogen receptor antagonism dependent on genetic variations of metabolic enzymes and the estrogen receptor. Early breast cancer patients who were randomized to receive tamoxifen, followed by exemestane within the Tamoxifen Exemestane Adjuvant Multinational (TEAM) Trial were genotyped for five CYP2D6 alleles. CYP2D6 genotypes and phenotypes were related to the occurrence of hot flashes as adverse event (AE) during the first year of tamoxifen use (primary aim) and the time to the occurrence of hot flashes as AE during the complete time on tamoxifen (secondary aim). Additionally, exploratory analyses on 22 genetic variants of other metabolic enzymes and two common polymorphisms in the estrogen receptor-1 (ESR1) were performed. No association was found between the CYP2D6 genotype/ phenotype or any other genetic variant and hot flashes during the first year. Only higher age was related to a lower incidence of hot flashes in the first year (adjusted odds ratio 0.94, 95% CI 0.92-0.96; $p<0.001$). The ESR1 PvuII XbaI CG haplotype was associated with the time to the occurrence of hot flashes during the complete time on tamoxifen (CG/CG vs CG/other + other/other: adjusted hazard ratio 0.49, 95% CI 0.25-0.97; $p=0.04$). In conclusion, the CYP2D6 genotypes and phenotypes were not associated with the occurrence of hot flashes. Common polymorphisms in the estrogen receptor-1 might predict hot flashes as common tamoxifen side effect, although this finding needs replication.

INTRODUCTION

Hot flashes are a common side effect of tamoxifen treatment and are generally thought to be the result of decreased estrogen or increased gonadotropin concentrations.[1] Genetic variations of tamoxifen metabolizing enzymes leading to decreased concentrations of the active metabolites 4-OH-tamoxifen and more importantly endoxifen may therefore lead to a decrease of vasomotor symptoms by insufficiently blocking the estrogen receptor.[2, 3] In addition, genetic variants of the estrogen receptor may also lead to inefficient receptor antagonism by tamoxifen and consequently less frequent and severe hot flashes. Hot flashes have been related to efficacy of adjuvant anastrozol and tamoxifen treatment.[4, 5] Genetic variants of metabolic enzymes or the estrogen receptor that predict hot flashes may therefore also predict treatment efficacy. However, if efficient estrogen receptor antagonism leads to more side effects including vasomotor symptoms, this may lead to early tamoxifen discontinuation or worse adherence.[3] Instead of improving efficacy, a decrease in compliance because of side effects may paradoxically cause a reduction in treatment effect.[6] Patients who are aware of their genetic profile that accurately predicts more side effects, but also a more favorable outcome may be better motivated to comply with the tamoxifen therapy. Because Cytochrome P450 2D6 (CYP2D6) is the major enzyme responsible for the formation of endoxifen, previous pharmacogenetic research on tamoxifen side effects has mainly focused on the CYP2D6 genotype.[2, 3, 7] Recent genotyping studies on CYP2D6, using DNA derived from tumor blocks, have been criticized because loss of heterozygosity (LOH) in tumors may have led to false CYP2D6 genotype assignment.[7-11] In future studies, in which such DNA is used to genotype CYP2D6, the influence of potential LOH should be ruled out.

The Tamoxifen Exemestane Adjuvant Multinational (TEAM) trial is a multinational trial comparing the sequence of 2.5 to 3 years of tamoxifen followed by exemestane (5 years of hormonal therapy in total) to 5 years of exemestane in early stage postmenopausal hormone receptor positive breast cancer patients.[12] In the current pharmacogenetic study we aimed to relate the CYP2D6 genotype / phenotype to hot flashes registered as adverse event (AE) during tamoxifen use in a Dutch cohort of the TEAM trial. Patients were excluded from our analysis when the possibility of a false CYP2D6 genotype, because of LOH in the tumor, could not be ruled out. Additionally, associations with other genetic variants of metabolic enzymes and the estrogen receptor were explored.

METHODS

Patients

Of in total 9,779 postmenopausal early breast cancer patients randomized in the TEAM trial from 2001 to January 2006, 2,753 were included in The Netherlands. Of these patients, 1,379 were assigned to the treatment arm with tamoxifen at a dose of 20 mg once daily with a planned switch to exemestane after 2.5 to 3 years. Tumor blocks were available for genotyping in 746 of these 1,379 patients (54.1%). The central TEAM datacenter in Leiden, The Netherlands, collected information on tumor and patient characteristics, including side effects that were registered on case record forms designed for data collection in the TEAM trial. This pharmacogenetic study was separately approved by the central medical ethics review board for The Netherlands at the Erasmus University Medical Center in Rotterdam.

Endpoints

The primary endpoint was the occurrence of hot flashes as AE during the first year of tamoxifen use. The secondary endpoint was the time to the first occurrence of hot flashes (TTFF) as tamoxifen AE. For both endpoints hot flashes were defined as the first registration of hot flashes or sweats as AE during tamoxifen use, regardless of the severity of the AE or the relation to the study drug that was reported. The severity grade and relation to the study drug were not taken into account because the accuracy of the reported variables was uncertain. Hot flashes were registered on an AE case report form with blank writing fields (without prespecified complaints).

Additionally, the relation between hot flashes that occurred during the first year of tamoxifen use and disease free survival during tamoxifen use (DFS-t) was studied. Disease free survival during tamoxifen use was defined as the time from the tamoxifen start date to a locoregional or distant recurrence, second breast cancer, death without recurrence or tamoxifen discontinuation.

Genotyping

Germline genetic variants in candidate genes of enzymes involved in the tamoxifen metabolism and of the estrogen receptor (Figure 6.1) were selected based on assumed clinical relevance, high allelic frequency or the assumption that nonsynonymous amino acid change leads to altered protein functionality. The polymorphisms included in our analyses are listed in Table 6.1. Genotyping was

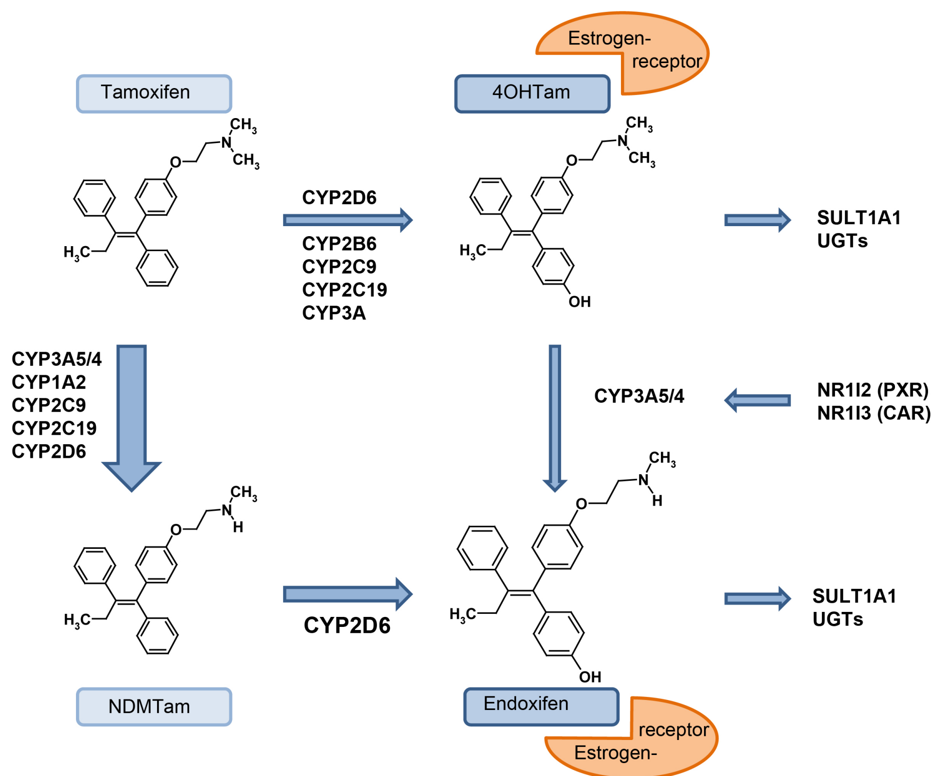


Figure 6.1 Tamoxifen metabolism. Abbreviations: 4OHTam, 4-hydroxytamoxifen; CYP, cytochrome P450 isoenzyme; SULT, sulfotransferase; UGT, UDP-glucuronosyltransferase; NDMTam, N-desmethyltamoxifen; NR1, nuclear receptor subfamily 1; PXR, pregnane X receptor; CAR, constitutive androstane receptor.

performed on formalin fixed paraffin embedded tumor (FFPE) tissue as described previously.[13] Briefly, from 3 slides of 20 μm, DNA was extracted with the Maxwell forensic DNA isolation kit (Promega, Leiden, The Netherlands). Before genotyping a pre-amplification step was used to increase the percentage of successfully genotyped samples without loss of reliability and with minimal use of DNA mass.[13] For genotyping Taqman assays (Applied Biosystems, Foster City, CA, USA) were used on the Biomark (Fluidigm, San Fransisco, USA). In case of failure of genotyping using the Taqman based method, pyrosequencing was performed (Qiagen, Chatsworth, CA, USA) on a Pyrosequencer 96 MA (Biotage, Uppsala, Sweden). Two SNPs in PXR (10799 G>A, 10620 C>T), three SNPs in CAR (45518 C>T, 47537 A>C, 47636 T>G) and the two ESR1 SNPs PvuII and XbaI were also analyzed in haplotype.

CYP2D6 genotypes were translated to predicted phenotypes (extensive, intermediate or poor metabolizer) as described in the Supplementary Methods section.

Loss of heterozygosity

The ratio between tumor and germline DNA in a sample derived from FFPE tumor tissue, differs between samples. A high percentage of tumor DNA may result in falsely called genotypes because of loss of heterozygosity in the tumor. If a certain germline homozygous CYP2D6 genotype was assumed while in fact one of the alleles has been lost in tumor but not in normal tissue a false test is the result.[8] To avoid such incorrect interpretation of CYP2D6 genotyping results, three microsatellite markers D22S276, D22S2284 and D22S423 near the CYP2D6 gene on chromosome 22q13 with a high frequency of heterozygosity (>80%) were additionally determined (Supplemental Table S6.1). The chance that a patient is homozygous for all three markers would be less than 0.203=0.8%. Thus, in nearly all patients including those with a homozygous germline CYP2D6 genotype, heterozygosity should be demonstrated for ≥1 microsatellite markers. We hypothesized that LOH of the CYP2D6 gene would also lead to LOH of the microsatellites given the proximity of the markers to the 22q13 locus. Heterozygosity for one of these microsatellite markers then validates a true homozygous germline CYP2D6 genotype tested in the same tumor block. Patients with a homozygous CYP2D6 genotype were excluded from our CYP2D6 analysis if influence of LOH on the CYP2D6 genotype in the tumor block could not be ruled out (i.e. in case of "homozygosity" of all microsatellite markers). Further details can be found in the Supplementary Methods section.

Statistical analysis

For comparison of proportions and means, χ^2 statistics and the Student's t-test were used, respectively. Logistic regression analysis was used to assess whether the occurrence of hot flashes within the first year of tamoxifen use differed with respect to age at diagnosis, adjuvant chemotherapy, body mass index (BMI) and the selected genetic variants. In case no hot flashes were registered, a patient was required to have at least one year of follow-up time. Cox regression analysis was used to assess whether time to hot flashes (TTHF) differed with respect to age at diagnosis, adjuvant chemotherapy, body mass index (BMI) and the selected genetic variants. Patients were censored at the time of tamoxifen discontinuation or loss to follow-up during tamoxifen use. Genetic variants were initially tested in a general model (2 degrees of freedom). If this test resulted in a p-value≤0.1, the genetic variant was fitted and the most appropriate model (gene-dose, dominant or recessive) was selected.

Covariates were included in a multivariable model if they were of assumed clinical significance or had a univariable p-value<0.05. The distributions of time to hot flashes were estimated overall using the Kaplan-Meier method. A log-rank test was used to assess the association between the genetic variant and the time to hot flashes. All results from the multivariable Cox regression analysis with a p-value<0.05 were considered significant. No correction for multiple testing for the 24 genetic variants other than CYP2D6 was applied, since this was an exploratory analysis.

In an additional landmark analysis the hot flashes that occurred during the first year of tamoxifen use were related to disease free survival during tamoxifen use (DFS-t) after one year using a Cox regression analysis. Hot flashes were treated according to the landmark method using one year as a landmark. Patients with a breast cancer event or those who were censored in the first year following tamoxifen initiation were excluded from this analysis. Statistical analyses were performed using SPSS 20.0 (SPSS Inc., Chicago, IL).

RESULTS

Tumor blocks were collected from 746 patients enrolled in the TEAM trial and randomized to tamoxifen followed by exemestane in 59 of the 69 participating Dutch hospitals. The 746 patients were similar to the whole group of Dutch patients randomized in the sequential arm of tamoxifen followed by exemestane (n=1,379) with regard to mean age, type of surgery, tumor stage, nodal status, tumor grade, adjuvant chemotherapy and radiotherapy (p>0.05, data not shown). Four patients were ineligible because of an ER/PgR negative primary tumor (n=4). The primary analysis therefore was performed on the 742 eligible patients. Twenty-nine genetic variants were successfully genotyped using Taqman assays except for CYP2D6*3 which was genotyped with pyrosequencing.[13] Genotype frequencies of 10 selected genetic variants showed deviation from Hardy Weinberg equilibrium, but were still considered appropriate to analyze, because they did not differ from the frequencies previously reported in literature or on the NCBI website (Table 6.1). With a median follow-up time of 2.5 years until tamoxifen discontinuation, 206 patients (28%) experienced hot flashes during tamoxifen use. Before starting tamoxifen, 25.5% of patients received adjuvant chemotherapy. Patients who developed hot flashes had received chemotherapy more often (32.0 vs. 23.0%; p=0.01). The mean age was 66.1 (44.8 – 90.7) years. Patients experiencing hot flashes were younger than patients without vasomotor symptoms (62.7 (SD=9.4) vs. 67.4 (SD=7.9) years; p<0.001). Of the baseline patient characteristics age and adjuvant chemotherapy were selected for multivariable analysis based on a p-value<0.05 and assumed clinical significance (Table 6.1).

Table 6.1 Genetic variants of metabolic enzymes and the estrogen receptor 1 and clinical variables associated with hot flashes as adverse event in the 1st year of tamoxifen use (univariable logistic regression with 2 degrees of freedom)

Gene	Allele name	Genetic variant	RS number	χ^2 test: HW-equilibrium	Logistic regression: p-value
CYP2D6	*3	2549 A/del	rs4986774	0.07	0.72
	*4	1846 G/A	rs3892097	11.17 [a]	0.31
	*6	1707 T/del	rs5030655	20.97 [a]	0.36
	*14	1758 G/A	rs5030865	0.00	1.00
	*41	2988 G>A	rs28371725	32.30 [a]	0.94
CYP2D6 phenotype	*3,*4,*6,*14,*41+ CYP2D6 inhibitor				0.48
CYP2C9	*2	3608 C/T	rs1799853	0.16	0.77
	*3	42614 A/C	rs1057910	15.00 [a]	0.81
CYP2C19	*2	19154 G/A	rs4244285	4.23 [a]	0.52
	*17	-806 C/T	rs12248560	27.91 [a]	0.83
CYP2B6	*6	516 G/T	rs3745274	4.79 [a]	0.17
	*8	415 A>G	rs12721655	0.04	0.71
CYP3A5	*3	6986 A>G	rs776746	4.21 [a]	0.98
UGT1A4	*2	70 C/A	rs6755571	1.87	0.37
		-163 G/A	rs3732218	0.28	0.99
		-219 T/C	rs3732219	1.18	0.94
UGT1A8	*2	518 C/G	rs1042597	1.27	0.64
UGT2B7		-840 G/A	rs7438135	0.32	0.87
UGT2B15	*2	253 G/T	rs1902023	21.04 [a]	0.08 [b]
NR1I2 (=PXR)		8055 C/T	rs2276707	0.92	0.19
		7635 A/G	rs6785049	0.02	0.78
		-24113 C/T	rs2276706	0.64	0.56
		-25385 C/T	rs3814055	0.00	0.48
	[c]	10620 C/T	rs1054190	0.06	0.73
	[c]	10799 G/A	rs1054191	0.10	0.48
NR1I3 (=CAR)	[c]	47636 T/G	rs4073054	0.39	0.83
	[c]	45518 C/T	rs2307424	0.81	0.70
	[c]	47537 A/C	rs2307418	1.30	0.54
ESR1	PvuII	453-397 T/C	rs2234693	0.00	0.51
	XbaI	453-351 A/G	rs9340799	8.96 [a]	0.54
	CG haplotype [d]				0.31
	CA haplotype [d]				0.91
	TA haplotype [d]				0.52
Age					<0.001
Chemotherapy					0.002
BMI					0.24

[a] not in Hardy Weinberg equilibrium but genotype frequencies in accordance with previous literature/ NCBI
[b] no suited model (gene-dose, dominant, recessive) was fitted [c] also analyzed as haplotype, but p>0.1 [d] analyzed as ESR1 PvuII XbaI haplotype
Abbreviations: CYP, cytochrome P450 isoenzyme; BMI, body mass index; HW, Hardy Weinberg; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; NR1, nuclear receptor subfamily 1; PXR, pregnane X receptor; CAR, constitutive androstane receptor; ESR1, estrogen receptor 1

Neither the separate CYP2D6 genotypes nor the predicted CYP2D6 phenotypes (the combination of *3, *4, *6, *14, *41 alleles and concomitant CYP2D6 inhibitor use) were associated with the occurrence of hot flashes during the first year (Table 6.1). In the exploratory analyses no other genetic variant of a metabolic enzyme or the ESR1 was associated with hot flashes in year one (Table 6.1). For the UGT2B15*2 genotype no suitable model could be fitted, although the logistic regression analysis using a general model (2 degrees of freedom) resulted in a p-value≤0.1. This is explained by the observation that the patients with a heterozygous UGT2B15*2 genotype more often experienced hot flashes than patients with the homozygous wildtype and variant type genotypes. Higher age was related to a lower incidence of hot flashes in the first year (adjusted odds ratio 0.94, 95% CI 0.92-0.96; p<0.001, Table 6.2).

Neither the separate CYP2D6 genotypes, nor the CYP2D6 phenotypes were associated with time to the first occurrence of hot flashes (TTFF), although the CYP2D6 poor metabolizers (PMs) experienced less hot flashes than intermediate (IMs) and extensive metabolizers (EMs) (p=0.78; Figure 6.2). For the CYP2D6 phenotype analysis 21 (6 events) of 611 patients (3.4%) were excluded after performing an additional microsatellite analysis, because the possibility of LOH causing false CYP2D6 genotype assignment could not be ruled out. Including these patients for the primary or secondary analysis did not importantly change the results (data not shown).

In the exploratory analyses, only the ESR1 CG haplotype was associated with a longer TTFF. The Kaplan Meier curve of the ESR1 CG haplotype according to a recessive model is shown in Figure 6.3. Patients with the ESR1 CG/CG haplotype had a 51% reduction in hot flashes compared to the other haplotypes (adjusted hazard ratio 0.49, 95% CI 0.25-0.97; p=0.040; Table 6.3). The separate ESR1 PvuII (CC vs CT+TT: adjusted HR 0.66, 95% CI 0.43-1.03; p=0.065) and XbaI (GG vs AG+AA:

Table 6.2 Logistic regression analysis for the occurrence of hot flashes as adverse event in the 1st year of tamoxifen use

	Univariable			Multivariable Adjusted for age and chemotherapy		
	OR	95% CI	p	OR	95% CI	p
Age	0.94	0.92-0.96	<0.001	0.94	0.92-0.96	<0.001
Previous chemotherapy						
No	1.00	Reference		1.00	Reference	
Yes	1.83	1.26-2.66	0.002	0.95	0.60-1.48	0.81

adjusted HR 0.60, 95% CI 0.35-1.02; p=0.057) genotypes were not associated with TTFF, but showed a trend for significance. Higher age was associated with a reduction in hot flashes (adjusted HR 0.95, 95% CI 0.93-0.97; p<0.001, Table 6.3). Hot flashes occurring in the first year of tamoxifen use were not related to DFS-t (unadjusted hazard ratio 0.82, 95% CI 0.39-1.71; p=0.59).

CYP2D6 phenotypes: TTFF

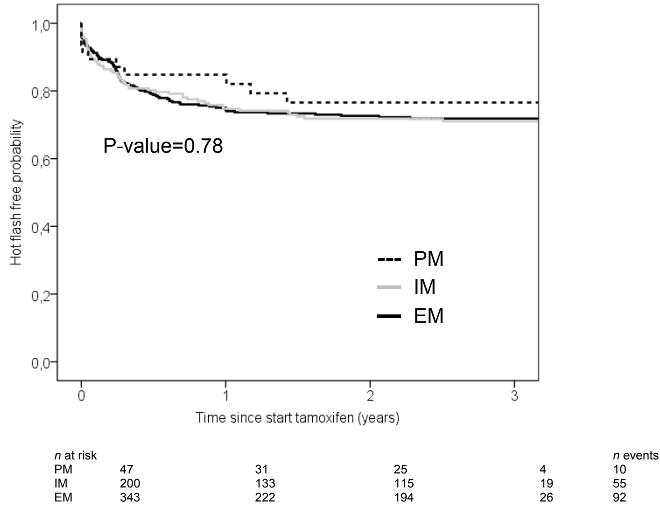
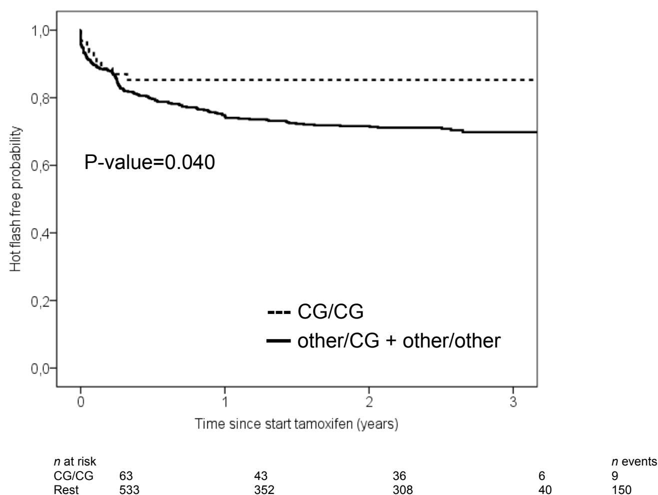


Figure 6.2 Kaplan Meier probabilities of the predicted CYP2D6 phenotypes (based on *3, *4, *6, *14, *41 alleles and concomitant CYP2D6 inhibitor use) for the time to the first occurrence of hot flashes as adverse event. Patients were excluded if influence of LOH on assigned genotype could not be ruled out. TTFF, time to the first occurrence of hot flashes; PM, poor metabolizer; IM, intermediate metabolizer; EM, extensive metabolizer; LOH, loss of heterozygosity.

Table 6.3 Cox regression analysis for the time to the first occurrence of hot flashes as adverse event

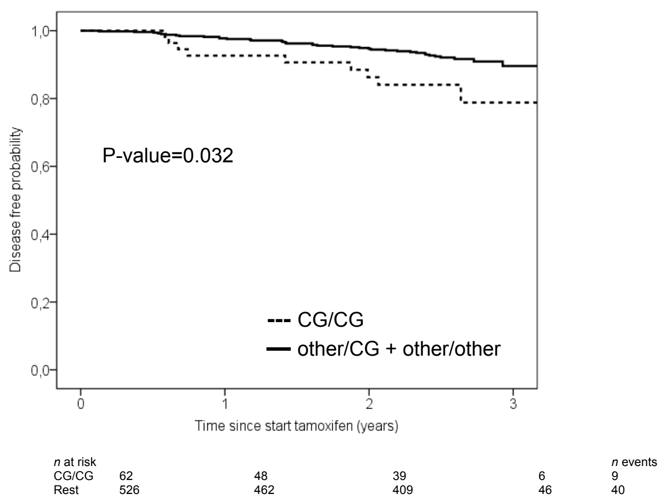
	Univariable			Multivariable		
				Adjusted for age and chemotherapy		
	HR	95% CI	p	HR	95% CI	p
Age	0.95	0.94-0.97	<0.001	0.95	0.93-0.97	<0.001
Previous chemotherapy						
No	1.00	Reference		1.00	Reference	
Yes	1.49	1.11-2.00	0.008	0.89	0.61-1.31	0.57
ESR1 haplotype CG						
CG/other + other/other	1.00	Reference		1.00	Reference	
CG/CG	0.50	0.26-0.98	0.045	0.49	0.25-0.97	0.040

A. ESR1 PvuII XbaI haplotype CG: TTFF



n at risk					*n* events
CG/CG	63	43	36	6	9
Rest	533	352	308	40	150

B. ESR1 PvuII XbaI haplotype CG: DFS-t



n at risk					*n* events
CG/CG	62	48	39	6	9
Rest	526	462	409	46	40

Figure 6.3 Kaplan Meier probabilities of the ESR1 PvuII XbaI haplotype (recessive model) for: (A) the time to the first occurrence of hot flashes as adverse event; (B) Disease Free Survival during tamoxifen use. TTFF, time to the first occurrence of hot flashes; DFS-t, disease free survival during tamoxifen use.

DISCUSSION

In the current study no association between the CYP2D6 genotypes or phenotypes and hot flashes in tamoxifen treated early breast cancer patients was found, which is in line with a previous report on a prospective patient cohort.[14] These findings are in contrast with a recent publication, in which a higher frequency of hot flashes was demonstrated in PMs and IMs compared to EMs, contrary to the hypothesis.[7] The latter study has been heavily criticized for using tumor blocks as a DNA source for CYP2D6 genotyping. The deviation from the Hardy Weinberg equilibrium of the CYP2D6*4 genotype in that study led to the criticism that loss of heterozygosity (LOH) of CYP2D6 in tumor tissue may cause false genotyping results.[8, 11] In the current study we excluded those patients of whom we were uncertain that LOH may have caused false genotype assignment. To our best knowledge, this is the largest study in a homogeneous well documented trial population thus far, in which CYP2D6 genotype and phenotype have been related to hot flashes using reliable genotype data.

An additional exploratory analysis of 24 genetic variants in 11 candidate genes encoding for metabolic enzymes and the estrogen receptor-1 (ESR1) was performed. We hypothesized that inefficient estrogen receptor antagonism caused by genetic variants of various tamoxifen metabolizing enzymes and the estrogen receptor leads to fewer estrogen dependent side effects, such as hot flashes. In the primary analysis no genetic variant was related to the occurrence of hot flashes during the first year of tamoxifen use. In the secondary analysis using the time to the first occurrence of hot flashes, the ESR1 PvuII XbaI CG/CG haplotype reduced the risk of hot flashes by half. In this prospective trial, information on side effects and factors that may influence the occurrence of hot flashes was well documented. None of the possible confounding factors, such as age, chemotherapy and BMI importantly affected the direction or significance of the ESR1 CG haplotype effect. The inconsistency between the results of the primary and secondary analyses may partly be due to an increase in number of events in the TTFF analysis (141 vs. 159 events). Furthermore, not only the occurrence of hot flashes, but also the time until a patient develops hot flashes as adverse event of tamoxifen may be determined by the level of estrogen receptor antagonism. Still, both analyses are underpowered to detect a 50% reduction of hot flashes occurring in the first year or during the complete time on tamoxifen (TTFF). Alternatively, the association that was found may be caused by chance.

We tested a variety of genetic variants and did not adjust for multiple testing. The need for adjusting for multiple testing is depending on the prior chance of finding a true association, which is influenced by the plausibility of the hypothesis and the amount of previous research pointing in the same direction. Previous publications reported on a potential association between

tamoxifen metabolism, tamoxifen efficacy and side effects.[2, 3, 7, 15-17] Nevertheless, we did not find any association between genetic variants of metabolic enzymes leading to lower active metabolite concentrations and hot flashes. This may be explained by the model based assumption that even in case of poor metabolism caused by an inactive CYP2D6 enzyme, more than 99% of the estrogen receptor is still bound by tamoxifen and its metabolites.[18] Active tamoxifen metabolites would therefore effectively antagonize the estrogen receptor in poor and extensive metabolizers to an equal extent. This explanation is consistent with all the reports on negative associations between CYP2D6 genotype and clinical outcome.[7, 19-25] Interestingly, functional polymorphisms in the estrogen receptor could diminish effective estrogen receptor binding and antagonism even in abundance of active tamoxifen metabolites. The influence of these ESR polymorphisms on tamoxifen efficacy may therefore be independent of tamoxifen metabolism. The precise effects of these polymorphisms on estrogen receptor binding, estrogen dependent gene expression and breast cancer growth in the presence of sufficient levels of endoxifen remain to be elucidated. Still, previous reports on a positive association between ESR polymorphisms and tamoxifen side effects including hot flashes strengthen our findings.[26-28]

Genetic variants in the ESR1 gene may not only predict fewer hot flashes, but also impaired tamoxifen efficacy. In a previous report on the same patient cohort an increasing number of ESR1 PvuII C alleles was associated with worse disease free survival.[23] The Kaplan Meier curve for disease free survival according to the CG haplotype is shown in Figure 6.3b. The same PvuII C allele in the CG haplotype is related to longer time to the first occurrence of hot flashes.

Finally, in the current study hot flashes appearing in the first year of tamoxifen use were not related to DFS-t. Previously, a positive association between the occurrence of hot flashes and decreased breast cancer recurrence was reported.[5] The reason for this discrepancy may be the fact that in our study probably too few events were recorded (45 DFS events occurring after the first year of tamoxifen use). Another explanation may be that hot flashes lead to a decrease in adherence to the tamoxifen therapy. In the current study however, data on tamoxifen adherence are lacking.

In conclusion, the CYP2D6 genotypes and phenotypes were not associated with the occurrence of hot flashes as common tamoxifen side effect. Common polymorphisms in the estrogen receptor-1 might predict hot flashes. Especially in light of earlier reports on a possible association between the ESR1 PvuII polymorphism, side effects and clinical outcome, these findings deserve replication.[23, 29]

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SUPPLEMENTARY DATA

CYP2D6 predicted phenotypes

CYP2D6 genotypes were translated to predicted phenotypes (extensive, intermediate or poor metabolizer). By definition, the CYP2D6 intermediate metabolizer phenotype predicted by genotype consisted of patients homozygous for a decreased activity allele (e.g. *41/*41) or heterozygous for an absent activity allele (e.g. *1/*4 and *41/*4). A patient could only be classified to a certain CYP2D6 phenotype if genotyping was successfully done for the CYP2D6 alleles with a reported frequency in Caucasians of more than 5%. In case of an allele frequency of less than 5% a missing genotyping result for that allele was accepted. For that specific allele the wild type was assumed. For example, if in a patient the assay for CYP2D6*4 resulted in a heterozygous (Wt/Vt) genotype but no result was available for the less frequent *3 allele (allele frequency=3%), the patient was considered to have a *1/*4 genotype and was thus classified as an intermediate metabolizer. Additionally, concomitant use of a CYP2D6 inhibitor could reclassify the CYP2D6 phenotype predicted by genotype.[30]

Loss of heterozygosity

DNA samples were pre-amplified for the three microsatellite markers as described by Fletcher et al.[31] Each PCR reaction consisted of 1 pmol of each primer, 4 µl Qiagen Hotstar PCR mastermix (Qiagen, Venlo, The Netherlands), 3 µl DNA in total volume of 8 µl. PCR conditions for pre-amplification were as follows: 15 minutes at 95°C, 20 cycles at 94°C-55°C-72°C for respectively 20, 20 and 60 seconds. PCR was finalized by 10 minutes at 72°C. Next, to the PCR products 100 µl sterile water was added and 1 µl was used for second round PCR using primers listed in Supplemental Table

Supplemental Table S6.1 Microsatellite markers for loss of heterozygosity analysis

Marker	Primers for second round PCR 5’-3’	Expected size
D22S276	AAATGGGCTTGTAAAGAAAAATA* AAATATGAAGTACTTCTTACCAC	165 +/- 18bp
D22S284	GAGCAAGACCCTGTCTCAAGA* ACAGCAAAATGATATTAGTTTGAGC	88 +/- 16bp
D22S423	GAGTGAGTGACTGAGTAAATGTAGTG* ATCCCTGAAATACACATATATGTAC	200 +/- 26bp

* Primers are labeled with FAM fluorescent dye at 5’-end

S6.1. Each microsatellite marker was separately amplified by PCR. Each reaction consisted of 2.5 pmol reverse and forward primer (of which one primer was labeled with FAM-fluorescent dye) 5 µl Qiagen Hotstar PCR mastermix (Qiagen, Venlo, The Netherlands), 1µl diluted pre-amplified DNA in total volume of 10µl. PCR conditions for pre-amplification were as follows: 15 minutes at 95°C, 35 cycles at 94°C-55°C-72°C for 30 seconds each step and PCR was finalized by 10 minutes at 72°C. To PCR product 100 µl sterile water was added and 1 µl was used for fragment length analysis using ABI-3130 and peakscanner software according to manufacturers prescription (Life Technologies, Nieuwerkerk aan den IJssel, The Netherlands).

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