Impairment of endoxifen formation in tamoxifen-treated premenopausal breast cancer patients carrying reduced-function CYP2D6 alleles

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Aims: Tamoxifen is bioactivated to endoxifen by polymorphic CYP2D6-dependent metabolism. Here, endoxifen levels were compared to CYP2D6 diplotypes, tentative target concentrations and side effects.

Methods: In total, 118 Swedish premenopausal breast cancer patients diagnosed 2006–2014, with on-going postoperative tamoxifen treatment January 2017, were included. Biobanked DNA from peripheral blood was used for CYP2D6 genotyping by TaqMan real-time polymerase chain reaction (CYP2D6*1, *3, *4, *5, *6, *9, *10, *41, *1xN). Plasma levels of tamoxifen and 3 major metabolites were quantified by liquid chromatography–tandem mass spectrometry. Clinical information on treatment and side effects was retrospectively obtained from medical records.

Results: In the final analysis of 114 patients, a clear relationship between CYP2D6 genotype and plasma endoxifen levels was evident. Low endoxifen (1.6–5.2 ng/mL), i.e. below the suggested threshold for clinical efficacy, was found in all patients with 2 reduced-function alleles, 2 null-alleles, or a null/reduced-function combination. CYP2D6*41 was the most common reduced-function allele (82%) and 17 of 21 CYP2D6*41-carriers exhibited a lower CYP2D6 activity than predicted from published guidelines. No difference in endoxifen levels was observed between carriers of 2 null-alleles vs patients homozygous for CYP2D6*41 or the corresponding heterozygous combination (P = .338). In patients with endoxifen levels <5.9 ng/mL (36/114), side effects were either mild or absent. At higher endoxifen levels moderate-to-severe side effects were reported in a concentration-dependent manner.

Conclusion: Significantly reduced endoxifen levels were observed not only in all homozygous carriers of CYP2D6 null-alleles, but also in carriers of 2 reduced-function alleles. This finding may be highly relevant for future, genotype-based dose considerations.
INTRODUCTION

Tamoxifen has been the standard endocrine therapy for oestrogen receptor-positive breast cancer for many decades. For premenopausal patients, tamoxifen is the standard endocrine treatment in both the adjuvant and palliative settings, as these patients cannot use aromatase inhibitors unless they are combined with ovarian suppression.1

Postoperative tamoxifen treatment for 5 years substantially reduces the rate of breast cancer recurrence and breast cancer-related mortality.2 Ten years of tamoxifen reduces the risk of recurrence and mortality even further and many women are now recommended this extended treatment.3

Tamoxifen is a weak antioestrogen and requires hepatic biotransformation to more potent metabolites—most importantly endoxifen—by hepatic cytochrome P450 2D6 (CYP2D6). The predominant metabolic pathway is demethylation by CYP3A4/5 to N-desmethyl-tamoxifen, which is further oxidized by CYP2D6 to endoxifen (the Z-enantiomer of 4-hydroxy-N-desmethyl-tamoxifen). In the second major pathway, CYP2D6 hydroxylates tamoxifen to 4-hydroxytamoxifen (with a minor contribution from CYP2C9, CYP2C19 and CYP2B6), which is further metabolized mainly by CYP3A4/5 to endoxifen.4

CYP2D6 is highly polymorphic with >100 known allelic variants.5 Interindividual variation in tamoxifen activation by polymorphic CYP2D6 has been suggested to directly influence the outcome of tamoxifen treatment so that patients carrying a poor metabolizer genotype benefit much less from tamoxifen treatment.6–9 The importance of pre-emptive CYP2D6 genotyping in clinical practice is, however, still controversial, partly due to contradictory results in some retrospective studies.10–12 Clearly, more work will be required to better understand the clinical value of CYP2D6 genotyping in relation to adjuvant treatment and the possibility of individualized dosing of tamoxifen.

CYP2D6 metabolizer status has traditionally been divided into 4 phenotypes: poor metabolizers (PM); intermediate metabolizers (IM); extensive metabolizers (normal/EM); and ultrarapid metabolizers (UM).13 The overall idea would be to use this patient stratification for individualized dose recommendations based on CYP2D6 genotype. However, intermediate metabolizers include a wide range of different allele combinations that clearly differ in their corresponding impact on the individual CYP2D6 metabolic activity.13–17 and, for some variant alleles, this might even be explained by substrate-dependent alterations in metabolic activity.18 It follows that the conventional approach to ascribe all reduced-function CYP2D6 alleles an anticipated 50% reduction of metabolic capacity compared to normal/fully functional alleles, is indeed simplistic and should be open for corrections over time.13 An example is the recent suggestion by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG), to downgrade the predicted activity of the CYP2D6∗10 allele from 0.5 to 0.25.17 In the context of long-term adjuvant treatment with tamoxifen, it is believed that such specific corrections are very important in order not to overestimate the CYP2D6 activity in carriers of reduced-function alleles as this would mean a risk of underestimating the risk of poor endoxifen formation at standard dosage.

Even though some hospital laboratories already offer measurements of tamoxifen and its metabolites in plasma,20 there is still no established concentration range to aim for.21–26 Two previous studies have suggested a threshold for tamoxifen efficacy of plasma endoxifen at 5.9 ng/mL21 and 5.2 ng/mL22 respectively. In theory, patients with lower endoxifen levels might then potentially benefit from higher tamoxifen intake than the standard oral daily dose of 20 mg in order to generate adequate levels of active metabolites. To better understand this, prospective studies are required that compare genotype-based dosing with standard treatment and—importantly—with sufficiently long follow-up time. It is already clear that endoxifen plasma concentrations differ between patients with normal CYP2D6
genotype and patients defined as poor metabolizers, i.e. carrying 2 null alleles.\textsuperscript{21,26} However, less is known about the impact of reduced-function alleles of CYP2D6 on endoxifen formation.

In the present study, the primary aim was to investigate the relationship between different CYP2D6 genotypes and plasma concentrations of tamoxifen metabolites in premenopausal breast cancer patients on adjuvant treatment. The specific interest in premenopausal patients came from our previous observation that the impact of CYP2D6 genotype on tamoxifen treatment outcome was derived mainly from premenopausal women.\textsuperscript{8} We then hypothesized that formation of endoxifen was especially critical in women with higher systemic oestrogen exposure, and that it would be relevant to characterize the relationship between CYP2D6 genotype and endoxifen formation in younger patients.

The focus was primarily on reduced-function CYP2D6 alleles and in particular CYP2D6*41, which is the most common reduced-function variant among Swedes and other Europeans.\textsuperscript{27} Another aim was to explore the relationship between endoxifen levels and reported side effects during ongoing treatment.

2 | METHODS AND MATERIALS

2.1 | Patients

Since 2006, DNA from peripheral blood has been collected and biobanked from newly diagnosed breast cancer patients at the Karolinska University Hospital and at the South General Hospital, Stockholm, Sweden. A detailed description of the selection of study population is depicted in Figure S1. From the regional and national (INCA) breast cancer registries, we identified 1249 breast cancer patients undergoing surgery January 2006–January 2014 in Stockholm who initiated postoperative treatment with adjuvant tamoxifen at the Departments of Oncology at Karolinska University Hospital/South General Hospital, with available DNA.

Data on menopausal status, tumour parameters, body mass index, treatment, concomitant medication with CYP2D6 inhibitors, compliance and reported side-effects from the start of tamoxifen until the time of sample collection of tamoxifen metabolites and follow-up was obtained from medical records. Patients were considered postmenopausal 1 year after their last menstrual period or after bilateral oophorectomy. If there was unequivocal information in the records that the patient had stopped taking the treatment prematurely, she was classified as noncompliant.

Of the 1249 patients, 511 were premenopausal at baseline. Of these, 196 women still had ongoing adjuvant tamoxifen treatment in January 2017. The base of the present study consisted of 190 patients, still residing in the Stockholm area, who were invited to participate in the study by providing a blood sample for quantification of tamoxifen metabolites. Of the eligible patients, 118 were willing to participate in the study and were included after providing informed consent. The sample used for endoxifen measurement was collected after the patient agreed to participate in this study. Two patients with inadequate blood samples and 2 patients with undetermined CYP2D6 genotype were excluded. Thus, 114 patients were included in the analysis.

This cohort or dataset (Table 1) has not been analysed or published before.

2.2 | Ethics committee approval

The study was approved by the ethical review board at Karolinska Institutet (Stockholm, Sweden Dnr 2014/427–31 and 2016/1184–31). Patients agreeing to participate in the study consented to publication of the results in a scientific paper.

2.3 | Genotyping procedure

Analysis of CYP2D6 variant alleles was performed using TaqMan-based real time polymerase chain reaction assays rigorously validated and implemented for routine clinical pharmacogenetic analyses at Dianonshemmet Hospital in Oslo, Norway. The CYP2D6 genotyping panel was based on the recommendations by Schroth et al.\textsuperscript{28} and included the noncoding (null) variants CYP2D6*3 (rs35742686), CYP2D6*4 (rs3892097), CYP2D6*5 (gene deletion) and CYP2D6*6 (rs5030655), the decreased-function variants CYP2D6*9 (rs5030656), CYP2D6*10 (rs1065852) and CYP2D6*41 (rs28371725), and the increased-function variant CYP2D6*1XN. Without detection of any of the above variant alleles, the genotype was defined as CYP2D6*1/*1. In the copy number analysis, samples with CYP2D6 gene deletions were identified and interpreted as CYP2D6*5. The copy number analysis made it possible to distinguish between 0–1–2–3–4 allele copies but did not reveal whether CYP2D6*1, CYP2D6*4 or CYP2D6*41 was duplicated. With regards to the latter limitation, an inconclusive genotyping result with regards to predicted activity (i.e. CYP2D6*1/*4, CYP2D6*1/*41, or CYP2D6*4/*41, in combination with CNA = 3 or 4) would lead to study exclusion.

2.4 | Bioanalytical determination of endoxifen, N-desmethyl-tamoxifen, Z-4-hydroxy-tamoxifen and tamoxifen

Blood samples from each patient were collected in tubes containing EDTA anticoagulant and centrifuged at 1500 × g for 10 minutes. The plasma was transferred into polypropylene tubes and stored at –80°C until analysis. Plasma concentrations of tamoxifen, its 2 active metabolites Z-4-hydroxy-tamoxifen and Z-4-hydroxy-N-desmethyl-tamoxifen (earlier in the text and hereafter referred to as endoxifen), as well as N-desmethyl-tamoxifen (DM-tamoxifen) were measured by ultra-performance liquid chromatography–tandem mass spectrometry (TSQ Quantiva with Dionex Ultimate 3000 system, Thermo Scientific, Waltham, MA, USA). The analytical method was validated according to the Guideline on bioanalytical method validation
This method was developed in the Karolinska University Hospital Clinical Pharmacology laboratory, and a more detailed description is provided in Supplementary material.

2.5 | Predicted vs observed CYP2D6 activity

The predicted CYP2D6 activity was based on activity scores in CPIC and DPWG guidelines for the diplotypes null/null, null/CYP2D6*41, CYP2D6*41/*41, CYP2D6*1/null, CYP2D6*1/*41, CYP2D6*1/*1, CYP2D6*1/*1x2 and CYP2D6*1/*1x3, respectively. Importantly, the total activity of the normal genotype CYP2D6*1/*1 with 2 fully functional alleles was set to 2.0. The observed CYP2D6 activity was defined as the plasma concentration ratio between endoxifen and DM-tamoxifen, as this would take into account interindividual differences in demethylation capacity, mainly derived from CYP3A4, and thereby better isolate interindividual differences in CYP2D6-dependent 4-hydroxylation. The association between predicted and observed CYP2D6 activity was presented using linear regression. The regression line was constructed based on patients without reduced-activity alleles, and the 5 cases that carry CYP2D6*9 or CYP2D6*10 were excluded from the comparison in order to focus on CYP2D6*41.

2.6 | Side effects to tamoxifen in relation to plasma levels of endoxifen

Data on side effects of tamoxifen treatment from start of tamoxifen until the time of sample collection of tamoxifen metabolites was...
2.7 | Statistical analyses

The distribution of endoxifen concentrations for each predicted CYP2D6 genotype group was graphically investigated using boxplots and the proportion of patients with a potentially subtherapeutic concentration <5.9 ng/mL was calculated. The linearity between predicted CYP2D6 activity according to CPIC and DPWG¹⁹ and observed CYP2D6 activity was investigated in a scatterplot with predicted activity on the x axis and observed ratio between endoxifen and DM-tamoxifen on the y axis as a measure of inter-individual differences in CYP2D6-dependent 4-hydroxylation.⁴ A regression line indicating perfect linearity was included in the plot to support interpretation. Due to current uncertainties regarding the enzyme activity associated with reduced-activity CYP2D6 variants, only null alleles, the wild-type allele (CYP2D6*1, with or without duplications) and the reduced-activity CYP2D6*41 allele were included in the linear regression. For carriers of the CYP2D6*41 alleles, the proportion of individuals with an observed CYP2D6 activity below the regression line (i.e. a lower activity than predicted by CPIC and DPWG¹⁹) was calculated. For symmetrically distributed activities, valid predictions would be expected to result in a proportion close to 0.5, while a considerably higher proportion could indicate systematic under-prediction of the activity in these patients. This deviation was formally tested by means of the test of given proportions (prop.test), comparing the observed proportion to 0.5.

Differences in endoxifen levels between homozygous carriers of null alleles vs patients homozygous for CYP2D6 red/red, or CYP2D6 red/null genotypes were compared by means of the Kruskal-Wallis test.

Finally, the proportion of patients presenting with side effects was compared between the endoxifen concentration quintiles (described above) by means of the Fisher’s exact test.

In all analyses, P-values (2-sided test) <.05 were considered statistically significant.

Statistical analyses were performed using R 3.6.1 (R Core Team, R Foundation for Statistical Computing Vienna, Austria, 2019 [https://www.R-project.org]).

3 | RESULTS

3.1 | Baseline characteristics

In total, 114 patients were available for analysis in the study. Baseline characteristics are presented in Table 1. The median age was 46.9 years at the time of breast cancer diagnosis. Mean duration of tamoxifen treatment at blood sampling was 4.7 years. A majority (76.3%) had received tamoxifen only as adjuvant endocrine therapy while 17% of patients had switched from a combination of tamoxifen and goserelin and 1 patient still used this combination. A minority of patients (6.1%) had previously switched to an aromatase inhibitor but later restarted on tamoxifen. According to medical records, no patients were comedicated with drugs described as clinically relevant CYP2D6 inhibitors.

3.2 | Concentrations of tamoxifen metabolites and CYP2D6 genotype distribution

Table 2 presents the concentration of endoxifen and CYP2D6 genotype distribution in the study population as well as the CYP2D6 allele frequencies. As expected from a mainly Caucasian patient population, CYP2D6*41 was the most common variant among reduced-function alleles (82%), and CYP2D6*41 was found in combination with a CYP2D6 null allele in 5.3% of included patients (relevant for the discussion below). Two percent of the patients were defined as CYP2D6 UM, 47% as EM, 44% as IM and 7% as PM according to current guidelines from CPIC/DPWG.¹⁹ A more detailed description of the plasma level of tamoxifen and corresponding metabolites is provided in Table S1.

3.3 | Plasma endoxifen concentration in relation to CYP2D6 genotype

The relationship between major groups of CYP2D6 genotypes and plasma endoxifen levels is presented in Figure 1. Here, it is evident that most carriers of the CYP2D6*1 allele had endoxifen concentrations in plasma that were higher than the suggested threshold for tamoxifen efficacy at 5.9 ng/mL.²¹ However, 32% of all patients in the study had endoxifen concentrations below this level. Importantly, not only carriers of 2 null alleles (null/null) but also patients with 2 reduced-function alleles (red/red), or a null allele in combination with a reduced-function allele (null/red), had endoxifen concentrations clearly below this level (1.6–5.2 ng/mL). In fact, the endoxifen plasma concentration amongst patients with 2 reduced function alleles (defined as IM according to CPIC and DPWG) were essentially at the same level as patients classified as PM by carrying 2 null alleles. There were no statistical differences (P = .338) in endoxifen levels between carriers of 2 null alleles; median (IQR) 3.4 (2.3–4.1) ng/mL vs patients genotyped as CYP2D6*41/*41; 2.1 (1.9–2.2) ng/mL, or CYP2D6*41/null; 3.0 (2.6–3.2) ng/mL.
3.4 | Predicted vs observed CYP2D6 activity

Figure 2 shows the predicted CYP2D6 activity, based on CPIC and DWPG latest guidelines,\textsuperscript{19} vs the observed CYP2D6 activity as measured by the individual ratio between plasma concentrations of endoxifen to N-desmethyl-tamoxifen. 17/21 (81\%) of CYP2D6*41 carriers were below the regression line, i.e. had a significantly lower CYP2D6 activity than predicted (P > .01). In line with this finding, it was evident that the CYP2D6 metabolic ratio in patients genotyped as CYP2D6*1/*41 was at a similar level as patients genotyped as CYP2D6*1/null.

3.5 | Side effects

Seventy percent of the included patients experienced side effects to tamoxifen, i.e. during ongoing therapy for many years, and most commonly concerned hot flashes (52\%). Reported side effects in relation to tamoxifen, including hot flashes, breast tenderness, arthralgia, and myalgia, were more frequent among patients with CYP2D6*4/*5 and CYP2D6*5/*5 genotypes compared to those with CYP2D6*1/*1 or CYP2D6*1/*1x2 genotypes. These findings highlight the importance of considering genetic variability in drug metabolism when prescribing tamoxifen for breast cancer patients.

### Table 2

The plasma level of endoxifen in 114 breast cancer patients listed according to CYP2D6 genotype and corresponding phenotype according to Pharmacogenetics Implementation Consortium and the Dutch Pharmacogenetics Working Group.\textsuperscript{19}

| CYP2D6 genotype | n (% of 114) | Endoxifen (ng/mL) median (IQR) |
|-----------------|--------------|--------------------------------|
| CYP2D6*1/*1     | 2 (1.8)      | 12.8 (9.7–16.0)                |
| CYP2D6*1/*1 n > 2 | 37 (32.5)    | 10.10 (7.5–13.5)               |
| CYP2D6*1/*9     | 2 (1.8)      | 11.7 (10.6–12.7)               |
| CYP2D6*1/*10    | 2 (1.8)      | 9.80 (9.2–10.2)                |
| CYP2D6*1/*41    | 13 (11.4)    | 9.28 (5.8–9.9)                 |
| CYP2D6*1/*3     | 2 (1.8)      | 5.92 (5.6–6.2)                 |
| CYP2D6*1/*4     | 33 (28.9)    | 7.24 (4.0–10.5)                |
| CYP2D6*1/*5     | 6 (5.3)      | 8.40 (4.5–10.2)                |
| CYP2D6*4/*10    | 1 (0.9)      | 4.7 (–)                        |
| CYP2D6*4/*41    | 4 (3.5)      | 3.01 (2.7–3.3)                 |
| CYP2D6*5/*41    | 1 (0.9)      | 3.04 (–)                       |
| CYP2D6*6/*41    | 1 (0.9)      | 1.50 (–)                       |
| CYP2D6*41/*14   | 2 (1.8)      | 2.10 (1.9–2.2)                 |
| CYP2D6*3/*4     | 2 (1.8)      | 3.81 (3.1–4.5)                 |
| CYP2D6*4/*4     | 3 (2.6)      | 4.00 (3.4–4.1)                 |
| CYP2D6*4/*5     | 1 (0.9)      | 4.1 (–)                        |
| CYP2D6*4/*6     | 1 (0.9)      | 1.99 (–)                       |
| CYP2D6*5/*5     | 1 (0.9)      | 1.55 (–)                       |
| CPIC/DPWG phenotype | n (% of 114) | Endoxifen (ng/mL) median (IQR) |
| CYP2D6 UM       | 2 (1.8)      | 12.8 (9.7–16.0)                |
| CYP2D6 EM       | 54 (47.4)    | 9.60 (7.5–12.2)                |
| CYP2D6 IM       | 50 (43.9)    | 6.45 (3.5–9.2)                 |
| CYP2D6 PM       | 8 (7.0)      | 3.35 (2.3–4.1)                 |

IQR, interquartile range

### Figure 1

Plasma endoxifen concentrations in relation to major groups of CYP2D6 genotypes with regards to the combination of fully functional (CYP2D6*1), reduced activity (red) and null alleles (see methods). The dashed line indicates a previously suggested therapeutic threshold at 6.0 ng/mL for clinical efficacy.\textsuperscript{21}

### Figure 2

Predicted vs observed CYP2D6 activity with focus on CYP2D6*41. The predicted CYP2D6 activity was based on activity scores in Pharmacogenetics Implementation Consortium and the Dutch Pharmacogenetics Working Group guidelines for the diplotypes null/null, null/*41, CYP2D6*1/*41, CYP2D6*1/*1, CYP2D6*1/*1x2 and CYP2D6*1/*1x3, respectively. The total activity of the normal genotype CYP2D6*1/*1 with 2 fully functional alleles was set to 2.0. The observed CYP2D6 activity was defined as the plasma concentration ratio of endoxifen over N-desmethyl-tamoxifen (see methods). The regression line was constructed based on patients carrying wild-type (*1), null and *41 alleles, and the 5 cases that carry CYP2D6*9 or CYP2D6*10 have been excluded to simplify the comparison. For additional visual clarity, a random jitter has been added to the predicted activity in the plot, but not in the statistical analyses.
to individual endoxifen levels are presented in Figure 3. Almost half of the patients with no or only mild side effects had endoxifen levels <5.9 ng/mL, implying that the absence of side effects is closely linked to potentially subtherapeutic levels of endoxifen. Patients with endoxifen concentrations in strata 3 and 4 (endoxifen > 10.2 ng/mL) were significantly more likely to report severe side effects than patients in strata 1 and 2 \( (P = .001) \). All patients in the strata 4 (endoxifen > 14.6 ng/mL) reported side effects, of which 63% were considered mild-to-moderate and 38% severe.

4 | DISCUSSION

In this tamoxifen-treated breast cancer cohort of premenopausal women, low plasma levels of endoxifen were observed not only in patients with 2 null-alleles but also in patients with 2 reduced-function alleles and in patients that carry 1 reduced-function allele in combination with a null allele (Figure 1). This suggest an overall impact of reduced-function alleles of CYP2D6 on endoxifen formation that is greater than expected from current activity score assignments. More specifically, it was evident in the present material that the carriers of the most common reduced-function allele in Caucasians, CYP2D6*41, had significantly lower CYP2D6 metabolic activity than predicted from CPIC and DPWG guidelines, as illustrated in Figure 2. Interestingly, a recent study based on >1000 Norwegian samples from routine therapeutic drug monitoring of the antidepressant venlafaxine arrived at a similar conclusion regarding a surprisingly great impact of the CYP2D6*41 allele on the individual metabolic capacity.

It follows that the group of poor bioactivators of tamoxifen in clinical practice might be substantially larger than now understood from the definition of PM, which is currently restricted to carriers of 2 null alleles. In several studies, such PM patients have been associated with a worse prognosis than other genotypes during adjuvant treatment with tamoxifen, but perhaps the conservative PM definition will fail to detect that additional CYP2D6 genotypes with very low metabolic activity also could be at high risk of treatment failure.

There is as yet no established therapeutic target range for the individual endoxifen concentration in plasma. The relationship between CYP2D6 genotype, steady-state concentration of endoxifen and clinical effects clearly needs to be further clarified. The published studies showing that low endoxifen concentrations predicted a poorer outcome were both based on a retrospective material. Madsen and co-workers divided observed plasma levels of endoxifen into 5 strata, where patients in the lowest quintile (20% of patients) had a poorer outcome than the other quintiles. The corresponding cut-off of endoxifen in plasma of around 5.9 ng/mL is therefore purely a biostatistical cut-off from that large collection of patient samples, leaving the predictive value for treatment outcome quite uncertain at this stage. A recent prospective study failed to find any link between CYP2D6 genotype, endoxifen levels and relapse in breast cancer. However, a potential explanation for this negative finding could be that 6.4-year follow-up after 2.5-year median duration of tamoxifen treatment would be too short to detect an impact of variable CYP2D6-dependent tamoxifen metabolism.

Importantly, none of the patients with endoxifen levels <5.9 ng/mL had severe side effects of tamoxifen. It was revealed that almost half of all patients without side effects or with only mild symptoms, were exposed to potentially subtherapeutic levels of endoxifen. The clinical experience is that side effects to tamoxifen are quite common. Indeed, in our study population the great majority of patients (70%) reported side effects. As shown in Figure 3, patients with endoxifen concentrations endoxifen >10.2 ng/mL (i.e. corresponding to strata 3–4 in the Madsen study) and 29% of the present study population) had significantly more severe side effects than patients with lower endoxifen levels. These findings are in line with a recent investigation where dose reduction of tamoxifen from 20 to 10 mg daily with a corresponding drop in endoxifen plasma levels from approximately 10 to 5 ng/mL, was associated with less problems of
hot flushes. More evidence for a concentration-dependent risk of adverse effects to the standard dose of tamoxifen comes from recent Swedish data, showing that patients genotyped as CYP2D6 UM, and thereby expected to have higher endoxifen levels at the standard dose of tamoxifen seemed to experience more severe side effects and that this would increase the risk of early treatment discontinuation during the first 6 months.

Our study has some important limitations. We believe that the inclusion of patients with a long treatment duration (mean of 5 years) caused a selection bias towards cases with higher tolerability to tamoxifen treatment. Patients with serious adverse effects should therefore be under-represented and the proportion of patients in different concentration ranges and reported side-effects (Figure 3) might well look different during the first months after treatment initiation, as discussed above. Other limiting factors include the retrospective collection of data regarding compliance and incomplete information on concomitant medication with CYP2D6 inhibitors from medical records. However, with regards to adherence, it needs to be remembered that informed consent for study inclusion was dependent on continued drug intake and with the expressed purpose to measure drug concentrations. We have no reason to assume that this attracted participants who already had discontinued treatment and, more importantly, we did indeed observe tamoxifen levels in all samples that matched previously reported concentrations at 20 mg daily. Finally, an obvious limitation of this study is the limited size, and therefore many of our statistically significant findings will require confirmation in larger study settings. Conversely, this premenopausal patient population is well-characterized from a clinical point of view (Table 1) and it is questionable whether inclusion of also postmenopausal women would help to draw more firm conclusions. It may be argued that tolerability to tamoxifen looks different between pre- and postmenopausal women. In addition, the pharmacokinetics of tamoxifen bioactivation might also differ between the 2 groups. Interestingly, it was reported that the metabolism of the antidepressant venlafaxine, like tamoxifen, a drug substrate for both CYP2D6 and CYP3A4, differed between CYP2D6 genotypes in an age-dependent fashion, possibly explained by a reduced CYP3A4 activity in postmenopausal women. Taken together with our previous notion that CYP2D6 genotype appeared to be more important for tamoxifen treatment outcome in premenopausal women we believe that more work should specifically address treatment optimization in younger patients, as they have fewer therapeutic options. Thus, we consider our study exploratory in order to initiate much larger studies that based on sample size calculation will include sufficient numbers of premenopausal patients for the clarification of the relevance of CYP2D6 genotyping in tamoxifen-taking premenopausal breast cancer patients.

Both in the present study and in earlier reports patients with higher concentrations of endoxifen were predominantly carriers of CYP2D6*. However, a considerable variability among CYP2D6* carriers was evident (Figure 1) and, in analogy with earlier observations, some patients genotyped as extensive metabolizers were associated with low plasma levels of endoxifen. The explanation for this is still unknown but could relate to rare genetic variants of CYP2D6 that escape detection by current allele-specific polymerase chain reaction methodology. So, not only is it still controversial whether dosing of tamoxifen should be individualized but also if such a strategy should be based on CYP2D6 genotype or on individual endoxifen measurements. It seems quite clear, however, that genotyping may help to identify the great majority of patients that generate only low levels of endoxifen and that a confirmatory plasma concentration measurement may be a logical follow-up to any genotype-based decision on dose. Several previous studies have investigated whether a dose escalation from 20 to 40 mg of daily tamoxifen in CYP2D6 PM or IM patients results in higher plasma endoxifen. Indeed, this turned out to be true but the absolute effect in poor metabolizers appeared more limited and resulted in still potentially subtherapeutic concentrations of endoxifen. Based on the results from the present investigation, it is suggested that a dose increase to 40 mg daily would be insufficient not only in PM subjects but also in the subgroup of IM subjects that carry 2 reduced-function alleles or 1 reduced-function allele in combination with a null allele.

To summarize our findings in a relatively limited cohort of tamoxifen-treated breast cancer patients, a profound impairment of endoxifen formation was evident not only in patients with CYP2D6 null alleles but also in patients with reduced-function CYP2D6 alleles, most importantly CYP2D6*41. This might be of significant relevance for genotype-based dosing of tamoxifen. Future studies with larger sample sizes are required to better define the critical plasma concentrations of endoxifen required to secure therapeutic efficacy and whether individualized dosing of tamoxifen based on CYP2D6 genotype will help to further improve patient compliance and clinical outcome.

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Competing Interests
There are no competing interests to declare.

Contributors
Concept and study design: L.T., J.D.L., S.M., E.E. Provision of patient samples S.M., L.T., J.B. Provision of analytical methodology and collection of data: G.A., P.H., E.M., M.K.K., J.D.L. Assembly and interpretation of data: L.T., S.M., J.D.L., E.E., E.M., M.K.K., J.B. Manuscript
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DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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