Phenotyping in oncology

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The use of the ^{13}C-dextromethorphan breath test for phenotyping CYP2D6 in breast cancer patients using tamoxifen: Association with *CYP2D6* genotype and serum endoxifen levels

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ABSTRACT

Purpose: Adjuvant therapy with tamoxifen significantly reduces breast cancer recurrence and mortality in estrogen receptor positive disease. CYP2D6 is the main enzyme involved in the activation of the prodrug tamoxifen into the anti-estrogen endoxifen. Endoxifen is thought to be a main determinant for clinical efficacy in breast cancer patients using tamoxifen. As the large interindividual variation in endoxifen levels is only partly explained by *CYP2D6* genotype, we explored the use of the ^{13}C-dextromethorphan breath test (DM-BT) for phenotyping CYP2D6 and to predict serum steady-state endoxifen levels as a marker for clinical outcome in breast cancer patients using tamoxifen.

Methods: In 65 patients with early breast cancer using tamoxifen, CYP2D6 phenotype was assessed by DM-BT. *CYP2D6* genotype using Amplichip and serum steady-state levels of endoxifen were determined. Genotype was translated into the gene activity score and into ultrarapid, extensive, heterozygous extensive, intermediate or poor metabolizer CYP2D6 predicted phenotype.

Results: CYP2D6 phenotype determined by the DM-BT explained variation in serum steady-state endoxifen levels for 47.5% (R^2=0.475, $p<0.001$). Positive and negative predictive values for a recently suggested threshold serum level of endoxifen (5.97 ng/ml) for breast cancer recurrence rate were 100% and 90% respectively for both CYP2D6 phenotype by DM-BT (delta-over-baseline on t=50 minutes (DOB_{50}) values of 0.7–0.9) and genotype (*CYP2D6* gene activity score of 1.0).

Conclusion: DM-BT might be, along with *CYP2D6* genotyping, of value in selection of individualized endocrine therapy in patients with early breast cancer, especially when concomitant use of CYP2D6 inhibiting medication alters the phenotype.

Trial registration: The Netherlands Trial Register (NTR1509).

INTRODUCTION

Tamoxifen has been used for more than three decades in the adjuvant treatment of localized estrogen receptor positive (ER+) breast cancer. Five years of adjuvant tamoxifen reduces 15-year breast cancer recurrence and mortality by nearly a third in women with ER+ breast cancer. However, in one third of patients disease recurs, suggesting non-response to the antiestrogen action of tamoxifen [1]. The interpatient variability in response to tamoxifen might be due to estrogen receptor density in the tumor [1], specific estrogen receptor alfa splice variants (like ERα36) [2] and variability in biotransformation to the active species endoxifen.

Tamoxifen has limited affinity for the ER and is considered to be a prodrug [3]. CYP2D6 and CYP3A are thought to be the most important enzymes for biotransformation of tamoxifen into the active metabolites 4-hydroxy-tamoxifen (4-OH-TAM) and endoxifen.Other isoforms that are involved in the metabolism of tamoxifen include CYP2C19, CYP2B6, CYP2C9 and CYP1A2 [4]. Both 4-OH-TAM and endoxifen have a 30 to 100-fold higher affinity for the ER than tamoxifen [5] and exhibit the same strong anti-estrogen potency [5-7].

Concentrations of tamoxifen and its metabolites in tumor tissues are significantly correlated to their serum levels [8]. Serum endoxifen levels are 10 to 12 times higher than 4-hydroxy-tamoxifen levels, suggesting that endoxifen is the most important metabolite of tamoxifen [7;9;10].

Studies correlating the interindividual response to tamoxifen and pharmacogenetics of the most important enzymes involved in the biotransformation of tamoxifen and metabolites are conflicting [11;12]: some studies show decreased breast cancer recurrence-free survival in CYP2D6 poor metabolizers (PM) predicted phenotype compared with extensive metabolizers (EM) predicted phenotype [13-16], whilst others fail to show any association [17-20], although results from the large Arimidex, Tamoxifen, Alone or in Combination (ATAC) trial [19] and the Breast International Group (BIG) 1-98 Trial [20] have been questioned regarding insufficient quality of pharmacogenetic data [21]. One study demonstrated even a better recurrence-free survival in CYP2D6 PMs [22].

Variation in response to tamoxifen has been attributed to variations in endoxifen concentrations in carriers of variant alleles. Patients with CYP2D6 PM predicted phenotype have 2 to 4-fold lower endoxifen levels compared to patients with EM predicted phenotype [16;23;24;24;25]. However the variation in endoxifen [25] and (Z)-endoxifen [26] (the active stereo-isomer of endoxifen) serum concentrations is explained by *CYP2D6* genotype for only 23% [25] and 39% [26] of patients on tamoxifen therapy respectively. Among CYP2D6 poor metabolizers, 93% had (Z)-endoxifen levels below IC_{90} values, underscoring the role of CYP2D6 metabolism in tamoxifen

bioactivation [26]. A minimal threshold concentration has recently been suggested, above which endoxifen reduces the risk of recurrence of breast cancer [23]. Therefore, endoxifen levels may serve as a predictor for clinical outcome.

Many drugs inhibit CYP2D6 activity. Co-administration of the strong CYP2D6 inhibitor paroxetine decreases plasma concentration of endoxifen by 58–72% in CYP2D6 EMs [10;24;25]. However, studies on concomitant use of strong CYP2D6 inhibiting drugs with tamoxifen show inconsistent results with respect to breast cancer recurrence and mortality risk [27-32]. Therefore, it is thought that, in addition to the *CYP2D6* genotype, the presence of many exogenous/environmental (epigenetic) factors interact and contribute to the phenotype.

Dextromethorphan (DM) metabolism has similarities with tamoxifen metabolism and has been used as a phenotyping probe [33]. DM is metabolized to dextrorphan (DXO) through *O*-demethylation by CYP2D6, to 3-methoxymorphinan (MEM) through *N*-demethylation by CYP3A, and to 3-hydroxymorphinan (HYM) through *N,O*-didemethylation by both enzymes [33].

A ^{13}C-dextrometorphan breath test (DM-BT) has been developed to rapidly and selectively assess CYP2D6 phenotype [34].

Assessing CYP2D6 phenotype, which includes both genetic, epigenetic, and environmental factors would be potentially better than using *CYP2D6* genotype to predict breast cancer recurrence rate by means of serum steady-state endoxifen levels. The aim of this study was to explore the utility of the DM-BT in clinical practice to evaluate CYP2D6 phenotype and to predict serum steady-state endoxifen levels in breast cancer patients using tamoxifen.

METHODS

Study patients

A total of 65 patients were recruited from the multicenter, prospective CYPTAM study (NTR1509) aiming to relate *CYP2D6* genotype and endoxifen serum levels to tamoxifen efficacy.

Women 18 years and older with early stage estrogen receptor positive breast cancer who were on adjuvant tamoxifen therapy of tamoxifen at a daily dose of 20 mg for at least two months (to ensure steady-state concentrations of tamoxifen and metabolites), were eligible. Excluded from the study were patients unwilling to fast overnight, to abstain from alcohol for at least 24 hours prior to the DM-BT, with known allergy to DM, with grade ≥ 3 liver or renal impair-

ment, hypercapnia, concomitant use of MAO-inhibitors or medication slowing gastrointestinal motility.

Study drugs

Clinical trial material grade ^{13}C-DM (API) was synthesized by Cambridge Isotope Laboratories (Andover, Massachusetts, U.S.A.) as a powder meeting USP standards. Production of the drug substance meets Good Manufacturing Practice (GMP) guidelines. The oral liquid formulation (2.08 mg/mL) was manufactured under GMP conditions in the GMP facility of Confab Laboratories Inc. in Montreal, Canada.

Two Alka Seltzer Gold® tablets (anhydrous citric acid, potassium bicarbonate and sodium bicarbonate) were administered to patients prior to ^{13}C-DM ingestion. These tablets were used to increase gastrointestinal motility leading to faster absorption and transport of the substrate ^{13}C-DM to the liver to be metabolized to dextrorphan and $^{13}CO_2$ [34].

Study design

The protocol was approved by the institutional review boards of all three participating study sites, and all patients provided written informed consent.

The study was registered in The Netherlands Trial Register (NTR1509, www.trialregister.nl).

A tube of ten mL of blood was collected up to 12 hours before the next dose of tamoxifen, centrifuged and stored at -20°C for serum analysis of tamoxifen and metabolites [35]. Prior to the administration of Alka Seltzer solution and ^{13}C-DM (0.5 mg/kg with a maximum dose of 60 mg), patients provided a baseline breath sample in a 1.3 L breath bag. Fifty minutes after administration of ^{13}C-DM, a second breath sample was collected. Patients were monitored during 50 minutes and adverse events were recorded.

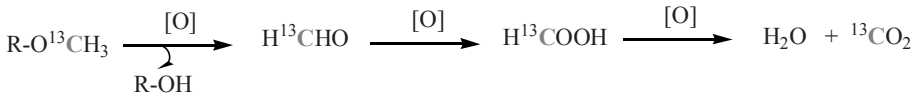
Analysis of *CYP2D6* genotype and translation to predicted phenotype

Blood samples were collected to determine *CYP2D6* genotype by the Amplichip array (Amplichip, Roche). *CYP2D6* genotype was translated into an ultrarapid (=UM e.g. XN *1), extensive (=EM, e.g. *1/*1), heterozygous extensive (=hetEM, e.g. *1/*4), intermediate (=IM, e.g. *41/*41) or poor metabolizer (=PM, *4/*4) predicted phenotype. CYP2D6 activity score was determined for each patient according to the method introduced by Gaedigk [36].

CYP2D6 gene activity score was tested for association with CYP2D6 phenotype by DM-BT. Predicted phenotype and CYP2D6 gene activity score were tested for association with endoxifen levels and other metabolites of tamoxifen. Two patients using CYP2D6 inhibiting medication were excluded from association analysis between CYP2D6 activity score or predicted phenotype and endoxifen serum levels.

Breath sample analysis and quantification of CYP2D6 phenotype

Isotopically labeled CO_2 breath test have been investigated as minimally invasive procedures for estimating drug biotransformation activity [37]. ^{13}C-labeled DM-BT is dependent on CYP2D6 *O*-demethylation. The released methyl group is involved in the formation of $^{13}CO_2$ that is released in expired breath over time:



$^{13}CO_2$ and $^{12}CO_2$ in exhaled breath samples is measured by IR spectrometry using the FDA approved POCone IR spectrometer (Photal Otsuka Electronics, Japan). The amount of $^{13}CO_2$ present in breath samples is expressed as a delta over baseline ratio (DOB) that represents a change in the $^{13}CO_2/^{12}CO_2$ ratio of breath samples collected before and after ^{13}C-dextromethorphan ingestion [38].

Delta-over-baseline after 50 minutes (DOB_{50}) values were calculated from baseline and postdose (t=50 min.) breath samples:

> DOB_{50}=1000x[($^{13}CO_2/^{12}CO_2$) Postdose - [($^{13}CO_2/^{12}CO_2$) Baseline]/R_{PDB} where DOB is expressed in units of Δ per mil (‰), and R_{PDB}=0.012373 is $^{13}C/^{12}C$ in PDB (international standard Pee Dee Belemnite)

DOB_{50} values were tested for association with serum levels of tamoxifen and other metabolites.

Calculation of sensitivity, specificity, positive and negative predictive values

We chose the reported threshold serum endoxifen concentration of 5.97 ng/mL, above which the recurrence rate of breast cancer appeared to be lower [23] to calculate sensitivity, specificity, positive (PPV) and negative predictive values (NPV) for gene activity scores and DOB_{50} values (Table 4.2) as follows:

Sensitivity = true positives/(true positives+false negatives)

Specificity = true negatives/(true negatives+false positives)

PPV = true positives/(true positives+false positives)

NPV=true negatives/(true negatives+false negatives).

Analysis of serum levels of tamoxifen and metabolites

Serum levels of tamoxifen, 4-hydroxytamoxifen, N-desmethyltamoxifen and endoxifen were analyzed by a validated HPLC-MS/MS assay [35].

Statistical analysis

Pharmacokinetic data were presented as mean values with standard deviation and 95% confidence interval or as median with a range, as indicated. To test significance of difference in mean endoxifen levels between predicted phenotype subgroups, F-test was used. In correlation analysis, levels of tamoxifen and metabolites as well as DOB_{50} values were additionally log transformed, to correct for outliers. To test association between gene activity score (ordinal scale) with serum steady state concentrations of tamoxifen and metabolites the non-parametric Spearman rank correlation test was used. To test association between DOB_{50} values (continuous scale) and serum steady state concentrations of tamoxifen and metabolites Pearson's correlation coefficient was used. Multiple testing has been corrected for by Bonferroni method by dividing α=0.05 by the total number of tests (n=30). All statistical tests were two-sided. Statistical analysis was performed with SPSS version 15.0.

RESULTS

Study population

Sixty-six female patients with resected localized breast cancer were enrolled in the study. All patients were in follow-up, and at the time of study enrollment, no recurrence of disease was documented. One patient was excluded because of undetectable levels of tamoxifen and metabolites, reflecting non-compliance to tamoxifen. The mean age of patients was 56.5 years (39–84 years). All patients were taking tamoxifen for at least two months with a mean of 21.2 months (range 11–46 months).

Adverse events

One patient experienced grade 1 nausea after administration of ^{13}C-DM which disappeared after 50 minutes of observation.

Endoxifen levels

Mean endoxifen levels were 16.85 (95% CI 14.52–19.28 ng/mL, SD 6.52) in EMs predicted phenotype (n=30), which were significantly ($p<0.001$) higher than in hetEMs (n=23; 10.71; 95% CI 9.00–12.41, SD 3.95 ng/mL), IMs (n=4; 5.63 95% CI 4.49–6.76, SD 0.71ng/mL), and PMs (n=8; 3.48 95% CI 2.33–4.62, SD 1.37 ng/mL).

Two patients with EM predicted phenotype concomitantly used CYP2D6 inhibiting medication (citalopram and paroxetine) and had endoxifen levels of 10.6 and 6.6 ng/mL. These levels are in the 2.5% lowest range of endoxifen levels in EMs and match mean endoxifen levels in hetEMs and IMs indicating possible phenoconversion.

Correlation between CYP2D6 gene activity, CYP2D6 phenotype by DM-BT and serum endoxifen levels

Mean DOB_{50} was 2.82 (range 1.2–4.4) for EMs (n=30), 2.77 for hetEM (n=23 range 0.9–4.5), 0.73 for IMs (n=4; range 0.4–1.1) and 0.18 for PMs (n=8; range 0–0.5).

CYP2D6 gene activity score explained 48.7% of variance in the log transformed CYP2D6 phenotype by DM-BT (R^2=0.487, $p<0.001$, n=63, Figure 4.1), and 57.8% of variance in log endoxifen serum levels (R^2=0.578, $p<0.001$, n=63 (Figure 4.2a)). Correlations between *CYP2D6* gene activity score and serum concentrations of tamoxifen, 4-hydroxytamoxifen and desmethyl-tamoxifen are shown in Table 4.1.

Correlation between CYP2D6 phenotype by DM-BT and endoxifen serum levels

Log-transformed CYP2D6 phenotype predicted variance in log endoxifen serum levels for 47.5% (R^2=0.475, $p<0.001$, n=65, Figure 4.2b). Two patients with EM predicted phenotype who concomitantly used CYP2D6 inhibiting medication, had low DOB_{50} values of 1.7 and 1.6 (mean DOB_{50} value: 2.82 for EMs) possibly due to phenoconversion, stressing the value of phenotyping in this group.

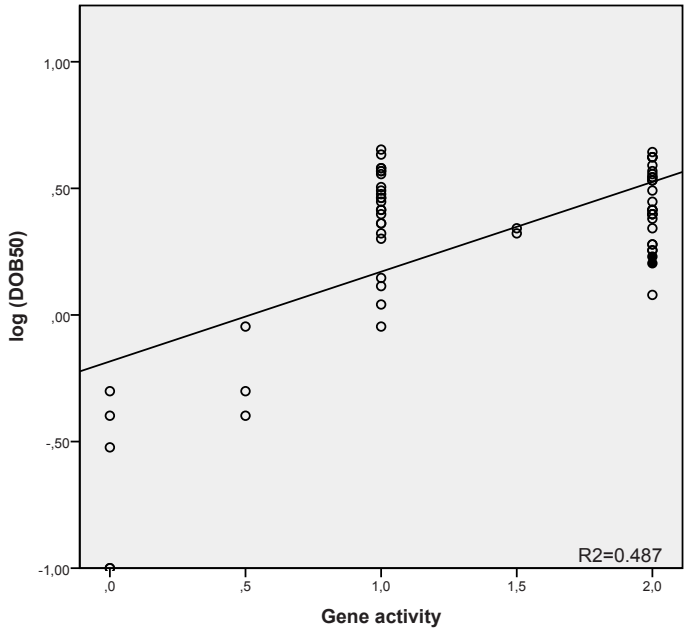


Figure 4.1 Correlation between CYP2D6 gene activity score and log transformed CYP2D6 phenotype by ^{13}C-dextromethorphan breath test (DM-BT) (N=63).
Abbrevations: DOB_{50}, Delta-over-baseline at t=50 min.
• = patient using CYP2D6 inhibiting medication.

Predictive value of CYP2D6 phenotype by DM-BT for suggested threshold endoxifen serum concentration

DOB_{50} value of less than 0.8 had a NPV of 90% for suggested subtherapeutic endoxifen serum levels (<5.97 ng/mL) [23]. Only one subject had a false negative (FN) result, which means that despite a low DOB_{50} value of 0.8 or less, the subject had endoxifen serum levels in the therapeutic range (Figure 4.3). PPV for suggested therapeutic endoxifen serum levels (≥5.97 ng/mL) was 100% for DOB_{50} cut-off values of 0.7–0.9 (Table 4.2). No subjects had a false positive (FP) result (i.e DOB_{50} value >0.8 despite suggested subtherapeutic (<5.97 ng/mL) endoxifen serum levels).

Predictive value of CYP2D6 (from genotype) predicted phenotype for suggested threshold serum endoxifen concentration

PPV for suggested therapeutic endoxifen serum levels (≥5.97 ng/mL was 100% and NPV for suggested subtherapeutic endoxifen serum levels (<5.97 ng/mL) was 90% for gene activity cut-off score of 1.0.

Table 4.1 Correlations (R^2*) between *CYP2D6* gene activity score(a), CYP2D6 clinical phenotype, determined by DM-BT (b), log transformed phenotype, determined by DM-BT (c) and (log-transformed) serum concentrations levels of tamoxifen and metabolites

Drug or metabolite pharmacokinetic parameter	Mean	SD	(a) Correlation (R^2¶) with CYP2D6 gene activity score	(b) Correlation (R^2*) with phenotype DOB_{50}	(c) Correlation (R^2*) with Log transformed DOB_{50}
TAM conc. (ng/mL)	128.8	42.0	0.001 (p=0.981, n=63)	0.009 (p=0.455, n=65)	0.034 (p=0.151, n=62)
END conc. (ng/mL)	12.34	6.93	0.578 (p<0.001£, n=63)	0.517 (p<0.001£, n=65)	0.511 (p<0.001£, n=62)
4-OH-TAM conc. (ng/mL)	2.371	0.941	0.298 (p<0.001£, n=63)	0.110 (p=0.007, n=65)	0. 132 (p=0.004, n=62)
N-Desmethyl-TAM conc. (ng/mL)	256.3	88.3	0.138 (p=0.0028, n=63)	0.171 (p<0.001£, n=65)	0.033 (p=0.154, n=62)
(END/N-desmethyl-TAM) ratio	0.0534	0.053	0.659 (p<0.001£, n=63)	0.347 (p<0.001£, n=65)	0.306 (p<0.001£, n=62)
Log TAM conc. (ng/mL)	2.089	0.136	0 (p=0.981, n=63)	0.002 (p=0.699 n=65)	0.048 (p=0.085, n=62)
Log END conc. (ng/mL)	1.016	0.274	0.578 (p<0.001£, n=63)	0.404 (p<0.001£, n=65)	0.475 (p<0.001£, n=62)
Log 4-OH-TAM conc. (ng/mL)	0.3437	0.164	0.298 (p<0.001£, n=63)	0.121 (p=0.004 n=65)	0. 166 (p<0.001£, n=62)
Log N-Desmethyl-TAM conc. (ng/mL)	2.386	0.140	0.138 (p=0.003, n=63)	0.144 (p=0.0018, n=65)	0.032 (p=0.162 , n=62)
Log (END/N-Desmethyl-TAM) ratio	-0.1370	0.324	0.659 (p<0.001£, n=63)	0.494 (p<0.001£, n=65)	0.501 (p<0.001£, n=62)

Abbrevations: conc., concentration; DOB_{50}, Delta-over-Baseline on t=50 min; END, endoxifen; N-Desmethyl-TAM, N-Desmethyltamoxifen; 4-OH-TAM, 4-hydroxytamoxifen; TAM, tamoxifen.

R^2*A Pearson's correlation coefficient was used to evaluate associations between DOB_{50} and tamoxifen pharmacokinetics.

R^2 A Spearman's correlation coefficient was used to evaluate associations between gene activity score and tamoxifen pharmacokinetics.

All statistical tests were two sided.

£ Significant correlation: each individual test was tested at a significance level of α/n=0.05/30=0.0017 (Bonferroni correction).

DISCUSSION

This is the first prospective phenotype study, in which the DM-BT was used to correlate CYP2D6 phenotype with endoxifen serum levels in breast cancer patients, who use tamoxifen in the adjuvant setting. The study shows that CYP2D6 phenotype determined by DM-BT correlates well with endoxifen serum levels. The correlation we found between CYP2D6 phenotype by DM-BT and log-transformed endoxifen serum levels (R^2=0.475, p<0.001, n=65) was almost similar to the correlation (R^2=0.518, p<0.001, n=35) between CYP2D6 phenotype determined by log DM

Table 4.2 Sensitivity, specificity, positive and negative predictive values of *CYP2D6* gene activity and DOB_{50} cut-off values for threshold (5.97 ng/mL) endoxifen serum concentrations (n=65)

Genotype or phenotype test cut-off values	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Gene activity <0.5	98.2	77.8	96.5	87.5
Gene activity <1.0	98.2	100	100	90
DOB_{50} <0.4	98.2	50	91.5	83.3
DOB_{50} <0.5	98.2	70	94.7	87.5
DOB_{50} <0.6	98.2	90	98.2	90.0
DOB_{50} <0.7	98.2	100	100	90.0
DOB_{50} <0.8	98.2	100	100	90.0
DOB_{50} <0.9	98.2	100	100	90.0
DOB_{50} <1.0	94.6	100	100	75.0
DOB_{50} <1.1	94.6	100	100	75.0
DOB_{50} <1.2	92.6	100	100	69.2

Abbreviations: DOB_{50}, Delta-over-Baseline on t=50 min; NPV, negative predictive value; PPV, positive predictive value.

serum levels (0 to 6 hours) and log-transformed AUC of endoxifen (0 to 24 hours), found by Graan et al. [39] in patients using tamoxifen for invasive breast cancer.

We preferred *CYP2D6* gene activity score, which incorporates the activity of each *CYP2D6* allele (0, 0.5, 1, or 2) [36], instead of the genotype, to correlate with pharmacokinetic parameters, as it better aggregates alleles with similar functionality.

Our study population included widely representative phenotypes. The CYP2D6 predictive phenotypes (i.e 46.2% EMs, 41.5% IMs (=hetEMs and true IMs combined) and 12.3% PMs) almost approached normal distribution in the Caucasian population [40]. In addition, endoxifen levels in EMs were significantly higher than in hetEMs, IMs, and PMs, consistent with results of other studies [24;25].

We observed a large interpatient variability of phenotype results in patients with hetEM (DOB_{50} range 0.9–4.5) and EM (DOB_{50} range 1.2–4.4) predicted phenotypes. This finding is in line with the large interpatient variability in the original DM-BT validation study with 30 healthy subjects [34] and might be due to analytical variations in DM-BT results as well as intersubject variations in absorption and first-pass biotransformation of ^{13}C-DM. Although patients who used medication slowing gastrointestinal motility (such as morphine) were excluded from the study, there still could be a large intra- and interindividual variation in biotransformation.

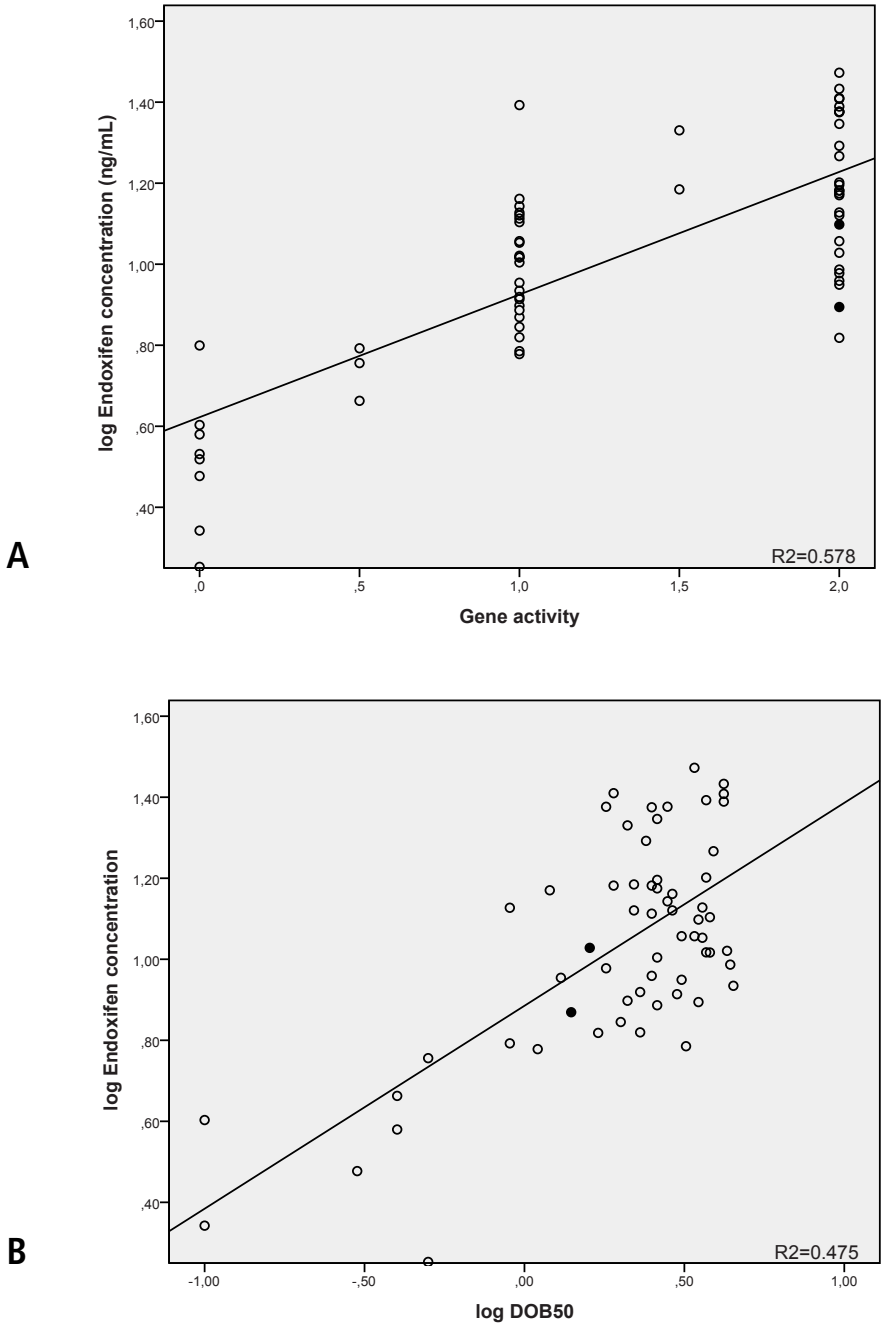


Figure 4.2 (A) Correlation between CYP2D6 gene activity score and log-transformed endoxifen serum levels (N=63). (B) Correlation between log-transformed CYP2D6 phenotype determined by ^{13}C-dextromethorphan breath test (DM-BT) and log-transformed endoxifen serum levels (N=62).

Abbrevations: DOB_{50}, Delta-over-baseline at t=50 min.

• = patient using CYP2D6 inhibiting medication.

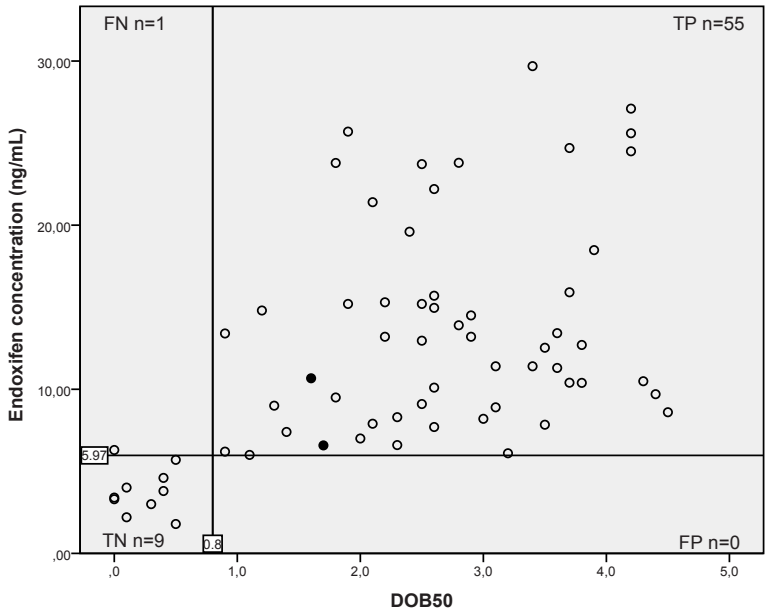


Figure 4.3 True positive (TP), false positive (FP), true negative (TN) and false negative (FN) DOB_{50} results for threshold serum endoxifen level of 5.97 ng/mL with DOB_{50} cut-off value of 0.8 (N=65).
Abbrevations: DOB_{50}, Delta-over-baseline at t=50 min.
• = patient using CYP2D6 inhibiting medication.

Although it has been suggested that, by application of specific validation criteria, DM is one of the best CYP2D6 phenotyping drugs [41], DM-BT has been minimally validated. The initial DM-BT study by Leeder [34] showed a good correlation between breath test results determined at two separate days (R^2=0.537). DM-BT results in 30 healthy subjects correlated well with urinary ^{13}C-dextromethorphan/dextrorphan (DM/DX) ratio, an alternative way to phenotype CYP2D6 activity. By defining CYP2D6 PM phenotype as DOB_{50} ≤0.5, DM-BT sensitivity to detect poor metabolizers was 100%, and specificity was 95% with 95% accuracy using either genotype or DM/DX ratio as the "golden" standard [34].

To additionally validate DM-BT, we performed a validation study (data not shown) and demonstrated reproducibility of the DM-BT in 6 EM patients by repeating the test for three times in each patient. *Intra*day analytical variation in DM-BT DOB_{50} results of three different breath samples, measured on one single day was 7.2, 11.2 and 11.5% respectively. *Inter*day analytical variation in DM-BT DOB_{50} results of one single breath sample, measured on 10 separate days was 11.7%. The validation study also showed excellent correlation of DOB_{50} with $AUC_{0–120}$ (DOBxtime

(‰xmin)) (R^2=0.8342) , suggesting that single time point breath sample collection at 50 minutes post ingestion of ^{13}C-DM is reliable to determine CYP2D6 phenotype (unpublished results).

We observed almost identical mean DOB_{50} values in hetEMs (2.77) and EM (2.82). However, from a genetic point of view, we expected higher DOB_{50} values in EMs than in hetEMs, because of the presence of twice as many active alleles in the former group. A "ceiling" effect for DOB_{50} values observed in the EM group might be due to dose restriction of ^{13}C-DM, as it is likely that their metabolic capacity exceeds the administered substrate resulting in lowered DOB_{50} values. For purpose of further validation, studies on using a higher fixed dose of ^{13}C-DM (e.g. 50 mg) as opposed to a weight adjusted dose (0.5 mg/kg) are warranted to investigate the potential ceiling effect in DM-BT results.

A factor contributing to the actual phenotype is the concomitant use of CYP450 inhibiting drugs. Although no specific DM-BT studies investigating phenoconversion in patients using CYP2D6 inhibitors or inducers have been performed yet, our study showed that the two patients with EM predicted phenotype who concomitantly used medication known to inhibit CYP2D6, had substantial lower DOB_{50} values. Besides, endoxifen levels were lower than expected in these two patients in line with results from other studies [10;24;25].

Additional studies confirm the effect of CYP2D6 inhibitors and inducers on DM metabolism. De Graan et al. [39] performed a CYP2D6 phenotyping study with oral DM in patients with (metastatic) breast cancer. In this study, there was one patient who used the potent CYP2D6 inhibitor paroxetine and had likewise low endoxifen levels and low DM AUC. Moreover, in a recent article by Schoedel et al. [42] addition of paroxetine to DM and quinidine 30 mg/30 mg twice daily increased steady-state plasma concentrations of DM, stressing the impact of CYP2D6 inhibition on DM pharmacokinetics.

Because of a potential advantage of assessing CYP2D6 phenotype, which includes both genetic, epigenetic, and environmental factors, above CYP2D6 genotype, the aim of this study was to explore the utility of the DM-BT in clinical practice.

Despite the potential advantage of phenotype over genotype, correlation between log-transformed CYP2D6 phenotype, determined by DM-BT and log-transformed endoxifen serum levels was not better but comparable to correlation between *CYP2D6* gene activity score and log transformed endoxifen serum levels.

In our study only 2 patients concomitantly using CYP2D6 inhibitors were included. Due to phenoconversion in these patients, correlation of CYP2D6 phenotype with endoxifen levels might

improve when more patients with CYP2D6 inhibiting medication would have been included. The best correlation was between clinical CYP2D6 phenotype by DM-BT and endoxifen/N-desmethyltamoxifen ratio, which is a direct measure for CYP2D6 mediated metabolism, indicating the high affinity of ^{13}C-DM for CYP2D6. Labeling the substrate DM with ^{13}C at both *O*-CH_3 (CYP2D6) and *N*-CH_3 (CYP3A4) group would probably yield an even better correlation between DOB_{50} and endoxifen trough levels since both CYP2D6 and CYP3A4 are involved in conversion tamoxifen to endoxifen.

We demonstrated that ^{13}C-DM was a safe phenotype probe, as no serious adverse events were observed.

Our study showed that both CYP2D6 genotype test as well as phenotype test by DM-BT might be useful in clinical decision making and confirms the study results of Wu et al. [43] who found that a composite model including both *CYP2D6* genotype and CYP2D6 inhibiting co-medication can be used to predict endoxifen concentrations.

The suggested serum endoxifen cut-off level of 5.97 ng/mL as a marker for reduced breast cancer recurrence rate by Madlensky [23] needs to be reproduced in larger studies in future. The DM-BT phenotype test along with the genotype test offers the possibility to discriminate between therapeutic and subtherapeutic endoxifen serum levels using an optimal chosen cut-off CYP2D6 activity score of 1.0 or optimal DOB_{50} cut-off values of 0.7–0.9.

Nevertheless, in addition to CYP2D6 genotype test, the DM-BT might be of significant value in the outpatient clinic setting when decisions are made on initiation of hormonal therapy in patients with localized breast cancer. In the future, in case of DM-BT result of $DOB_{50} \geq 0.8$, the clinician might choose tamoxifen in the adjuvant treatment. When DM-BT result corresponds to $DOB_{50} < 0.8$, the clinician might consider to start with an aromatase-inhibitor (AI) instead of tamoxifen. Alternatively, genotype [44;45] or phenotype guided dose escalation of tamoxifen might be explored when AI is contra-indicated. However, large clinical studies with focus on efficacy and safety of increased tamoxifen dosing are warranted. Theoretically, endoxifen is the antiestrogen of choice, being the most potent substrate of the estrogen receptor and because biotransformation is not CYP450 dependent. Endoxifen is also not susceptible to drug-drug interactions [46]. Orally administered endoxifen was rapidly absorbed, had good tolerability and was safe in healthy volunteers [47]. However, efficacy and safety of orally administered endoxifen should be investigated in a large phase III clinical trial.

Conclusion

In conclusion, CYP2D6 phenotype by DM-BT correlated well with endoxifen serum levels and serum endoxifen/NDMT ratios. The large variability in CYP2D6 phenotype by DM-BT between breast cancer patients exhibiting the same predicted phenotype might be in part due to variability in absorption and first-pass metabolism of DM, which needs further research. From a clinical point of view, CYP2D6 genotype test and phenotype DM-BT might be of equal value in supporting decisions about individualized pharmacotherapy unless comedications alter the phenotype, in which case the phenotype test is preferred.

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