# *In vivo* assessment of the metabolic activity of CYP2D6 diplotypes and alleles

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## WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- *CYP2D6* is genetically polymorphic leading to dramatic variability in metabolic activity. Phenotype classification systems collapse diplotypes into phenotypes or assign activity scores based on half number increments. These assumptions were tested to assess whether reclassification and recalibration is necessary.

## WHAT THIS STUDY ADDS

- The IM phenotype is comprised of diplotypes that are distinct from each other and the other phenotypes. The empirically estimated relative activities of the four allele types and individual *EM* and *IM* alleles are different from those previously assumed. CYP2D6 phenotype classification systems require additional calibration to improve individualized dosing of CYP2D6 dependent drugs.

## AIMS
A prospectively enrolled patient cohort was used to assess whether the prediction of CYP2D6 phenotype activity from genotype data could be improved by reclassification of diplotypes or alleles.

## METHODS
Three hundred and fifty-five patients receiving tamoxifen 20 mg were genotyped for *CYP2D6* and tamoxifen metabolite concentrations were measured. The endoxifen : N-desmethly-tamoxifen metabolic ratio, as a surrogate of CYP2D6 activity, was compared across four diplotypes (*EM/IM, EM/PM, IM/IM, IM/PM*) that are typically collapsed into an intermediate metabolizer (IM) phenotype. The relative metabolic activity of each allele type (*UM, EM, IM,* and *PM*) and each *EM* and *IM* allele was estimated for comparison with the activity scores typically assigned, 2, 1, 0.5 and 0, respectively.

## RESULTS
Each of the four IM diplotypes have distinct CYP2D6 activity from each other and from the EM and PM phenotype groups (each $P < 0.05$). Setting the activity of an *EM* allele at 1.0, the relative activities of a *UM, IM* and *PM* allele were 0.85, 0.67 and 0.52, respectively. The activity of the EM alleles were statistically different ($P < 0.0001$), with the *CYP2D6*2 allele (scaled activity = 0.63) closer in activity to an *IM* than an *EM* allele. The activity of the *IM* alleles were also statistically different ($P = 0.014$).

## CONCLUSION
The current systems for translating *CYP2D6* genotype into phenotype are not optimally calibrated, particularly in regards to IM diplotypes and the *2 allele. Additional research is needed to improve the prediction of CYP2D6 activity from genetic data for individualized dosing of CYP2D6 dependent drugs.

# Introduction

CYP2D6 is one of the primary enzymes responsible for the metabolic activation or inactivation of exogenous compounds. It is estimated that approximately 25% of drugs are metabolized by CYP2D6 [1], including many analgesics, antidepressants and cytotoxics. There is substantial inter-patient variability in CYP2D6 activity, which is partly caused by genetic variation [2]. The *CYP2D6* gene is highly polymorphic, with over a hundred allelic variants [3] and gene multiplications, deletions and other structural variants [4].

CYP2D6 activity, or phenotype, can be used to individualize dosing of certain drugs [5, 6]. The extensive genetic heterogeneity in *CYP2D6* makes phenotype prediction very complicated. The most common *CYP2D6* alleles have been grouped by functional activity classifications with descending activity: ultra-rapid (*UM*), extensive (*EM*), intermediate (*IM*) or poor (*PM*) metabolism. A given patient has two alleles, giving them 10 possible allelic combinations, or diplotypes (*UM/UM*, *UM/EM*, *EM/ EM*, etc.). These diplotypes are collapsed into four phenotypes, UM, EM, IM or PM, using either a translation table or a quantitative activity scoring system [7, 8].

Tamoxifen is a selective oestrogen receptor modulator used primarily in the treatment of oestrogen receptor positive breast cancer, and is the first line for use in pre-menopausal patients [9, 10]. Tamoxifen and its major metabolite N-desmethyl-tamoxifen are weakly anti-oestrogenic, but are metabolically activated via CYP2D6 to the more potent anti-oestrogen endoxifen [11, 12]. Patients with lower CYP2D6 metabolic activity have lower conversion of N-desmethyl-tamoxifen to endoxifen, which may be associated with decreased treatment effectiveness [13, 14]. An immense research effort has been dedicated to determining whether patients with low activity *CYP2D6* genotypes receive less benefit from tamoxifen treatment [15–18]. However, the results of these studies are highly controversial [19–21] and at this time *CYP2D6* genotyping is not recommended prior to tamoxifen initiation.

In a previous manuscript, we confirmed that steady-state endoxifen exposure is dependent on CYP2D6 phenotype and demonstrated that increasing the tamoxifen dose to 40 mg in IM (but not PM) patients normalizes the endoxifen concentrations with that of EM (or UM) patients remaining on 20 mg [22]. Although the clinical usefulness of genotype-guided tamoxifen dosing remains unproven, this large dataset of steady-state metabolite data and comprehensive *CYP2D6* genotyping could be useful for assessing CYP2D6 phenotype prediction systems. The purpose of this analysis was to determine whether any of the diplotypes currently classified as IM phenotype should be reclassified, and to estimate the relative activity of each allele type (*UM*, *EM*, *IM*, *PM*) and each *EM* and *IM* allele to assess the calibration of the activity scoring systems currently in use.

# Methods

## Clinical cohort

Data included in this analysis were collected during the expansion phase of a prospective *CYP2D6*-genotype-guided tamoxifen dose escalation study, Lineberger Comprehensive Cancer Center (LCCC) 0801. The objective of the clinical study was to determine whether increasing the daily tamoxifen dose to 40 mg in patients with *CYP2D6* genotypes that predict reduced activity CYP2D6 phenotype (IM or PM as defined below) would normalize their endoxifen concentrations with that of patients with high predicted CYP2D6 activity phenotypes (EM or UM) treated with the standard 20 mg dose. Patients were treated with the genotype-determined dose for the 4 month duration of the study. Details of the inclusion criteria, study methodology and primary findings for the original 122 patient cohort have been previously published [22]. Briefly, women 18 years or older who had been taking tamoxifen 20 mg for at least 4 months were eligible for enrolment. Relevant exclusion criteria included ECOG performance status >2, impaired kidney or liver function or concurrent use of a moderate or strong CYP2D6 inhibitor. This is the initial analysis of an expansion cohort that brought the total study enrolment to 500 patients. Due to a modification to the endoxifen assay occurring between analysis of the original and expansion cohort samples, only patients from the expansion cohort were eligible for this analysis (Supplementary Figure S1). This study was approved by the institutional review boards of the University of North Carolina at Chapel Hill and all other participating institutions. All patients signed informed consent prior to participation.

## Genotyping and CYP2D6 phenotype activity prediction

Genomic DNA was purified from whole blood collected at study enrolment and genotyped in a CLIA-certified laboratory using the AmpliChip® CYP450 test (Roche Diagnostics, Indianapolis, IN, USA). This chip can identify 20 distinct *CYP2D6* alleles and seven known duplications (denoted by XN). Each of these alleles can be categorized by activity: *UM*, *EM*, *IM* or *PM* (Table 1A), and the combination of any two alleles represents the patient's diplotype. These diplotypes are then collapsed into four phenotype categories (UM, EM, IM, or PM) (Table 1B). This particular system for converting diplotype to phenotype is identical to that used in the prospective clinical study to determine which patients remained at 20 mg (UM and EM) and which received a dose increase (IM and PM). The phenotype classification system was based on the most convincing evidence of association between genotype and tamoxifen treatment outcomes from Schroth *et al.* [18]. Because there were few UM patients (*n* = 5) this phenotype was excluded from phenotype comparisons. An additional four patients who carried *UM* alleles, but

## Table 1A

Transformation of CYP2D6 genotype to allelic activity

| Activity | Genotypes | Allele |
|---|---|---|
| High | *1XN, *2XN, *35XN | UM |
| Normal | *1, *2, and *35 | EM |
| Reduced | *9, *10, *17, *29, *36, *41, *17XN, *41XN | IM |
| None | *3, *4, *5, *6, *7, *8, *11, *15, *19, *20, *40, *4XN | PM |

## Table 1B

Transformation of diplotype to activity phenotype

| Phenotype | Diplotypes |
|---|---|
| UM* | UM/UM†, UM/EM† |
| EM | EM/EM, UM/IM†, UM/PM† |
| IM | EM/IM, EM/PM, IM/IM, IM/PM |
| PM | PM/PM |

*Excluded from phenotype comparisons †Excluded from diplotype comparisons

were not UM phenotype, were excluded from diplotype analyses which include only three phenotypes comprised of six diplotypes (EM: *EM/EM,* IM: *EM/IM, EM/PM, IM/IM, IM/PM* and PM: *PM/PM*).

### Tamoxifen and metabolite concentrations

Blood samples were collected from each patient at study enrolment, when all patients had been on 20 mg tamoxifen for at least 4 months. Steady-state plasma concentrations of tamoxifen (Z isomer only), (Z)-4-hydroxy-tamoxifen (4-OH-tam), N-desmethyl-tamoxifen (N-DM-tam, Z isomer only) and (Z)-4-hydroxy-N-desmethyl-tamoxifen (endoxifen; 10% (E), 90% (Z)) were measured using a h.p.l.c.-MS/MS (API 3200) assay method developed using diphenhydramine as the internal standard and liquid–liquid extraction as previously described with minor modification [22]. Tamoxifen, 4-OH-tam, N-DM-tam, endoxifen and diphenhydramine were stable for 28 h and the lower limit of quantification was 0.3 ng ml$^{-1}$ for each analyte using 200 µl of plasma. Variability was minimized in the method by using glass tubes and formic acid as part of the mobile phase. E isomers of each analyte eluted prior to the Z isomer. This analysis focuses primarily on the baseline (at study enrolment, all patients on 20 mg) metabolic ratio of endoxifen to N-DM-tam (end : N-DM-tam). However, endoxifen concentrations at baseline and at 4 months (UM and EM patients on 20 mg, IM and PM on 40 mg) are used in some secondary analyses.

### Statistical methods

Endoxifen and end : N-DM-tam ratio descriptive data are reported using medians and standard deviations. Baseline demographic comparisons across phenotype groups (excluding UM) were carried out via Fisher's exact (categorical data) and Kruskal–Wallis (continuous data) tests. Comparisons of metabolite concentrations across phenotypes and diplotypes (excluding *UM* alleles) and estimation of the percentage of variance explained was carried out via analysis of variance (ANOVA) and Kruskal–Wallis tests. The activity score modeling was performed using linear regression with each allele (*UM, EM, IM,* or *PM*) its own parameter. After initial modelling the activity of an *EM* allele was fixed to 1.00 and the relative activity of all other alleles were scaled accordingly. The analysis was then rerun with the *EM* (*1, *2, *35) and *IM* (*9, *10, *17, *29, *41/*41XN) alleles included individually to enable independent estimation of the N-DM-tam to

## Table 2

Demographic data for all eligible patients ($n = 355$) and broken down by CYP2D6 phenotype

| | | All patients ($n = 355$) | UM ($n = 5$) | EM ($n = 119$) | IM ($n = 214$) | PM ($n = 17$) | P value* |
|---|---|---|---|---|---|---|---|
| **Age (years)** | Median | 52 | 49 | 52 | 52 | 46 | |
| | Range | 24–95 | 43–61 | 30–95 | 24–88 | 38–78 | 0.17 |
| **Weight (kg)** | Median | 71.0 | 69.8 | 71.6 | 71.1 | 70.0 | |
| | Range | 32.1–149.1 | 48.6–78.7 | 44.2–149.1 | 32.1–135.4 | 47.6–140.7 | 0.93 |
| **Race** | White | 83.0% | 80% | 89.0% | 78.8% | 94.1% | |
| | Black | 14.7% | 20% | 6.8% | 19.8% | 5.9% | |
| | Other | 2.3% | 0% | 4.2% | 1.4% | 0% | 0.006 |
| **Months on tamoxifen prior to study** | Median | 10.1 | 10.1 | 11.1 | 9.9 | 13.1 | |
| | Range | 3.4–116.3 | 6.2–16.5 | 3.8–116.3 | 3.4–72.1 | 3.7–39.8 | 0.64 |
| **CYP2D6 weak inhibitor use** | Citalopram | 4.0% | 0% | 7.6% | 1.9% | 5.9% | 0.03 |
| | Escitalopram | 4.0% | 20% | 5.9% | 2.4% | 5.9% | 0.16 |
| | Venlafaxine | 9.1% | 0% | 11.8% | 8.0% | 5.9% | 0.53 |
| | Other | 0.9% | 20% | 0.8% | 0.5% | 0% | 1.00 |

*Comparison across EM, IM and PM phenotype patients (UM not included)

endoxifen metabolic activity of each allele. Allele activity was compared within the EM and IM allele types using Kruskal–Wallis tests to determine whether any allele was distinct from the group. Within each allele type the mean activity of each individual allele was compared with that of the most common allele within that group, *1 for EM alleles and *41/*41XN for IM alleles.

## Results

After exclusion of six patients with inconclusive genotyping, 355 patients with EM ($n = 119$), IM ($n = 214$), PM ($n = 17$), or UM ($n = 5$) predicted CYP2D6 phenotype were eligible for inclusion in these analyses (Supplementary Figure S1). Demographic data including age, weight, race, time on tamoxifen prior to study consent and concomitant CYP2D6 weak inhibitor use for the entire cohort and by CYP2D6 phenotype can be found in Table 2. As expected, race was unevenly distributed across CYP2D6 phenotypes ($P = 0.006$), as was the use of weak CYP2D6 inhibitors ($P = 0.01$). All other demographic factors were similar ($P > 0.05$). At baseline, with all patients receiving 20 mg tamoxifen, CYP2D6 phenotype was a significant predictor of steady-state endoxifen concentration ($P < 0.0001$, $r^2 = 0.11$, Figure 1A) and end : N-DM-tam ratio ($P < 0.0001$, $r^2 = 0.19$, Figure 1B). Because predicted CYP2D6 phenotype explained 80% more variance in the metabolic ratio, compared with endoxifen alone, the end : N-DM-tam ratio was the focus of subsequent analyses.

### Baseline differences between IM diplotypes

The 214 IM patients were further subdivided into four distinct diplotypes (EM/IM: $n = 69$, EM/PM: $n = 89$, IM/IM: $n = 19$, IM/PM: $n = 37$). The CYP2D6 activity, estimated by the end : N-DM-tam ratio when all patients were treated with 20 mg, varied significantly between these diplotypes ($P < 0.0001$). As expected, the ratio decreased with decreasing activity of the component alleles, from the highest activity EM/IM patients (median end : N-DM-tam ratio = 0.061) to the lowest activity IM/PM patients (end : N-DM-tam ratio = 0.024). When considering the end : N-DM-tam ratio in the IM phenotype group only, the diplotypes explained approximately 7.6% of the variance. Similarly, the diplotypes were significantly associated with baseline endoxifen concentration, though the amount of variance explained was somewhat lower ($r^2 = 0.047$, $P = 0.0027$).

### Baseline differences between IM diplotype extremes and EM or PM phenotypes

Across CYP2D6 diplotypes, the end : N-DM-tam ratio decreased with decreasing diplotype activity, as expected ($P < 0.0001$, Figure 2). Compared with EM phenotype (EM/EM) patients, the IM phenotype patients with the highest activity diplotype (EM/IM) had significantly lower median
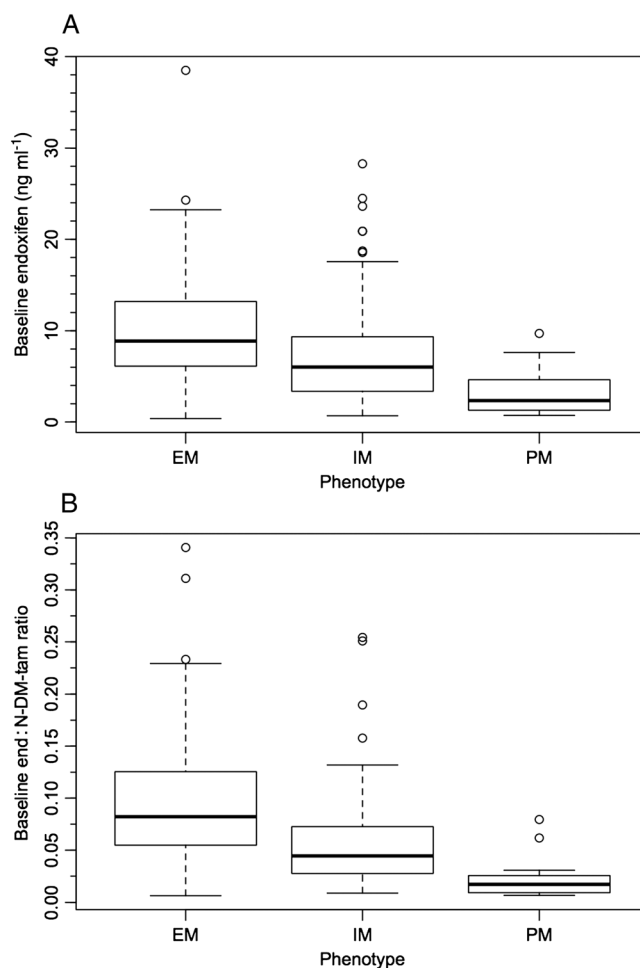


## Figure 1

A) Baseline endoxifen concentration by CYP2D6 phenotype. Decreasing CYP2D6 activity phenotype was associated with decreased steady-state concentration of endoxifen (Kruskal–Wallis $P < 0.0001$). CYP2D6 phenotype explained more than 10% of the variability in endoxifen concentration. B) Baseline end : N-DM-tam ratio by CYP2D6 phenotype. Decreasing CYP2D6 activity phenotype was associated with decreased steady-state ratio of endoxifen to N-desmethyl-tamoxifen (Kruskal–Wallis $P < 0.0001$). CYP2D6 phenotype explained more than 18% of the variability in end : N-DM-tam ratio

baseline end : N-DM-tam ratios (0.061 vs. 0.082, $P = 0.0004$, Table 3) and endoxifen concentration (7.9 ng ml$^{-1}$ vs. 8.9 ng ml$^{-1}$, $P = 0.033$, Supplementary Table S1, Figure S2). Similarly, when compared with PM (PM/PM) patients at baseline, the IM diplotype group with the lowest activity (IM/PM) had a greater end : N-DM-tam ratio (0.024 vs. 0.017, $P = 0.029$) and a trend toward greater endoxifen concentration (4.0 ng ml$^{-1}$ vs. 2.4 ng ml$^{-1}$, $P = 0.068$).

### Differences between IM diplotypes and EM phenotype at 4 months

As previously reported, the endoxifen concentration for the IM phenotype group on 40 mg was similar to that of the EM group on 20 mg at the 4 month time point [22], but the PM group on 40 mg did not achieve normalized
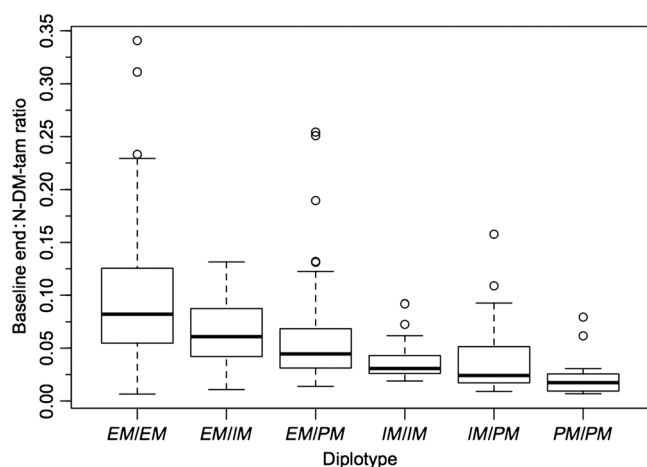
## Figure 2

Baseline end : N-DM-tam ratio by CYP2D6 diplotype. Decreasing CYP2D6 diplotype was associated with decreased steady-state ratio of endoxifen to N-desmethyl-tamoxifen (Kruskal–Wallis $P < 0.0001$). Within the IM phenotype, the patients with the highest (EM/IM) and lowest (IM/PM) activity diplophenoytpes were distinct from patients with EM (EM/EM) or PM (PM/PM) activity, respectively

concentration. The 4 month endoxifen concentration for the EM phenotype (EM/EM) patients on 20 mg was compared with that of the IM diplotypes on 40 mg. Only the EM/PM and IM/IM diplotypes had similar endoxifen concentrations after dose increase ($P > 0.05$), the EM/IM patients had significantly greater endoxifen concentrations (13.1 ng ml$^{-1}$ vs. 8.2 ng ml$^{-1}$, $P < 0.0001$), and the IM/PM patients remained significantly lower (5.8 ng ml$^{-1}$ vs. 8.2 ng ml$^{-1}$, $P = 0.0009$) (Supplementary Table S2).

### Modelling relative activity of allele types and alleles

Using each patient's two CYP2D6 alleles and their baseline end : N-DM-tam metabolic ratio the relative activity of each allele type (UM, EM, IM, PM) was estimated. Fixing the activity of an EM allele to 1.00, the relative activity of a UM, IM, and PM allele was estimated to be 0.85, 0.67 and 0.52, respectively (Table 4). The activity score modelling

## Table 4

Activity for each allele type relative to an EM allele fixed at 1.00

| Allele type | n | Median | Standard deviation | Scaled activity* |
|---|---|---|---|---|
| UM | 9 | 0.056 | 0.053 | 0.85 |
| EM | 390 | 0.066 | 0.053 | 1.00 |
| IM | 149 | 0.044 | 0.034 | 0.67 |
| PM | 162 | 0.034 | 0.039 | 0.52 |

*Scaled to fix EM activity = 1.00 Note: Numbers of alleles do not match up with previous tables due to the inclusion of UM allele carriers in this analysis but not others.

analysis was performed a second time with each EM (*1, *2, *35) and IM (*9, *10, *17, *29, *41/*41XN) allele included as an independent parameter. Individual alleles in the EM group had distinct metabolic activity ($P < 0.0001$). Fixing the wild-type (*1) allele activity to 1.00, the *2 allele had an estimated scaled activity of 0.63, which was significantly different from the *1 allele ($P = <0.0001$, 95% CI 0.021, 0.041) while the *35 allele had activity of 1.03, which was similar to *1 (Figure 3). The individual IM alleles also had distinct metabolic activity ($P = 0.014$). Comparing each allele to the activity of the most common *41/*41XN allele (activity = 0.53) only *17 had a significantly different activity estimate (activity = 0.39, 95% CI 0.005, 0.030, $P = 0.021$). The scaled activity of the other alleles ranged from 0.58–0.85 (Table 5). The scaled activity of the UM and PM alleles changed marginally to 0.71 and 0.43.

## Discussion

The original intent of this prospective genotype-guided tamoxifen escalation study was to determine whether increasing IM or PM patients to 40 mg achieved endoxifen concentrations similar to EM patients maintained on 20 mg. As a composite phenotype group the IM patients achieved similar endoxifen concentrations, but the PM

## Table 3

Comparison of baseline endoxifen : N-desmethyl-tamoxifen ratio by CYP2D6 diplotype

| Phenotype | Diplotype | n (%) | Baseline end : N-DM-tam ratio | | P value* vs. EM/EM | 95% CI† vs. EM/EM | P value* vs. PM/PM | 95% CI‡ vs. PM/PM |
| | | | Median | Standard deviation | | | | |
|---|---|---|---|---|---|---|---|---|
| EM | EM/EM | 115 (33%) | 0.082 | 0.058 | NA | NA | <0.0001 | (0.058, 0.087) |
| IM | EM/IM | 69 (20%) | 0.061 | 0.030 | 0.0004 | (0.018, 0.044) | <0.0001 | (0.029, 0.054) |
| | EM/PM | 89 (26%) | 0.045 | 0.043 | <0.0001 | (0.024, 0.052) | <0.0001 | (0.021, 0.048) |
| | IM/IM | 19 (5%) | 0.031 | 0.019 | <0.0001 | (0.044, 0.72) | 0.0032 | (0.001, 0.028) |
| | IM/PM | 37 (11%) | 0.024 | 0.033 | <0.0001 | (0.041, 0.072) | 0.029 | (0.001, 0.030) |
| PM | PM/PM | 17 (5%) | 0.017 | 0.020 | <0.0001 | (0.058, 0.087) | NA | NA |

*value is based on the non-parametric Wilcoxon rank-sum test †95% confidence interval for the difference in means (EM/EM – comparator diplotype) ‡95% confidence interval for the difference in means (comparator diplotype – PM/PM)
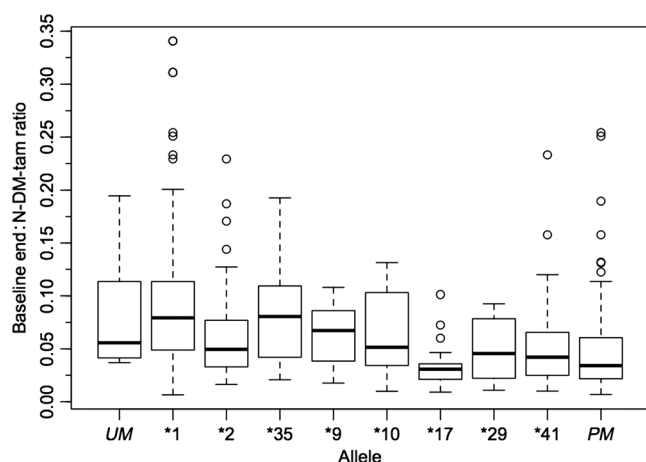
## Figure 3

Baseline end : N-DM-tam ratio by CYP2D6 allele. The *EM* and *IM* alleles were analyzed separately to estimate their individual activity. The *EM* alleles (*1, *2, *35) generally had greater activity. However, the *2 allele had activity more similar to an *IM* or *PM* allele. The *IM* alleles represent a continuum of activity that spans from activity similar to an *EM* allele (*9) to activity similar to a *PM* allele (*17). The *UM* alleles were combined due to small numbers and the *PM* alleles were combined because they create non-functional protein or no protein at all, and thus have no activity.

## Table 5

Activity for each *EM* and *IM* allele relative to a wild-type *1 allele fixed at 1.00

| Allele type | Allele | n | Median | Standard deviation | Scaled activity* |
|---|---|---|---|---|---|
| UM | Composite | 9 | 0.056 | 0.053 | 0.71 |
| EM | *1 (wild-type) | 250 | 0.079 | 0.058 | 1.00 |
| | *2 | 109 | 0.050 | 0.036 | 0.63 |
| | *35 | 31 | 0.081 | 0.046 | 1.03 |
| IM | *9 | 20 | 0.067 | 0.029 | 0.85 |
| | *10 | 11 | 0.051 | 0.044 | 0.65 |
| | *17 | 21 | 0.031 | 0.022 | 0.39 |
| | *29 | 10 | 0.046 | 0.031 | 0.58 |
| | *41/*41XN | 87 | 0.042 | 0.035 | 0.53 |
| PM | Composite | 162 | 0.034 | 0.039 | 0.43 |

*Scaled to fix *1 activity = 1.00 Note: Numbers of alleles do not match up with previous tables due to the inclusion of *UM* allele carriers in this analysis but not others.

patients remained below EM patients [22]. In this analysis the IM diplotypes were analyzed separately. Doubling the dose successfully achieved concentrations similar to those of EM patients only for the *EM/PM* and *IM/IM* diplotypes. The highest activity *EM/IM* patients may require a smaller dose increase, as their endoxifen concentrations exceeded those of the EM group. In some phenotype classification systems the *EM/IM* and *EM/PM* diplotypes are collapsed with the EM, not the IM, phenotype. These

patients had endoxifen concentrations lower than the *EM/EM* patients, suggesting that they may be underdosed if they were considered EM patients and did not receive a dose adjustment. Alternatively, this would prevent potential over-treatment in the *EM/IM* patients. Increasing the lowest activity *IM/PM* patients to the maximum FDA-approved 40 mg dose is insufficient to achieve endoxifen concentrations similar to that of EM patients. Though genotype-guided tamoxifen dose escalation is not clinically warranted at this time, our results indicate that *CYP2D6* genotype-guided treatment algorithms should avoid collapsing the distinct IM diplotypes into a composite IM phenotype group.

Steady-state concentrations of tamoxifen metabolites were used to evaluate CYP2D6 activity scoring systems that categorize patients into four phenotypes (UM, EM, IM, PM). The metabolic ratio of end : N-DM-tam was used as it was nearly twice as reflective of CYP2D6 activity as endoxifen alone, and may be more predictive of tamoxifen treatment efficacy [14]. These results further suggest that the IM phenotype is comprised of four diplotypes (*EM/IM, EM/PM, IM/IM, IM/PM*) that have metabolic activity distinct from each other and from the other CYP2D6 phenotype groups (EM or PM). Several CYP2D6 activity scoring systems that return sometimes discordant phenotypes have been proposed [23]. The Clinical Pharmacogenetics Implementation Consortium (CPIC), which publishes genetics-guided treatment recommendations [24], has endorsed a system that assigns each allele an activity score of 0 (*PM*), 0.5 (*IM*) or 1 (*EM*), which is multiplied in the event of a gene duplication [5]. In this system *EM/PM* (1 + 0 = 1.0) and *IM/IM* (0.5 + 0.5 = 1.0) patients would have equivalent activity, but *EM/PM* patients had on average 45% greater activity in this analysis (end : N-DM-tam 0.045 *vs*. 0.031, $P = 0.018$). Using a scaled regression model the activity for an *IM* allele (0.67) was similar to that (0.5) used in existing systems. Interestingly, the empirical activity estimate of a *PM* allele (0.52) was much greater than previously assumed. PM alleles have no CYP2D6 enzyme activity as they are gene deletions or non-functional truncated proteins. The activity estimated in this study is likely attributable to alternative pathways for endoxifen formation including CYP3A4/5 and CYP2C9/19 [25].

The activity of each *EM* and *IM* allele was then estimated and scaled to the activity of the wild-type *1 allele. The relatively common *2 genotype (allele frequency in this cohort =15.4%) is typically characterized as an *EM* allele (activity = 1.0) but had an activity of only 0.63, which is much closer in activity to an *IM* (0.67) allele. The *in vitro* conversion of N-DM-tam to endoxifen for *CYP2D6*2 has been estimated as only 21% of the wild-type (*1) enzyme [26] and a dextromethorphan pharmacokinetic study reported that *CYP2D6*2 had approximately 40% of the activity of the wild-type [27]. Recently, the *2 SNP was reported to alter exonic splicing and reduce gene expression [28]. Our results for tamoxifen bioconversion are

consistent with prior results for dextromethorphan metabolism, further supporting the need for reclassification of *2 as an intermediate function allele. Unlike *2, the *35 *EM* allele had activity very similar to *1 (activity = 1.03) and should continue to be considered a fully functional allele. There was a wide range of activity within the *IM* alleles, with estimates as low as 0.39 (*17) and as high as 0.85 (*9). There is statistical evidence that these alleles have distinct activity but the small number of some alleles makes it difficult to confirm which actually differ. Similar to the previously discussed results for *2, ideally the activity for each allele would be estimated *in vitro* and confirmed in healthy controls in a pharmacokinetic study using targeted pharmacogenetic enrolment.

There are several important limitations of this analysis. Tamoxifen is metabolized by several enzymes and the conversion to endoxifen has an alternate pathway. The estimates in this analysis, such as the 'activity' seen in non-functional *PM* alleles, reflect not just CYP2D6 activity but also this alternative pathway. Unfortunately, due to the small number of patients with UM phenotype or *UM* alleles, it was not possible to assess whether the activity of these diplotypes (UM: *UM/UM* and *UM/EM*, EM: *UM/IM* and *UM/PM*) are distinct, should continue to be collapsed into UM and EM phenotypes, or should be collapsed into a single EM phenotype. Another limitation of this analysis is the allele coverage of the AmpliChip CYP450 Test™. This test only reports that a copy number variant was detected, not the actual number of copies of the gene, and is unable to detect certain rare variants [29]. Additionally, some newly discovered functionally consequential variants [30] and the 2988G > A variant that defines the *41 haplotype [28] are missing from this panel. This causes some *41 alleles to be erroneously called *2, which would artificially depress the estimate of *2 activity in this analysis. One final limitation is that the genotype explained a relatively small proportion of the variability in end : N-DM-tam ratio (18.6%), compared with that in prior analyses (53–69%) [14, 25]. Log-transformation marginally increased the variability explained (24%) but to improve interpretation of results this was not done for the reported analyses. Several other factors including inhibitor co-administration, patient size, and perhaps even the season of sample collection, may influence systemic concentrations of tamoxifen metabolites [31]. Patients taking strong or moderate CYP2D6 inhibitors were excluded from this study but these other factors were not accounted for in drug dosing or in this analysis. Additional clinical and genetic factors, including variants in other enzymes and transporters [32], will be included in follow-up analyses attempting to validate and extend a previously published endoxifen prediction algorithm [31].

In conclusion, pharmacogenetic–pharmacokinetic analysis of steady-state tamoxifen metabolite concentrations confirms that predicted CYP2D6 phenotypes are comprised of metabolically distinct diplotypes that should not be collapsed in genotype-guided dosing algorithms. Furthermore, currently used activity scoring systems are not optimally calibrated, specifically in regards to the *2 genotype which does not have full activity. Due to the inconsistent findings regarding the relationship of *CYP2D6* genotype and tamoxifen efficacy, it is unclear whether these results will be useful for personalizing dosing of tamoxifen. However, these results highlight the need for further research to calibrate optimally CYP2D6 phenotype systems to guide personalized dosing of other CYP2D6-dependent drugs such as codeine [5] and tricyclic anti-depressants [6].

## Competing Interests

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1**
Consort diagram

**Figure S2**

**Table S1**
Comparison of endoxifen concentrations at baseline by CYP2D6 diplotype

**Table S2**
Comparison of endoxifen concentrations at 4 months by CYP2D6 diplotype