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Tamoxifen Pharmacogenetics: CYP2D6 and Other Variables Influencing Tamoxifen and Tamoxifen Metabolite Exposure

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by

Simone Mizy Tchu

DISSERTATION

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ABSTRACT

Tamoxifen Pharmacogenetics: *CYP2D6* and Other Variables Influencing Tamoxifen and Tamoxifen Metabolite Exposure

Simone Ming Tchu

Tamoxifen is a selective estrogen receptor modulator (SERM) that is used for the treatment of estrogen receptor positive (ER^+) breast cancer, most commonly as an adjuvant for the prevention of disease recurrence. Several retrospective studies suggest that functional *CYP2D6* polymorphisms are associated with the clinical outcome of tamoxifen adjuvant therapy; variants that confer reduced enzymatic activity are associated with poorer outcomes. The biological basis for this association is that tamoxifen is a pro-drug and CYP2D6 metabolism is important for the formation of the potent anti-estrogenic metabolite, endoxifen. The general goal of this work was to investigate the effect of *CYP2D6* polymorphisms and other variables on tamoxifen and tamoxifen metabolite exposure in order to clarify the best use of clinical tamoxfien pharmacogenetic test data.

An LC-MS/MS assay was developed and validated for the quantitation of tamoxifen, N-desmethyltamoxifen, and endoxifen in human serum. The assay was found to be robust for the measurement of these analytes at physiologically relevant concentrations. In collaboration with the Women's Healthy Eating and Living (WHEL) study group, serum endoxifen concentrations were shown to be associated with the clinical outcome of tamoxifen adjuvant therapy, and a

sub-therapeutic endoxifen risk group was defined. Extensive *CYP2D6* genotypes were determined for WHEL study subjects using the Roche P450 AmpliChip, and an analysis was performed to determine the extent to which genotype cutoffs define the sub-therapeutic endoxifen risk group among Caucasian subjects. Of subjects who carried two null *CYP2D6* alleles, approximately 72% fell into the sub-therapeutic endoxifen risk group, but this accounted for less than a quarter of all subjects within the risk group. Poor metabolizer phenocopies were present in all genotype groups. In addition, inter-ethnic differences in serum tamoxifen and tamoxifen metabolites were determined between Caucasian, Asian, Hispanic, and African American subjects within the WHEL cohort, with Asians and Hispanics exhibiting higher median endoxifen concentrations. Finally, an association study was performed in order to determine if variants in candidate genes (*UGT2B7, ABCC2, CYP2C19*) influence serum endoxifen concentrations in Caucasian subjects. Statistically significant associations were determined, but are unlikely to be clinically relevant due to small effect.

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CHAPTER 1

Tamoxifen Pharmacogenetics, Breast Cancer Treatment, and Clinical *CYP2D6* Testing

1.1 Introduction

Tamoxifen is a selective estrogen receptor modulator (SERM) that is approved for the use of all stages of estrogen receptor positive (ER^+) breast cancer, and is most frequently used in the adjuvant setting for the prevention of recurrence. While a five year course of tamoxifen adjuvant therapy is associated with a 49% reduction in recurrence, 20-30% of patients still experience breast cancer relapse [1]. Thus, it is of interest to identify factors associated with tamoxifen treatment failure in order to further tailor breast cancer treatment. Several retrospective clinical studies suggest an association between *CYP2D6* genotype and the outcome of tamoxifen adjuvant therapy; carriers of functional variants that confer lower enzymatic activity tend to have poorer clinical outcomes. The biological basis for this association is that tamoxifen is a pro-drug, the CYP2D6 enzyme is important for the formation of the active metabolite 4-hydroxy-N-desmethyltamoxifen (endoxifen), and that reduced endoxifen formation has a negative impact on drug efficacy. There is clinical interest in using pharmacogenetic testing to make treatment decisions for breast cancer patients who are eligible for endocrine therapy. A major question is if, and how, this knowledge can be applied to

the clinical setting to improve the outcomes of breast cancer patients with estrogen receptor positive tumors.

1.2 Breast Cancer Diagnosis and Treatment

Over 200,000 women are diagnosed with breast cancer each year in the United States. According to the Center for Disease Control (CDC), it is the most common cancer in all races of women as well as the second leading cause of cancer death (the first in Hispanic women) [2]. In recent decades, improvements in imaging as well as an aggressive public health campaign for breast cancer screening have resulted in increased detection of early stage breast cancer. Notably, the percentage of women presenting with non-invasive breast cancer has increased from 5% to 30%. This, and innovations in therapy, have led to a decrease in breast cancer mortality over the past decade. Despite this progress, treatment remains a challenge and the majority of women experience relapse. Breast cancer is a heterogeneous disease; cancer cells vary in terms of proliferative drivers and patterns of genetic alterations. Response to therapy is highly variable, providing both a challenge and opportunity to personalize treatment.

The current standard treatment for early breast cancer includes surgery, either lumpectomy or mastectomy, to remove the primary tumor. Axillary lymph nodes are examined by a pathologist for signs of cancer cell invasion to determine the likelihood of metastasis. Sections of tumor tissue are stained for molecular markers in order to determine the best treatment strategy. The standard markers that are assessed in

breast tumors are estrogen receptor alpha (ER), the progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu); more extensive molecular profiling is available, such as the OncotypeDx test from Genomic Health, but is not yet standard practice. Depending on tumor characteristics and the presence of tumor cells in the lymph nodes, a breast cancer patient may undergo chemotherapy and/or radiation therapy. Tumors that are negative for all three markers often show aggressive growth and have a poor prognosis. Tumors that express Her2/neu are treated with the drug Herceptin (Trastuzumab), which interferes with Her2 signaling – the driving force for proliferation in this type of tumor. ER/PR positivity suggests the tumor will be susceptible to adjuvant endocrine therapy, which can include five years of tamoxifen, five years of aromatase inhibitor (AI), or tamoxifen followed by AI over the course of five years. The majority of breast cancer patients (74%) are post-menopausal, and the majority of these women have ER^+ tumors (73%). ER^+ tumors occur less frequently in the pre-menopausal population, although more than half of these tumors are ER^+ (54%).

1.3 Breast Cancer Adjuvant Endocrine Therapy

The goal of endocrine therapy for ER^+ breast cancer is to block the proliferative effect of estrogens on tumor cells by one of two mechanisms: 1) inhibiting the binding of estrogen to the estrogen receptor or, 2) blocking the synthesis of estrogens from androgens by the aromatase (CYP19) enzyme. Tamoxifen inhibits breast tumor cell proliferation by the first mechanism, but is described as a selective estrogen receptor modulator (SERM) because its effects on ER signaling are tissue dependent; tamoxifen

acts as an estrogen receptor agonist in bone and in the uterus. This effect is likely due to differences between tissues in ER subtype expression and co-regulators. As their name suggests, AIs block the activity of the aromatase enzyme. In post-menopausal women, conversion of androstenedione to estrone by the aromatase enzyme in peripheral tissues is the major source of circulating estrogen. AIs are able to suppress this process by 97-99% in post-menopausal women. The aromatase inhibitors that are approved for the treatment of breast cancer include Anastrozole (Arimidex), Letrozole (Femara) and Exemestane (Aromasin).

For decades, tamoxifen was the gold standard of adjuvant endocrine therapy for both pre- and post-menopausal breast cancer patients. However, this is no longer the case for post-menopausal patients, which make up the majority of women who are diagnosed with hormone receptor (HR) positive breast tumors. Several large clinical studies have been published which suggest a five year course of AI is slightly more effective than five years of treatment with tamoxifen at preventing breast cancer recurrence in post-menopausal women with early HR^+ breast cancer [3-5]. In addition, sequenced endocrine therapy (tamoxifen followed by an AI) is also more effective than tamoxifen alone in this population [6]. Aromatase inhibition alone is not appropriate for pre-menopausal breast cancer patients due to continued ovarian production of estrogen. Research is underway to determine if a combination of AI and ovarian suppression (OS) using Gosrelin is comparable to tamoxifen therapy in the adjuvant setting in pre-menopausal patients [7]. Pending study results, tamoxifen continues to be

the mainstay of adjuvant endocrine therapy for pre-menopausal breast cancer patients with HR^+ disease.

Although both tamoxifen and AIs are designed to prevent estrogen signaling in breast tumors they have different side effect profiles. Tamoxifen has tissue specific estrogenic or anti-estrogenic effects while AIs eliminate or reduce estrogen signaling throughout the body. Vasomotor symptoms are the most common side effect of tamoxifen and can have a significant impact on quality of life. Increased bone mineral density and improved cholesterol profile are positive estrogenic effects of tamoxifen. However, tamoxifen therapy is also associated with some rare, but troubling side effects such as the proliferation of endometrial cells and an increased risk for endometrial cancer, as well as an increased risk for thromboembolic events. Arthralgias and myalgias are the most troubling side effect of AIs and can be quite severe in some patients. Side effects are a major reason for discontinuation of adjuvant endocrine therapy. On average, AIs are slightly more effective than tamoxifen in terms of recurrence free survival, although overall survival is approximately the same. However, an individual may benefit more from, or be more likely to adhere to, one therapy than another.

1.4 ER-α Testing, Tamoxifen, and the Birth of Targeted Breast Cancer Therapy

Breast cancer treatment has evolved in parallel with our scientific understanding of cancer biology, and continues to be driven forward by the potential for improved clinical outcomes and greater treatment tolerability. Remarkably, the discovery of ER-α, the development of assays for ER-α expression in breast tumors, and the clinical testing and

FDA approval of tamoxifen as a drug for the treatment of ER-α positive breast cancer, all occurred within a time period of two decades (1958-1977).

Before the discovery of the estrogen receptor (ER-α) by Elwood Jensen (presented in 1958) [8], or even the characterization of estrogen by Edgar Doisy (published 1923) [9], research by George Beatson (published in 1896) [10] suggested that "ovarian secretions" drive the growth of certain breast tumors. Beatson published a case report in which the removal of the ovaries in three premenopausal breast cancer patients resulted in remission. Thereafter, ovarian ablation became a treatment available to premenopausal breast cancer patients. However, only one third of patients responded to this treatment, and until the discovery and characterization of ER-α, it was not possible to identify subjects who would be likely to benefit from this procedure.

ER-α was the first of 48 nuclear hormone receptors (in humans) to be described, a discovery which was integral to elucidating the mechanisms by which estrogen and other hormones exert their effects on tissues. This discovery also led to the ability to assay for ER-α expression in tissues -- a major advance in breast cancer medicine (see Figure 1.1). Jensen took advantage of recent advances in radioisotope chemistry and the detection of tritium; the tritiated estrogen synthesized in his laboratory was an essential tool for the purification and characterization of ER-α, as well as for the detection of ER-α in breast tumors prior to the availability of anti- ER-α antibodies for histological assessment [11]. In the early 1970s, Jensen was able to show that the tumors of metastatic breast cancer patients were more likely to respond to endocrine therapy (removal of the ovaries in premenopausal women and removal of the adrenals and/or

pituitary of post-menopausal women) if they expressed a certain threshold of ER-α (see Figure 1.2). Testing for ER-α expression in tumors allowed for the exclusion of patients who were unlikely to respond to endocrine therapy, thereby enriching the population of responders to 60% from 30%.

Craig Jordan was researching a pharmacological approach to breast cancer endocrine therapy using an orphan drug from ICI Pharmaceuticals – a drug that was eventually re-named tamoxifen. In collaboration with Elwood Jensen, he was able to demonstrate tamoxifen's efficacy in patients with ER^+ tumors. Thus, tamoxifen treatment was able to achieve the same effect as surgical or chemical castration. In 1977, tamoxifen was approved for the treatment of metastatic breast cancer. While chemotherapy had been an available and effective treatment for breast cancer patients since the 1960's, the major benefit of tamoxifen was its comparatively mild side effect

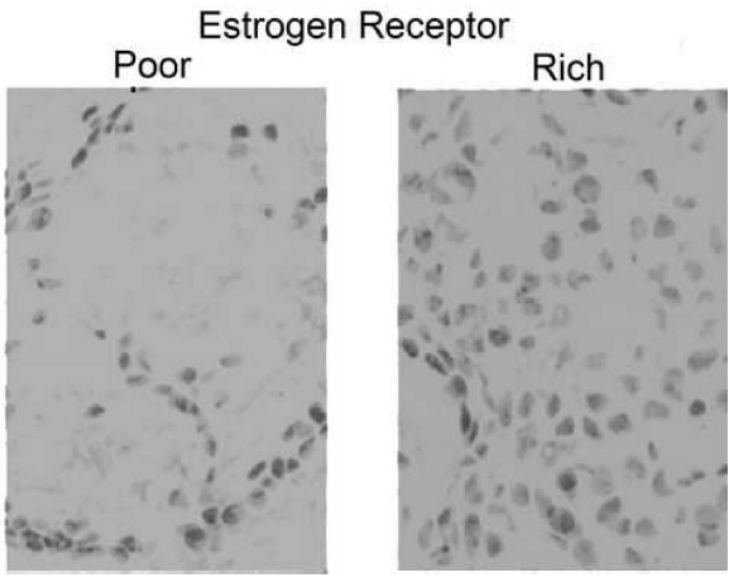

Figure 1.1. Examples of antibody staining of breast tumor tissue for ER-α expression. The sample on the left shows low expression of ER-α while the sample on the right shows high expression of the receptor. [11]

profile, enabling the drug's long term use in the prevention setting. Tamoxifen is approved for the treatment of all stages of breast cancer, as an adjuvant therapy, as well as for the prevention of breast cancer in women who are at high risk. By the mid-1980s, evaluation of estrogen receptor expression in tumors became a standard part of care for breast cancer patients. The addition of PR evaluation in tumors increased the response rate to tamoxifen to 70-75%; PR expression suggests hormonal signaling pathways remain intact within tumor cells. These were major advancements in the field of breast cancer treatment, but the question remains: Why are 25-30% of ER^+/PR^+ breast tumors not responsive to tamoxifen and how can we identify patients who are unlikely to benefit from tamoxfien therapy?

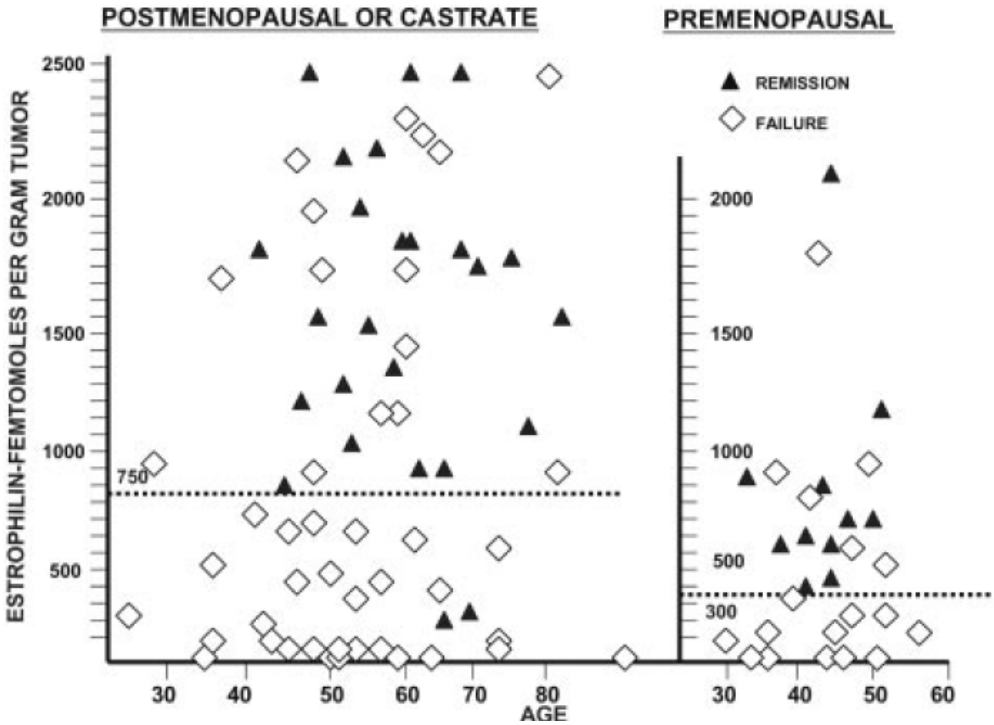

Figure 1.2. Response to endocrine therapy in pre- and post- menopausal breast cancer patients with advanced disease based on the level of ER expression in the primary tumor. [12]

1.5 Clinical Literature Summary

1.5.1 Clinical Tamoxifen Pharmacogenetics in the Non-Metastatic Adjuvant Setting

Patients taking tamoxifen as an adjuvant for breast cancer prevention represent the largest population of patients taking this drug. Clinical interest in *CYP2D6* testing for breast cancer patients arose when Goetz *et al.* published a study which found an association between *CYP2D6* genotype and the outcome of tamoxifen adjuvant endocrine therapy; subjects with the *CYP2D6* *4/*4 poor metabolizer genotype had worse relapse-free time (RF-time; P = .023) and disease-free survival (DFS; P = .012), although there was no difference in overall survival [13]. However, in the same year, a study by Nowell et al. [14] found no association between *CYP2D6* genotype and clinical outcome, and a study by Wegman et al. found that carriers of the *CYP2D6**4 variant who had taken tamoxifen versus no endocrine treatment had a decreased risk of recurrence (relative risk = 0.28, 95% CI = 0.11-0.74, p = 0.0089) [15]. Since these findings, over twenty studies have been published that examine the relationship between *CYP2D6* genotype and the outcome of tamoxifen adjuvant therapy in non-metastatic breast cancer patients, but it is still unclear if an association between *CYP2D6* genotype and clinical outcome actually exists. Thus, it is no surprise that there is still debate over whether to perform CYP2D6 testing for the breast cancer population.

Of the published studies, there are nine [13, 16-24] which found an association between CYP2D6 reduced metabolizer genotypes and reduced efficacy, two for which the opposite association was found [15, 25], and ten in which no association was

determined [14, 26-34]. There is one publication in which there is a positive association in a subgroup of patients who were treated with tamoxifen monotherapy but not for subjects who underwent combination therapy [21]. The most recent study by Schroth et al. [19] includes the population from an earlier publication by Schroth et al. [23] as well as several publications by Goetz et al. [13, 35, 36], and they found a positive association between CYP2D6 genotype and clinical outcome. .

Several causes of discrepant study findings have been proposed. Studies have been heterogeneous in term of the number of *CYP2D6* variants assayed, and genotyping comprehensiveness may be one factor that influences whether or not a positive association between *CYP2D6* genotype and outcome is determined. An analysis by Schroth et al. demonstrated the effect of insufficient genotyping, limited to the *4 allele, versus comprehensive genotyping that includes all of the variants tested by the Roche P450 AmpliChip, on study power, hazard ratio, and the percentage of subjects classified as EM, IM, or PM within their cohort [22]. Their study population consisted of 492 German subjects, taken from a larger population of 1361 breast cancer patients, for which sufficient amount and quality of DNA was available for extensive genotyping. Dramatic increases in study power and hazard ratio were observed with comprehensive versus insufficient genotyping; power increased from 7.8% to 63.2%, and hazard ratio increased from 1.33 ($p = .58$) to 2.87 ($p = .006$). In terms of CYP2D6 metabolizer group assignment, the percentage of PMs increased from 5.5%-8.3%, the percentage of IMs increased from 32.7% - 54.1%, and the percentage of EM decreased from 61.8% - 37.6%. In addition, Thompson *et al.* found that, only with extensive genotyping (AmpliChip) was

the association between *CYP2D6* and outcome statistically significant [20]. Thus, the extent of *CYP2D6* genotyping may have a significant impact on the ability to detect an association between genotype and outcome. Of the studies which did not find an association between *CYP2D6* genotype and poor clinical outcome, five genotyped only the *4 variant [14, 15, 25, 30, 33]and two (Asian populations) genotyped only the *10 variant [28, 29]. Of studies that found a positive association, there were two which genotyped only the *4 variant [13, 18]and one which genotyped the *10 variant [16](Asian population). A greater proportion of studies which found a positive association had extensive genotyping (4 or more variants) [17, 19, 20, 24], although extensive genotyping was certainly not absent among the negative studies [31, 32].

DNA source, which can affect genotype data quality, has been variable. Several studies used DNA extracted from archived, paraffin-fixed tumor blocks, as opposed to DNA extracted from whole blood or buffy coat, because this was the only source of DNA available. While the results of these studies have varied, one concern is that the use of such DNA may have resulted in sub-standard genetic data, and therefore questionable study findings. DNA derived from tumor tissue may be problematic because frequent losses of heterozygosity in breast cancer cells for 22q13, where *CYP2D6* is located, have been reported [37], which could result in inaccurate genotype data. The largest of the studies which used DNA derived from tumor blocks, which included 4393 subjects from the Breast International Group 1-98 trial, found no association between *CYP2D6* genotype and the clinical outcome of tamoxifen adjuvant therapy [27]. A letter to JNCI from Nakamura et al. [38] in response to this article, brought to attention a significant

departure from Hardy-Weinberg equilibrium for two very important SNPs, the ones which define the *4 and *41 variants which are the most common null and reduced function alleles in Caucasians, respectively. The letter urged the retraction of this study and the reanalysis of other studies that used DNA derived from tumor blocks. This would include another recent, negative study, which used a subset of postmenopausal patients with early stage HR^+ breast cancer taken from the Arimidex, Tamoxifen, Alone, or in Combination (ATAC) trial (N = 1203 patients: anastrozole group, n = 615 patients; tamoxifen group, n = 588 patients)[26].

The use of tamoxifen monotherapy versus combination therapy (tamoxifen plus chemotherapy and/or radiation therapy) may be one factor that has affected study results, as has been suggested by Kiyotani et al. [21]. In a study of breast cancer patients who received combination therapy (n =167), no significant association between *CYP2D6* genotype and recurrence-free survival was observed. However, a positive association between *CYP2D6* genotype and outcome was observed in patients who received tamoxifen monotherapy (n=282). The authors point out that, in the majority of early studies which found no association between CYP2D6 and outcome, patients who received both tamoxifen and chemotherapy were included in study populations. However, in three negative studies a tamoxifen monotherapy sub-group analysis was performed, and none of these analyses indicated a significant association between *CYP2D6* genotype and outcome [25, 28, 33]. Thus, the inclusion of subjects who received chemotherapy in addition to tamoxifen does not fully explain these discrepant results.

Patient drug-taking behavior is another factor that is likely to influence study results, but is notoriously difficult to account for accurately. The expected duration and dosage (40 mg/day or 20 mg/day) of tamoxifen therapy is inconsistent between studies. Duration of tamoxifen therapy is important because maximum benefit is derived from a five year course of therapy [1], whereas the current standard dose is generally 20 mg/day. In addition to this, actual patient adherence and persistence is likely to vary. Generally, a patient must take at least 80% of prescribed doses to be considered adherent. In the outpatient setting, poor adherence is a known issue for many types of drug therapy and tamoxifen is no exception. Adherence may be as low as fifty percent by year four of treatment [39]. One year after commencement of tamoxifen therapy, the non-persistence rate may be as high as 22.1% [40] (non-persistence defined as 180 consecutive with no tamoxifen supply). Only one of the published studies accounted for adherence, using prescription data, and found that 14% of their study population took less than 80% of the prescribed tamoxifen doses [20]. By regrouping non-adherent subjects into a decreased metabolizer category, the hazard ratio increased from 2.57 to 3.02. Non-adherence may be reducing the observable effect of *CYP2D6* genotype on outcome in other studies. Co-medication with CYP2D6 inhibitors, such as SSRIs, is another factor to consider since this has been shown to reduce plasma endoxifen concentration [41]. One might expect efficacy to be reduced as well. Several studies accounted for the use of inhibitors in their analyses [17, 18, 20, 32, 33], but only Newman *et al.* found a significant relationship between inhibitor use and clinical

outcome [17]. However, like tamoxifen, it is difficult to accurately account for co-medication use among study subjects.

1.5.2 Association Between Serum Endoxifen Concentration and Clinical Outcome

CYP2D6 functional variants influence the metabolism of tamoxifen, specifically the formation of the potent anti-estrogenic metabolite endoxifen, and this is the proposed mechanism by which these variants affect clinical outcome. Thus, an important question to ask is whether or not serum endoxifen concentrations, not just *CYP2D6* genotype, are associated with clinical outcomes. To date, there has been only one study published that has addressed this question, which was performed by the Women's Healthy Eating and Living (WHEL) study group in collaboration with our laboratory, which was responsible for determining serum concentrations of tamoxifen, N-desmethyltamoxifen and endoxifen for subjects in the study who had taken tamoxifen for 3 or more months[42]. The primary goal of the WHEL study group was to determine whether or not a dietary intervention improved outcomes of early breast cancer patients. As part of their study, they banked not only several hundred DNA samples, but also serum samples, thus enabling the assessment of tamoxifen, N-desmethyltamoxfen, and endoxifen levels in their study subjects. In addition, they had clinical outcome data, such as five year recurrence rates. The lack of collection of these types of samples and data from subjects by other study groups interested in breast cancer outcomes is one factor that has likely prevented the publication of similar studies. Thus, the availability of banked biological specimens, long-term endpoint data, patient reported covariates

(age, race, BMI, etc.) and tumor characteristics in a sizeable study population (1370 subjects were used in the analysis), allowed the WHEL study group to determine if serum endoxifen concentrations are associated with clinical outcomes in subjects taking tamoxifen as adjuvant therapy. In addition to serum measurments of tamoxifen, NDTam, and endoxifen, *CYP2D6* genotype was determined using the Roche P450 AmpliChip. There was not an independent association between *CYP2D6* genotype and clinical outcome. However, serum endoxifen concentrations were associated with outcome, and *CYP2D6* genotype was one factor that was found to be associated with serum endoxifen concentrations, in addition to BMI and serum tamoxifen concentration. Like *CYP2D6* genotype, neither BMI nor serum tamoxifen concentration were independently associated with outcome. A threshold effect was determined for endoxifen using split-regression analysis. Subjects with serum endoxifen concentrations above 5.97 ng/mL, which represented the top four quintiles of endoxifen concentration, had a 30% lower risk of additional breast cancer events (HR = 0.70, 95% CI, 0.52–0.94) when data was adjusted for tumor stage and grade. Compliance and CYP2D6 inhibitor use are two factors not accounted for in the study, which may have contributed significantly to low serum endoxifen concentrations within the cohort. Thus, serum endoxifen concentrations do, indeed, appear to be associated with the clinical outcome of tamoxifen adjuvant therapy within this cohort. Additional studies are required in order to replicate these findings.

1.6 Challenges of CYP2D6 Activity Prediction

The *CYP2D6* gene is localized on chromosome 22q13.1 and encodes a polypeptide of 497 amino acids. Approximately 20-25% of drugs in clinical use are metabolized, at least in part, by the CYP2D6 enzyme [43]. Over 80 different allelic variants have been described which include full activity alleles, reduced function alleles, non-functional alleles, and gene duplications which result in "ultra-rapid" metabolism (see Table 1.1). The resulting genotypes are somewhat complex, leading to additional challenges for the prediction and description of CYP2D6 metabolic phenotype. Clinical pharmacology studies, which have often utilized the CYP2D6 probe substrates debrisoquine, sparteine or dextromethorphan, have been used to interrogate the wide range of CYP2D6 activity observable within populations. Urinary metabolite data (i.e. dextromethorphan/dextrorphan ratios) is used to describe CYP2D6 activity. The traditional phenotypes that have resulted from these studies are poor (PM), intermediate (IM), extensive (EM), and ultrarapid (UM) metabolizers (see Table 1.3). Enzymatic activity is predicted by a subject's highest function allele [44]. For example, a subject carrying one null and one full activity allele is classified as EM, while a carrier of one reduced function and one null allele is classified as IM. Carriers of one or more full function alleles are classified as EM and are considered phenotypically "normal", while carriers of one reduced function and one null allele or two reduced function alleles are classified as IM, and carriers of two null alleles are classified as PM. Subjects with functional *CYP2D6* gene amplification are considered UM, and the copy number can vary from 2-13 [45], with each copy increasing enzymatic activity. In addition to this

classification system, a CYP2D6 activity score has been proposed by Gaedigk *et al.*, in which a full activity allele is assigned a score of 1, a reduced activity allele is assigned a score of 0.5 and an inactive allele is assigned a score of zero [46]. The sum of allele scores results in the CYP2D6 activity score. It is intended to be "a 'user-friendly' tool that allows translation of genotype into a qualitative measure of phenotype." One can see how it would be simpler to report and explain this type of scoring system in the clinical setting. However, due to potential substrate dependent effects of CYP2D6 variants, modifications in the score system may be warranted based upon the drug of interest. Very extensive genotyping explains approximately 60% of the variation in dextropmethorphan metabolism [46] using the activity score system. Borges *et al.* elected to represent genotype predicted metabolizer group by merely listing the activity of each allele [47]. Variants may confer full (EM), reduced (IM), null (PM), or increased (UM) metabolism. Thus, a subject with one full activity allele and one null allele would fall into the EM/PM category. Clearly, there is potential for confusion considering the nomenclature of the classical categorization system is used in a different way. However, one advantage is that it doesn't make assumptions about the relative activity of each allele. This is therefore the categorization system used in the following chapters.

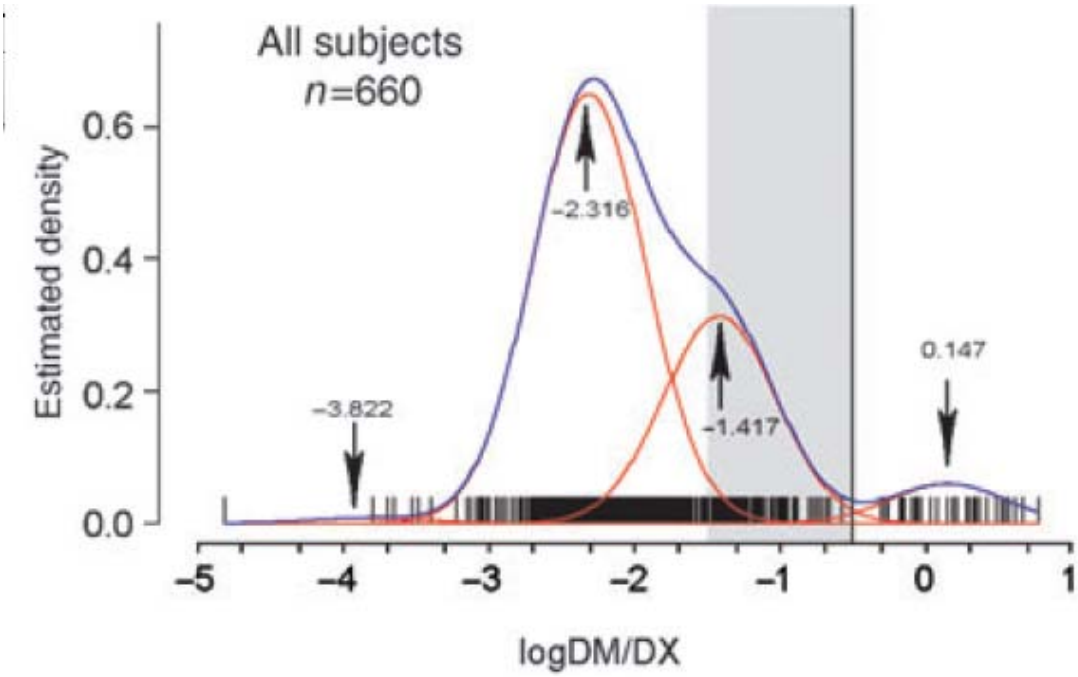

Figure 1.3: CYP2D6 activity using dextromethorphan as a probe drug [46]. The log of the urinary metabolic ratio of dextromethorphan to dextorphan (logDX/DM) is used to describe CYP2D6 activity within the study population, which consists of Caucasian (n= 362) and African American subjects (n = 274). The blue line is the summary line, while the red line marks the observable population clusters.

Due to the multitude of functional variants, very extensive genotyping is required in order to make reasonable predictions of CYP2D6 activity. The method and extent of genotyping may be a source of discrepancy between clinical research studies on CYP2D6 and the outcome of tamoxifen adjuvant therapy. When *CYP2D6* genotyping is performed clinically, the methods used and the variants tested are somewhat variable. The potential effect of this variability on clinical care is unknown, but of concern because of the potential for misclassification of metabolizer status. The Roche P450 AmpliChip was the first FDA approved pharmacogenetic test, and it provides extensive genotyping of both *CYP2D6* and *CYP2C19* variants. The AmpliChip was

developed by Affymetrix and licensed by Roche Diagnostics. It is one method by which a clinical study or clinical laboratory may interrogate *CYP2D6* polymorphisms, however there are less expensive and less extensive methods that are used. Roche Diagnostics recognized the potential for the clinical use of the AmpliChip for tamoxifen pharmacogenetics, and provided genotyping and AmpliChips to several tamoxifen pharmacogenetics studies, including the WHEL study. Thus, the tools are in place to offer clinical pharmacogenetic testing of *CYP2D6* variants for patients with ER^+ disease. However, if and how this data should be used for clinical care is still up to debate.

Table 1.1. *CYP2D6* Polymorphisms and Their Predicted Enzymatic Activity by Population [48]

Allele	Predicted Enzymatic Activity	Japan	China	Caucasian EU	Caucasian US	Black American	Black African	Amerindian	Saudi Arabia	Turkey
*1	Normal	42-43%	23%	33-37%	37-40%	29-34%	28-56%	66%	*	37%
*2	Normal	9-13%	20%	22-33%	26-34%	20-27%	11-45%	19%	*	35%
*3	None	*	1%	1-4%	<2%	<1%	<1%	0%	*	0%
*4	None	<1%	0-1%	12-23%	18-23%	7-9%	1-7%	4%	4%	11%
*5	None	5-6%	6%	2-7%	2-4%	6-7%	1-6%	4%	<1%	15%
*6	None	*	*	<2%	3%	<1%	0%	1%	*	7%
*9	Reduced	*	*	0-3%	7%	<1%	0%	0%	*	<1%
*10	Reduced	39-41%	50-70%	1-2%	4-8%	3-8%	3-9%	1-17%	<1%	6%
*17	Reduced	*	*	<1%	*	15-26%	9-34%	*	<1%	<1%
*41	Reduced	*	*	20%	*	*	*	*	*	*
*1XN	Increased	<1%	*	<1%	<1%	1%	3%	*	*	<1%
*2XN	Increased	<1%	1%	<2%	<1%	1%	3%	*	10%	<1%
*4XN	None	*	*	<1%	<1%	2%	1%	*	*	<1%

1.7 Tamoxifen Metabolism and Transporters

In the United States, the standard dose of tamoxifen for adjuvant endocrine therapy is currently 20 mg/day, independent of age and body weight. Tamoxifen is 98% bound to albumin, resulting in a long half-life. Steady-state concentrations of tamoxifen are achieved after 3–4 weeks [49, 50]. The metabolism of tamoxifen is complex, and there are many metabolites present in the tissues and biological fluids of patients taking the drug. The major route of elimination of tamoxifen and its metabolites is in the feces, with approximately 20% excreted in the urine [49].

1.7.1 Phase I metabolism

Several members of the cytochrome P450 superfamily are involved in the oxidative metabolism of tamoxifen. CYP3A4/5 and CYP2D6 play prominent roles. Recent tamoxifen pharmacogenetic studies that have measured serum metabolite levels have focused on the phase I metabolites N-desmethytamoxifen (NDTAM), 4-hydroxytamoxifen (4OHTam) and 4-hydroxy-N-desmethyltamoxifen (endoxifen) [13, 51, 52] (see Figure 1.4). The most abundant tamoxifen metabolite is N-desmethyltamoxifen (NDTam), which can be found at concentrations 2-3 fold higher than that of tamoxifen. Like tamoxifen, NDTam is highly bound to albumin. NDTam has potency similar to that of tamoxifen and is produced when tamoxifen is metabolized by CYP3A4 and CYP3A5. Steady state levels of NDTam are attained after approximately 8 weeks of tamoxifen therapy [50]. 4OHTam is a primary metabolite that is produced when tamoxifen is metabolized by CYP2D6, CYP2B6, CYP2C9, or CYP2C19. While 4OHTam is present at

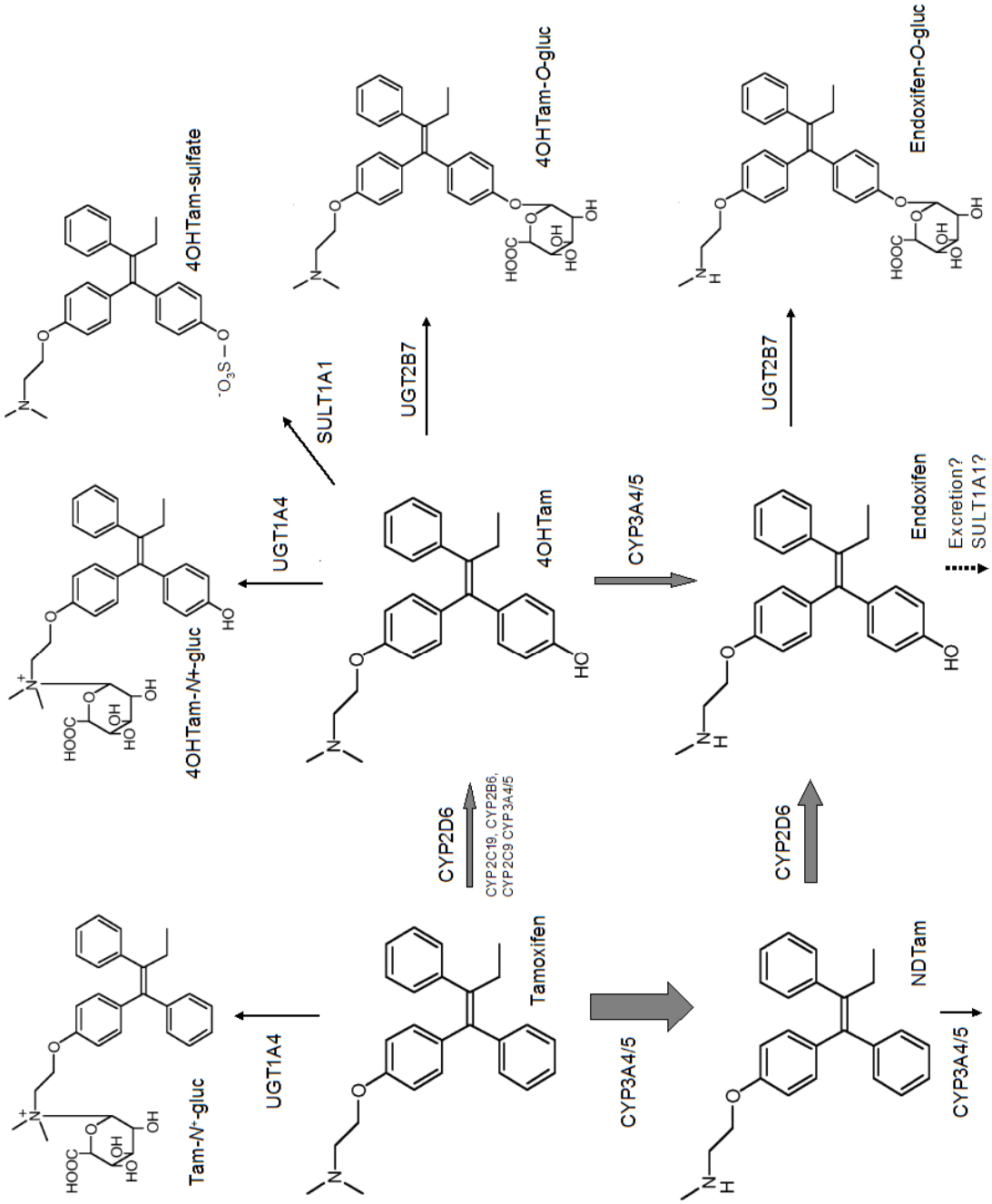

Figure 1.4. Tamoxifen Metabolism

low concentrations in plasma, it has a higher affinity for ER-α and is a more potent anti-estrogen (30-100X) than tamoxifen in ERα$^+$ MCF-7 cells [53, 54]. Currently endoxifen is thought to be the major mediator of tamoxifen's pharmacological effect because, on average, it is more abundant than 4OHTam in plasma (~6X) and has similar anti-estrogenic potency in ERα$^+$ MCF-7 cells. The hydroxylation of NDTam by CYP2D6 is the major pathway of formation of endoxifen. 4OHtam and endoxifen are subject to phase II metabolism [55-59].

1.7.2 Phase II metabolism

Glucuronidation and sulfation may be important for the inactivaton of tamoxifen and its metabolites. Recent *in vitro* studies by Sun *et al.* suggest that UGTs 1A10, 2B7, and 1A8 exhibit high capacity for *trans*-endoxifen glucuronidation, as well as *trans*-4OHtam glucuronidation [55]. In the study, kinetic analyses of endoxifen and 4OHtam *O*-glucuronidation were performed using lysates of nine different cells lines that over-express individual UGTs. Of the UGTs examined, UGT2B7 was able to conjugate endoxifen with high activity, and unlike UGT1A10 and 1A8, it is highly expressed in the liver where it can have a major influence on serum endoxifen concentration. UGT1A4 is capable of tamoxifen and 4OHTam *N*-glucuronidation. However, the N-demethylated tamoxifen metabolites, NDTam and endoxifen, are not subject to *N*-glucuronidation. Endoxifen-gluc and 4OHtam-gluc are highly enriched in urine and bile, and tamoxifen-gluc also appears to be present in these biological fluids [49]. Both endoxifen-gluc and 4OHtam-gluc have been detected by LC-MS/MS directly in the urine of women taking

tamoxifen [60]. The direct detection of sulfated tamoxifen metabolites in biological fluids has not been published, so the relevance of sulfation to the inactivation of endoxifen is somewhat unclear. Further research is warranted. However, SULT1A1 does exhibit high activity for 4OHTam sulfation *in vitro* [59]. Of the phase II metabolic enzymes, the published data suggests that UGT2B7 is the most likely to influence serum endoxifen concentrations because it is highly expressed in the liver, capable of endoxifen glucuronidation, and the metabolite endoxifen-gluc is detectable in human biological fluids.

1.7.3 Drug efflux transporters

P-glycoprotein (Pgp), multidrug resistance protein 1 (MRP1), multidrug resistance protein 2 (MRP2), and breast cancer resistance protein (BCRP) are well characterized multidrug efflux pumps that are members of the ATP-binding cassette (ABC) superfamily. ABC multi-drug transporters are known to influence the absorption, distribution, metabolism, and excretion (ADME) of their substrates. In particular, these transporters have been implicated in numerous studies of cancer resistance mechanisms. Tamoxifen is known to inhibit the transport of known Pgp substrates and has been shown to activate the ATP-ase activity of P-glycoprotein which is generally indicative of transport [61, 62]. Neither tamoxifen, NDTam, nor 4OHTam were substrates for Pgp when assayed in Caco-2 cell monolayers in a transwell system [63]. However, rats that were exposed to tamoxifen for 12-days, versus untreated rats, exhibited an increase in hepatic Pgp expression and an increase in biliary excretion of injected C14-tamoxifen and polar metabolites, as measured by HPLC and scintillation

counting [64]. These results suggest that tamoxifen and its metabolites may be substrates for Pgp, or possibly another efflux transporter that is expressed apically by hepatocyes and is also induced by tamoxifen exposure. Tamoxifen has also been described as a weak BCRP inhibitor [65], but whether or not it is a substrate for this transporter is unclear from the literature. There is little to no published data on transport of tamoxifen or its metabolites by multidrug resistance proteins. However, in a study by Kauffmann *et al.*, rhesus monkeys that were dosed with tamoxifen (25 mg/kg/day) for seven days prior to sacrifice had a marked increase in hepatic MRP2 expression compared to monkeys treated with vehicle alone [66]. It must be noted that the dose of tamoxifen given to monkeys in this study was super-therapeutic (20X or more); the normal dosage of tamoxifen is 0.3-1.2 mg/kg/day in humans. In addition, a recent study by Choi *et al.* suggests that repeated exposure to high doses of tamoxifen can induce MRP2 expression in MCF-7 cells [67]. Thus, transport of tamoxifen and its metabolites by MRP2 is an interesting possibility that remains to be tested. In addition, there have been no published studies on the efflux transporters and phase II tamoxifen metabolites, although these metabolites are known to be excreted into the urine and bile. While Pgp does not transport conjugated metabolites, conjugated metabolites are common substrates for the efflux transporters BCRP, MRP1, and MRP2. The Phase II tamoxifen metabolites, mainly glucuronidated 4OHTam and endoxifen, are potential substrates for these transporters.

1.8 Overall Significance and Research Project

At present, there is an incredible amount of interest from patients, clinicians, and diagnostic companies in the use of *CYP2D6* testing to optimize adjuvant endocrine therapy for breast cancer patients. Because ER^+ breast cancer is a common disease, a very sizeable patient population could potentially benefit from this pharmacogenetic intervention. Not only is *CYP2D6* genotyping feasible in the clinical setting, it is currently being performed and reported by certified clinical laboratories. FDA approved diagnostics, like the Roche P450 AmpliChip, are available. The question is not *if* this testing can be done, but whether or not it *should* be done for the breast cancer population. The answer to this question may only be apparent after the completion of several prospective clinical trials. However, in order to guide these research efforts, it is useful to identify which interventions based upon patient genotype and/or other testing are likely to be beneficial; this was the general goal of this thesis project. Of major importance was to address the issue of the endoxifen hypothesis – to determine whether or not serum endoxifen concentrations, and not merely *CYP2D6* genotype, are associated with the outcome of tamoxifen adjuvant therapy. In order to address this question, an LC-MS/MS assay was developed and validated for the measurement of tamoxifen and tamoxifen metabolites in serum. Using this assay and the measurements made by our laboratory, the WHEL study group was able to show that serum endoxifen concentrations are associated with the clinical outcome of tamoxifen adjuvant therapy. In addition, they were able to define a sub-therapeutic endoxifen population at increased risk for recurrence. Included in this thesis is an assessment of how well

CYP2D6 genotype predicted metabolizer status defines the sub-therapeutic endoxifen risk group for Caucasian subjects from the WHEL cohort. This was done using serum tamoxifen and tamoxifen metabolite data from the WHEL cohort, in addition to the extensive CYP2D6 genotyping data from the Roche P450 AmpliChip that was performed for these study subjects. In addition, the ethnic diversity and large number of study subjects within the WHEL cohort allowed for a comparison of serum tamoxifen and tamoxifen metabolite levels, as well as *CYP2D6* genotypes, between different ethnic populations. Finally, it was of interest to see if variants in genes that may be involved in tamoxifen metabolism are associated with serum endoxifen concentration and the endoxifen:N-desmethyltamxifen metabolic ratio within the WHEL cohort.

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Chapter 2

Development, Validation, and Application of a Liquid Chromatography Tandem Mass Spectrometry Method for the Quantitation of Tamoxifen, N-Desmethyltamoxifen, and Endoxifen in Human Serum

2.1 Introduction

Tamoxifen is a selective estrogen receptor modulator that is used in the treatment of all stages of estrogen receptor positive breast cancer, as well as for the prevention of breast cancer in women who are at high risk for developing the disease. Tamoxifen is widely used as an adjuvant – after the removal of the primary tumor for the prevention of recurrence, at the standard dose of 20 mg/day. However, response to this treatment is variable and approximately 30-50% of patients in the adjuvant population experience relapse [1]. Variation in response to therapy may be due to tumor specific factors, such as differences in gene expression within the tumor cells, or to patient specific factors, such as germline genetic variability or patient behavior.

Polymorphisms in *CYP2D6* that result in decreased enzymatic activity have been associated with poorer clinical outcomes in patients treated with tamoxifen in some [2-6], but not all studies [6-10]. Tamoxifen is considered a pro-drug since hepatic metabolism by the CYP2D6 enzyme results in the formation of potent 4-hydroxylated metabolites [11-17] (Figure 1). These 4-hydroxylated metabolites exhibit increased binding affinity for the estrogen receptor alpha, and are thus more potent competitive inhibitors of estrogen signaling than tamoxifen itself. Both 4-hydroxytamoxifen and 4-hydroxy-N-desmethyltamoxifen (endoxifen) are present in the serum of patients taking

tamoxifen. However, because average concentrations of endoxifen are six times higher than 4-hydroxytamoxifen, endoxifen is thought to be responsible for the majority of tamoxifen's anti-estrogenic effects [18].

Any factor that results in reduced serum concentrations of endoxifen could potentially compromise tamoxifen efficacy. *CYP2D6* genotype appears to predict less than 30% of the variation in endoxifen concentrations that has been observed [19]. Co-medication with potent CYP2D6 inhibitors has been shown to influence serum endoxifen concentrations. While co-medication with potent CYP2D6 inhibitors is contraindicated for patients taking tamoxifen, research suggests that drug label recommendations related to impaired bioactivation of pro-drugs are more likely to be ignored than recommendations related to adverse drug reactions [20]. When both CYP2D6 inhibitors and *CYP2D6* variants are considered, less than 50% of variation in endoxifen concentration is explained. [18, 19]. Thus, there are other factors, yet to be identified, that influence serum endoxifen levels. These factors may include the use of herbal medications that interfere with tamoxifen metabolism, or genetic variation that influences the elimination of endoxifen. In terms of patient behavior, compliance to adjuvant endocrine therapy is known to be poor in both premenopausal and postmenopausal patients, and this may be an additional cause of treatment failure [21-23]. Measurement of serum tamoxifen and NDTam concentrations yields additional information about the cause of low serum endoxifen concentrations. A low ratio of endoxifen to NDTam suggests low CYP2D6 activity while low serum tamoxifen concentrations may be an indicator of poor compliance. Thus, the ability to measure

tamoxifen, NDTam, and endoxifen is important for clinical tamoxifen pharmacogenetic studies, and potentially for therapeutic drug monitoring in the clinical setting.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is well suited for the quantification of tamoxifen and its metabolites in serum. For this reason, a quantitative LC-MS/MS assay was developed in our laboratory in order to support ongoing clinical tamoxifen pharmacogenetic studies. Identification of the analytes of interest using LC-MS/MS is based upon: (a) resolution of tamoxifen and its metabolites using reverse phase chromatography, and (b) identification of metabolites using electrospray ionization (ESI) and a selected reaction monitoring (SRM) tandem mass spectrometry. Many tamoxifen metabolites, in addition to NDTam and endoxifen, are present in the serum of patients taking tamoxifen. Both LC retention time and identification of an SRM precursor:product ion pair are very important for quantitation since there are multiple tamoxifen metabolites that may either have the same transition or similar retention times, but not both. Drugs and metabolites with different SRM precursor:product ion pairs do not need to be completely resolved from one another for accurate quantitation, enabling shorter run times than HPLC would allow.

2.2 Materials and Methods

2.2.1 Reagents

The drug standards tamoxifen (Sigma Aldrich), N-desmethyltamoxifen (Toronto Research Chemicals) and endoxifen (Toronto Research Chemicals), as well as the deuterated internal standards D5-tamoxifen, D5-N-desmethyltamoxifen, and D5-endoxifen (Toronto Research Chemicals) were prepared as concentrated methanolic

stocks and stored at -70°C in tinted vials. Concentrated master mixes containing tamoxifen, ND-Tam, and endoxifen, which were used for the preparation of known calibrators and quality control material, were prepared using analytical glassware and positive displacement pipettes. These master mixes were stored at -70°C in tinted vials. An internal standard (IS) mix, containing D5-endoxifen, D5-tamoxifen, and D5-N-desmethyltamoxifen, was prepared in methanol and stored at -70°C. Drug free human serum, used for the preparation of calibrators and QC material was obtained from Biological Specialty Corp. and stored at -70ºC until use. Formic acid, ammonium formate, LC-MS grade water, LC-MS grade acetonitrile, and LC-MS grade methanol, were obtained from Fisher Scientific and used for the preparation of mobile phase. Oasis MCX extraction cartridges were purchased from Waters.

2.2.2 Clinical Samples

Seventy eight serum and DNA samples were banked as part of a study on Genetic Predictors of Tamoxifen Response (CHR: H11480-25504-04; Dr. Elad Ziv). At the time of sampling, the duration of tamoxifen therapy was between 17-63 months and all subjects are presumed to have reached steady-state tamoxifen concentrations. The population consisted of 44% pre-menopausal women and 56% post-menopausal women. The ages of the subjects range from 35-83 years (average 57.8 years). Of the 75 subjects for which race/ethnicity data is available, 62 were Caucasian (82.7%), 10 were Asian (13.3%), 2 were African American (2.7%) and 1 was Latina (1.3%).

2.2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS)

An Applied Biosystems 3200 QTRAP, a triple quadrupole/linear ion trap (LIT) mass spectrometer, equipped with a Turbo Ion-Spray ionization source, was used for mass spectrometry. In order to optimize settings for the assay, the quantitative optimization feature in the Analyst 1.4.2 software (Applied Biosystems Sciex), which tests a series of assay conditions to optimize detection of the analytes of interest, was used. The analytes of interest were infused into the mass spectrometer with a syringe pump at a concentration of 100 ng/mL at a rate of 5 µL/min in a mixture of 50:50 mobile phase A: mobile phase B. The information-dependent acquisition feature (IDA) of the Analyst Software was used to perform selected reaction monitoring as a survey scan, followed by a product ion scan under varied conditions, for each analyte in order to optimize detection parameters. Compounds were infused at a concentration of 100 ng/mL at a rate of 5 µL/min into the mass spectrometer using a syringe pump during optimization.

Liquid chromatography was performed using an Agilent 1200 series LC system. The analytical column used was a Waters X-terra MS C18 column, 3.5 µm (2.1 mm x 150 mm), which was preceded by a Waters 2.1 x 10 mm C18 guard column and pre-column (MacMod). Mobile phase A consisted of 5 mM ammonium formate buffer, pH 4.5 + 2% methanol, while mobile phase B consisted of 70:20:10 acetonitrile:methanol:50 mM ammonium formate buffer, pH 4.5 (by volume). During LC method optimization, glycerophosphocholines (GPChos), which are present in many biological samples, were monitored in order to ensure the separation of these interfering substances from the

analytes of interest. As has been described by Little et al, these were monitored using the following conditions: declustering potential (165), entrance potential (10), collision energy (7), and collision cell exit potential (5V) using a mass transition of *m/z* 184 → 184 in the positive ion electrospray mode (ref). Gradient elution was used to resolve analytes (min 0-7), and was followed by a column wash (min 7.1-9) and re-equilibration (min 9.1-11). See table 2.1 for the chromatography gradient. A volume of 15 μL was injected for each sample. A needle wash in mobile phase B was performed before each injection.

Table 2.1 Liquid Chromatography Gradient.

Time (min)	*Flow rate (μL/min)*	*%MPA*	*%MPB*
0	350	65	35
0.5	350	65	35
0.6	350	50	50
4	350	40	60
4.1	350	30	70
6	350	25	75
7	350	25	75
7.1	350	0	100
8	600	0	100
9	800	0	100
9.1	500	65	35
11	350	65	35

2.2.4 Extraction Efficiency

Stocks (10X) for high and low analyte concentrations were prepared in methanol for the extraction efficiency study. Two sample sets, consisting of four 200 μL serum aliquots, were prepared for each concentration tested: (1) serum spiked with 20 μL of methanol, and (2) serum spiked with high (HI) or low (LO) drug standards. Serum samples were diluted with 800 μL of 0.5 mM ammonium formate, pH 3.0. Samples were extracted with Waters 1cc MCX columns according to the manufacturer's instructions, with minor modifications. Briefly, the 1cc MCX extraction cartridges were attached to a Visiprep (Sigma) vacuum manifold. Under gentle vacuum, cartridges were conditioned with 1 mL of MeOH, equilibrated with 1 mL of ddH_2O, and then 1 mL of sample was applied. Columns were washed once with 1 mL of 2% formic acid in ddH_20, followed by a wash of 1 mL of MeOH. Sample was then eluted from the extraction cartridges using freshly prepared 5% ammonium hydroxide in MeOH. The extracts were collected in 5 mL polystyrene tubes. After extraction, sample set 1 was spiked with 20 μL of either the HI or LO 10X stock, while sample set 2 was spiked with 20 μL of methanol. Low concentrations for tamoxifen, NDTam, and endoxifen were spiked at 10 ng/mL, 20 ng/mL, and 1.75 ng/mL respectively. High concentrations for tamoxifen, NDTam, and endoxifen were spiked at 200 ng/mL, 400 ng/mL and 35 ng/mL respectively. Extracts were dried in an N-EVAP under a gentle stream of nitrogen at 40°C. Samples were resuspended in 100 μL of 80:20 MpA:MpB and transferred to 300 μL polypropylene autosampler vials for analysis. Peak area counts from samples spiked with standards before and after extraction were compared in order to determine extraction efficiency.

2.2.5 Ion Suppression

Ion suppression profiles were obtained by post-column infusion of the compounds of interest. Three different serum aliquots, which were taken from leftover clinical samples from the San Francisco General Hospital (SFGH) Clinical lab, were extracted using the procedure listed above. Extracted serum samples, as well as an MpA blank, were injected and run through the LC column using the method described, while analytes were infused, post column, with a syringe pump using a T-in connector before delivery into the MS.

2.2.6 Limit of Quantitation and Assay Linearity

Nine concentration points plus blank were analyzed in order to determine the lower limit of quantitation (LLOQ) of the assay. Drug-free serum was spiked with a master mix containing tamoxifen, N-desmethyltamoxifen, and endoxifen and a set of two-fold serial dilutions were made. Concentrations ranged from 0.125 – 25 ng/mL for endoxifen, 1.25 – 250 ng/mL for tamoxifen, and 2.5 – 500 ng/mL for N-desmethyltamoxifen. In addition to this dilution series, calibrators were extracted and run (see table 2.2). Over the series of four days, one set of six calibrators was extracted and analyzed each day, and linearity was assessed. Prior to extraction, samples were spiked with 80 μuL of internal standard mix diluted in 0.5 mM ammonium formate, pH 3.0. Samples were extracted as described above. Dilution series were run on the instrument from lowest to highest concentration, with a blank after the highest concentration to determine background. Least-squares linear regression was used to fit

calibration curves, using the reciprocal of the squared concentration ($1/x^2$) as the weighing factor.

Table 2.2 Calibrator Concentrations (ng/mL)

Calibrator#	*Tamoxifen*	*NDTam*	*Endoxifen*
1	7.5	15	1.25
2	15	30	2.5
3	30	60	5
4	60	120	10
5	120	240	20
6	240	480	40

2.2.7 Precision and Accuracy

Quality control material, which consisted of aliquots of serum spiked with high and low concentrations of the analytes of interest, was prepared and stored at -70°C until use. Low concentrations for tamoxifen, NDTam, and endoxifen were spiked at 10 ng/mL, 20 ng/mL, and 1.75 ng/mL respectively. High concentrations for tamoxifen, NDTam, and endoxifen were spiked at 200 ng/mL, 400 ng/mL and 35 ng/mL respectively. Four high QC and four low QC serum aliquots were thawed, spiked with internal standard and extracted as described, and analyzed, in addition to standard curve material (see table 2.2 for concentrations), on each of the four days of precision

testing. The concentrations of the quality control samples were calculated using the calibration standards that were processed in parallel. The difference between the nominal and the measured concentrations was used to determine the accuracy of the assay.

Applied Biosystems Analyst Software was used for quantitation. The ratio of the peak area of each analyte in the calibration material to that of the peak area of the respective internal standard is used in order to generate a standard curve across concentrations for the analytes of interest. Analyst software integrates both the analyte peak area and the internal standard peak area for all analytes in unknown samples and then calculates the ratio of analyte to internal standard peak area. The software then references the standard curve in order to determine analyte concentrations in the unknown samples.

2.2.8 Sample Stability

Assessment of sample stability after extraction and re-suspension in mobile phase was performed. A standard curve plus three high, three medium and three low concentration QC samples were extracted, run on the instrument, and allowed to sit overnight in the refrigerated (4°C) autosampler. For tamoxifen, QC material concentrations were 10 ng/mL, 50 ng/mL and 200 ng/mL. For NDTam, QC material concentrations were 20 ng/mL, 100 ng/mL and 400 ng/mL. For endoxifen, QC material concentrations were 1 ng/mL, 5 ng/mL and 20 ng/mL. Extracted calibrators and QC samples were then stored in the freezer (-20° C) and re-run to determine if there was a difference in overall signal and calculated concentration from the initial analysis.

2.2.9 LC-MS/MS Method Application

Serum concentrations of tamoxifen, ND-Tam, and endoxifen were determined using the method described in the 78 banked TAMGEN serum samples. These samples were run over a series of three days. A set of four high and four low QC samples, as well as a six point calibration curve, was extracted and run with each sample set.

2.2.10 Genotyping

The *CYP2D6* *4 (1846G>A, rs3892097), *10 (100 C>T, rs1065852), and *41 (2988G>A, rs28371725) alleles were genotyped using commercially available TaqMan Drug Metabolism (DM) genotyping assays (Applied Biosystems), specifically the C__27102431_B0, C__11484460_40, and C__34816116_20 assays for the 78 banked TAMGEN DNA samples. Samples were prepared in 384 well optical plates in 5 μL reaction volumes according to the manufacturer's instructions. Plates were run on an ABI PRISM 7900HT Sequence Detection System at the UCSF Genomics Core Facility. As the 100T allele, which is used to identify the *10 variant, is part of the *CYP2D6**4 haplotype, the 100T allele was classified as *10 in the absence of 1846A.

2.2.11 Statistics

Statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. The D'Agostino & Pearson omnibus normality test was used to determine if serum concentrations of tamoxifen, NDTam, and endoxifen concentrations were normally distributed. Median values, as well as the 25th and 75th percentile values are reported in this study for analyte concentrations. Kruskall-Wallis and Mann-Whitney tests were used to

determine differences between groups, and a p-value < 0.05 was considered statistically significant.

2.3 Results and Discussion

2.3.1 LC-MS/MS

Based upon the quantitative optimization that was performed using Analyst software, the following optimized conditions were used for positive mode electrospray ionization: curtain gas, 35.0; ion spray voltage, 5500; ion source gas 1, 60.0; ion source gas 2, 35.0; and temperature, 700°C. Compound dependent parameters were determined using the IDA feature. The protonated molecular $[M+1]^+$ ions for the known standards tamoxifen (*m*/*z* 372), NDtam (*m*/*z* 358), and endoxifen (*m*/*z* 374), and the internal standards used in the assay, D5-tamoxifen (*m*/*z* 377), D5-NDtam (*m*/*z* 363), and D5-endoxifen (*m*/*z* 379) were the predominant ions obtained by the Q1 scan (*m*/*z* 40–500). In terms of the product ions detected during optimization, only one major product ion could be detected for each analyte under the conditions tested. The proposed patterns of fragmentation for tamoxifen (372→72), NDtam (358→58), and endoxifen (374→58) are listed in Figure 2.2. Additional compound dependent parameters determined from optimization can be found in Table 2.3.

The development of the liquid chromatography portion of the assay presented various challenges. In addition to the analytes of interest, for which pure standards were available, there were several other metabolites that were consistently present in the serum of subjects who were taking tamoxifen, but not in drug-free serum samples. The clinical significance of these metabolites, if any, is not known. One such metabolite

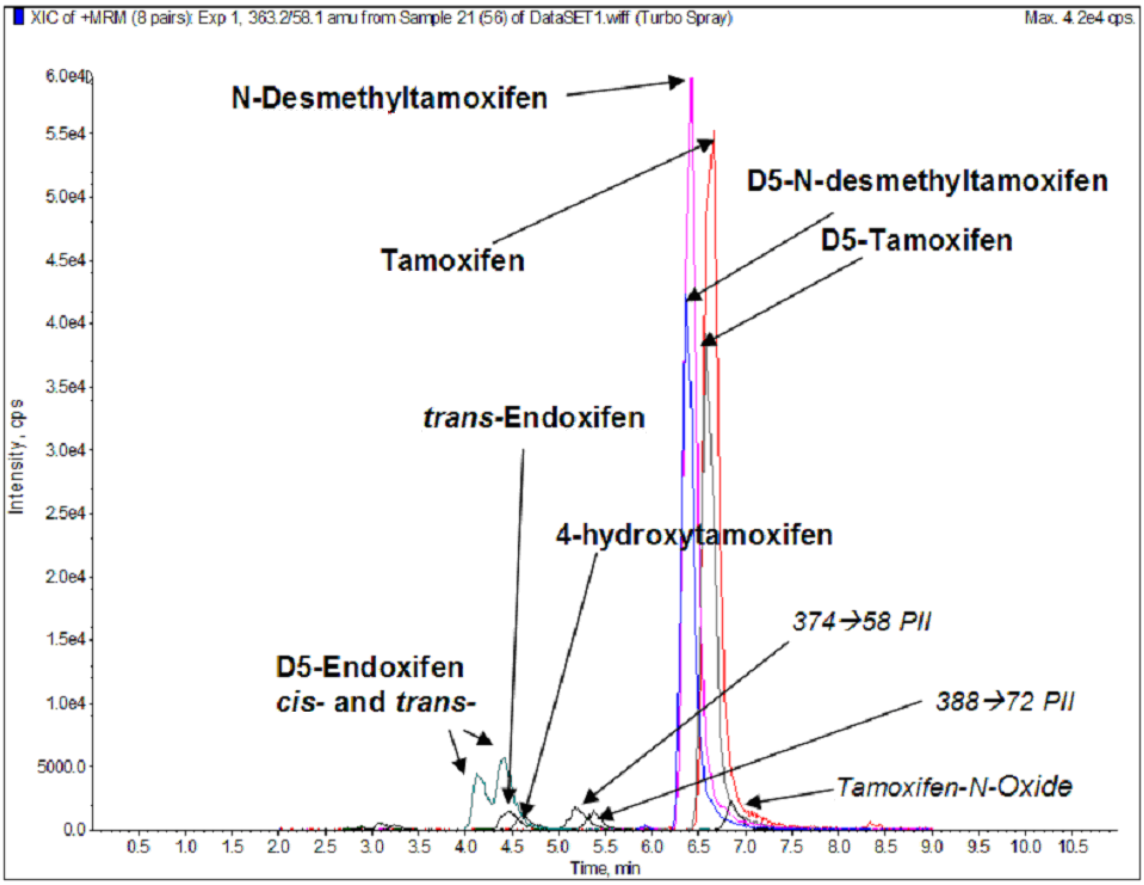
had the same transition as endoxifen (374→58), so it was therefore very important to resolve it from endoxifen in order to ensure specificity of detection. In the final LC method, endoxifen elutes at 4.5 min, while this additional peak elutes at 5.3 min. In addition, both endoxifen and D5-endoxifen are made up of *E*- and *Z*- isoforms, which elute off of the column in two peaks. The major form of endoxifen present in serum is the *Z*-isoform, although a small *E*-isoform peak is generally present. Analyst software was able to integrate this double peak in the vast majority of samples, but it was necessary to check the peak integration for all chromatograms, and sometimes perform manual integration. For a representative extracted ion chromatogram, see Figure 2.1. Early in assay development, it was apparent that GPChos co-eluting with the later eluting analytes, tamoxifen and NDTam, were interfering with detection such that within-day assay precision was suboptimal. Altering chromatography to resolve GPChos from the analytes, and including a column wash with a high percentage of MpB, appeared to dramatically improve assay precision.

2.3.2 Ion Suppression

No significant ion suppression was observable, when comparing the three matrix samples and MpA blank, at the retention times of the analytes of interest during post-column infusion. The liquid chromatography gradient was designed such that analytes would elute well after the first two minutes of the run, during which ion suppression was apparent.

Table 2.3 MS-MS Compound Dependent Parameters

Analyte	*Q1*	*Q3*	*Declustering Potential (V)*	*Entrance Potential (V)*	*Collision Energy (V)*	*Cell Exit Potential (V)*
Tamoxifen	372.2	72.1	51	8	45	4
N-desmethyltamoxifen	358.5	57.9	41	4.5	41	4
Endoxifen	374.2	58.0	46	4.5	43	6
D_5-tamoxifen	377.3	72.1	56	4	79	4
D_5-N-desmethyltamoxifen	363.2	58.1	71	2.5	55	4
D_5-endoxifen	379.3	58.0	41	8	41	4



*Metabolites in italics were detected, but concentrations were not measured in assay.

Figure 2.1 Representative Extracted Ion Chromatogram

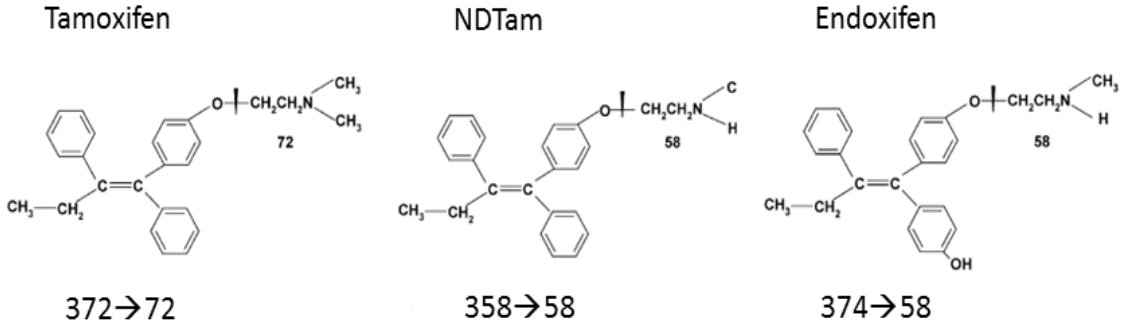


Figure 2.2 Proposed Fragmentation of Tamoxifen, NDTam, and Endoxifen

2.3.3 Extraction Efficiency

The extraction efficiency for each analyte was determined at two different concentrations. For tamoxifen, the extraction efficiencies at 10 ng/mL and 200 ng/mL were 88.9% and 69.6% respectively. For NDTam, extraction efficiencies for the 20 ng/mL and 400 ng/mL concentrations were 97.5% and 71.9%, respectively. Extraction efficiencies of endoxifen at 1.75 ng/mL and 35 ng/mL were 85.7% and 71.2%, respectively. For a summary of the extraction efficiency data, see Table 2.4. Extraction efficiencies at the lower concentrations for all three analytes are good, but somewhat less than expected for the higher concentrations tested. Nonetheless, these values are acceptable for the intended purpose of the assay.

Table 2.4 Extraction Efficiency

Analyte (conc)	*Avg. Peak Area, Extracted*	*Avg. Peak Area, Spiked in Matrix*	*Extraction Efficiency (%)*
Tamoxifen (10 ng/mL)	2.59E+05	2.91E+05	**88.9**
Tamoxifen (200 ng/mL)	1.95E+06	2.79E+06	**69.6**
NDTam (20 ng/mL)	2.74E+05	2.83E+05	**97.5**
NDTam (400 ng/mL)	1.93E+06	2.69E+06	**71.9**
Endoxifen (1.75 ng/mL)	4.41E+04	5.15E+05	**85.7**
Endoxifen (35 ng/mL)	4.36E+05	6.12E+05	**71.2**

2.3.4 Linearity and Limit of Quantitation

The criterion for the LLOQ for the assay is, (1) for the calculated concentration of the calibrator to be within 80-120% of nominal concentration, and (2) for the calculated concentration to be over two times that of background levels. For Tamoxifen, NDTam and endoxifen, 5 ng/mL, 10 ng/mL and 0.5 ng/mL were determined to be the LLOQs, respectively. Assay linearity was robust, with R^2 values greater than 0.99 for all analytes on all four days of testing.

2.3.5 Accuracy and Precision

Assay performance data is summarized in Table 2.5. The inter-assay precision data for tamoxifen, NDTam, and endoxifen at high and low concentrations were well under the allowable 15% of CV. Ideally, inter-assay accuracy should be within 85-115% of nominal concentration. This is the case for the analytes tested at high and low concentrations with the exception of the high concentration of NDTam, which measures at 83.6% of the nominal concentration. Because calibrators and QC materials were prepared on different days from separate methanolic stocks, and because the %CV for the 400 ng/mL NDTam is quite good at 3.06%, this issue of accuracy is likely due to differences in preparation between the calibrators and QC materials.

2.3.6 Sample Stability

After storage in a refrigerated auto-sampler overnight and a week of freezer storage, QC samples showed minimal deviation in measured concentration from the initial analysis. High, medium, and low concentrations in comparison to the initial measurement were as follows: tamoxifen (96.3%, 102%, 101%), NDTam (112%, 108%,

Table 2.5 Accuracy and Precision

Analyte	*Nominal Concentration (ng/mL)*	*Mean Measured Concentration (ng/mL)*	*Inter-assay Accuracy (%)*	*Inter-assay Precision (%CV)*
Tamoxifen	10.0	11.4	114	8.85
	200	185	93.0	4.77
NDTam	20.0	19.0	94.8	3.57
	400	334	83.6	3.06
Endoxifen	1.75	1.73	98.7	6.48
	35.0	32.9	94.1	3.00

and 105%), and endoxifen (91.3%, 100% and 97.2%). Extracted samples are generally considered stable if 115%-85% of the initial concentration is recovered. In terms of absolute signal, peak areas of QC samples were decreased to 50-64% that of the initial analysis. This decrease in signal may be due to degradation and/or the binding of analytes to autosampler vials. One must therefore re-run calibrators along with samples after storage in order to get accurate quantitation. A thorough assessment of other aspects of sample stability has been performed by Tenuissen et al.[24]. They found that three freeze thaw cycles of serum spiked with standards led to a minimal change (less than 4% deviation) in the calculated concentrations of tamoxifen, NDTam, and endoxifen.

2.3.7 Clinical Sample Screening

Concentrations of tamoxifen, NDTam, and endoxifen, as well as the [END]/[NDTam] ratio, were not normally distributed. Median values were 81.6 ng/mL, 186 ng/mL, 7.06 ng/mL, and 4.28E-02, respectively. For a summary of the LC-MS/MS screening data, see Table 2.6. Significant inter-patient variation in tamoxifen and tamoxifen metabolite concentrations were determined within the sample set screened. Serum endoxifen concentrations were the most variable. CYP2D6 metabolism is known to be highly variable, and the major pathway for endoxifen formation, so these results are as expected.

Genotypes for the *CYP2D6* *1, *4, *10, and *41 were determined for all 78 subjects using TaqMan allelic discrimination assays. The *4 allele results in an absence of CYP2D6 activity (PM), while the *10 and *41 alleles result in reduced activity (IM). In the absence of these variants, alleles were classified as *1, which is wild-type. See Table 2.7 for the distribution of *CYP2D6* genotypes. Genotype was used to predict CYP2D6 activity and subjects were classified into six different metabolizer groups: EM/EM, EM/IM, IM/IM, EM/PM, PM/IM, and PM/PM. Median, maximum, minimum, 25th percentile, and 75th percentile values for concentrations of tamoxifen, NDTam, and endoxifen were determined for these groups (see Table 2.8 and Figure 2.3). Previous publications do not indicate a relationship between *CYP2D6* genotype and serum tamoxifen concentrations, however there was a significant difference in tamoxifen concentration between metabolizer groups ($p=0.0008$). Median tamoxifen concentrations were significantly ($p < 0.05$) higher in the IM/IM group in comparison to

all other groups, with the exception of EM/EM. There was also a significant difference in NDTam concentrations between metabolizer groups (p=0.0002), with the IM/IM group exhibiting significantly higher concentrations ($p < 0.05$) than all other groups. This difference cannot be explained by differences in CYP2D6 activity as predicted by the genotyping that was performed. In terms of serum endoxifen concentrations, there was a significant difference between metabolizer groups ($p < 0.0001$). Subjects in the

Table 2.6 Summary of Tamoxifen, NDTam, and Endoxfien Concentrations

	[TAM] (ng/mL)	*[NDTam] (ng/mL)*	*[END] (ng/mL)*	*[END]/[NDTam]*
Minimum	20.9	55.2	1.46	7.86E-03
25% Percentile	58.8	129	4.55	2.40E-02
Median	81.6	186	7.06	4.28E-02
75% Percentile	113	220	10.6	6.32E-02
Maximum	238	535	26.1	1.42E-01

Table 2.7 Summary of CYP2D6 Genotype Data

Metabolizer Group (predicted phenotype)	*Genotypes*	*#*	*%*
EM/EM (Extensive)	*1/*1	22	28.2
EM/PM (Intermediate)	*1/*4	22	28.2
EM/IM (Intermediate)	*1/*10	4	5.13
	*1/*41	14	18.0
IM/PM (Intermediate)	*41/*4	5	6.41
IM/IM (Intermediate)	*10/*10	4	5.13
	*41/*41	1	1.28
	*10/*41	2	2.56
PM/PM (Poor)	*4/*4	4	5.13

Table 2.8 Concentrations of Tamoxifen, NDTam, Endoxifen, and [END]/[NDTam] Ratio by Metabolizer Group

	PM/PM	*PM/IM*	*PM/EM*	*IM/IM*	*IM/EM*	*EM/EM*
Number of values	4	7	6	22	17	22
[TAM] (ng/mL)						
Minimum	27.9	52.4	93.0	20.9	37.4	46.7
25% Percentile	32.8	68.7	98.0	54.2	59.6	76.9
Median	61.4	82.9	145	66.5	82.8	101
75% Percentile	79.7	101	209	78.6	97.2	143
Maximum	81.2	121	238	219	223	160
[NDTam] (ng/mL)						
Minimum	76.7	150	242	55.2	81.1	57.7
25% Percentile	89.8	176	269	108	140	178
Median	177	189	322	133	171	207
75% Percentile	236	211	486	183	198	238
Maximum	239	299	535	359	266	282
[END] (ng/mL)						
Minimum	1.46	2.95	3.82	1.63	3.30	2.40
25% Percentile	1.53	2.98	4.72	4.52	6.54	7.19
Median	2.52	3.51	6.34	6.12	9.01	11.1
75% Percentile	3.98	4.89	7.55	8.67	12.2	17.9
Maximum	4.21	7.19	8.95	13.1	19.9	26.1
[END]/[NDTam]						
Minimum	1.35E-02	1.50E-02	1.32E-02	7.86E-03	3.30E-02	1.17E-02
25% Percentile	1.38E-02	1.68E-02	1.35E-02	3.59E-02	3.87E-02	3.82E-02
Median	1.61E-02	1.99E-02	1.72E-02	4.47E-02	5.34E-02	6.39E-02
75% Percentile	1.87E-02	2.40E-02	2.30E-02	5.61E-02	7.64E-02	8.44E-02
Maximum	1.90E-02	2.41E-02	2.47E-02	8.82E-02	1.16E-01	1.42E-01

PM/PM group had significantly lower median concentrations ($p < 0.05$) of endoxifen in comparison to all groups except PM/IM. A trend towards higher serum endoxifen concentration with increasing predicted metabolizer status was observed, as expected.

A caveat of the genotype-phenotype correlation that was performed is that, in the absence of the *4, *10 and *41, alleles were classified as *1 by default. While the variants tested are expected to account for the majority of variation in our population, *CYP2D6* is highly polymorphic and there are many functional, but less common, variants that may affect endoxifen formation. Testing for these additional variants could

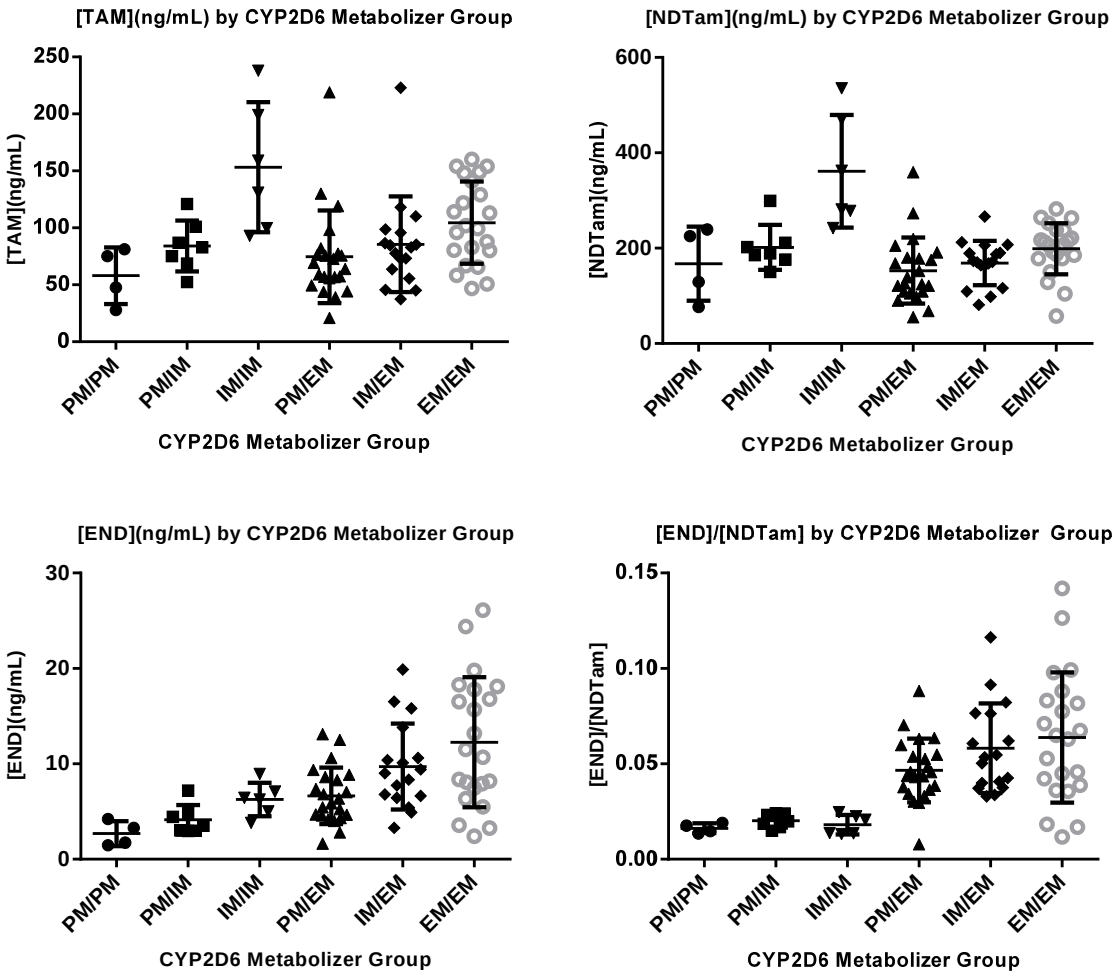


Figure 2.3 Concentrations of Tamoxifen, NDTam, Endoxifen, and [END]/[NDTam] Ratio by Metabolizer Group

potentially result in the re-classification of some EM/EM, EM/IM, or EM/PM individuals within the sample set. For this reason, more extensive genotyping is highly desirable. In addition, compliance and co-medication data was not available for this cohort, and these factors are known to alter serum endoxifen concentration [18].

2.4 Conclusion

A quantitative LC-MS/MS assay for the measurement of tamoxifen, NDTam, and endoxifen was developed and validated. The assay was used to screen 78 clinical study samples, and these results were correlated with *CYP2D6* genotype data [25]. The results of the genotype-phenotype correlation replicate previous findings that relate endoxifen concentration and [END]/[NDTam] ratio to *CYP2D6* genotype. In addition, the LC-MS/MS assay was shown to produce robust, quantitative data for tamoxifen, NDTam, and endoxifen within the range of concentrations observed within the clinical samples screened. Thus, our methods are suitable to address further tamoxifen pharmacogenetics research.

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Chapter 3

Association between CYP2D6 Genotype and Serum Tamoxifen and Tamoxifen Metabolite Concentrations in the Women's Healthy Eating and Living Cohort:

Assessment of Interethnic Differences and Analysis of CYP2D6 Genotype Test Cutoffs

3.1 Introduction

Tamoxifen is a selective estrogen receptor modulator (SERM) that has been used for several decades in the treatment of ER positive breast cancer. A five year course of tamoxifen adjuvant therapy in early breast cancer patients reduces the risk of recurrence by 47% [1]. In some but not all studies, functional polymorphisms in the *CYP2D6* gene have been associated with the outcome of tamoxifen adjuvant therapy; variants which lead to lower CYP2D6 enzyme activity have been associated with shorter recurrence free survival [2, 3]. The proposed biological basis for this association is that tamoxifen is a pro-drug, and the CYP2D6 enzyme is important for the production of 4-hydroxy-N-desmethyltamoxifen (endoxifen), the active metabolite to which the pharmacological activity of tamoxifen has been attributed [4-7].

The technology for *CYP2D6* genotyping in the clinical setting is currently available, and there is interest from both patients and clinicians in the use of *CYP2D6* genotyping to guide adjuvant endocrine therapy. In addition, there are direct-to-consumer testing services and diagnostic companies that are currently marketing related services and products. However, there is no consensus on how *CYP2D6* genotype should be used clinically to guide adjuvant endocrine therapy. Indeed,

whether or not *CYP2D6* genotype is truly associated with the outcome of tamoxifen adjuvant therapy is still subject to debate; there are conflicting results among the retrospective studies that have been performed thus far. Ultimately, prospective clinical trials are required in order to demonstrate the clinical utility of interventions based upon patient *CYP2D6* genotype. Nonetheless, data that is currently available can provide guidance on the best use of *CYP2D6* genotype data for clinical intervention with tamoxifen.

The Women's Healthy Eating and Living (WHEL) cohort is comprised of over 3000 women, recruited from seven clinical sites between 1995 and 2000, who were diagnosed with early breast cancer [8]. Many of these subjects underwent tamoxifen adjuvant therapy and are included in this analysis. While the majority of WHEL subjects are Caucasian, there are a greater number of Asian, Hispanic, and Black American subjects within the cohort than in other studies which have been published on tamoxifen pharmacogenetics [9]. It is of interest to determine if, and how, minority populations differ from Caucasians in terms of serum tamoxifen and tamoxifen metabolite concentrations and their relationships to *CYP2D6* genotypes. In general, it is important to know if clinical decisions based upon studies that were performed mainly in Caucasians actually apply to other ethnicities. While several studies have investigated tamoxifen pharmacogenetics in Asian populations from Asia [10-14], the Asian American population has been underrepresented thus far in the literature. And, as happens often in clinical studies, there is an unfortunate dearth of published studies on tamoxifen pharmacogenetics in African American and Hispanic American populations. The diversity

of the WHEL dataset, in which all samples were assessed with the same analytical methods, allows for direct comparison of tamoxifen pharmacogenetic data across ethnicities.

While it is no longer a question that *CYP2D6* genotype is associated with serum endoxifen concentrations, it is unclear what genotype cutoffs, based upon predicted CYP2D6 metabolism, should be used to define an "at-risk" group of patients with low endoxifen levels. More *CYP2D6* genotypes and serum tamoxifen, N-desmethyltamoxifen, and endoxifen concentrations are available for Caucasian WHEL subjects than in any other published dataset. In addition, the WHEL tamoxifen pharmacogenetic study is currently the only published study to find an association between serum endoxifen concentration and breast cancer recurrence in women undergoing tamoxifen adjuvant therapy [9]. Thus, the WHEL dataset provides a good opportunity to assess the ability of genotype cutoffs to predict inclusion in a low endoxifen risk group.

These two separate, but important, questions were addressed in the following analysis. Associations beteween CYP2D6 genotype and phenotype with serum concentrations of tamoxifen and its major metabolites were tested.

3.2 Materials and Methods

3.2.1 Study Population

Study subjects were participants in the WHEL study, a randomized controlled dietary trial of women with early stage breast cancer. Between 1995 and 2000, the WHEL trial enrolled 3088 breast cancer survivors who had been treated for Stages I (T1c)-IIIA breast cancer, were 2–48 months from initial breast cancer diagnosis and were between the ages of 18 and 70 years at diagnosis. Study subjects were enrolled from the following sites: University of California, San Diego and Davis, Stanford University, Kaiser Permanente in Oakland and Portland, University of Arizona at Tucson and the MD Anderson Cancer Center. The study was approved by each institution's review board and informed consent was obtained from all participants. At the time of initial enrollment in the WHEL study, EDTA whole blood and serum were collected from each study participant and stored at -80°C. These samples were used for the current analysis.

Of the 3088 WHEL participants, 1799 subjects reported taking tamoxifen at the time of blood collection. Analytical measurements of tamoxifen, endoxifen, and N-desmethyltamoxifen were made for these subjects using liquid chromatography tandem mass spectrometry (LC-MS/MS). Of these subjects, 179 had been taking tamoxifen for less than three months at the time of sampling. These subjects were excluded from analysis as they were not expected to have achieved steady-state levels of tamoxifen and tamoxifen metabolites. Of the remaining subjects, the following were excluded due

to small numbers: those self-identified as American Indian (1), mixed race (20), Pacific Islander (8), and other ethnicity (9).

3.2.2 DNA Isolation and Determination of CYP2D6 Genotypes

DNA was extracted from archival blood samples (QIAcube robot with QIAamp DNA blood Mini Kit, Qiagen, Valencia, CA, USA). DNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), according to manufacturers' instructions. The Roche AmpliChip P450 assay [15] was used to genotype the following *CYP2D6* variants: *1, *1xN, *2, *2xN *3, *4, *4xN, *5, *6, *7, *8, *9, *10, *10XN, *11, *15, *17, *19, *20, *29, *35, *35xN *36, *40, *41, and *41xn. The assay is a micro-array based genotyping system. The main steps of the assay are DNA amplification, fragmentation, labeling, hybridization, staining, and scanning. The array contains >15,000 oligonucleotide probes, resulting in a high level of specificity and reliability. Data analysis software was used to determine *CYP2D6* genotype on the basis of the pattern of hybridization of labeled DNA product to the oligonucleotide probes. *CYP2D6* genotyping was performed on anonymized samples at Roche Molecular Diagnostics laboratories, (Pleasanton, CA). Operators were blinded to clinical data.

3.2.3 Determination of Tamoxifen Metabolite Concentrations in Serum

Tamoxifen was obtained from Sigma Aldrich. Endoxifen, N-desmethyltamoxifen, D5-tamoxifen, D5-endoxifen, and D5-N-desmethyltamoxifen were obtained from Toronto Research Chemicals. HPLC grade H_20, methanol, and acetonitrile were obtained from Fisher Chemicals.

Endoxifen, Tamoxifen, and N-desmethyltamoxifen concentrations were determined for serum samples using liquid chromatography tandem mass spectrometry (LC/MS-MS). A 1200 series Agilent HPLC system was used for detection and quantification. The analytical column used in the assay was a Waters xTerra MS C18, 3.5µm (2.1x100 mm), and the guard column was a Waters 2.1x10 mm C18 column. Column compartment temperature was maintained at 40°C during the run. Mobile Phase A (MpA) for the assay was composed of 5 mM Ammonium Formate, pH 4.5, + 1% methanol, while Mobile Phase B (MpB) was composed of 70:20:10 Acetonitrile: Methanol: 50 mM Ammonium Formate, pH 4.5. A sample volume of 15 µL was injected for analysis. Reverse phase gradient elution was used to resolve analytes (see Chapter 2).

An Applied Biosystems 3200 Q-trap Tandem Mass Spectrometer was used for analyte detection. The MRM transitions used to track analytes and internal standards were as follows: Tamoxifen: 372/72; N-desmethyltamoxifen: 358/58; 4-hydroxytamoxifen: 388/72; endoxifen: 374/58; D5-tamoxifen: 377/72; D5-N-desmethyltamoxifen: 363/58; D5-endoxifen: 379/58. The following conditions were used for positive mode electrospray ionization: curtain gas, 35.0; ion spray voltage, 5500; ion source gas 1, 60.0; ion source gas 2, 35.0; and temperature, 700°C.

Sample extractions were performed at the University of California San Diego Moores Cancer Center. An 80X stock of internal standard (IS) mix consisting of 200 ng/mL D5-endoxifen, 4 µg/mL D5-N-desmethyltamoxifen, and 1 µg/mL D5-tamoxifen

was prepared in methanol and stored at -80°C. Prior to the extraction procedure, IS stock was diluted in 0.5 mM Ammonium Formate, pH 3.0, and 800 μL of diluted IS was added to 200 μL of archived serum sample. Waters MCX 1cc solid phase extraction cartridges were used to exact the samples according to the manufacturer's instructions. Eluate was collected in microfuge tubes and dried in a speed-vac. Samples were shipped overnight on dry ice to the University of California San Francisco and stored at -80°C prior to analysis. Samples were resuspended in a 20:80 mixture of MpB:MpA and transferred to autosampler vials.

Analyte concentration was determined by comparing the Peak Area:IS Area ratio to that of a five point standard curve. The standard curve was made by spiking known amounts of standard into drug-free serum. Standard curve, quality control (QC) material, and drug-free blank were extracted with each set of test samples. Three high (HI) and three low (LO) QC samples were run with a sample batch size of approximately forty samples per run. Concentrations of calibrators were as follows: endoxifen (25 ng/mL, 20 ng/mL, 12.5 ng/mL, 5 ng/mL, 2 ng/mL), tamoxifen (250 ng/mL, 200 ng/mL, 125 ng/mL, 50 ng/mL, 20 ng/mL) and N-desmethyltamoxifen (500 ng/mL, 400 ng/mL, 250 ng/mL, 100 ng/mL, 40 ng/mL). Concentrations of known standards in HI QC were 20 ng/mL endoxifen, 150 ng/mL tamoxifen, and 300 ng/mL N-desmethyltamoxifen. Concentrations of known standards in LO QC were 3 ng/mL endoxifen, 50 ng/mL tamoxifen, and 75 ng/mL N-desmethyltamoxifen. D5-Tamoxifen was used as the internal standard for tamoxifen, D5-N-desmethyltamoxifen was used as the internal standard for N-desmethyltamoxifen, and D5-endoxifen was used as the internal

standard for endoxifen and 4-hydroxytamoxifen. Analyst Software (Applied Biosystems) was used for the determination of analyte concentration. Within and between day coefficient of variations were <15% for all analytes. Analysis was performed by an operator who was blinded to *CYP2D6* genotype. The limits of quantitation for the assay are 1 ng/mL for endoxifen, 10 ng/mL for tamoxifen, and 20 ng/mL of N-desmetyltamoxifen.

3.2.4 Sensitivity, Specificity, PPV, and NPV Calculations

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) calculations were performed using MedCalc for Windows, version 12.4.0 (MedCalc Software, Mariakerke, Belgium) using the Caucasian dataset. Sensitivity = (true positives)/(true positives + false negatives), and is the probability that the test result will be positive when a subject is at risk. Specificity = (true negatives)/(true negatives + false positives), and is the probability that a test result will be negative when a subject is not at risk. PPV = (true positives)/(true positives + false positives), and is the probability that a subject is a risk when the test is positive. NPV = (true negatives)/(true negatives + false negatives), and is the probability that a subject is not at risk when the test is negative.

3.2.5 Statistical Analysis

The D'Agostino & Pearson omnibus normality test was used to determine if the values for BMI, age, and months of tamoxifen treatment at blood draw, when grouped by ethnicity, were normally distributed. Values were not normally distributed for all

ethnic groups, with one or more groups violating the normality test for BMI, age, and months on tamoxifen at blood draw. Thus, the non-parametric Kruskall-Wallis and Mann-Whitney tests were used to determine differences between groups. Likewise, data was not normally distributed for tamoxifen, N-desmethyltamoxifen, or endoxifen concentrations by CYP2D6 metabolizer group, nor were variances the same between genotype groups; these are both requirements for parametric tests. Thus, the Kruskall-Wallis and Mann-Whitney tests were used to determine differences between groups. Median values, as well as the 25th and 75th percentile values are reported in this study instead of the average and standard deviation; these values are more informative for data that is not normally distributed. Statistical analyses were performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

3.3 Results

3.3.1 Comparison of Subject Demographics

Within the WHEL cohort, African Americans (50 years) and Hispanics (52 years) were significantly younger ($p = 0.0008$ and $p = 0.047$, respectively) than Caucasians (55 years), while Asians had the same median age (55 years) as Caucasians. In terms of BMI, African Americans (28.0) had a significantly higher BMI ($p = 0.012$) than Caucasians (25.9), whereas median BMI for Asians (24.2) was significantly lower ($p = 0.0017$). There was no significant difference in BMI between Caucasians and Hispanics (26.6). At the time of blood collection, Hispanics (13 months) had taken tamoxifen for a significantly (p

= 0.0026) shorter time than Caucasians (18 months). However the Caucasian population did not differ significantly from the Asian (23 months), or African American (18 months) populations in terms of months of tamoxifen therapy at the time of blood draw.

Table 3.1. Demographic Information by Ethnicity. Differences in age, BMI, and months of tamoxifen treatment at the time of blood draw, by ethnicity.

Ethnicity		CAU	ASN	AA	HISP
# of subjects (%)		1375 (88.1%)	59 (3.8%)	45 (2.9%)	81 (5.2%)
Age	Minimum	28	41	32	32
	25th Percentile	49	49	43.5	47
	Median	55	55	50***	52*
	75th Percentile	61	59	56.5	61
	Maximum	74	72	70	71
BMI	Minimum	16	15.4	20.8	18.2
	25th Percentile	22.9	21.6	25.25	23.3
	Median	25.9	24.2**	28*	26.6
	75th Percentile	30.3	27.2	31.05	30.5
	Maximum	64.1	36.1	53.6	46.9
Months of Tam at Blood Draw	Minimum	3	3	3	3
	25th Percentile	9	12	9.5	8
	Median	18	23	18	13**
	75th Percentile	30	33	27.5	21.5
	Maximum	57	42	43	48

***, **, and *** indicate p-values of less than 0.05, 0.005, and 0.0001 respectively comparing each ethnic group to Caucasians**

3.3.2 CYP2D6 Allele Frequencies

CYP2D6 allele frequencies and genotypes of Caucasian, Asian, African American, and Hispanic groups were determined by Roche P450 AmpliChip genotyping. Alleles are grouped according to the predicted enzymatic activity of their protein products in table 3.2; extensive metabolizer (EM), intermediate metabolizer (IM), poor metabolizer (PM),

and ultrarapid metabolizer (UM) denote full, reduced, null, and increased enzymatic activity. This is not to be confused with traditional classification of CYP2D6 genotypes which uses similar nomenclature to indicate predicted enzymatic activity. The frequencies of *CYP2D6* alleles, by ethnicity, were similar to what has been published in the literature. The frequency of null alleles (PM) in Caucasians was the highest (26.3%) of the ethnic groups that were examined (see figure 3.1 and table 3.2). The*4 variant accounted for 76.6% of the null alleles, out of the 11 null alleles detected, not including *4xN. The frequency of null alleles was much lower in the other ethnic groups. Within the Hispanic population, the frequency of null alleles was only 9.3%, while it was 11.9% in Asians and 12.2% in African Americans. Reduced function alleles (IM) were found to be most frequent among the African American (44.4%) and Asian (41.5%) populations. The *17 allele was the most common reduced function allele in African Americans (47.5%), followed by *41 (30%), *29 (20%), and *10 (2.5%). The *29 allele was present in the Hispanic population at a frequency of 0.62%, while it was not detected in either the Caucasian or Asian groups. The *17 variant was present in Caucasians and Hispanics at a very low frequency (0.29% and 0.62% respectively) and not detected in Asians; the *17 and *29 alleles are specific to populations of African descent. As for the Asian group, the *10 allele accounted for all but one (48/49 = 98%) of the reduced function variants, the one other being the *41 allele. Reduced function alleles were present at a frequency of 15.2% and 16.0% in the Caucasian and Hispanic groups, respectively. Full activity (EM) alleles were highest among the Hispanic population (71.6%), followed by the Caucasian (56.9%), Asian (45.8%), and African American (43.3%) groups. As

expected, the *1 variant was the most common full activity allele for all ethnic groups. Allele amplifications (UM) were somewhat rare, overall, and were not observed in the African American population. The highest frequency of UM was observed among Hispanics (3.09%), followed by Caucasians (1.64%) and Asians (0.43%).

Table 3.2. *CYP2D6* Allele Frequencies by Ethnicity. CYP2D6 variants were determined using the Roche P450 AmpliChip Assay. Variants are classified by predicted activity and their frequencies are listed by ethnicity.

Variant	Activity	CAU		ASN		AA		HISP	
		#	Freq	#	freq	#	freq	#	freq
*1	EM	954	34.69%	43	36.44%	30	33.33%	78	48.15%
*2	EM	441	16.04%	11	9.32%	9	10.00%	28	17.28%
*35	EM	159	5.78%					9	5.56%
*41XN	EM*	10	0.36%					1	0.62%
total	EM	1564	56.87%	54	45.76%	39	43.33%	116	71.60%
*3	PM	42	1.53%						
*4	PM	554	20.15%	3	2.54%	4	4.44%	12	7.41%
*5	PM	81	2.95%	8	6.78%	5	5.56%	1	0.62%
*6	PM	35	1.27%						
*7	PM	1	0.04%						
*14	PM			1	0.85%				
*15	PM	2	0.07%						
*19	PM	1	0.04%						
*36	PM	1	0.04%	2	1.69%				
*40	PM					1	1.11%		
*4XN	PM	6	0.22%			1	1.11%	2	1.23%
total	PM	723	26.29%	14	11.86%	11	12.22%	15	9.26%
*9	IM	70	2.55%					3	1.85%
*10	IM	38	1.38%	48	40.68%	1	1.11%	6	3.70%
*17	IM	8	0.29%			19	21.11%	1	0.62%
*29	IM					8	8.89%	1	0.62%
*41	IM	302	10.98%	1	0.85%	12	13.33%	15	9.26%
total	IM	418	15.20%	49	41.53%	40	44.44%	26	16.05%
*1XN	UM	17	0.62%	1	0.85%				
*2XN	UM	26	0.95%					5	3.09%
*35XN	UM	2	0.07%						
total	UM	45	1.64%	1	0.43%	0	0.00%	5	3.09%

*41xN is listed as an EM allele in this analysis. While *41 is known to result in reduced CYP2D6 activity, copy number of *41xN cannot be determined using the Roche P450 AmpliChip. The few subjects carrying *41xN grouped well with EM on the basis of [END]/[NDTam] ratio, and were thus given the assignment of EM.

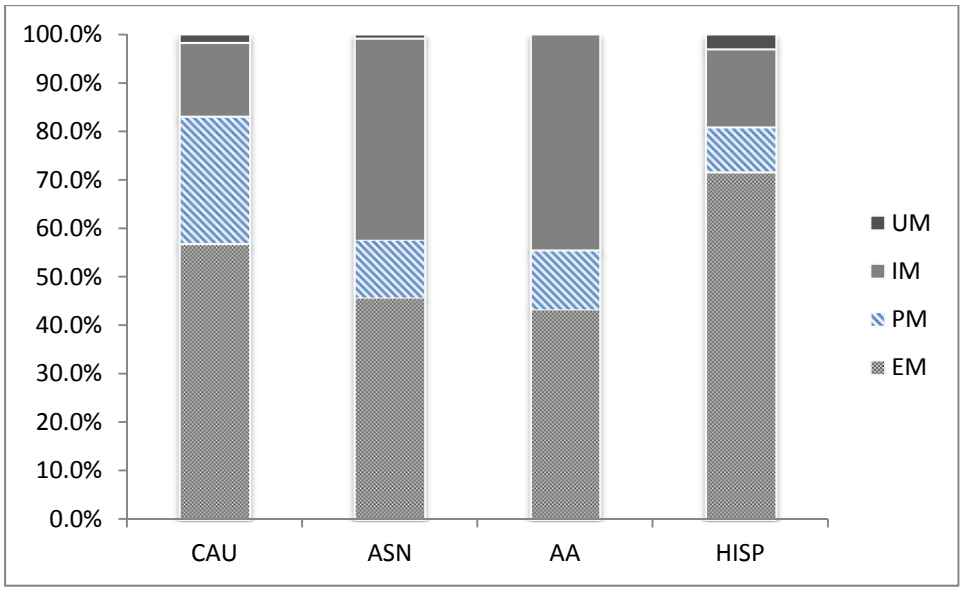


Figure 3.1. Distribution of Predicted CYP2D6 Allele Activity by Ethnicity

3.3.3 CYP2D6 Genotypes and Metabolizer Groups

Of the Caucasian subjects included in the current study (n=1375), 84 distinct genotypes were observed. This impressive number of distinct *CYP2D6* genotypes can be attributed to both the large number of Caucasian subjects included in the study, as well as the extensive coverage of *CYP2D6* variants assayed by the Roche P450 AmpliChip. Fewer distinct genotypes were observed among the other race/ethnicity categories, in part due to smaller numbers of subjects in these groups, as well as less genetic variation in CYP2D6. Among African American subjects (n=45), 18 different genotypes were observed. Within the Hispanic group (n=81), 27 different genotypes were determined. Within the Asian population (n=59), 15 different genotypes were determined. For a list of the genotypes observed as well as their frequencies, see table 3.3.

Subjects were separated by ethnicity and classified into the following *CYP2D6* genotype predicted metabolizer groups: PM/PM, IM/PM, IM/IM, EM/PM, EM/IM, and EM/EM+UM. PM/PM comprised 6.7% of the Caucasian group, but were absent from the other ethnic groups (see Table 3.3). EM/PM were much more common in the Caucasian population that in other ethnic groups; the frequency of EM/PM was 30% among Caucasians, 14.8% among Hispanics, 5.1% among Asians, and 4.4% among African Americans. EM/EM occurred at the highest frequency in the Hispanic population (56.8%), followed by the Caucasian (33.7%), Asian (23.7%), and African American (16.4%) groups. Because of the high frequency of IM alleles among Asian and African Americans, the majority of these subjects were carriers of one or more IM alleles. EM/IM made up 46.7% of African Americans, 39.0% of Asians, 19.8% of Hispanics, and 16.4% of Caucasians. IM/IM comprised 15.3% of the Asians, 11.1% of African Americans, 3.7% of Hispanics, and 2.62% of Caucasians. IM/PM made up 20.0% of African Americans, 15.3% of Asians, 8.3% of Caucasians, and 2.5% of Hispanics. UM were rare in all populations, with a frequency of 2.11% in Caucasians, 2.5% in Hispanics, 1.7% in Asians, and none detected in African Americans.

Table 3.3. CYP2D6 genotype frequencies and predicted enzymatic activity by ethnicity

Caucasian

Geno	class	#	freq
*1XN/*6	UM/PM	1	0.07%
*1XN/*9	UM/IM	1	0.07%
*1XN/*41	UM/IM	1	0.07%
*2XN/*3	UM/PM	1	0.07%
*35XN/*4	UM/PM	1	0.07%
*1/*41XN	EM/EM	2	0.15%
*2/*41XN	EM/EM	2	0.15%
*35/*41XN	EM/EM	2	0.15%
*35/*35	EM/EM	3	0.22%
*1XN/*4	UM/PM	3	0.22%
*2XN/*41	UM/IM	3	0.22%
*2XN/*4	UM/PM	5	0.36%
*2/*35	EM/EM	23	1.67%
*2/*2	EM/EM	33	2.40%
*1/*35	EM/EM	56	4.07%
*1/*2	EM/EM	159	11.6%
*1/*1	EM/EM	168	12.2%
total	full act	464	33.7%
*10/*35	EM/IM	1	0.07%
*17/*2	EM/IM	2	0.15%
*35/*9	EM/IM	2	0.15%
*1/*17	EM/IM	3	0.22%
*1/*10	EM/IM	8	0.58%
*10/*2	EM/IM	9	0.65%
*2/*9	EM/IM	11	0.80%
*1/*9	EM/IM	20	1.45%
*35/*41	EM/IM	26	1.89%
*2/*41	EM/IM	47	3.42%
*1/*41	EM/IM	97	7.05%
total	EM/IM	226	16.4%

Caucasian Cont.

geno	class	#	freq
*1/*7	EM/PM	1	0.07%
*1/*19	EM/PM	1	0.07%
*35/*4XN	EM/PM	1	0.07%
*41XN/*5	EM/PM	1	0.07%
*2/*4XN	EM/PM	2	0.15%
*3/*35	EM/PM	2	0.15%
*35/*6	EM/PM	2	0.15%
*4/*41XN	EM/PM	3	0.22%
*2/*6	EM/PM	4	0.29%
*35/*5	EM/PM	5	0.36%
*2/*3	EM/PM	9	0.65%
*1/*3	EM/PM	11	0.80%
*2/*5	EM/PM	13	0.95%
*1/*6	EM/PM	14	1.02%
*35/*4	EM/PM	31	2.25%
*1/*5	EM/PM	35	2.55%
*2/*4	EM/PM	86	6.25%
*1/*4	EM/PM	192	14.0%
total	EM/PM	413	30.0%
*9/*9	IM/IM	1	0.07%
*10/*9	IM/IM	2	0.15%
*10/*41	IM/IM	3	0.22%
*41/*9	IM/IM	11	0.80%
*41/*41	IM/IM	19	1.38%
total	IM/IM	36	2.62%

Caucasian Cont.

geno	class	#	freq
*10/*36	IM/PM	1	0.07%
*15/*17	IM/PM	1	0.07%
*4XN/*9	IM/PM	1	0.07%
*3/*9	IM/PM	2	0.15%
*17/*4	IM/PM	2	0.15%
*4/*41	IM/PM	3	0.22%
*5/*9	IM/PM	3	0.22%
*10/*5	IM/PM	3	0.22%
*3/*41	IM/PM	5	0.36%
*41/*5	IM/PM	5	0.36%
*41/*6	IM/PM	5	0.36%
*10/*4	IM/PM	11	0.80%
*4/*9	IM/PM	15	1.09%
*4/*41	IM/PM	58	4.22%
total	IM/PM	115	8.36%
*3/*3	PM/PM	1	0.07%
*3/*6	PM/PM	1	0.07%
*4/*5	PM/PM	1	0.07%
*5/*5	PM/PM	1	0.07%
*6/*6	PM/PM	1	0.07%
*15/*4	PM/PM	1	0.07%
*4/*4XN	PM/PM	2	0.15%
*5/*6	PM/PM	2	0.15%
*4/*6	PM/PM	4	0.29%
*3/*4	PM/PM	9	0.65%
*4/*5	PM/PM	11	0.80%
*4/*4	PM/PM	58	4.22%
total	PM/PM	92	6.69%
*2/*35XN	UM	1	0.07%
*1XN/*35	UM	1	0.07%
*2XN/*35	UM	1	0.07%
*2/*2XN	UM	3	0.22%
*1XN/*2	UM	4	0.29%
*1/*1XN	UM	6	0.44%
*1/*2XN	UM	13	0.95%
total	UM	29	2.11%

Table 3.3 cont.

Asian

Genotype	class	#	freq
*2/*35	EM/EM	1	1.7%
*2/*2	EM/EM	1	1.7%
*1/*2	EM/EM	3	5.1%
*1/*1	EM/EM	9	15.3%
Total	full act	14	23.7%
*2/*41	EM/IM	1	1.7%
*1/*10	EM/IM	19	32.2%
*10/*2	EM/IM	3	5.1%
Total	EM/IM	23	39.0%
*2/*5	EM/PM	1	1.7%
*1/*4	EM/PM	2	3.4%
Total	EM/PM	3	5.1%
*10/*41	IM/IM	1	1.7%
*10/*10	IM/IM	8	13.6%
Total	IM/IM	9	15.3%
*10/*14	IM/PM	1	1.7%
*10/*4	IM/PM	1	1.7%
*10/*5	IM/PM	7	11.9%
Total	IM/PM	9	15.3%
*1/*2XN	UM	1	1.7%
Total	UM	1	1.7%

African American

genotype	class	#	freq
*1/*1	EM/EM	3	6.7%
*1/*2	EM/EM	5	11.1%
total	full act	8	17.8%
*1/*10	EM/IM	1	2.2%
*2/*41	EM/IM	2	4.4%
*1/*29	EM/IM	5	11.1%
*1/*17	EM/IM	5	11.1%
*1/*41	EM/IM	6	13.3%
*17/*2	EM/IM	2	4.4%
total	EM/IM	21	46.7%
*1/*4	EM/PM	2	4.4%
total	EM/PM	2	4.4%
*29/*41	IM/IM	1	2.2%
*17/*17	IM/IM	4	8.9%
total	IM/IM	5	11.1%
*29/*5	IM/PM	1	2.2%
*29/*40	IM/PM	1	2.2%
*17/*4	IM/PM	1	2.2%
*17/*4XN	IM/PM	1	2.2%
*4/*41	IM/PM	1	2.2%
*41/*5	IM/PM	2	4.4%
*17/*5	IM/PM	2	4.4%
total	IM/PM	9	20.0%

Hispanic

genotype	class	#	freq
*1/*41XN	EM/EM	1	1.2%
*2XN/*5	UM/PM	1	1.2%
*2/*35	EM/EM	2	2.5%
*2/*2	EM/EM	2	2.5%
*2XN/*41	UM/IM	2	2.5%
*1/*35	EM/EM	4	4.9%
*1/*2	EM/EM	10	12.3%
*1/*1	EM/EM	24	29.6%
total	full act	46	56.8%
*1/*9	EM/IM	1	1.2%
*1/*29	EM/IM	1	1.2%
*1/*10	EM/IM	1	1.2%
*1/*17	EM/IM	1	1.2%
*35/*41	EM/IM	1	1.2%
*10/*35	EM/IM	1	1.2%
*10/*2	EM/IM	1	1.2%
*1/*41	EM/IM	4	4.9%
*2/*41	EM/IM	5	6.2%
total	EM/IM	16	19.8%
*1/*4	EM/PM	5	6.2%
*1/*4XN	EM/PM	1	1.2%
*35/*4	EM/PM	1	1.2%
*2/*4	EM/PM	5	6.2%
total	EM/PM	12	14.8%
*41/*41	IM/IM	1	1.2%
*10/*9	IM/IM	2	2.5%
total	IM/IM	3	3.7%
*41/*4XN	IM/PM	1	1.2%
*10/*4	IM/PM	1	1.2%
total	IM/PM	2	2.5%
*1/*2XN	UM	1	1.2%
*2/*2XN	UM	1	1.2%
total	UM	2	2.5%

3.3.4 Tamoxifen and Tamoxifen Metabolite Concentrations

Of the 1375 Caucasian subjects eligible for analysis, there were 12 individuals who claimed to be taking tamoxifen for >3 months at the time of blood draw, but had non-quantitative or undetectable serum concentrations of tamoxifen and/or tamoxifen metabolites. Likewise, there was one African American subject and one Asian subject with a similar discrepancy. Non-quantitative values were set to zero. Note that the limits of quantitation for tamoxifen, N-desmethyltamoxifen and endoxifen are less than 10% of the median values observed for these analytes within the cohort. These subjects were most likely not taking tamoxifen at the time. However, this data remains in the analyses since low values are of particular interest in this study.

Serum endoxifen concentrations were compared between Caucasian (12.2 ng/mL), Asian (16.9 ng/mL), African American (10.9 ng/mL), and Hispanic (17.9 ng/mL) subjects within the WHEL cohort. Among these subjects, a statistically significant difference ($p = 0.0001$) in serum endoxifen concentrations was determined. In relation to Caucasians, significantly higher serum concentrations of endoxifen were observed in both the Asian ($p = 0.001$) and Hispanic ($p = 0.0001$) populations. However, there was no significant difference between African American and Caucasians within the WHEL cohort.

Serum tamoxifen concentrations were also found to be significantly different ($p = 0.0013$) among these ethnic groups. While the African American (121 ng/mL) and Hispanic (125 ng/mL) populations did not differ from the Caucasians (128 ng/mL), Asians

(154 ng/mL) were found to have significantly higher ($p = 0.0002$) serum tamoxifen concentrations.

Serum N-desmethyltamoxifen concentrations were significantly ($p = 0.0001$) different between the Caucasian (236 ng/mL), Asian (289 ng/mL), African American (231 ng/mL), and Hispanic (237 ng/mL) groups. Asians were found to have significantly higher N-desmethyltamoxifen concentrations than Caucasians ($p < 0.0001$), while African American and Hispanics did not differ significantly from Caucasians.

In terms of the [END]/[NDTam] metabolic ratio, a significant difference ($p < 0.0001$) among the ethnic groups was also apparent. While Asian (0.058) and African American (0.052) metabolic ratios did not differ significantly from the Caucasian (0.054) group, the value for Hispanics (0.076) was significantly higher ($p < 0.0001$) than that of Caucasians.

Based upon the differences in allele frequencies, demographic information, and serum tamoxifen and tamoxifen metabolite concentrations, it was determined that further analyses of the association between serum concentration of tamoxifen and its metabolites in relation to CYP2D6 genotype should be performed for each ethnic group separately.

TABLE 3.4. Tamoxifen and Tamoxifen MetaboliteConcentrations by Ethnicity

Ethnicity		CAU	ASN	AA	HISP
# of subjects (%)		1375 (88.1%)	59 (3.8%)	45 (2.9%)	81 (5.2%)
END (ng/mL)	Minimum	0	0	0	2.05
	25th Percentile	7.02	9.71	5.91	12.3
	Median	12.2	16.3**	10.5	17.9***
	75th Percentile	18.6	24.3	15.4	25.9
	Maximum	61.4	73.7	32.4	60.2
TAM (ng/mL)	Minimum	0	0	0	10
	25th Percentile	92.9	127	93.3	92.5
	Median	128	154**	121	125
	75th Percentile	169	187	148.5	165
	Maximum	728	452	234	546
NDTAM (ng/mL)	Minimum	0	27	0	44.8
	25th Percentile	183	235	151.5	171
	Median	236	289***	231	227
	75th Percentile	308	351	312.5	271
	Maximum	869	704	400	575
[END]/[NDTAM]	Minimum	0.00E+00	0.00E+00	1.51E-02	1.53E-02
	25th Percentile	2.91E-02	2.95E-02	2.54E-02	4.99E-02
	Median	5.42E-02	5.81E-02	5.22E-02	7.56E-02***
	75th Percentile	8.20E-02	9.05E-02	7.43E-02	1.11E-01
	Maximum	3.51E-01	2.76E-01	1.07E-01	7.92E-01

END, endoxifen; TAM, tamoxifen; NDTAM, N-desmethyltamoxifen *, **, and * indicate p-values of less than or equal to 0.05, 0.005, and 0.0001 respectively, in comparison to the Caucasian population**

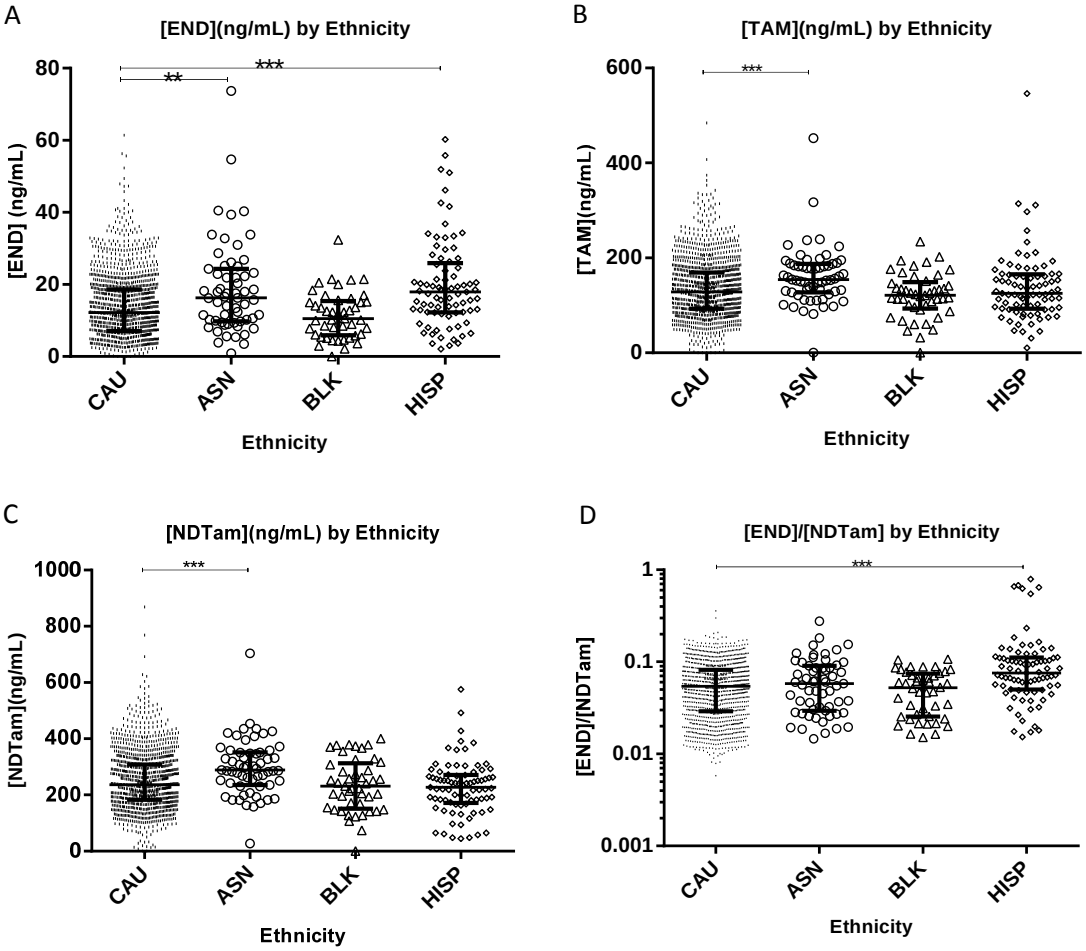


FIGURE 3.2. Tamoxifen and Tamoxifen Metabolites by Ethnicity. Median values with the interquartile range are shown for: (A) endoxifen concentration (ng/mL), (B) tamoxifen concentration (ng/mL), (C) N-desmethyltamoxifen concentration (ng/mL), and (D) ratio of [endoxifen]/[N-desmethytamoxifen] concentrations. *, **, and *** indicate p-values of less than or equal to 0.05, 0.005, and 0.0001 respectively, in comparison to the Caucasian population**.**

3.3.5 Genotype-Phenotype Correlations

Variants were assigned to groups on the basis of genotype-predicted CYP2D6 enzymatic activity according to standard convention. The rare variant, *41xN, was treated as a full activity allele. Subjects carrying an amplification of a full activity variant in addition to a null variant were grouped with subjects carrying two full activity alleles. Subjects were categorized into six CYP2D6 activity groups for analysis on the basis of the predicted activity of each of the two alleles: null/null (PM/PM), reduced/null (IM/PM), reduced/reduced (IM/IM), full/null (EM/PM), full/reduced (EM/IM), and full/full + full activity allele amplification (EM/EM)+(UM).

Categorized by CYP2D6 metabolizer group, subjects did not differ significantly in terms of age, BMI, or months of tamoxifen treatment at blood draw in the Caucasian, Asian, African American, or Hispanic populations.

Table 3.5. Serum Endoxifen Concentration (ng/mL) by CYP2D6 Metabolizer Group and Ethnicity

CYP2D6 Met Group		PM/PM	IM/PM	IM/IM	EM/PM	EM/IM	EM/EM +UM
CAU	#	92	116	35	413	226	493
	Minimum	0	1.06	2.94	0	0	0
	25th %-ile	3.49	5.07	6.49	8.17	8.12	11.0
	Median	**4.69**	**6.45**	**8.06**	**12.0**	**12.4**	**17.6**
	75th %-ile	6.39	9.41	10.4	16	17.8	24.3
	Maximum	21.9	31.2	16.7	38.8	52.3	61.4
ASN	#	0	9	9	3	23	15
	Minimum		0	7.78	9.34	3.84	11.6
	25th %-ile		4.4	8.89	9.34	14.0	17.5
	Median		**6.01**	**10.6**	**9.91**	**18.0**	**23.2**
	75th %-ile		9.14	13.4	24.3	25.2	39.4
	Maximum		12.4	73.7	24.3	33.8	54.7
AA	#	0	9	5	2	21	8
	Minimum		2.15	2.97	12.3	0	8.46
	25th %-ile		4.02	5.44	12.3	6.78	14.4
	Median		**5.43**	**8.06**	**14.2**	**11.9**	**18.5**
	75th %-ile		7.63	11.4	16.1	15.1	21.3
	Maximum		12.8	12.6	16.1	21.5	32.4
HISP	#	0	2	3	12	16	48
	Minimum		6.19	4.66	2.05	3.66	3.59
	25th %-ile		6.19	4.66	5.28	12.1	14.0
	Median		**7.23**	**15.3**	**11.7**	**15.3**	**20.0**
	75th %-ile		8.26	23.8	18.5	18.6	30.6
	Maximum		8.26	23.8	29.7	51.9	60.2

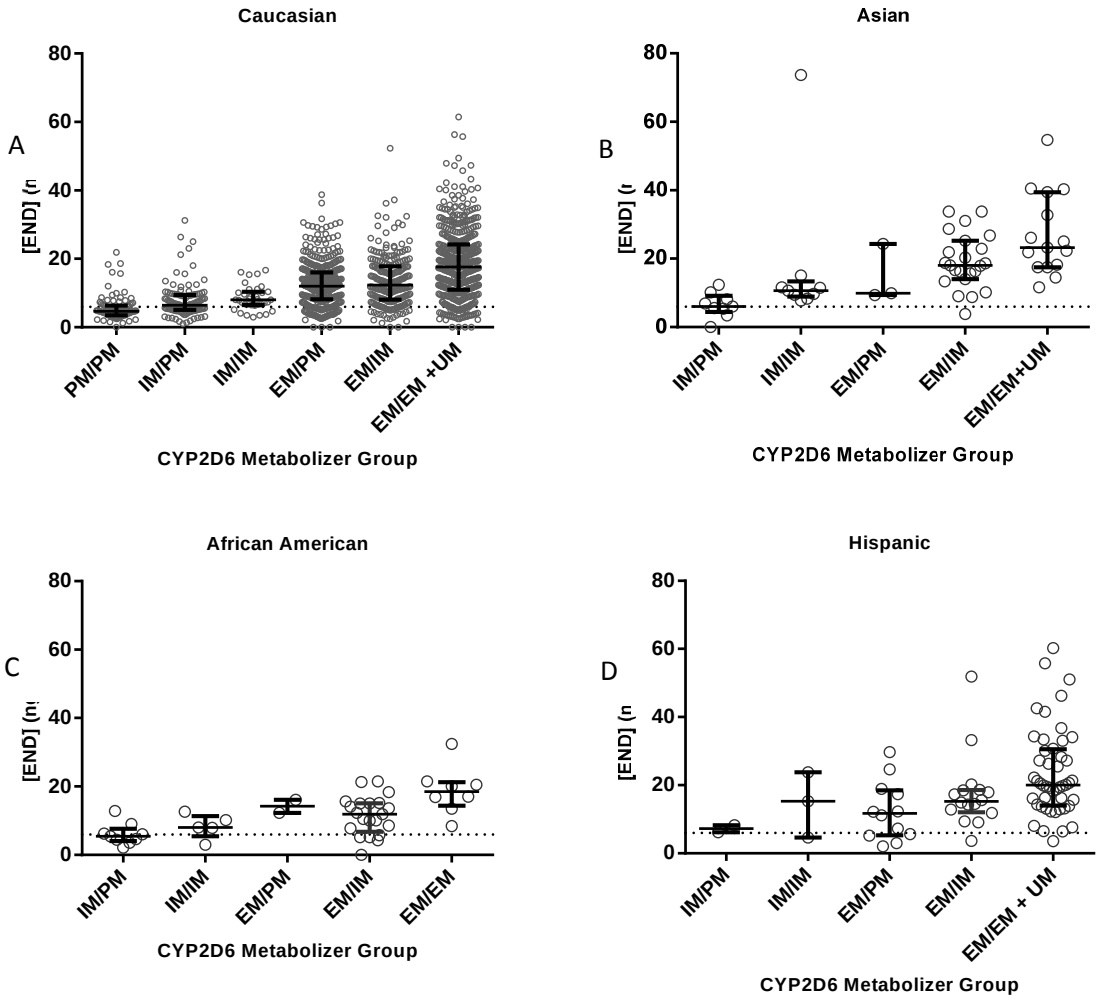


Figure 3.3. Serum Endoxifen Concentration (ng/mL) by CYP2D6 Metabolizer Group. Median values with the interquartile range are shown for: (A) Caucasian, (B) Asian, (C) African American, and (D) Hispanic populations.

Median serum endoxifen concentrations for the six CYP2D6 activity groups in the Caucasian population were as follows: 4.69 ng/mL (PM/PM), 6.45 ng/mL (IM/PM), 8.06 ng/mL (IM/IM), 12.0 ng/mL (EM/PM), 12.4 ng/mL (EM/IM), 17.6 ng/mL (EM/EM +UM). A statistically significant difference among these groups was determined. Four groups were significantly different ($p < 0.05$) from each other: PM/PM, IM/PM + IM/IM, EM/PM

+EM/IM, EM/EM+UM. In the Asian population, no PM/PM subjects were observed and only three EM/PM subjects were present due to the low frequency of PM alleles. Median serum endoxifen concentrations for Asians for the observed metabolizer groups were as follows: 6.01 ng/mL (IM/PM), 10.6 ng/mL (IM/IM), 9.91 ng/mL (EM/PM), 18.0 ng/mL (EM/IM) and 23.2 ng/mL (EM/EM+UM). Similar to the Asian group, no PM/PM subjects were detected and only two EM/PM subjects were observed within the African American group due to the low frequency of PM alleles. Median serum endoxifen concentrations for the observed African American metabolizer groups were as follows: 5.43 ng/mL (IM/PM), 8.06 ng/mL (IM/IM), 14.2 ng/mL (EM/PM), 11.9 ng/mL (EM/IM) and 18.5 ng/mL (EM/EM+UM). In the Hispanic population, only two IM/PM and three IM/IM subjects were detected due to the low number of IM alleles within this population. In addition, there were no PM/PM subjects. Median serum endoxifen concentrations for the observed Hispanic metabolizer groups were as follows: 7.23 ng/mL (IM/PM), 15.3 ng/mL (IM/IM), 11.7 ng/mL (EM/PM), 15.3 ng/mL (EM/IM) and 20.0 ng/mL (EM/EM+UM).

Table 3.6. Serum Tamoxifen Concentration (ng/mL) by CYP2D6 Metabolizer Group and Ethnicity

CYP2D6 Met Group		PM/PM	IM/PM	IM/IM	EM/PM	EM/IM	EM/EM +UM
CAU	#	92	116	35	413	226	493
	Minimum	0	18.3	75.3	0	0	0
	25th %-ile	93.7	98.0	99	96.9	90.6	88
	Median	**134**	**137**	**144**	**130**	**122**	**126**
	75th %-ile	165	180	172	178	161	165
	Maximum	326	484	231	407	728	374
ASN	#	0	9	9	3	23	15
	Minimum		0	111	152	87.8	96.1
	25th %-ile		94.8	156	152	112	134
	Median		**130**	**182**	**177**	**148**	**177**
	75th %-ile		150	231	198	194	187
	Maximum		184	317	198	452	227
AA	#	0	9	5	2	21	8
	Minimum		48.9	46.1	115	0	60.9
	25th %-ile		94.1	89.6	115	74.1	96.1
	Median		**134**	**164**	**123**	**113**	**125**
	75th %-ile		165	176	130	131	188
	Maximum		234	176	130	191	202
HISP	#	0	2	3	12	16	48
	Minimum		127	115	10	45.4	30.8
	25th %-ile		127	115	61.8	102	92.7
	Median		**139**	**136**	**105**	**149**	**122**
	75th %-ile		150	546	160	165	166
	Maximum		150	546	211	257	314

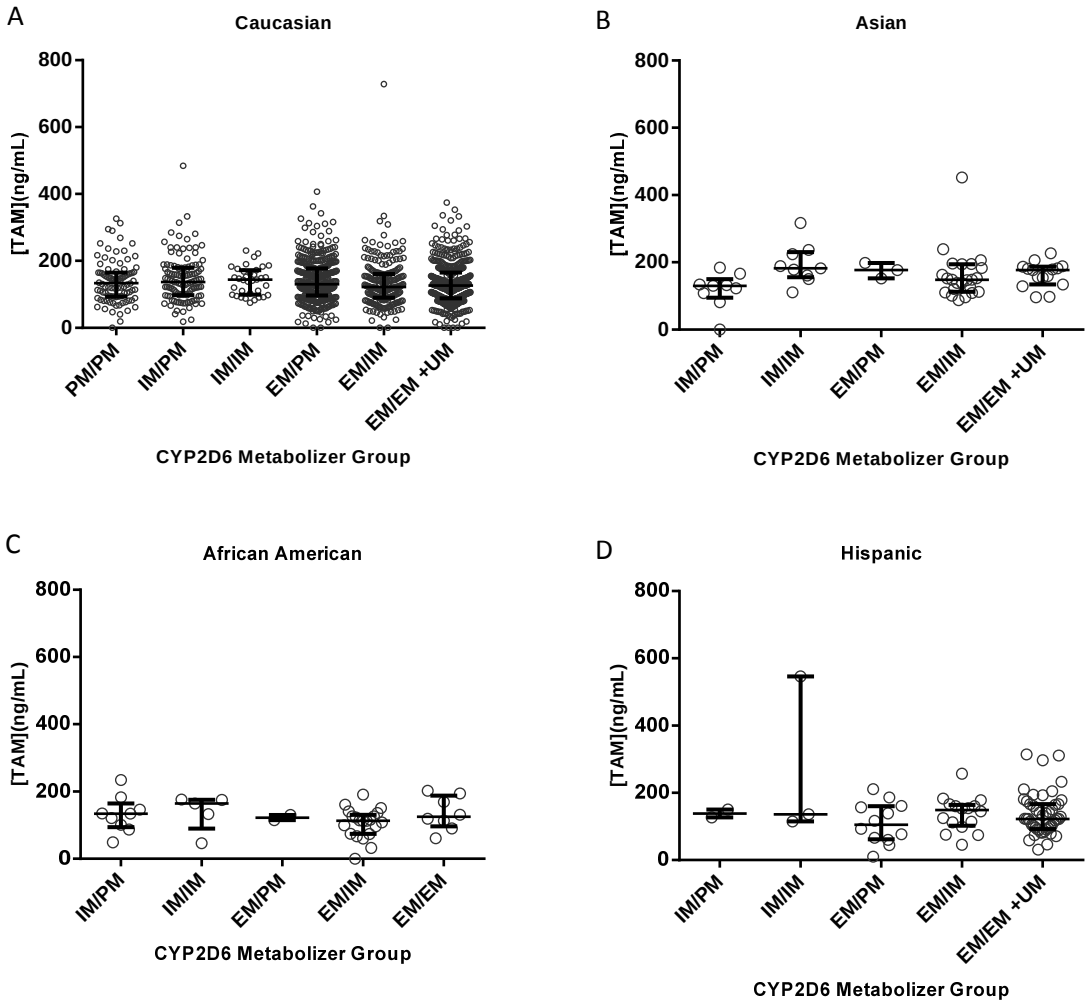


Figure 3.4. Serum Tamoxifen Concentration (ng/mL) by CYP2D6 Metabolizer Group. Median values with the interquartile range are shown for (A) Caucasian, (B) Asian, (C) African American, and (D) Hispanic populations.

For the Caucasian population, the median serum tamoxifen concentrations for the CYP2D6 metabolizer groups were as follows: 134 ng/mL (PM/PM), 137 ng/mL (IM/PM), 144 ng/mL (IM/IM), 130 ng/mL (EM/PM), 122 ng/mL (EM/IM), and 126 ng/mL (EM/EM + UM). These values were not significantly different from one another. Median serum tamoxifen concentrations in the Asian population for the observed metabolizer groups were: 130 ng/mL (IM/PM), 182 ng/mL (IM/IM), 177 ng/mL (EM/PM), 148 ng/mL

(EM/IM) and 177 ng/mL (EM/EM+UM). There was a significant difference between these values (p = 0.048), with IM/PM subjects having significantly (p = 0.011) lower serum tamoxifen concentrations than the other metabolizer groups. For African American subjects, median serum tamoxifen concentrations were: 134 ng/mL (IM/PM), 164 ng/mL (IM/IM), 123 ng/mL (EM/PM), 113 ng/mL (EM/IM) and 125 ng/mL (EM/EM+UM). There was no significant difference between these values. Median serum tamoxifen concentrations for the Hispanic population for the metabolizer groups were: 139 ng/mL (IM/PM), 136 ng/mL (IM/IM), 105 ng/mL (EM/PM), 149 ng/mL (EM/IM) and 122 ng/mL (EM/EM + UM). No significant difference between these values was determined.

Table 3.7. Serum N-desmethyltamoxifen Concentrations (ng/mL) by CYP2D6 Metabolizer Group and Ethnicity

CYP2D6 Met Group		PM/PM	IM/PM	IM/IM	EM/PM	EM/IM	EM/EM +UM
CAU	#	92	116	35	413	226	493
	Minimum	69.2	36.1	175	0	0	0
	25th %-ile	223	217	212	202	179	163
	Median	**292**	**298**	**281**	**253**	**226**	**208**
	75th %-ile	375	363	357	324	298	261
	Maximum	628	692	494	869	769	544
ASN	#	0	9	9	3	23	15
	Minimum		27.0	267	332	158	170
	25th %-ile		199	306	332	199	242
	Median		**285**	**369**	**350**	**270**	**286**
	75th %-ile		378	423	420	329	319
	Maximum		435	454	420	704	395
AA	#	0	9	5	2	21	8
	Minimum		107	123	148	0	127
	25th %-ile		182	188	148	145	198
	Median		**283**	**374**	**181**	**204**	**218**
	75th %-ile		374	379	214	260	316
	Maximum		400	379	214	329	367
HISP	#	0	2	3	12	16	48
	Minimum		272	240	44.8	64.4	48.1
	25th %-ile		272	240	143	219	150
	Median		**316**	**305**	**217**	**249**	**209**
	75th %-ile		360	575	260	289	263
	Maximum		360	575	492	364	428

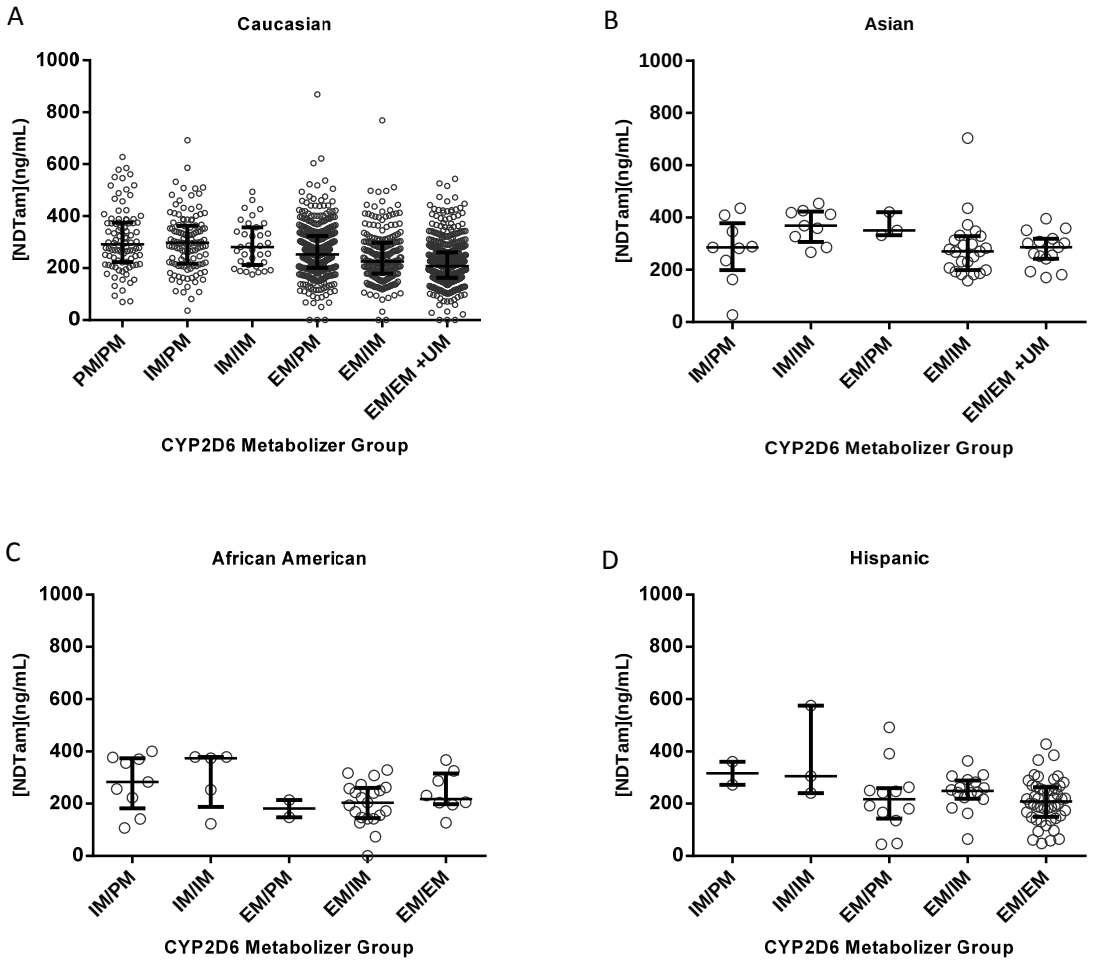


Figure 3.5. Serum N-desmethyltamoxifen Concentration (ng/mL) by CYP2D6 Metabolizer Group. Median values with the interquartile range are shown for (A) Caucasian, (B) Asian, (C) African American, and (D) Hispanic populations.

The median serum N-desmethyltamoxifen concentrations in the Caucasian population for the CYP2D6 metabolizer groups were as follows: 292 ng/mL (PM/PM), 298 ng/mL (IM/PM), 283 ng/mL (IM/IM), 253 ng/mL (EM/PM), 226 ng/mL (EM/IM), 208 ng/mL (EM/EM + UM) (see table). There are four significantly ($p < 0.05$) different groups: PM/PM + IM/PM + IM/IM, EM/PM, EM/IM, EM/EM + UM within this population. There is a trend towards lower serum N-desmethyltamoxifen concentrations with increasing

CYP2D6 activity, an association which has been reported previously in the literature. In the Asian population, median serum N-desmethyltamoxifen concentrations were: 285 ng/mL (IM/PM), 369 ng/mL (IM/IM), 350 ng/mL (EM/PM), 270 ng/mL (EM/IM) and 286 ng/mL (EM/EM+UM). N-desmethyltamoxifen concentrations were determined to be significantly lower for EM/IM than other metabolizer groups. For African Americans, median serum N-desmethyltamoxifen concentrations were: 283 ng/mL (IM/PM), 374 ng/mL (IM/IM), 181 ng/mL (EM/PM), 204 ng/mL (EM/IM) and 218 ng/mL (EM/EM+UM). No significant difference in N-desmethyltamoxifen concentrations between metabolizer groups was determined for this population. For the Hispanic population, median serum N-desmethyltamoxifen concentrations were: 316 ng/mL (IM/PM), 305 ng/mL (IM/IM), 217 ng/mL (EM/PM), 249 ng/mL (EM/IM) and 209 ng/mL (EM/EM+UM). No significant difference in N-desmethyltamoxifen concentration between these groups was determined.

Previous studies have used the ratio of endoxifen concentration to N-desmethyltamoxifen concentration ([END]/[NDTAM]) to represent CYP2D6 metabolic activity. This ratio normalizes absolute levels of metabolites, which are quite variable within the populations, yielding a value which is, hypothetically, a better representation of CYP2D6 metabolism than serum endoxifen concentration alone. Median values for [END]/[NDTam] ratio within the Caucasian population were as follows: 0.016 (PM/PM), 0.023 (IM/PM), 0.028 (IM/IM), 0.048 (EM/PM), 0.056 (EM/IM), 0.088 (EM/EM+UM) (see table). Increasing ratio was observed with increasing predicted CYP2D6 metabolic

Table 3.8. Endoxifen to N-desmethyltamoxifen Metabolic Ratio by CYP2D6 Metabolizer Group and Ethnicity

CYP2D6 Met Group		PM/PM	IM/PM	IM/IM	EM/PM	EM/IM	EM/EM +UM
CAU	#	92	116	35	410	224	490
	Minimum	0.0E+00	8.0E-03	1.2E-02	8.8E-03	0.0E+00	0.0E+00
	25th %-ile	1.3E-02	1.8E-02	1.9E-02	3.5E-02	4.3E-02	6.1E-02
	Median	**1.6E-02**	**2.2E-02**	**2.9E-02**	**4.8E-02**	**5.6E-02**	**8.9E-02**
	75th %-ile	2.0E-02	2.9E-02	3.8E-02	6.4E-02	7.3E-02	1.1E-01
	Maximum	1.3E-01	1.1E-01	8.0E-02	1.5E-01	1.7E-01	3.5E-01
ASN	#	0	9	9	3	23	15
	Minimum		1.5E-02	2.0E-02	2.2E-02	1.7E-02	3.8E-02
	25th %-ile		1.9E-02	2.6E-02	2.2E-02	4.8E-02	6.1E-02
	Median		**2.5E-02**	**2.7E-02**	**2.8E-02**	**6.5E-02**	**9.2E-02**
	75th %-ile		3.1E-02	3.4E-02	7.3E-02	9.1E-02	1.4E-01
	Maximum		3.7E-02	2.8E-01	7.3E-02	1.2E-01	1.8E-01
AA	#	0	9	5	2	20	8
	Minimum		1.5E-02	2.1E-02	7.5E-02	2.2E-02	4.7E-02
	25th %-ile		1.6E-02	2.3E-02	7.5E-02	4.6E-02	6.3E-02
	Median		**2.0E-02**	**2.7E-02**	**7.9E-02**	**5.9E-02**	**8.5E-02**
	75th %-ile		2.5E-02	3.2E-02	8.3E-02	7.1E-02	8.9E-02
	Maximum		3.5E-02	3.4E-02	8.3E-02	1.1E-01	1.1E-01
HISP	#	0	2	3	12	16	48
	Minimum		1.7E-02	1.5E-02	1.8E-02	3.8E-02	1.8E-02
	25th %-ile		1.7E-02	1.5E-02	3.3E-02	5.1E-02	7.0E-02
	Median		**2.4E-02**	**2.7E-02**	**4.5E-02**	**6.2E-02**	**9.9E-02**
	75th %-ile		3.0E-02	9.9E-02	6.9E-02	7.1E-02	1.4E-01
	Maximum		3.0E-02	9.9E-02	6.3E-01	2.3E-01	7.9E-01

activity. A statistically significant difference ($p < 0.0001$) was observed between CYP2D6 genotype groups and all metabolizer groups differed significantly from one another ($p < 0.05$). For the Asian population, the median [END]/[NDTam] metabolic ratios were 0.025 (IM/PM), 0.027 (IM/IM), 0.028 (EM/PM), 0.065 (EM/IM) and 0.092 (EM/EM+UM). Like the Caucasian population, the metabolite ratio increased with higher predicted CYP2D6 activity. The IM/PM, IM/IM, and EM/PM groups were not significantly different from one another; the EM/PM group was too small ($n = 3$) for proper statistical analysis. However, the IM/EM group had significantly ($p < 0.0001$) larger ratios that IM/PM + IM/IM. The EM/EM + UM group had significantly higher ratios ($p = 0.035$) than the IM/EM group. For the African American population, the median [END]/[NDTam] ratios

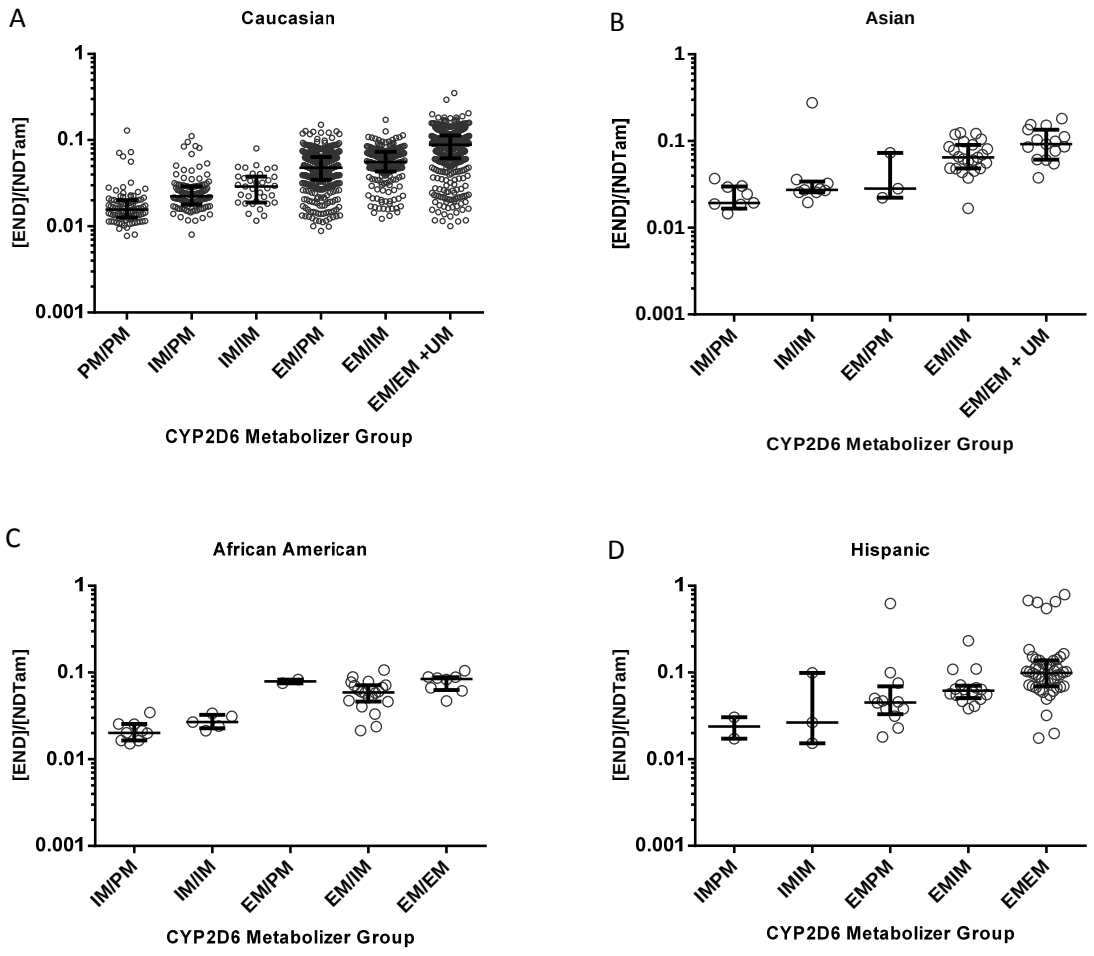


Figure 3.6. [END]/[NDTam] Ratio by CYP2D6 Metabolizer Group. Median values with the interquartile range are shown for (A) Caucasian, (B) Asian, (C) African American, and (D) Hispanic populations.

by metabolizer group were: 0.020 (IM/PM), 0.027 (IM/IM), 0.079 (EM/PM), 0.059 (EM/IM) and 0.085 (EM/EM+UM). The IM/PM and IM/IM groups did not differ significantly from one another, but the IM/EM ratio was found to be significantly larger ($p < 0.0001$) than that of IM/PM + IM/IM. The ratio was significantly ($p = 0.038$) greater for the EM/EM group in comparison to EM/IM. The EM/PM group ($n = 2$) was too small for statistical analysis. For the Hispanic population, the median [END]/[NDTam]

metabolic ratios were 0.024 (IM/PM), 0.027 (IM/IM), 0.045 (EM/PM), 0.062 (EM/IM) and 0.099 (EM/EM+UM). The EM/PM, IM/IM and IM/PM groups did not differ from one another significantly, though it should be noted that the IM/IM and IM/PM groups were quite small (n=2 and n=3, respectively). The metabolic ratio for IM/EM was significantly different from EM/PM ($p = 0.047$), and EM/EM +UM was significantly different from IM/EM ($p = 0.001$). Thus, the trend of higher ratio with higher predicted CYP2D6 activity was consistent between ethnic groups. Interestingly, the subjects with the six highest [END]/[NDTam] ratios within the entire cohort were Hispanic. Their genotypes and ratios were: *1/*1 and 0.55, *1/*4XN and 0.63, *1/*2 and 0.64, *1/*1 and 0.66, *1/*1 and 0.68, and *1/*1 and 0.79.

3.3.6 CYP2D6 Metabolizer Status and the Sub-therapeutic Endoxifen Risk Group

Madlensky et al. reported poorer outcomes of tamoxifen adjuvant therapy for subjects in the lowest quintile of serum endoxifen concentration (≥ 5.97ng/mL); subjects with endoxifen concentrations above this level had a 26% lower breast cancer recurrence rate [9]. The African American group had the highest frequency of subjects with serum endoxifen concentrations under 5.97 ng/mL (10/45 = 0.22, 95% CI: 0.37-0.11), followed by Caucasians (276/1375 = 0.20, 95% CI: 0.22-0.18), Hispanics (7/81 = 0.09, 95% CI: 0.17-0.04), and Asians (5/59 = 0.09, 95% CI: 0.19 – 0.03). Thus, Hispanics have a lower frequency of subjects in the sub-therapeutic endoxifen risk group than Caucasians. Larger numbers of Asians and African Americans may be required in order to show significant differences in the frequency of at risk subjects in comparison to the

other ethnic groups. The data does suggest a lower frequency of at risk subjects among Asians versus Caucasians, although there is a slight overlap of confidence intervals between the two groups. Of the Asian subjects in the risk group, four were IM/PM (*10/*5) and one was EM/IM (*1/*10). Of the Hispanics, one was IM/IM (*10/*9) four were EM/PM (1= *1/*4, 3 = *2/*4), one was EM/IM (*10/*9) and one was EM/EM (*2/*35). Of the African Americans, five were IM/PM (1 = *29/*5, 2= *41/*5, 1 = *17/*5, and 1 =*17/*4xN), one was IM/IM (*17/*17), and four were EM/IM (2 = *1/*41, 1 = *2/*41, 1= *1/*17, and 1= *2/*17).

Of interest is to determine the optimal CYP2D6 metabolizer group cutoff for predicting inclusion in this sub-therapeutic endoxifen risk category. Using data from the Caucasian subjects, sensitivity, specificity, positive predictive value, and negative predictive value were determined for the following potential CYP2D6 genotype test cut-offs for inclusion of subjects in the sub-therapeutic endoxifen risk group: PM/PM, IM/PM, IM/IM, and EM/PM. (Table 3.10)

TABLE 3.9. Low Endoxifen Risk Group by CYP2D6 Metabolizer Group for Caucasian Subjects

CYP2D6 Met Group	# in Met Group	[END] < 5.97 ng/mL	% of Met Group	% of low END
EM/EM +UM	493	53	10.8%	19.2%
EM/IM	226	32	14.2%	11.6%
EM/PM	413	69	16.7%	25.0%
IM/IM	36	7	19.4%	2.5%
IM/PM	115	49	42.6%	17.8%
PM/PM	92	66	71.7%	23.9%
Total	1375	276	20.1%	100.0%

Using the PM/PM metabolizer status as the test cut-off, sensitivity is 23.9%, specificity is 97.6%, the PPV is 71.7%, and the NPV is 83.6%. By extending the test cutoff to PM/IM, the sensitivity is increased to 41.7%, specificity is 91.6%, PPV is decreased to 55.6%, and NPV is 86.2%. By setting the test cutoff to IM/IM, very similar values for sensitivity, specificity, PPV and NPV as PM/IM result, such that the 95% confidence intervals overlap. By designating EM/PM as the test cutoff, a sensitivity of 69.2%, a specificity of 57.7%, a PPV of 29.1%, and an NPV of 88.2% are achieved. Thus, by setting the test cutoff to a more active CYP2D6 metabolizer group, the sensitivity increases at the expense of PPV and specificity.

While the majority of PM/PM subjects fall into the low-endoxifen risk group, this only accounts for a minority of subjects with sub-therapeutic endoxifen concentrations. The rest of this category is comprised of subjects from the other metabolizer groups. As one might expect, the percentage of subjects within the risk group decreases with increasing CYP2D6 metabolizer status: IM/PM (42.6%), IM/IM (19.4%), EM/PM (16.7%), EM/IM (14.2%), and EM/EM + UM (10.8%). Importantly, there is no metabolizer group within the cohort that lacks subjects that fall into the sub-therapeutic endoxifen risk category.

Table 3.10. Sensitivity, specificity, positive and negative predictive values of CYP2D6 metabolizer group cutoffs to define the sub-therapeutic endoxifen risk group. PPV = positive predictive value. NPV = negative predictive value. 95% confidence intervals are shown in parentheses.

test cutoff	sensitivity (95% CI)	specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
PM/PM	23.9% (19.0% - 29.4%)	97.6% (96.6% - 98.5%)	71.7% (61.4% - 80.6%)	83.6% (81.5% - 85.6%)
IM/PM	41.7% (35.8%-47.7%)	91.6% (89.8% - 93.2%)	55.6% (48.5% - 62.4%)	86.2% (84.1% - 88.1%)
IM/IM	44.2% (38.3% to 50.3%)	89.0% (87.0%-90.8%)	50.2% (43.7% - 56.7%)	86.4% (84.3%-88.3%)
EM/PM	69.2% (63.4%-74.6%)	57.7% (54.7%-60.6%)	29.1% (25.7%-32.7%)	88.2% (85.6%-90.5%)

3.3.7. Phenocopy of PM/PM in the Sub-therapeutic Endoxifen Risk Group

The majority of PM/PM subjects (71.7%) fell into the sub-therapeutic endoxifen risk group. Of all the PM/PM subjects, 93.5% (86/92) exhibit [END]/[NDTAM] ratios within the lowest quartile of the population, while a wide range of serum tamoxifen concentrations across all quartiles is observed (see figure 3.7 B). Thus, a dominant feature of the PM/PM phenotype is an [END]/[NDTAM] ratio within the lowest quartile. Subjects genotyped as PM/PM who exhibited high serum tamoxifen concentrations were able to attain serum endoxifen concentrations above the risk cutoff, despite low [END]/[NDTam] ratios. Of the PM/PM subjects who did not fall into the sub-therapeutic endoxifen risk group, there were several obvious outliers. One outlier was genotyped as *4/*4 and had a serum tamoxifen concentration within the lowest quartile but an [END]/[NDTam] ratio within the highest quartile. Four outliers exhibited both serum tamoxifen concentrations and [END]/[NDTam] ratios within the upper third quartiles of tamoxifen concentration and [END]/[NDTam] ratio, and their genotypes were *4/*4 (n=3) and *4/*5 (n=1). These five outliers exhibited the five highest serum endoxifen

concentrations of the PM/PM group. The majority of PM/PM subjects have low serum endoxifen concentrations and [END]/[NDTam] ratios, but may exhibit a wide range of serum tamoxifen concentrations.

Of all subjects who fell into the sub-therapeutic endoxifen risk group, 81.9% (226/276) exhibit [END]/[NDTAM] ratios within the lowest quartile of ratios and 46% (127/276) subjects exhibit serum tamoxifen concentrations within the lowest quartile of tamoxifen concentrations (see figure 3.7 A). There is overlap between these groups, with 29.3% (81/276) of these subjects exhibiting both tamoxifen concentrations and [END]/[NDTAM] ratios within the lowest quartile; low serum endoxifen concentrations can be attributed to both of these factors in these subjects. For subjects who exhibit [END]/[NDTam] ratios above the lowest quartile, the vast majority of serum tamoxifen concentrations fall within the lowest quartile; this profile differs from the major PM/PM phenotype. One can attribute low serum endoxifen concentrations to low serum tamoxifen levels, rather than CYP2D6 activity, in these subjects.

Subjects in the low endoxifen risk group from the IM/PM and IM/IM metabolizer groups exhibit mainly [END]/[NDTam] ratios within the first quartile, with a range of serum tamoxifen concentrations (figure 3.7 C), very similar to what is seen for PM/PM within the low endoxifen risk group (figure 3.7 B). This profile is present among subjects from all CYP2D6 metabolizer groups. However, subjects who exhibit [END]/[NDTam] ratios above the lowest quartile, but serum tamoxifen concentrations within the lowest

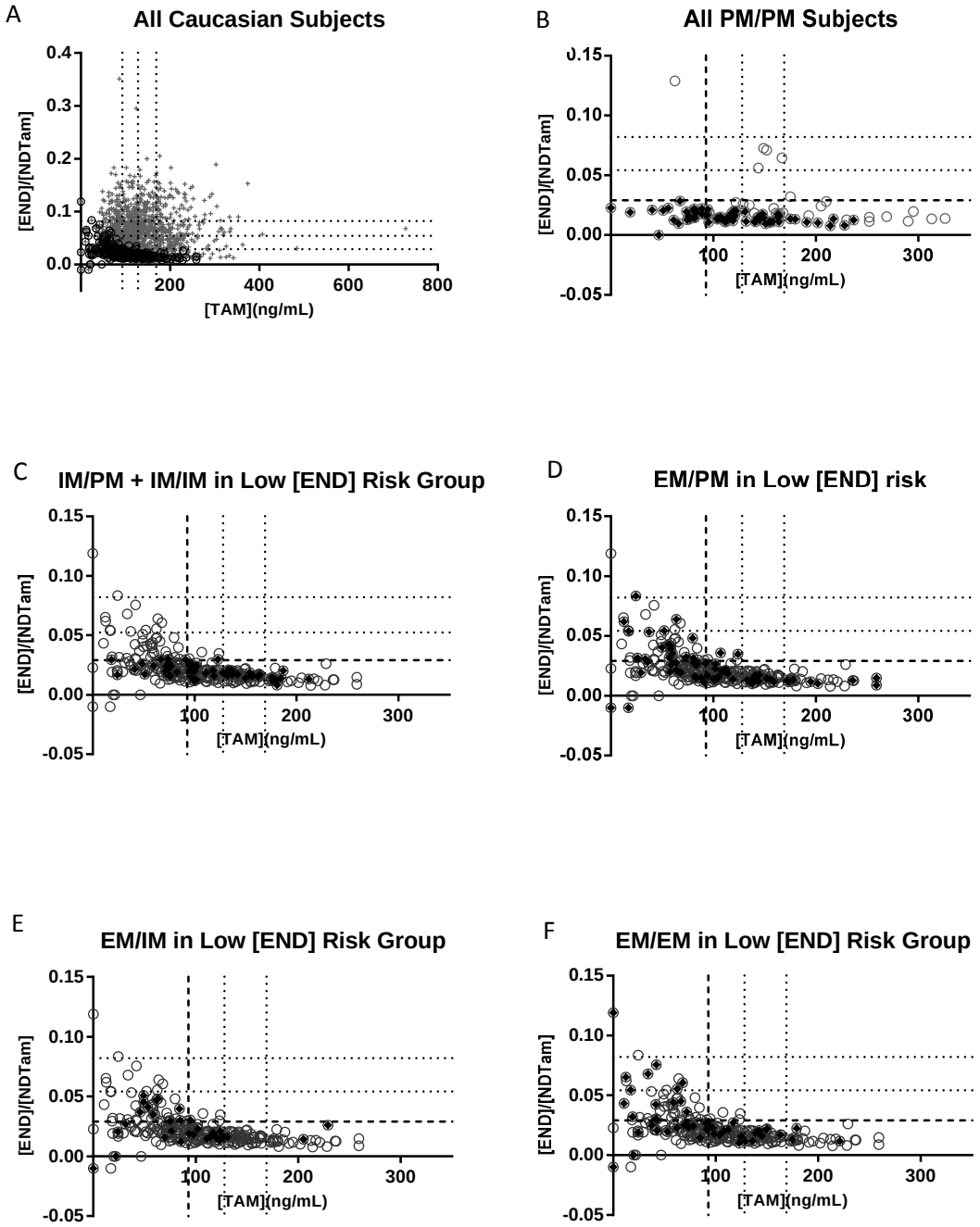
quartile come from metabolizer groups with higher predicted enzymatic activity – EM/PM, EM/IM, and EM/EM.

3.4 Discussion

In this study, extensive *CYP2D6* genotyping was performed using the Roche P450 AmpliChip, and tamoxifen, endoxifen, and N-desmethyltamoxifen serum concentrations were assessed using a quantitative LC-MS/MS assay in a sizeable population of subjects undergoing tamoxifen adjuvant therapy. *CYP2D6* genotypes were used to predict CYP2D6 enzymatic activity, and subjects were then classified into metabolizer groups.

Inter-ethnic differences in tamoxifen and tamoxifen metabolite concentrations were identified in this study. Both the Asian and Hispanic populations exhibited significantly higher median serum endoxifen concentrations than Caucasians. For the Asians, the median tamoxifen and N-desmethyltamoxifen concentrations were also higher, although the [END]/[NDTam] ratio was not significantly different from that of Caucasians. Asians appear to maintain higher concentrations of tamoxifen,

FIGURE 3.7. (Next Page) Tamoxifen Concentration versus [END]/[NDTam] Ratio. (A) All Caucasian subjects in grey, sub-therapeutic endoxifen group represented by black open circles; (B) All PM/PM in grey, PM/PM in sub-therapeutic risk group in black; (C) IM/IM + IM/PM in sub-therapeutic endoxifen risk group; (D) EM/PM in sub-therapeutic endoxifen risk group; (E) EM/IM in sub-therapeutic endoxifen risk group; (F) EM/EM in sub-therapeutic endoxifen risk group. For figures C-F, grey open circles represent all subjects in the sub-therapeutic endoxifen risk group while black diamonds are subjects from the indicated metabolizer group that fall within the risk group. Horizontal dotted lines represent the 25th %-ile, Median, and 75th %-ile for [TAM](ng/mL). Vertical dotted lines represent the 25th %-ile, Median, and 75th %-ile for [END]/[NDTam]. Points for which no [END]/[NDTam] ratios could be determined due to low non-quantitative values were assigned a ratio of -0.01 for the sake of visualization.



N-desmethyltamoxifen, and endoxifen at the standard 20 mg/day dose than Caucasians. One can infer from [END]/[NDTam] ratios that a higher rate of endoxifen formation did not appear to be responsible for this difference. As for the Hispanic population, the converse appeared to be true. While tamoxifen and N-desmethyltamoxifen

concentrations did not differ significantly from that of the Caucasian group, median [END]/[NDTam] ratios were significantly higher, suggesting higher median levels of endoxifen in the Hispanic group are driven by metabolism of N-desmethyltamoxifen into endoxifen. Hispanics had the highest percentage of EM/EM+UM CYP2D6 metabolizers of the ethnicities within the WHEL cohort. Notably, the six highest [END]/[NDTam] ratios were from subjects in the Hispanic group. These samples were assayed on different dates, and all samples had been blinded by the WHEL collaborators, therefore it is unlikely that these values are due to analytical error. In addition to a high [END]/[NDTam] ratio, they also exhibited relatively low serum tamoxifen concentrations. Unexpectedly, none of these subjects were UM. The CYP2D6 genotype data, which is quite extensive, does not explain the high [END]/[NDTam] ratios of these subjects. Therefore, some other genetic or environmental factor is likely to be responsible. Either the rate of endoxifen formation is higher, or the rate of its elimination is decreased. Further research is required in order to determine if these types of subjects are present in other Hispanic populations, and to identify the factors that have led to such high [END]/[NDTam] ratios. In addition, Hispanics appear to have a lower frequency of subjects in the sub-therapeutic endoxifen risk group than Caucasians. This is perhaps not surprising considering the low frequency of reduced and null CYP2D6 activity alleles within this group.

Interestingly, the distribution of genotype predicted CYP2D6 allele activity is almost the same for the Asian and African American populations. However, Asians appear to have noticeably higher median serum endoxifen concentrations in

comparison to the African Americans, despite this similarity. This suggests that ethnicity must be taken into account when attempting to use *CYP2D6* genotype to predict serum endoxifen concentration. This finding suggests that CYP2D6 genotype should not be used to make treatment decisions without considering the ethnicity of the patient. Further tamoxifen pharmacogenetic research in minority populations is required in order to determine optimal treatment guidelines based upon *CYP2D6* genotype. All ethnic groups analyzed exhibited substantial overlap in serum tamoxifen and tamoxifen metabolite concentrations between genotype predicted metabolizer groups. Thus, *CYP2D6* genotyping predicts some, but not all, variation in tamoxifen and tamoxifen metabolite concentrations in these groups.

Based upon analysis of genotype-phenotype correlation in the Caucasian population, it appears that CYP2D6 activity score does not suit this dataset. Some investigators have elected to classify *CYP2D6* alleles by an activity score, assigning the following scores to each allele: PM = 0, IM = 0.5, EM = 1, and UM= 2. Subjects are then classified by the sum of the two allele scores [16]. Notably, IM/IM (0.5 + 0.5 = 1) and EM/PM (1 + 0 = 1) groups are statistically different from one another, although both would be assigned an activity score of one. Thus, activity score does not result in the best categorization of subjects within this cohort by serum endoxifen concentration since the median serum endoxifen concentration of IM/IM is significantly lower than that of EM/PM subjects. Whether or not this has any clinical significance is difficult to determine at this time; neither IM/IM nor EM/PM are reasonable test cutoffs for predicting sub-therapeutic endoxifen concentrations, without any additional

information. Arguably, activity score does simplify description of predicted CYP2D6 activity, and may be more appealing in the clinical environment than others used to describe predicted CYP2D6 activity.

The primary intent of the WHEL study was to assess the impact of a dietary intervention on breast cancer recurrence. While the banked serum and DNA samples from WHEL provided a valuable resource for the study of tamoxifen pharmacogenetics, the lack of co-medication data at the time of serum collection is a limitation to the current study. Co-medication with CYP2D6 inhibitors has been shown to result in reduced plasma concentrations of endoxifen [17]. Before a role for CYP2D6 metabolism in tamoxifen efficacy was apparent, SSRIs such as paroxetine and fluoxetine were given to women taking tamoxifen for the treatment of hot flash. These drugs are also potent CYP2D6 inhibitors. During the time frame in which subjects were recruited to the WHEL study (1995-2000), it was not standard practice to prescribe SSRIs for the treatment of hot flash at WHEL study recruitment sites. However, the use of SSRIs for the treatment of depression within the cohort cannot be ruled out. Within the WHEL cohort, there were significant numbers of subjects who were PM/PM phenocopies in terms of [END]/[NDTam] ratios and serum tamoxifen concentrations within all other metabolizer groups within the Caucasian population. The use of CYP2D6 inhibitors may have contributed to some of the variation in endoxifen concentration and [END]/[NDTam] ratio that could not be explained by *CYP2D6* genotype, although the extent to which this is a contributing factor is unknown.

The intent of excluding individuals who were taking tamoxifen for less than 3 months at the time of serum collection was to limit analyses to subjects with "steady-state" levels of tamoxifen metabolites resulting from a 20 mg/day dosing regimen. However, compliance (adherence + persistence) to tamoxifen therapy is highly variable within the adjuvant setting. While compliance is expected to be high within the WHEL cohort, no measures of compliance were actually made for the study. There were definitely subjects within the cohort who had no detectable, or detectable but non-quantifiable, level of tamoxifen, N-desmethyltamoxifen and/or endoxifen. Above these levels, a continuous spectrum of tamoxifen and tamoxifen metabolite concentrations were observed. Thus, it is not difficult to imagine that certain subjects in the cohort were not fully compliant with the 20 mg/day dose of tamoxifen, and the extent of non-compliance was variable. However, without additional data, it would not be valid to set cutoffs for a non-compliant group. What one can say from the WHEL data is that low serum tamoxifen concentrations do contribute to sub-therapeutic endoxifen concentrations in individuals with otherwise moderate [END]/[NDTam] ratios; this phenotype differs from the majority of PM/PM subjects. Compliance is a real issue in the clinical setting, and the contribution of poor compliance to treatment failure may be quite significant [18-20]. One must find better ways to address this issue, both in clinical studies and clinical practice.

The Royal Dutch Pharmacist Association-Pharmacogenetics Working Group has published dose recommendations guidelines for tamoxifen based on *CYP2D6* genotype [21]. For patients carrying two inactive alleles, they recommend the use of aromatase

inhibitors rather than tamoxifen as adjuvant endocrine therapy. Similarly, it is recommended that patients who are IM/PM, IM/IM, and EM/PM consider aromatase inhibitors or avoid concomitant use of CYP2D6 inhibitors if they elect to use tamoxifen. Considering that aromatase inhibitor therapy is a good and, possibly superior, alternative to tamoxifen [19, 22-26], and these agents have replaced tamoxifen as the preferred adjuvant endocrine therapy in recent years, this advice seems very reasonable. Certainly, it would be of interest to see the clinical outcome of a genotype guided intervention to see if it is superior to treatment not based upon genotype. However, sequenced endocrine therapy consisting of tamoxifen therapy followed by aromatase inhibitor may have a greater benefit than aromatase inhibitor alone, and it would be good not to exclude patients who might benefit from this treatment course. Analysis of the WHEL data suggests that a large percentage of subjects who are IM/PM, IM/IM and EM/PM may still benefit from tamoxifen therapy. In order to make balanced decisions about how to treat these patients, more research needs to be done on factors that influence aromatase inhibitor efficacy. A recent study suggests that plasma Letrozole concentrations are associated with *CYP2A6* genetic variants [27], but whether or not this influences efficacy is currently unknown. And for both aromatase inhibitors and tamoxifen, it would be beneficial to know what factors, genetic and otherwise, predispose patients to side effects that lead to poor compliance. Vasomotor symptoms, a major side effect of tamoxifen therapy, have been associated with both *CYP2D6* genotype [2, 28] and higher serum endoxifen concentrations [29]. An association between musculoskeletal side effects related to aromatase inhibitor treatment and a

SNP in *TCL1A*, which influences interleukin 17 receptor A expression has been determined through a genome-wide association study [30]. Selection of therapy on the basis of predicted side effect severity, in addition to efficacy, could be a promising strategy.

The best hypothesis for the association between *CYP2D6* genotype and the outcome of tamoxifen adjuvant therapy is that CYP2D6 is important for the formation of endoxifen, and that exposure of tumor cells to endoxifen is necessary for tamoxifen efficacy. *CYP2D6* genotype is essentially a surrogate marker for exposure of tumor cells (micro-metasteses in the adjuvant setting) to tamoxifen. This type of direct measurement is not feasible in the clinical setting, but assessment of endoxifen concentrations in the serum or plasma of patients taking tamoxifen is possible. Therapeutic drug monitoring (TDM) of endoxifen and tamoxifen may be justified for patients who would not be excluded from tamoxifen therapy due to *CYP2D6* genotype, or in lieu of genotyping. Further research into the clinical utility of TDM for patients taking tamoxifen is warranted. In addition, the identification and validation of tumor-specific markers that predict response to tamoxifen continue to be of interest, and have the potential to significantly increase the value of TDM and/or genotyping strategies for determining optimal adjuvant endocrine therapy.

3.5 Conclusion

In conclusion, there are significant differences between ethnic groups within the WHEL cohort. Notably, Hispanics have a lower frequency of subjects in the sub-

therapeutic endoxifen risk group in comparison to Caucasians. This may also be true for Asians, but the data is inconclusive. In addition, the optimal genotype cutoff for defining a low endoxifen risk group, at least for Caucasians, appears to be the PM/PM genotype. However, this identifies only a fraction of subjects who exhibit sub-therapeutic serum endoxifen concentrations. Among "at risk" subjects with low serum endoxifen concentrations, there are both PM/PM phenocopies with low [END]/[NDTam] ratio as well as those with low serum tamoxifen concentrations, which may indicate poor compliance. Indeed, many subjects fall into both of these categories.

The metabolism of tamoxifen is complex, and *CYP2D6* genotype accounts for only some of the variation in serum endoxifen concentrations. Absorption of tamoxifen, elimination of endoxifen, CYP3A metabolism, co-medication, herbal supplements, compliance, and health status are factors that may influence steady-state endoxifen levels. At the present time, one could use *CYP2D6* genotyping to exclude poor metabolizers from tamoxifen therapy; the majority of subjects in this group exhibit very low serum endoxifen concentrations. However, it would be important for both doctors and patients to be aware that, besides CYP2D6 poor metabolizers, there are many other patients who may not benefit from tamoxifen therapy either. In other words, not being a CYP2D6 poor metabolizer does not ensure a patient will generate therapeutic levels of endoxifen; it does not ensure benefit from tamoxifen.

3.6 References

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Chapter 4

Contribution of *ABCC2*, *UGT2B7*, and *CYP2C19* Variants to Serum Endoxifen Concentration

4.1 Introduction

Endoxifen is considered the major active metabolite of the pro-drug tamoxifen, and CYP2D6 metabolism is an important step in the formation of endoxifen. A high degree of inter-individual variation in steady-state endoxifen concentrations has been observed among breast cancer patients taking tamoxifen[1]. Both the rate of formation and elimination are important determinants of steady-state endoxifen concentrations. *CYP2D6* genotype explains some, but not all, variation in serum endoxifen concentration. Thus it is of interest to determine additional factors that influence steady-state endoxifen concentrations. Genetic predictors are of particular interest. If shown to be predictive of response, additional genetic markers can be combined with *CYP2D6* testing in order to increase the utility of genetic testing for the optimization of adjuvant endocrine therapy.

Variable elimination of endoxifen may contribute to inter-individual differences in steady-state endoxifen concentrations that cannot be attributed to functional polymorphisms in *CYP2D6*. Glucuronidation is a major mechanism for the inactivation of many xenobiotics, and endoxifen glucuronides have been identified in the urine of breast cancer patients taking tamoxifen [2]. Thus, glucuronidation may play an important role in the inactivation and elimination of endoxifen. Recent *in vitro* studies by Sun et al. suggest that UGT2B7 is the major hepatic enzyme responsible for the

glucuronidation of endoxifen [3]. Glucuronidation eliminates the anti-estrogenic activity of these potent metabolites in cell models of ER^+ breast cancer [4]. In cell lines overexpressing wild-type or variant UGTs, it was found that $UGT2B7^{268Tyr}$ exhibited an 80% decrease($p<0.01$) in glucuronidation activity against *trans*-endoxifen in comparison to $UGT2B7^{268His}$ [5]. The minor allele frequency of this variant, *UGT2B7**2, is approximately 0.5 in Caucasians and it is important to determine if it has an influence on serum endoxifen concentration or [END]/[NDTam] ratio. Innocenti et al. recently identified six major *UGT2B7* haplotypes that account for 90% of genetic variation in Caucasians. One haplotype (frequency 0.12) was correlated with a 61% average increase in UGT2B7 activity for morphine glucuronidation in human liver microsomes, as well as increased *UGT2B7* transcript levels [6]. The ivs1985ag variant is unique to this haplotype. The effect of this variant on UGT2B7 activity has yet to be determined *in vivo*, but it is of interest to determine if it is associated with any difference in serum endoxifen concentration or [END]/[NDTam] ratio.

ATP binding cassette (ABC) efflux transporters may influence the absorption, distribution, metabolism, and excretion of tamoxifen and its metabolites. Therefore, genetic variation in genes that encode ABC efflux transporters may influence tamoxifen and tamoxifen metabolite levels. The multidrug resistance associated transporter MRP2 is of particular interest as repeated exposure to high doses of tamoxifen induces MRP2 expression in MCF-7 cells[7]. Several genetic variants in the gene that encodes this enzyme (*ABCC2*) alter activity [8-11], and it is of interest to see if these variants influence serum endoxifen concentrations or [END]/[NDTam] ratio.

In addition to *CYP2D6*, other genetic variants have been associated with clinical outcomes of adjuvant tamoxifen therapy by various groups, but these associations have yet to be replicated. It is of interest to determine if these variants are associated with endoxifen concentrations in the WHEL cohort. One example is *CYP2C19*17*. In a recent paper by Schroth et al. [12], heterozygotes and homozygotes for the *CYP2C19*17 (-806 C>T) genotype were reported to have improved relapse free survival (HR, 0.45; 95% CI, 0.21 to 0.92; P = .03) in comparison to non-carriers. In comparison to CYP2C19*1, CYP2C19*17 is associated with increased enzymatic activity. The biological basis for this association is thought to be due to increased production of potent tamoxifen metabolites. Additional variants for which *in vitro* or clinical data suggests an effect on tamoxifen metabolism were also investigated.

The objective of this study is to determine if there is a relationship between serum endoxifen concentration and [END]/[NDTam] ratio and genotypes in candidate genes as this suggests a biological mechanism for a possible association with clinical outcome.

4.2 Materials and Methods

4.2.1 Study Subjects and Genotyping

DNA was obtained from the Women's Healthy Eating and Living (WHEL) study group for Caucasian subjects taking tamoxifen for whom serum concentration measurements of tamoxifen, NDTam, and endoxifen were available. At the time of WHEL study enrollment, subjects provided a 5-ml sample of EDTA whole blood, which was stored at -80°C. Genomic DNA was extracted from these archival samples, using 200

μL of the buffy coat fraction (QIAcube robot with QIAamp DNA Blood Mini Kit, Qiagen, Valencia, CA, USA). *CYP2D6* genotyping had been performed on these subjects previously using the Roche P450 AmpliChip [13]. There were 940 DNA samples for which both an adequate quantity of DNA was available and the 260/280 ratio was above 1.7. DNA was transferred from vials into 96 well plates, and DNA concentration was normalized with the addition of ddH_2O to 10 μg/mL, using a TECAN robotic system at the UCSF DNA bank. Sequenom iPlex Gold genotyping was performed at UCSF Helen Diller Family Comprehensive Cancer Center Genome Analysis Core to interrogate the following variants: *ABCC2* rs3740066, rs1885301, rs17222723 and rs2273697, *UGT2B7* ivs1985ag and rs7439366, *CYP2C19* rs12248560, *HNF4A* rs1800961 and rs736823, *UGT1A4* s6755571, *ABCG2* rs2231142, and *CYP3A4* rs2740574. Several variants of interest could not be genotyped well using this assay platform. The Sequenom genotype data for *UGT2B7*2* (rs7439366) failed quality control, such that genotypes were out of Hardy-Weinberg Equilibrium. In addition, the call rate for *ABCC2* rs2273697 was less than 50% using the Sequenom assay. During initial testing, the *ABCC2* variant rs717620 was judged to be incompatible with Sequenom genotyping due to high dimer potential. As these variants were of special interest to the project, they were genotyped using TaqMan allelic discrimination assays using an ABI Prism 7900 Sequence Detector System. Applied Biosystems TaqMan Drug Metabolism Genotyping assays were used for *ABCC2* rs2273697 and rs717620, while probes and primers for *UGT2B7* rs7439366 genotyping were taken from Coulbault et al. [14]. For TaqMan genotyping, 10 ng of

DNA was dispensed into 384-well plates and dried. Concentrations of probes and primers used for the assay were 200 nmol/L and 900 nmol/L, respectively.

Of the 940 subjects who were genotyped, there were 50 who had taken tamoxifen for less than three months at the time of blood draw. Three months of tamoxifen therapy is required in order for tamoxifen, endoxifen and N-desmethyltamoxifen serum concentrations to reach steady-state so data for these subjects was excluded from further analysis. Eight subjects had tamoxifen, endoxifen, and/or N-desmethyltamoxifen measurements below the limit of quantitation and were excluded from further analysis. The *CYP2D6* genotypes of these subjects were *1/*1, *1/*2, *1/*3, *1/*4, *1/*41, *2/*41, *3/*4, and *4/*4. These subjects were excluded from further analysis, leaving 882 study subjects.

4.2.2 Determination of Tamoxifen Metabolite Concentrations in Serum

Serum concentrations of tamoxifen, N-desmethyltamoxifen, and endoxifen were measured using LC-MS/MS. For additional information on assay conditions, see chapters 2 and 3 of this dissertation.

4.2.3 Statistical Analysis

STATA/IC 12.1 was used for statistical analyses. Robust multiple regression was performed to determine the contribution of each *CYP2D6* variant to log([END]) and log([END]/[NDTam]). For the regression analysis, each variant was coded as 0 (absent), 1 (heterozygous) or 2(homozygous), which assumes co-dominant expression of alleles.

Coefficients from these analyses were used to generate *CYP2D6* variant scoring systems. In addition to these *CYP2D6* scoring systems, Gaedigk et al. developed a qualitative activity score system for *CYP2D6* variants based upon dextromethorphan to dextrorphan (DM/DX) urinary metabolic ratios in a large cohort of Caucasian and African American subjects. Using this system, a score of 0 is given to alleles associated with poor metabolism (*3, *4, *5, *6, and *7), 0.5 for intermediate metabolism (*9, *10, *17, *29, and *41), 1.0 for extensive metabolism (*1, *2, and *35), and 2.0 for ultra-rapid metabolism (*1XN,*2XN and *35XN, gene duplications). The score for each allele is added and the resulting sum is the CYP2D6 activity score. Univariate regression was used to correlate the resulting *CYP2D6* variant scoring systems, as well as the published CYP2D6 activity score by Gaedigk et al., with log([END]) and log([END]/[NDTam)]. Stepwise, forward multiple regression was used to determine the influence of the CYP2D6 genotype score, weight, age, duration of tamoxifen treatment at blood draw, and the genetic variants of interest on log([END]/[NDTam]) and log([END]). A P-value < 0.05 was considered significant. Semipartial correlation analysis was used to determine the relative contribution of variants shown to have a statistically significant effect ($p<0.05$) on log([END]/[NDTam]) and log([END]).

4.3 Results

4.3.1 Genotypes

CYP2D6 genotyping had been performed using the Roche AmpliChip in a previous study [13] for the 882 subjects included in this analysis. See Table 4.1 for the frequencies of *CYP2D6* variants within the population. Genotype, minor allele

frequencies, and p-value for deviation from HWE of the additional genetic variants that were assessed in this chapter are listed in Table 4.2. Call rates for genotypes were >97% for variants used in the following analyses.

Table 4.1 Frequency of CYP2D6 Variants

CYP2D6 Variant	Activity	#	Frequency
***1**	Full	609	34.5%
***2**	Full	275	15.6%
***35**	Full	104	5.90%
***3**	Null	26	1.47%
***4**	Null	358	20.3%
***5**	Null	47	2.66%
***6**	Null	23	1.30%
***7**	Null	1	0.06%
***15**	Null	1	0.06%
***19**	Null	1	0.06%
***3**	Null	1	0.06%
***4xN**	Null	5	0.28%
***9**	Reduced	46	2.61%
***10**	Reduced	24	1.36%
***17**	Reduced	3	0.17%
***41**	Reduced	204	11.6%
***1xN**	Ultrarapid	11	0.62%
***2xN**	Ultrarapid	19	1.08%
***35xN**	Ultrarapid	1	0.06%
***41xN**	Unknown	5	0.28%

Table 4.2 Genotype and minor allele frequencies (MAF) of investigated variants and test for deviation from Hardy Weinberg equilibrium (p HWE)

Gene	Variant	Major Homo	Het	Minor Homo	MAF	p HWE
ABCC2	rs3740066	342	423	117	0.37	0.441
ABCC2	rs1885301	279	420	182	0.44	0.301
ABCC2	rs17222723	748	126	6	0.08	0.783
ABCC2	rs717620	558	293	28	0.20	0.159
ABCC2	rs2273697	566	275	32	0.19	0.843
UGT2B7	ivs1985ag*	661	200	9	0.13	0.150
UGT2B7	rs7439366	228	434	199	0.48	0.786
CYP2C19	rs12248560	543	293	40	0.21	0.997
HNF4A	rs1800961	823	40	1	0.02	0.482
HNF4A	rs736823	779	89	6	0.06	0.055
UGT1A4	rs6755571	788	92	0	0.05	0.102
ABCG2	rs2231142	696	178	8	0.11	0.359
CYP3A4	rs2740574	824	57	1	0.03	0.989

*rs number not available. See Innocenti et al. for additional details [15].

4.3.2 Contribution of *CYP2D6* variants to log([END]) and log([END]/[NDTam])

Frequencies of *CYP2D6* variants in the population used are listed in Table 4.1. Robust multiple regression was used to determine the contribution of each *CYP2D6* variant to log([END]) and log([END]/[NDTam]). Coefficients for the *CYP2D6* variants, confidence intervals, and p-values from the analysis are listed in Table 4.3 for log([END]) and Table 4.4 for log([END]/[NDTam]). The coefficients which had statistically significant results ($p < 0.05$) were used to generate a scoring system for the *CYP2D6* alleles for prediction of log([END]) (score 1), and log([END]/[NDTam]) (score 2) respectively, thereby condensing all *CYP2D6* variants tested into single variables. Univariate regression was used to determine the contribution of score 1 to log([END]) and score 2 to log([END]/[NDTam]) and the Gaedigk activity score to both log([END]) and log([END]/[NDTam]). The Gaedigk

activity score r^2 values were 0.21 for log([END]) and 0.35 for log([END]/[NDTam]). The r^2 value for score 1 for the prediction of log([END]) was 0.24, and the r^2 value for score 2 for prediction of log([END]/[NDTam]) was 0.41. Thus, these scoring systems were able to explain slightly more variation in log([END]) and log([END]/[NDTam]) than the Gaedigk activity score.

Table 4.3 Robust linear multiple regression analysis of *CYP2D6* variants for prediction of log([END])

Variant	Coefficient	Std. Error	P-value	95% CI		
***1**	0.72	0.14	<0.001	0.44	-	1.00
***2**	0.60	0.14	<0.001	0.31	-	0.88
***35**	0.47	0.15	0.002	0.18	-	0.77
***3**	0.14	0.18	0.46	-0.22	-	0.50
***4**	0.20	0.15	0.179	-0.09	-	0.48
***5**	0.19	0.15	0.229	-0.12	-	0.49
***6**	0.07	0.18	0.688	-0.28	-	0.42
***7**	0.83	0.14	<0.001	0.55	-	1.11
***15**	-0.90	0.14	<0.001	-1.18	-	-0.62
***19**	-0.54	0.14	<0.001	-0.82	-	-0.26
***4xn**	-0.31	0.19	0.107	-0.68	-	0.07
***9**	0.46	0.16	0.004	0.15	-	0.77
***10**	0.19	0.28	0.5	-0.36	-	0.75
***17**	0.58	0.23	0.013	0.12	-	1.03
***41**	0.27	0.15	0.069	-0.02	-	0.55
***1xn**	0.92	0.29	0.001	0.35	-	1.49
***2xn**	0.97	0.20	<0.001	0.57	-	1.37
***35xn**	1.28	0.15	<0.001	1.00	-	1.57
***41xn**	0.83	0.25	<0.001	0.34	-	1.33
intercept	*1.50*	*0.28*	*<0.001*	*0.94*	-	*2.05*

Table 4.4 Robust linear multiple regression analysis of *CYP2D6* variants for prediction of log([END]/[NDTam])

Variant	Coefficient	Std. Error	P-value	95% CI		
***1**	1.08	0.12	<0.001	0.84	-	1.31
***2**	0.88	0.12	<0.001	0.65	-	1.11
***35**	0.81	0.13	<0.001	0.56	-	1.06
***3**	0.33	0.17	0.054	-0.01	-	0.66
***4**	0.34	0.12	0.005	0.10	-	0.57
***5**	0.33	0.13	0.012	0.07	-	0.59
***6**	0.17	0.16	0.291	-0.14	-	0.48
***7**	0.73	0.12	<0.001	0.49	-	0.96
***15**	-0.35	0.12	0.004	-0.58	-	-0.11
***19**	-0.15	0.12	0.198	-0.38	-	0.08
***4xn**	-0.20	0.24	0.405	-0.67	-	0.27
***9**	0.69	0.14	<0.001	0.42	-	0.96
***10**	0.53	0.23	0.025	0.07	-	0.98
***17**	0.83	0.16	<0.001	0.51	-	1.14
***41**	0.48	0.12	<0.001	0.24	-	0.71
***1xn**	1.29	0.26	<0.001	0.78	-	1.81
***2xn**	1.23	0.21	<0.001	0.82	-	1.65
***35xn**	1.46	0.12	<0.001	1.22	-	1.70
***41xn**	1.34	0.28	<0.001	0.79	-	1.88
intercept	*-4.51*	*0.23*	*<0.001*	*-4.97*	-	*-4.05*

4.3.3 Contribution of Additional Variables to log([END]) and log([END]/[NDTam])

CYP2D6 genotype score 2, weight, age, duration of tamoxifen treatment at blood draw, and the genetic variants of interest were considered as covariates for log([END]/[NDTam]). Stepwise regression analysis indicated that log([END]/[NDTam]) was dependent on the variables score 2 and *ABCC2* rs2273697, with a combined R^2 of 0.401. In order to determine the relative contribution of score 2 and *ABCC2* rs2273697 to R^2, semipartial correlation analysis was performed. See table 4.5 for parameter estimates. Note that the coefficient for *ABCC2* rs2273697 (-0.091) is quite small in

comparison to score 2 (0.945), so the effect is statistically significant but weak. The semipartial R^2 values for score 2 and *ABCC2* rs2273697 are 0.4129 and 0.0041, respectively. Thus, only score 2 contributes substantially to R^2.

Table 4.5. Multiple regression analysis of independent predictors of log([END]/[NDTam])

Entry into Model	Predictor Variable	Coefficient	Std. Error	95% CI			P-value	Semipartial R^2
-	Intercept	-4.382	0.064	-4.507	-	-4.257	< 0.001	n/a
1	Score 2	0.945	0.040	0.866	-	1.024	<0.001	0.4129
2	rs2273697	-0.091	0.034	-0.157	-	-0.024	0.008	0.0041

Table 4.6 Multiple regression analysis of independent predictors of log([END])

Entry into Model	Predictor Variable	Coefficient	Std. Error	95% CI			P-value	Semipartial R^2
-	Intercept	1.761	0.172	0.172	-	2.100	< 0.001	n/a
1	Score 1	0.945	0.040	0.866	-	1.024	<0.001	0.2384
2	Age	0.007	0.002	0.003	-	0.012	0.003	0.0083
3	Weight	-0.004	0.001	-0.006	-	-0.001	0.004	0.0070
4	rs2273697	-0.089	0.037	-0.161	-	-0.016	0.017	0.0040
5	rs12248560	0.076	0.035	0.006	-	0.145	0.033	0.0044

A similar analysis was undertaken to determine contribution of variables to log([END]). *CYP2D6* genotype score 1, weight, age, duration of tamoxifen treatment at blood draw, and the genetic variants of interest were considered as covariates for log([END]). Stepwise regression analysis indicated that log([END]) was dependent on the variables: score 1, age, weight, *ABCC2* rs2273697 and *CYP2C19* rs12248560, with a combined R^2 of 0.2514. See Table 4.6 for parameter estimates. Again, examination of the coefficients suggests that score 1 has the greatest impact on log([END]) since the

coefficients for age, weight, *ABCC2* rs2273697 and *CYP2C19* rs12248560 are relatively small in comparison . The semipartial R^2 values for score 1, age, weight, *ABCC2* rs2273697 and *CYP2C19* rs12248560 are 0.2384, 0.0083, 0.0070, 0.0040, and 0.0044, respectively. Thus, only score 1 contributes substantially to R^2.

4.4 Discussion

CYP2D6 is known to be an important determinant of serum endoxifen concentrations and [END]/[NDTam] ratio, and this is supported by the results of this study. However, none of the additional variants tested are likely to increase the utility of genetic testing for tamoxifen pharmacogenomics. While statistically significant associations were apparent, clinical significance is unlikely. Unfortunately these factors could not be assessed in the current study. The CYP2D6 scoring system developed by Gaedigk et al. performed well in terms of explaining variation in log([END]) and log([END]/[NDTam]) within the dataset, although the scoring systems based upon the coefficients of the *CYP2D6* variants from multiple regression analysis accounted for slightly more variation. This is not surprising since these genotype scoring systems are based directly on data from the cohort being assessed, and performance may not be as good in a different study population.

A statistically significant association between the *ABCC2* rs2273697 variant and log([END]/[NDTam]) and log([END]) was determined in the current study. However, partial correlation analysis indicates that *CYP2D6* genotype score accounts for the vast majority of the R^2 value from the multiple regression analysis, and that any effect of this

variant on serum endoxifen concentration or [END]/NDTam] ratio is quite small. It is of interest to see if this result can be replicated in a different population. No other *ABCC2* variants genotyped for this study were associated with log([END]/[NDTam]). Kiyotani et al. performed an association study to determine if CYP2D6 variants and single nucleotide polymorphisms in transporters were associated with the outcome of tamoxifen adjuvant therapy in a population of Japanese breast cancer patients [16]. In addition to the possible functional polymorphisms rs2032582 (2677G>T/A) and rs3213619 (-129T>C) in *ABCB1* and rs2273697 (1249G>A) in *ABCC2*, 51 tag-SNPs were assayed. Of the transporter variants tested, only the *ABCC2* rs3740065 tag-SNP was associated with clinical outcome. However, no significant difference between serum endoxifen concentrations and *ABCC2* rs3740065 genotype groups was apparent, leading the authors to hypothesize that this SNP, or a variant linked to this SNP, may affect tumor cell exposure to tamoxifen. Kitoyani did not find an association between *ABCC2* rs2273697 and outcome, which is not surprising since this variant would have a minor effect on endoxifen metabolism according to our findings. As we were not granted access to clinical outcomes for WHEL study subjects, it is not possible for us to determine if *ABCC2* rs2273697 or other variants are associated with clinical outcome. While this is of interest, it is unlikely that any associations found would be related to differences in endoxifen concentration or [END]/[NDTam] ratio that can be detected in serum samples.

In addition to *ABCC2* rs2273697*, CYP2C19* rs12248560 was associated with log([END]). Like *ABCC2* rs2273697, the effect of this variant on log([END]) is expected to

be very small. Schroth et al. has been the only group to find an association between *CYP2C19* rs12248560 and the clinical outcome of tamoxifen adjuvant therapy, and it is of interest to see if this result can be replicated in another cohort. If *CYP2C19* rs12248560 does indeed influence clinical outcomes, it is not likely to be due to its contribution to endoxifen formation; some other pathway may be involved.

No association between the *UGT2B7**2 variant or *UGT2B7* ivs1985ag and log(END) or log(END/NDTam) was observed in the current study. Our results agree with those of a recent study by Ahern et al., which aimed to determine if there was an association between functional polymorphisms in key UGTs (*UGT2B15**2, *UGT2B7**2, and *UGT1A8**3) and the recurrence rate among breast cancer survivors who were treated with tamoxifen [17]. There was no association between any of these variants and breast cancer recurrence. Endoxifen-gluc has been found in the urine of patients taking tamoxifen, so glucuronidation does take place *in vivo* and may be important for the elimination of this potent tamoxifen metabolite. However, there may be additional factors which override any effect functional UGT2B7, or other UGT, variants might have on serum endoxifen concentration or END/NDTam ratio. For example, endoxifen-gluc may be exposed to bacterial glucuronidase activity in the gut during enterohepatic re-circulation, converting it back into the active metabolite endoxifen. In addition, there are other endogenous and xenobiotic chemicals which are subject to glucuronidation that may influence the rate of glucuronidation of endoxifen.

During the preparation and writing of this chapter, several studies have been published which investigated the effect of additional genetic and non-genetic variables

on serum or plasma endoxifen concentration. In a study by Teft et al., the recently defined *CYP3A4*22* variant, as well as seasonal variation and vitamin D status, were associated with serum endoxifen concentration [18]. The *CYP3A4*22* variant was associated with higher serum endoxifen concentration, and accounted for some of the 30% of PM subjects who had serum endoxifen concentration above the sub-therapeutic cutoff. A study by Murdter et al. found that *CYP2C9*2* and **3* variants are associated with lower plasma concentrations of endoxifen [19], although Teft et al. also looked at these variants and found no association [18]. Lim et al. investigated the impact of *CYP2D6, CYP2C9, CYP3A5, and CYP2C19* variants on plasma endoxifen concentration and found that only *CYP2D6* genotype was significantly associated [20]. It is of interest to see if *CYP3A4*22, CYP2C9*2* and **3*, as well as seasonal variation and vitamin D status are associated with serum endoxifen concentration in other cohorts.

4.5 Conclusion

The results of this study emphasize the important role of *CYP2D6* genetic variants in determining serum endoxifen concentration and the [END]/[NDTam] metabolic ratio. However, it is also clear that some, but not all, variation in serum endoxifen concentration and [END]/[NDTam] metabolic ratio can be explained by *CYP2D6* genetic variants. While *ABCC2* rs2273697 and *CYP2C19* rs12248560 were associated with log([END]), and *ABCC2* rs2273697 was associated with log([END]/[NDTam]), the contribution of these variants to these phenotypic measures is likely to be very small and not clinically significant.

4.6 References

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Chapter 5

Conclusions and Perspectives

5.1 Conclusions and Perspectives

The overall purpose of this dissertation was to investigate the factors that influence serum tamoxifen and tamoxifen metabolite concentrations in breast cancer patients undergoing tamoxifen adjuvant therapy, and to examine the ability of *CYP2D6* genotype cutoffs to define a patient population at risk of recurrence due to low serum endoxifen concentrations. In doing so, the hope was to clarify the best use of *CYP2D6* genotyping for the optimization of adjuvant endocrine therapy for ongoing clinical studies and clinical trials. Arguably, the clinical use of *CYP2D6* genotyping is as controversial now as it was when this research commenced. Several prospective clinical trials on the use of *CYP2D6* genotyping for breast cancer patients may be required before a consensus is reached on whether or not *CYP2D6* testing should become clinical practice and how this data is used to optimize therapy.

An LC-MS/MS assay was developed and validated for the quantitation of serum concentrations of tamoxifen, N-desmethyltamoxifen, and endoxifen. A set of 78 banked serum samples from UCSF were analyzed using this assay, and *CYP2D6* *4, *41, and *10 variants were assessed using TaqMan allelic discrimination assays in the corresponding DNA samples. The LC-MS/MS assay was shown to be robust for the quantitation of these analytes at physiologically relevant concentrations. Genotype-phenotype analysis yielded the expected result – a correlation between genotype predicted CYP2D6 activity

and serum concentrations of endoxifen. In hindsight, it would have been advantageous to develop an assay that could quantitate common, potent, CYP2D6 inhibitors in addition to tamoxifen, N-desmethyltamoxifen, and endoxifen. For many of the tamoxifen pharmacogenetics studies, including the WHEL study, good co-medicaton data was not available for the majority of study subjects. Direct measurement of CYP2D6 inhibitors in serum is better proof than pharmacy records or patient reporting that a drug was taken during tamoxifen therapy. For studies which measured serum tamoxifen and tamoxifen metabolite concentrations and also accounted for CYP2D6 inhibitor usage, this data explained some of the variation in serum endoxifen concentration and [END]/[NDTam] ratio that could not be attributed to *CYP2D6* genotype [1, 2]. Depending on how the data was used, the impact of co-medication on [END]/[NDTam] ratio was modest to dramatic. Concurrent usage of potent CYP2D6 inhibitors, such as paroxetine, has been contraindicated for several years now due to tamoxifen pharmacogenetics research, yet it is still a factor that one should account for in current studies since the extent to which this advice is followed is unknown.

Serum tamoxifen, endoxifen, and N-desmethyltamoxifen concentrations were measured for the WHEL cohort. In contrast to the UCSF sample set that was analyzed for the assay validation, the WHEL cohort was much larger (n=1560 subjects taking tamoxifen), more extensive *CYP2D6* genotyping was performed using the Roche P450 AmpliChip, and recurrence data was available for the cohort. The WHEL study group used the data generated from the LC-MS/MS measurements to show that serum endoxifen concentrations were associated with the clinical outcome of tamoxifen

adjuvant therapy, and that subjects in the lowest quintile of serum endoxifen concentration were at increased risk for breast cancer recurrence [3]. For this dissertation, a detailed examination of interethnic differences in serum tamoxifen and tamoxifen metabolites was performed, and significant differences between groups were found. Further research comparing tamoxifen pharmacogenetics and metabolism in different ethnic populations is warranted in order to determine if research done in Caucasians is generalizable to other groups. In addition, CYP2D6 genotype metabolizer group assignments were assessed, in the Caucasian population, for their ability to define the low endoxifen risk group. The PM/PM metabolizer group was optimal for defining the at risk population, in the sense that it yielded the highest positive predictive value as a test cut-off (71.7%). However, the sensitivity was low, at only 23.9%. Almost as many subjects in the EM/EM group fall into the low endoxifen risk group as the PM/PM group, it is just that the proportion of subjects is lower. These results suggest that direct measurement of serum tamoxifen and tamoxifen metabolites could be more useful clinically than *CYP2D6* genotyping. This is possible in the clinical setting and is currently under investigation. In addition, regular clinical monitoring of patients would have the added benefit of allowing doctors to see if a patient has lapsed in compliance or if a new medication has altered tamoxifen metabolism. If it is endoxifen that is important for efficacy, then perhaps it is endoxifen concentration rather than *CYP2D6* genotype that should be assessed. Therapeutic drug monitoring (TDM) is an old idea that is not as "sexy" as using genetic data, but the strategy has shown itself to be useful clinically for the management of a variety of drugs. If TDM of tamoxifen and tamoxifen metabolites

were to be adopted clinically, it would be desirable to have the appropriate standard reference materials available so that measurements made by different laboratories are on the same scale and are comparable.

While *CYP2D6* genotype is an important determinant of serum endoxifen concentration and endoxifen formation, it does not account for all variation observed. The utility of genotyping to determine optimal adjuvant endocrine therapy would likely be enhanced if additional genetic variants were identified and validated. In addition to *CYP2D6* genotypes, variants in several candidate genes were examined to see if they were associated with serum endoxifen concentration and/or [END]/[NDTam] ratio. While *ABCC2* rs2273697 and *CYP2C19* rs12248560 were significantly associated with log([END]), and *ABCC2* rs2273697 was significantly associated with log([END]/[NDTam]), the contribution of these variants was very small, such that clinical relevance would be unlikely. Tamoxifen metabolism is complex, and there are genetic and non-genetic factors that influence metabolism that have yet to be identified. Thus far, factors that have a clear contribution to serum endoxifen concentration are *CYP2D6* genotype, co-medication with CYP2D6 inhibitors, and compliance. The factors that influence tamoxifen efficacy are even more complex and greater in number.

The premise of clinical testing of functional *CYP2D6* variants for breast cancer patients eligible for endocrine therapy is that poor metabolizer genotypes result in low CYP2D6 activity, low CYP2D6 activity leads to poor conversion of tamoxifen into endoxifen and therefore sub-therapeutic endoxifen concentrations in breast cancer

cells, and this result is of little or no pharmacological effect. While the argument is straightforward, it is also somewhat simplistic. The biological mechanisms involved in drug efficacy, especially in the highly mutable environment of cancer, are complex. Germ-line DNA variants are informative, but do not yield complete information regarding expression levels or activity of functional gene products *in vivo.* There are many steps that must occur in order for a DNA sequence to be properly expressed as a functional gene product. For instance, epigenetic factors can influence gene expression. Perinatal exposure of rats to phenobarbital has been shown to result in a permanent (imprinted) increase in expression of the drug metabolizing enzymes CYP2C6 and CYP2C7 [4], and early exposure to certain xenobiotics in humans may result in similar changes in drug metabolism. This type of alteration would not be detectable via analysis of DNA extracted from blood. In addition, the information contained in DNA sequence is, for the most part, static while drug metabolism is in a real-time flux. For instance, co-medications and diet are known to influence drug metabolism, and the effects of these inputs are normally transient. And, as always, compliance is an issue; no drug taken means no pharmacological activity.

In terms of the breast tumor cells, *CYP2D6* germline testing does not tell us anything about the responsiveness of tumor cells to tamoxifen and its metabolites. Nor do serum measurements of cancer drugs and their metabolites. Cancer cells are highly mutable and often acquire resistance over time to the drugs that are meant to kill them. This may occur through a variety of mechanisms, from alterations in the estrogen receptor signaling pathways to the efflux of tamoxifen and its metabolites. The

pharmacodynamic side of the drug action equation is an important, but missing, piece of the puzzle in predicting tamoxifen efficacy via *CYP2D6* testing. This would require assessment of the tumor itself, not just germline DNA variation. The criteria for adjuvant endocrine therapy is ER/PR expression, mainly because tumors that do not express these markers are generally not responsive to hormonal therapy. In addition, tumors that are positive for HER2, regardless of ER and PR expression, are generally not responsive to endocrine therapy, but are likely to respond to Herceptin. These tumors vary in terms of gene expression profiles, benefit from chemotherapy and endocrine therapy, and overall prognosis. Several research groups have sought to identify molecular profiles that predict benefit from adjuvant endocrine therapy within the $ER^+/PR^+/HER2^-$ population. Symmans et al. developed the sensitivity to endocrine therapy (SET) index which is based upon the expression profile of 165 genes that are co-expressed with *ESR1* [5]. In a validation cohort, the hazard ratio for distant relapse or death in a population of patients treated with tamoxifen only was 3.45 [95% CI: 1.12-11.9] for low/intermediate versus high SET score. The SET index had no prognostic value in two untreated cohorts, suggesting that benefit from endocrine therapy, not intrinsic prognosis, is predicted by SET. These results are preliminary, but promising. Perhaps this strategy, in combination with standard pathological tumor assessment or OncotypeDx testing, would help to further define a population for which endoxifen levels are an important predictor of tamoxifen efficacy.

While *CYP2D6* genotyping for tamoxifen pharmacogenetics is imperfect as a clinical test, there is increasing interest in the use of genetic data to improve human

health as we enter the age of "personal genomics". In September of 2011 the direct to consumer (DTC) DNA testing company, 23andMe, announced a pilot program to provide raw, Whole Exome Sequencing (WES) (80X coverage of 50 million DNA bases) data to some of its current customers for the price of $999 [6]. In October of 2011, Baylor College of Medicine opened a commercial, clinical laboratory that uses next generation sequencing technology (Ion Torrent) to provide Whole Exome Sequencing (WES) for patients [7]. Currently, it is only doctors faced with difficult "medical mysteries" who are using this service, but the lab has been able to identify causal genetic mutations in approximately 30% of cases. Thus, DNA sequencing is becoming increasingly available and affordable to the general population. In the not-so-distant future, it could become standard medical practice for individuals to have their entire genomes sequenced. While the cost of sequencing plummets, one must also consider the amount of time and money required to generate meaningful analyses of genetic data. It could be that, "It's a $1000 genome but a $10000 analysis"[7]. Interpretation of genetic data is not a simple task and, at present, our ability to generate sequence data vastly exceeds our ability to understand what it means for human health. However, one might predict that genetic data pertaining to monogenic diseases, as well as pharmacogenetic testing such as *CYP2D6* genotyping for selection of adjuvant endocrine therapy, will provide the most actionable information early on in terms of health management.

5.2 References

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